2019

Exploration of Sonic Hedgehog Gene Expression in Fathead Minnows (Pimephales promelas)

Brooke Renee Greiner

Exploration of sonic hedgehog gene expression in fathead minnows (*Pimephales promelas*)

BY
Brooke Renee Greiner

UNDERGRADUATE THESIS

Submitted in partial fulfillment of the requirement for obtaining

UNDERGRADUATE DEPARTMENTAL HONORS

Department of Biological Sciences along with the Honors College at
EASTERN ILLINOIS UNIVERSITY
Charleston, Illinois

2019

I hereby recommend this thesis to be accepted as fulfilling the thesis requirement for obtaining Undergraduate Departmental Honors

04/08/19
Date THESIS ADVISOR

April 8, 2019
Date HONORS COORDINATOR

4/10/19
Date DEPARTMENT CHAIR

Abstract

Pollutants, as a result of wastewater treatments, have been shown to have negative impacts on aquatic ecosystems. To better understand the possible consequences caused by effluents on ecosystems, it is important to examine ecotoxicology data. One of the most commonly used species for water quality testing is the fathead minnow, *Pimephales promelas*. Ecotoxicology can then be taken one step further to understand the effects of pollutants on a molecular level. Previous research had identified effluents as causes for abnormal minnow fin morphology. In order to collect additional data on development, tanks with fathead minnows were placed at the Charleston Wastewater Treatment plant effluent, the Decatur Wastewater Treatment Plant effluent, and control tanks at EIU's H.F. Thut Greenhouse. Fathead minnows were randomly sampled for about eight weeks and underwent immunofluorescence staining and imaging for sonic hedgehog (*shh*) gene expression. The data collected indicates that sonic hedgehog gene may not be responsible for fin bud development or is expressed at a different time. Expression of *shh* was only seen in three samples: two anal fin expressions in 23 dph (days post hatching) and one dorsal fin expression in 38 dph. I concluded that late expression of *shh* is not responsible for the differences seen in fin growth ratios. There are different possible explanations for not seeing late *shh* expression: it is expressed mainly during a different developmental period, pollutant impact during critical growth period, or upregulation of another pathway replacing *shh* expression. Follow up studies should explore the fathead minnow's critical period of *shh* expression and specific pollutants that might disrupt the *shh* pathways.

Introduction

Pollution has become a rising problem around the world and its negative effects on ecosystems have been extensively studied. When choosing a particular life stage to study, aquatic toxicologists focus on the most sensitive stages, embryonic and larval. Teratogens, a substance that causes malformation of embryos, not only effect embryonic development but can affect regeneration processes that occur in adult life (Weis and Weis, 1987). There are numerous

existing teratogens, and they tend to be nonspecific. When it comes to their effects, many teratogens produce the same effect but are a result of different pathways (Laale 1981). The impact of teratogens or pollutants depends on the timing and length of exposure, especially during critical periods. Namely, pollutants are most commonly observed to affect the skeletal axis and skeletal growth (Weis and Weis, 1987; Devlin et al., 1985).

Lepidotrichia, fin rays, are constructed of pairs of segmented concave hemirays facing each other and are formed by scleroblasts, which mineralizes the bone matrix to create an intramembranous bone. Growth of lepidotrichia occurs through the process of branching or elongation of lepidotrichia segments in a proximal to distal progression. It has been documented in zebrafish that fin growth is related to hedgehog signaling pathways, specifically sonic hedgehog (*shh*) (Avaron et al., 2006; Laforest et al., 1998).

The hedgehog pathways have been shown to be involved in limb and fin bud development. More specifically, a substantial amount of information has been gathered about the developmental and regenerative processes in zebrafish as its paired fin development contains many similarities to limb development in tetrapod species. Vertebrate hedgehog (*hh*) genes have three classes: *sonic* (*shh*), *Indian* (*ihh*), and *desert* (*dhh*). In most cases, vertebrate genomes include one member from each gene family (Avaron et al., 2006). The various *hh* expressions have shown some overlap, however, when it comes to the expression in early fin bud development, suggesting that *shh* is the only activated *hh* (Neumann et al., 1999). When studying *shh* expression in zebrafish, the focus is regularly on pectoral fin buds which are analogous to tetrapods with mirrored limbs. Data suggests *shh* is also involved in the development of various organs, proving *shh* to be a key factor in overall development (Sire and Akimenko, 2003).

The strength of *shh* expression in zebrafish varies throughout the developmental process. *Shh* first appears 26-28 hours post fertilization (hpf) in few cells of an early fin bud and, by 30 hpf, *shh* expression intensifies. Zebrafish *shh* expression is located in a position parallel to the zone of polarizing activity (ZPA) in tetrapod limb buds. ZPA is a signaling center responsible for

anteroposterior (A/P) axis patterning, which coordinates with the apical ectodermal ridge (AER) creating a mutual feedback loop of coordinated axis growth of the fin bud. The AER is responsible for proximodistal (P/D) growth of a limb bud (Neumann et al., 1999, Martin 1998) and loss of the AER could lead to loss of *shh* expression (Neumann et al., 1999). By 48 hpf, *shh* is expressed in an even wider domain and this expression sustains until approximately 3 days post fertilization (dpf) and begins to be downregulated until undetectable, occurring around 4 dpf (Laforest et al., 1998; Avaron and Akimenko, 2006). Expression of *shh* is typically in the posterior limb or fin bud mesenchyme to direct A/P and P/D patterning and growth during development and regeneration (Neumann et al., 1999).

Disruption of *shh* expression can have a variety of outcomes including disorganization of the A/P patterning and activation of *hox* genes in zebrafish. Not all *hox* genes are *shh* dependent. In particular, *hoxd-13*, *hoxa-13*, and *ptc* are completely dependent while *hoxd-10* appears to be independent of *shh* expression. On the other hand, *hoxd-11*, *hoxd-12*, *hoxa-10*, and *bmp-2* require strong expression. (Avaron and Akimenko, 2006; 24, Neumann et al., 1999; Riddle et al., 1993; Laufer et al., 1994) Research has also shown correlation between *hox* gene position and degree of dependence of *shh* (Neumann et al., 1999).

There are a variety of pollutants that could affect *shh* expression, one being retinoic acid (RA). RA is a commonly studied pollutant and has been documented to affect activation of *hox* genes and *shh* expression in different ways. In the ZPA, *shh* and RA are involved in *hox* gene signaling (Avaron and Akimenko, 2006; Capdevila and Belmonte, 2001; Helms et al., 1994; Lu et al., 1997; Tickle et al., 1982; Riddle et al., 1993; Krauss et al., 1993; Akimenko and Ekker, 1995) and the fast-acting effects of RA on *shh* hint at a direct correlation between the two causing morphogenic effects and delay or loss of fin bud development, not limited to the pectoral fin. As RA administration time increases, negative impacts on fin bud development increase. In the ZPA, *ptc1* likely codes for the *shh* signal receptor. It is believed a limited number of cells contain the potential to create a polarizing zone. (Avaron and Akimenko, 2006; Akimenko and Ekker, 1995;

Laforest et al., 1998). As a result, it could only take a small change in concentration or expression to produce strong effects.

Fathead minnows, *Pimephales promelas*, are one of the most commonly used small fish models in ecotoxicology in North America due to their presence in a variety of aquatic environments (Isaak, 1961; Devine, 1968; Eddy and Underhill, 1974; Held and Peterka, 1974). The USEPA has documented the well-defined stages of both the developmental and reproductive cycles (USEPA, 1987, 1996). The popular procedures are a 30-day early life stage, partial life cycle, and a full life cycle test. For an early life stage test, it typically starts with less than 24 hour old embryos and data collection continues until 30 days post hatch (USEPA, 1989; OECD,1992; ASTM, 2000). In comparison, the partial life test is mainly utilized when quick environmental regulation is necessary while the early life stage and full life cycle tests allow for a more comprehensive conclusion. Other than life assays, fathead minnows have played an important role in studying the effects of over 600 chemicals (Russom et al., 1997; Bradbury et al., 2003) and more recently researchers have been exploring the effects of chemical mixtures. Many studies have shown that laboratory toxicity reports can withhold strong indirect implications to be applied to regulatory aspects (USEPA, 1991). Although the fathead minnow has been widely used in ecotoxicology, there is limited knowledge in genomic research as more laboratories focus on the zebrafish (*Danio rerio*), a model species. Exploration into genomic research of the fathead minnow could allow for ecotoxicologists to expand their understanding which in turn could lead to better regulatory measures (Ankley and Villeneuve, 2006).

Recently, one of the members from the same laboratory at Eastern Illinois University ran a toxicology study examining the possible effects of the Charleston Wastewater Effluent and the Decatur Wastewater effluent on fish. The chemical makeup of the water was examined along with the effects of a disinfection treatment, the addition of chlorine and ammonia to reduce bacteria. When comparing conditions, length and weight were concluded to have little to no

significant differences except in the pelvic fin where Decatur Post-Disinfection had a higher pelvic fin ratio (Bogue, pers. Comm).

The goal of my study was to explore the role of *shh* in fin bud development in fathead minnows. I wanted to test whether timing or length of expression was the underlying cause behind differences seen in fin length. In the light of published zebrafish studies, I expect to see no signs of *shh* expression, expect possible faint expression in the samples collected from 30 dph (Avaron and Akimenko, 2006; Neumann et al., 1999; Laforest et al., 1998).

Methods

Study Animals

We obtained 1200 fathead minnows from Aquatic Research Organisms Inc. Two days post hatching, we housed the fish in six different tanks: two for the Charleston Wastewater Treatment Plant effluent, two for the Decatur Wastewater Treatment Plant effluent, and two control tanks at EIU's H.F. Thut Greenhouse. Each location had two constructed tanks, a Pre-Disinfection and Post-Disinfection tank and all tanks began with 200 fish. Disinfection included the addition of chlorine and ammonia to reduce bacteria. Effluent tanks held 350 gallons with a pump into and out of the tank designed to simulate the continuous flow of the effluent. Control tanks held 10 gallons with no continuous flow but received water changes every other day. We fed the fish frozen brine shrimp twice a day, Monday through Friday.

Sampling

Every week, for eight weeks, we randomly chose four fathead minnows from each tank. For preservation, we stored samples in 4% paraformaldehyde (PFA) and kept at -20°C.

Solution Preparation

We prepared 1X standard phosphate-buffered saline solution (PBS, Westerfield, 1995), 1X PBS containing 0.1% Tween-20 (PBST), and 1X PBST containing 0.3% Triton-X and 1% DMSO (PDT). We stored these solutions at 4°C. We prepared a blocking buffer using 1X PBST, 5 mL of

heat-activated fetal bovine serum and 2g bovine serum albumin and stored the blocking buffer at -20°C.

Immunofluorescence Staining

I removed the fathead minnow samples from -20°C storage and allowed time to thaw. In order to prevent background antibody staining from air exposure, the samples remained submerged throughout the entire staining process. Once thawed, I removed the 4% paraformaldehyde (PFA) and properly disposed of the waste. To permeabilize the fathead minnow membrane, I slowly added 1 mL of 100% methanol, stored in -20°C, and kept the samples overnight at -20°C.

After 12 hours, I removed the methanol and washed the samples. For all sample washes, I added 1 mL of 1X PDT and gently swirled the tube. Then I removed ~1 mL of solution, added another 1 mL of 1X PDT and gently swirled the tube. I completed the wash by rocking the samples at room temperature for 30 minutes. After 30 minutes, I removed ~1 mL of solution, and repeated the wash. Next, I added 500 µL of blocking buffer to the samples and left them to be carefully rocked at room temperature for an hour. For biomarking purposes, I added 100 µL of the anti-*shh* primary antibody, using a 1:250 dilution in PDT. I rocked the samples at room temperature for an hour and stored at 4°C overnight.

The next day, I removed the antibody solution and washed the samples twice with 1X PDT. After washed, I added 500 µL of blocking buffer to the samples and rocked them at room temperature for an hour. At this point, I captured benchmark pictures as a control to show any background antibody staining that may have occurred throughout the immunofluorescence staining procedure.

After I captured the benchmark pictures, I added 100 µL of the green fluorescing anti-rabbit IgG secondary antibody using a 1:200 dilution in PDT. To preserve antibody fluorescence, I individually covered each sample and kept it in a dark container. I rocked the samples at room temperature for an hour and stored them at 4°C overnight.

The following day, I removed the antibody solution and washed the samples twice with 1X PDT, and pictures were taken.

Immunofluorescence Imaging

I captured the pictures using an Olympus BX50 fluorescence microscope connected to a computer using Pixera Viewfinder version 3.0.1 with the fluorescence color set to WB, a blue emitted light to allow for green fluorescence. I consistently oriented all samples in the same direction and captured pictures for the dorsal, pelvic, anal, and caudal fins.

Results

Of the 24 fish sampled, only three samples displayed *shh* expression (Table 1) Two samples from 23 dph showed mild expression on the anal fin (Figure 1; Figure 2) and one sample from 38 dph showed mild expression on the dorsal fin (Figure 3). For the remaining 21 samples, *shh* expression was not present. All samples exemplified normal fin development through branching and elongation of hemirays.

Discussion

As pollution continues to be a rising issue, it becomes more important to understand the impact of different teratogens for various ecosystems. Understanding the mechanisms underlying toxic effects seen in aquatic taxa will advance our capacity to deal with pollutants. Gene expression studies serve as the most promising area to accomplish that. In this study, *shh* does not reappear at the four-week mark as seen in zebrafish and may not be responsible for increased pelvic fin ratio seen in Decatur Post-Disinfection samples. On the other hand, expression of *shh* is mainly seen in early developmental stages, often referred to as critical periods, but the earliest sample collected was 23 dph. The length and timing of *shh* expression during the critical period or early embryonic development is likely the leading cause of the fin development differences seen in the pelvic fin rather than expression differences at the four-week mark. In future studies, the collection days should be expanded to include more samples between 26 hpf to 28 dph as zebrafish studies have shown strongest *shh* expression during the first week with a reappearance

around four weeks. (Avaron and Akimenko, 2006; Neumann et al., 1999; Laforest et al., 1998). However, fin development differences could potentially be a result of a completely different pathway.

One could attribute fin differences being a result of a pollutant or teratogen, for example RA. The control samples had similar fin growth ratios along with no *shh* expression, ruling out significant pollutant effects on late fin development. It might be possible that a pollutant could be causing inhibition, but it is also equally possible that a pollutant could be causing an upregulation of *shh* expression. The *shh* dependent *hoxd-13*, *hoxa-13*, and *ptc* may be affected while genes not completely dependent, *hoxd-11*, *hoxd-12*, *hoxa-10*, *bmp-2*, and *hoxd-10*, might be inhibited or upregulated by a different substance or molecule not yet researched (Avaron and Akimenko, 2006; Riddle et al., 1993; Laufer et al., 1994; Neumann et al., 1999).

My data shows that *shh* expression is not consistently present in fathead minnows over 23dph. Recent work only showed differences in the pelvic fins (Bogue, pers. Comm.) and there were no indicators that late expression of *shh* is the main contributor to the pelvic fin differences. Given this data, *shh* is not completely ruled out for fin bud development in fathead minnows and its expression from 26 hpf to 28 dph should be studied along with the water chemistry to determine possible pollutants that could be affecting gene expression and regulation, whether it be *shh* or *hox* genes. Numerous zebrafish studies show a direct correlation of expression of *shh* to fin bud growth leading me to believe that differences in fathead minnow fin growth ratios are the outcome of critical period *shh* expression. Additional studies focusing on gene expression can help us understand how impacts on the ecosystem can leave its chemical footprint and affect fish development.

Tables and Figures

Table 1. Presence of Sonic Hedgehog (*shh*) gene expression in fathead minnow samples

		Anal Fin	Dorsal Fin	Pelvic Fin	Caudal Fin
23 DPH	Control Tank 2 - 10	No	No	No	No
	Control Tank 2 - 11	*Yes*	No	No	No
	Decatur Post-Disinfection - 10	No	No	No	No
	Decatur Post-Disinfection - 12	*Yes*	No	No	No
29 DPH	Control Tank 2 - 15	No	No	No	No
	Control Tank 2 - 16	No	No	No	No
	Decatur Post-Disinfection - 15	No	No	No	No
	Decatur Post-Disinfection - 16	No	No	No	No
38 DPH	Control Tank 2 - 17	No	No	No	No
	Control Tank 2 - 18	No	*Yes*	No	No
	Decatur Post-Disinfection - 17	No	No	No	No
	Decatur Post-Disinfection - 18	No	No	No	No
43 DPH	Charleston Post Disinfection - 21	No	No	No	No
	Charleston Post Disinfection - 22	No	No	No	No
	Decatur Post-Disinfection - 21	No	No	No	No
	Decatur Post-Disinfection - 22	No	No	No	No
50 DPH	Charleston Post Disinfection - 25	No	No	No	No
	Charleston Post Disinfection - 27	No	No	No	No
	Decatur Post-Disinfection - 25	No	No	No	No
	Decatur Post-Disinfection - 27	No	No	No	No
57 DPH	Charleston Post Disinfection - 29	No	No	No	No
	Charleston Post Disinfection - 30	No	No	No	No
	Decatur Post-Disinfection - 29	No	No	No	No
	Decatur Post-Disinfection - 30	No	No	No	No

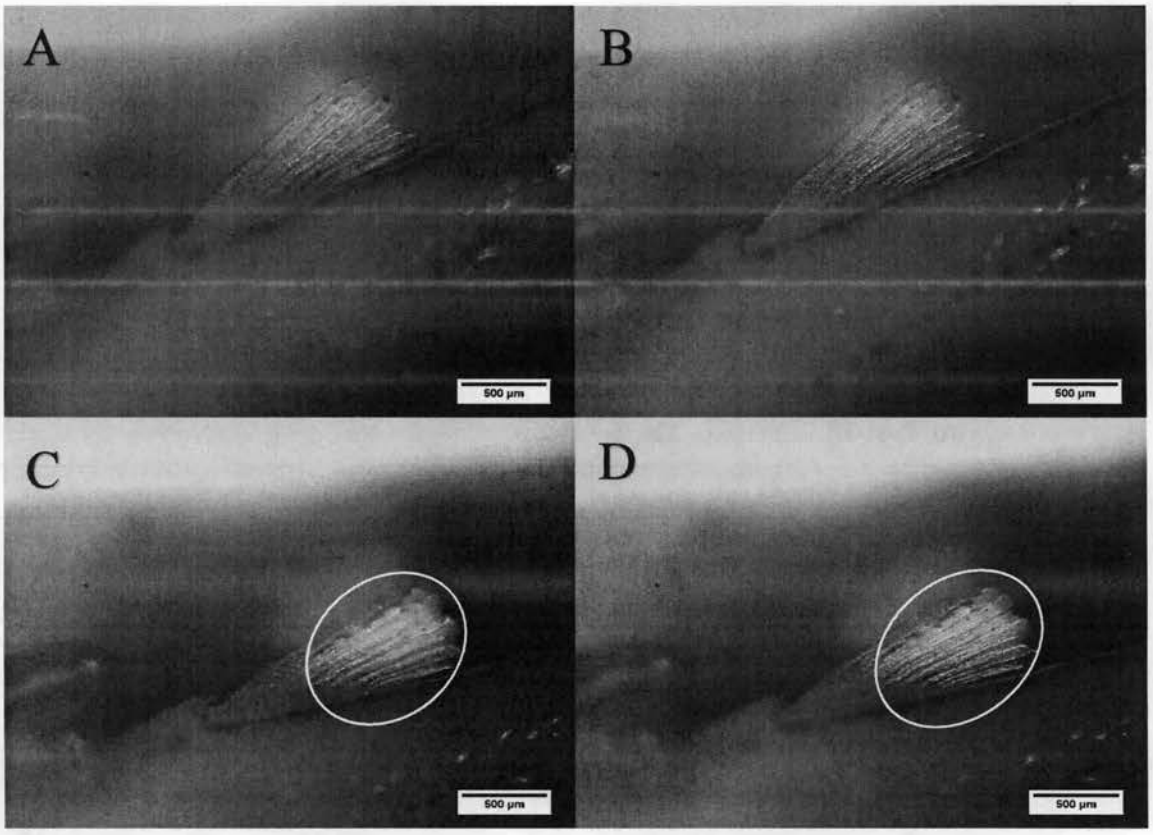

Figure 1. *shh* expression in the anal fin from Control Tank 2 fathead minnow sample 11. (A,B) Control/Background Fluorescence. (C,D) *shh* antibody expression.

Scale Bar – 500 µm

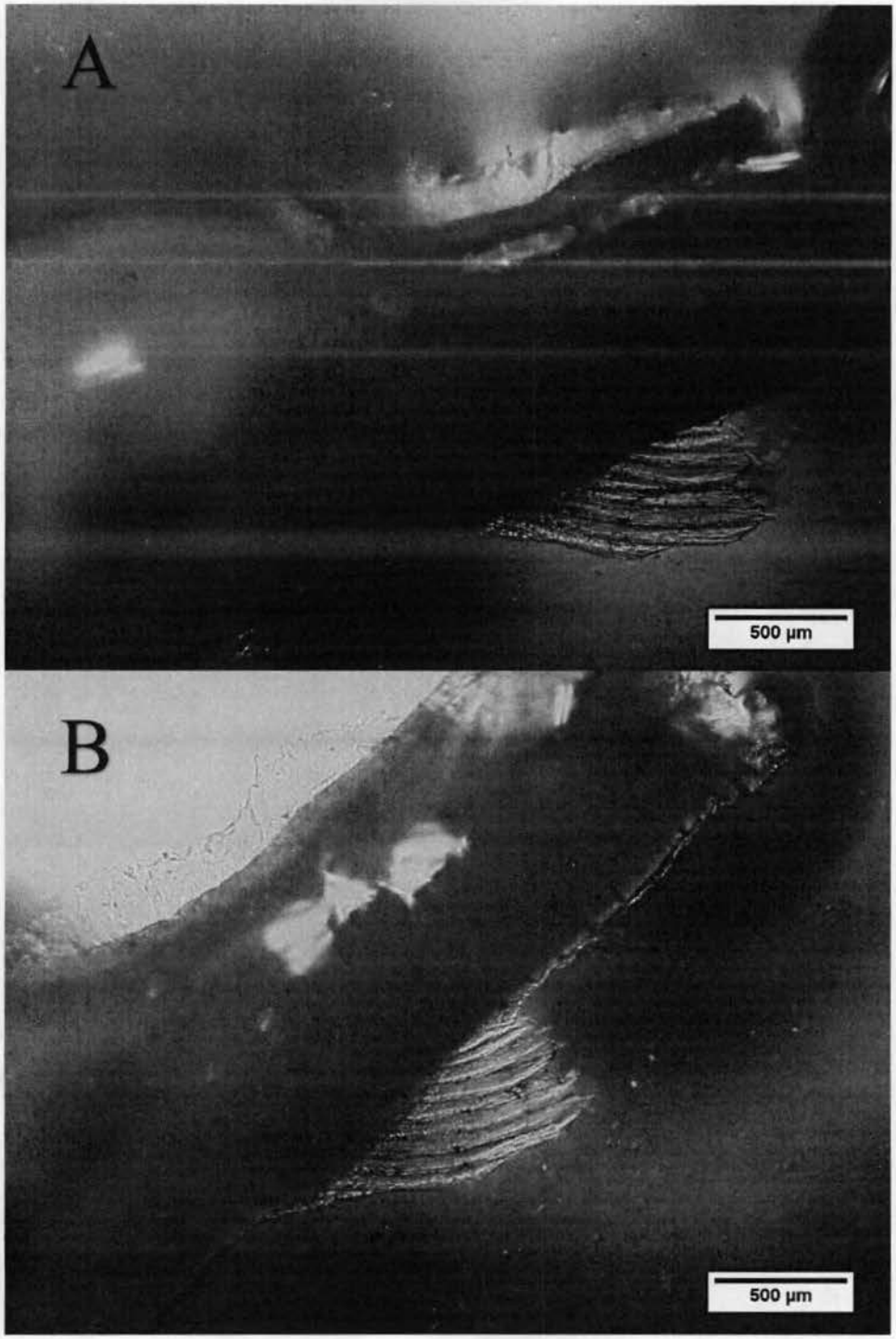

Figure 2. *shh* expression in the dorsal fin from Decatur Post-Disinfection fathead minnow sample 12. (A) Control/Background Fluorescence. (B) *shh* antibody expression.

Scale Bar – 500 µm

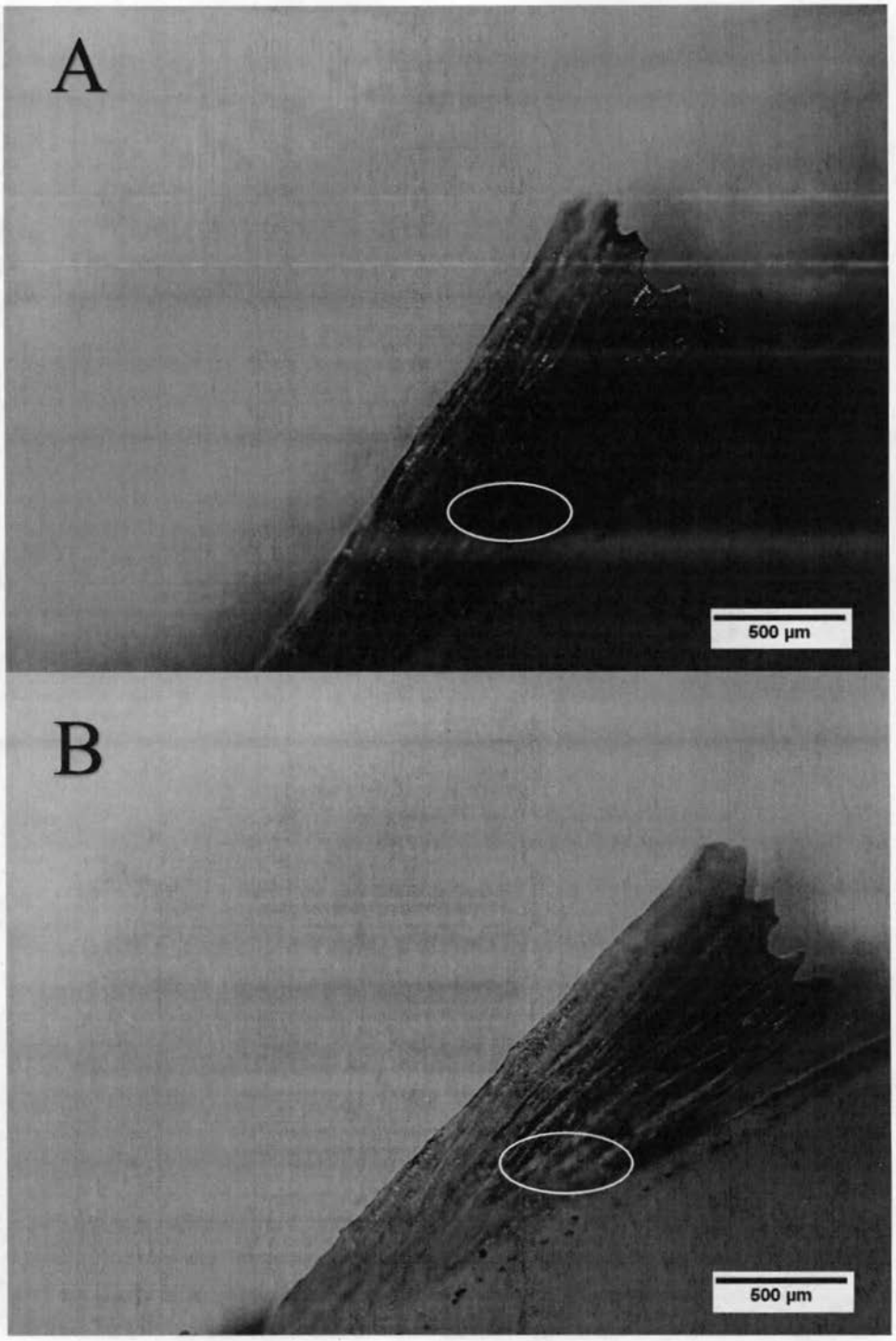

Figure 3. *shh* expression in the dorsal fin from Control Tank 2 fathead minnow sample 18. (A) Control/Background Fluorescence. (B) *shh* antibody expression. Scale Bar – 500 µm

References

Akimenko, M. A., & Ekker, M. (1995). Anterior duplication of the Sonic hedgehog expression pattern in the pectoral fin buds of zebrafish treated with retinoic acid. *Developmental biology*, *170*(1), 243-247.

Ankley, G. T., & Villeneuve, D. L. (2006). The fathead minnow in aquatic toxicology: past, present and future. *Aquatic Toxicology*, *78*(1), 91-102.

ASTM, (2000). Standard guide for conducting early life-stage toxicity tests with fishes. Method E 1241-98. In: Annual Book of ASTM Standards, vol. 11.05. West Conshohocken, PA, USA, pp. 941–967.

Avaron, F., Smith, A., & Akimenko, M. A. (2006). Sonic Hedgehog signalling in the developing and regenerating fins of zebrafish. In *Shh and Gli Signalling and Development* (pp. 93-106). Springer, New York, NY.

Bradbury, S.P., Russom, C.L., Ankley, G.T., Schultz, T.W., Walker, J.D., (2003). Overview of data and conceptual approaches for derivation of quantitative structure–activity relationships for ecotoxicological effects of organic chemicals. Environ. Toxicol. Chem. 22, 1789–1798.

Capdevila, J., & Belmonte, J. C. I. (2001). Patterning mechanisms controlling vertebrate limb development. *Annual review of cell and developmental biology*, *17*(1), 87-132.

Devlin, E. W. , Brammer, J. D. , and Puyear, R. L. Effects of toluene on fathead minnow (Pimephales promelas Rafinesque) development. Arch. Environ. Contam. Toxicol. 14: 595—603 (1985).

Devine, G., (1968). A study of the smallmouth bass in ponds with special consideration of minnows and decapods as forage. Thesis, University of Missouri, Columbia, MO, USA.

Eddy, S., Underhill, J.C., (1974). Northern Fishes. University of Minnesota Press, Minneapolis, MN, USA.

Held, J.W., Peterka, J.J., (1974). Age, growth, and food habits of the fathead minnow, Pimephales promelas, in North Dakota saline lakes. Trans. Am. Fish. Soc. 103, 743–756.

Helms, J., Thaller, C., & Eichele, G. (1994). Relationship between retinoic acid and sonic hedgehog, two polarizing signals in the chick wing bud. *Development*, *120*(11), 3267-3274.

Isaak, D., (1961). The ecological life history of the fathead minnow, Pimephales promelas (Rafinesque). PhD Thesis, University of Minnesota. St. Paul, MN.

Krauss, S., Concordet, J. P., & Ingham, P. W. (1993). A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. *Cell*, *75*(7), 1431-1444.

Laale, H. W. (1981) Teratology and early fish development. Am. Zool. 21: 517-533.

Laforest, L., Brown, C. W., Poleo, G., Géraudie, J., Tada, M., Ekker, M., & Akimenko, M. A. (1998). Involvement of the sonic hedgehog, patched 1 and bmp2 genes in patterning of the zebrafish dermal fin rays. *Development*, *125*(21), 4175-4184.

Laufer, E., Nelson, C. E., Johnson, R. L., Morgan, B. A. and Tabin, C. (1994). Sonic hedgehog and Fgf-4 act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. Cell 79, 993-1003.

Lu, H. C., Revelli, J. P., Goering, L., Thaller, C., & Eichele, G. (1997). Retinoid signaling is required for the establishment of a ZPA and for the expression of Hoxb-8, a mediator of ZPA formation. *Development*, *124*(9), 1643-1651.

Martin, G. R. (1998) The roles of FGFs in the early development of vertebrate limbs. *Genes Dev.* 12, 1571-1586.

Neumann, C. J., Grandel, H., Gaffield, W., Schulte-Merker, S., & Nusslein-Volhard, C. (1999). Transient establishment of anteroposterior polarity in the zebrafish pectoral fin bud in the absence of sonic hedgehog activity. *Development*, *126*(21), 4817-4826.

OECD, (1992). Fish early life-stage toxicity test. Guideline 204. In: OECD Guidelines for Testing of Chemicals, Section 2. OECD, Paris, France.

Riddle, R. D., Johnson, R. L, Laufer, E. and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. Cell 75, 1401-1416.

Russom, C.L., Bradbury, S.P., Broderius, S.J., Drummond, R.A., Hammermeister, D.E., (1997). Predicting modes of toxic action from chemical structure: acute toxicity in the fathead minnow (Pimephales promelas). Environ. Toxicol. Chem. 16, 948–967.

Sire, J. Y., & Akimenko, M. A. (2003). Scale development in fish: a review, with description of sonic hedgehog (*shh*) expression in the zebrafish (Danio rerio). *International journal of developmental biology*, *48*(2-3), 233-247.

Tickle, C., Alberts, B. M., Wolpert, L., & Lee, J. (1982). Local application of retinoic acid to the limb bud mimics the pattern of chick wing development. *Nature (Lond.)*, *296*, 564-565.

USEPA, (1996). Prehatching development of the fathead minnow Pimephales promelas Rafinesque. EPA/600/R-96/079. USEPA, Washington, DC.

USEPA, (1991). Technical support document for water quality-based toxics control. EPA/505/2-90/001. USEPA, Washington, DC.

USEPA, (1989). Pesticide assessment guidelines. Subdivision E, hazard evaluation: wildlife and aquatic organisms. EPA/540/09-82/024. USEPA, Washington, DC.

USEPA, (1987). Guidelines for the culture of fathead minnows Pimephales promelas for use in toxicity tests. EPA/600/3-87/001. USEPA, Duluth, MN.

Weis, J. S., & Weis, P. (1987). Pollutants as developmental toxicants in aquatic organisms. *Environmental Health Perspectives*, *71*, 77-85.

Westerfield, M. (1995). The zebrafish book. Eugene: University of Oregon Press.