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Studies on The Olfactory Biology and Behavioral Responses to Copper Exposure of The Native Indicator Species Delta Smelt (*Hypomesus transpacificus*)

By

PEDRO ALEJANDRO TRIANA GARCÍA

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In

Integrative Pathobiology

In the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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Committee in Charge

2021

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Dedication

This is dedicated to my lovely and supporting parents, Nubia Garcia, and Winston Torres. I am here thanks

to their sacrifice, hard work and teachings. I will always love you. I also want to dedicate this work to my

beautiful and amazing wife Monica Rincon. She has been always a source of support and encouragement

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Studies on The Olfactory Biology and Olfactory Responses to Copper Exposure of The Native Indicator Species Delta Smelt (*Hypomesus transpacificus*)

Abstract

The Delta Smelt (*Hypomesus transpacificus*) is a small Osmerid native to the San Francisco Bay Delta (Bay-Delta) in California. Delta Smelt population has declined dramatically since the species was declared as endangered in 1993, and now has been listed as critically endangered by the International Union for Conservation of Nature (IUCN). The Bay Delta is a highly modified ecosystem, and the Delta Smelt has been used as an indicator species to assess the overall health of this ecosystem. Recently, a wealth of evidence exists showing that anthropogenic intervention and change in ecosystems leads to alteration in the sensory perception of the environment by aquatic animals compromising survival in highly modified environments. One of the main modalities used by aquatic organisms to assess and survive in the wild is the olfactory system. The olfactory system is involved in pivotal functions such as recognition of predators, kin recognition, mating, foraging and migration. The olfactory system is highly susceptible to contaminants including copper, which is a common contaminant and a well-studied and measurable olfactory toxicant of fish. A link between the olfactory biology and the effects of common contaminants (i.e., copper) found in the Bay Delta on Delta Smelt is lacking. I studied the basic morphological characteristics of the olfactory organ (olfactory rosette) of Delta Smelt using histological, immunohistochemical and ultrastructural techniques; the olfactory mediated behavioral responses to predation related odorants using a behavioral standardized assay and tracking software; and finally, I evaluated morphological changes of the olfactory epithelium and behavioral responses to alarm cues after copper exposures using concentrations of 2, 8 and 32 μg/L and two exposure times (24 and 96 hours). The Delta Smelt can be classified as a macrosmatic fish, based on the morphological features of the olfactory rosette. This fish has multilamellar, paired olfactory rosettes containing a highly specialized olfactory epithelium. The olfactory epithelium was

composed by several populations of cells including sensory neurons with distinct morphology and immunocytochemical features. Delta Smelt have a highly sophisticated and sensitive response to predation related odorants. They detect alarm cues in a concentration dependent fashion using olfaction and display specific behaviors (escape responses and freezing) upon detection that all together are considered as olfactory mediated antipredator behaviors. Finally, I demonstrated using histopathological and immunohistochemical techniques that Delta Smelt olfactory epithelium is highly susceptible to copper exposure at concentrations commonly found in the Bay Delta and considered as sublethal. Moreover, there were differential effects on antipredator behaviors after exposure to copper for 24 and 96 hours. Fish exposed to 8 μg/L of copper for 24 hours showed severe damage to the olfactory epithelium and hyperexcitability when presented to alarm cues. At higher concentrations, the epithelium was severely damaged, the antipredator response was absent and there were signs of histological and behavioral toxicity. The results of these experiments demonstrate that Delta Smelt is a highly olfactory species and establishes that copper contamination can impair olfactory responses at environmentally relevant concentrations in this endangered fish.

Rationale for dissertation research

1. Presentation and justification for dissertation problem

Over the past decade, extensive efforts have been allocated to understand the drivers of population declines in aquatic organisms native to the San Francisco Bay Delta. Among fish, the Delta Smelt, a once abundant and now endangered fish, has been used as an indicator species to assess the overall health of this ecosystem (Moyle et al., 2016). Anthropogenic pollutants have been considered as drivers of Delta Smelt population declines, and the effects of such compounds at several biological organization levels have been widely studied (Connon et al., 2011, Connon et al., 2009, Hasenbein et al., 2016, Hobbs et al., 2017, Segarra et al., 2021, Teh et al., 2020). Among contaminants of concern found in the Bay Delta, copper has been well studied and toxic effects are well known (Grosell, 2011). Recently, copper has been regarded as an info disruptor (compounds that can potentially affect natural chemical communication systems), and its effects in sensory systems of fish are well studied (Lürling, 2012, Gosavi et al., 2020). Little is known about the olfactory system and olfactory mediated behavioral responses of Delta Smelt. Moreover, the concentrations at which copper can impact the olfactory ability of Delta Smelt are unknown. The main objective of this study was to determine the effects of copper exposure in the olfactory system and associated antipredator olfactory mediated behavioral responses in Delta Smelt. The study initiates by interrogating the olfactory ability of Delta Smelt. First, a thorough morphological characterization of fundamental morphological features of the olfactory organ (olfactory rosette) of this fish gave clue on how well developed the olfactory ability of this fish is. Second, a behavioral assay to evaluate the olfactory mediated antipredator behavioral response was developed to assess well conserved and robust behaviors important to fish survival in the environment. Finally, alterations in the morphology of the olfactory organ and antipredator behavioral responses were studied in Delta Smelt exposed to several environmentally relevant copper concentrations and two exposure times, establishing a link between tissue and behavioral responses to copper exposure at concentrations usually found in the

Bay Delta. My work provides basis to the study of olfactory biology and ecology of Delta Smelt and demonstrates that common contaminants at environmental concentrations can disrupt the sensory perception of this fish. These results are useful to establish water quality criteria specifically for this fish species and help in the conservation of habitats critical for species survival.

2. Specific objectives and integration of the dissertation

Chapter 1: Gross morphology, histology, and ultrastructure of the olfactory rosette of a critically endangered indicator species, the Delta Smelt, *Hypomesus transpacificus*

This chapter aims to answer the question: Do Delta Smelt have a well-developed olfactory system? The morphological features of the olfactory rosette of Delta Smelt were characterized using classical histological, immunohistochemical and ultrastructural approaches. It is well known that fish use the olfactory system for several aspects of life history critical for survival in the environment, and morphological features of well-developed olfactory systems in macrosmatic fish have been already investigated in iconic fish species such as salmonids (Hansen and Zielinski, 2005). Gross morphological features of the olfactory rosette were studied in several life stages of Delta Smelt and compared to those of already well studied macrosmatic fish species. The histological features of the olfactory epithelium were thoroughly described, and protocols for immunohistochemical identification of neuronal subpopulations were standardized. Moreover, ultrastructural features of the olfactory epithelium are discussed. Finally, the results are discussed in the context of structure regarding olfactory ability in Delta Smelt.

Chapter 2: The olfactory mediated alarm response of Delta Smelt (*Hypomesus transpacificus*) to different concentrations of skin extracts

In this chapter, I aimed to answer the questions: Do Delta Smelt respond to alarm cues derived from skin extracts with specific antipredator behaviors? And if so, at which concentrations of alarm cues there is a

recognizable antipredator response? The olfactory mediated antipredator response to alarm cues was characterized in Delta Smelt. Using a standardized behavioral assay and video tracking I described the characteristic behaviors displayed by Delta Smelt when exposed to skin extract (alarm cues). I developed a methodology for preparation and delivery of skin extracts at different concentrations in behavioral arenas and quantified behavioral responses using video recordings and tracking computer software. The alarm response of Delta Smelt is described in detail and implications for survival and managing of captive stocks are discussed. This study provides novel insight into the behavioral ecology of Delta Smelt and offers possibilities to develop pre-release protocols using groups of predator-trained Delta Smelt. This is the first study documenting the antipredator response of this species to different concentrations of skin extract.

Chapter 3: The effects of copper on the olfactory system of the endangered indicator species Delta Smelt (*Hypomesus transpacificus*): From tissues to behavioral responses.

Building on the studies in the first two chapters, I aimed to evaluate the effects of a common pollutant in the waters of the San Francisco Bay Delta, on the olfactory structure and function of Delta Smelt. I used copper, a well-known olfactory toxicant however understudied in the context of Delta Smelt toxicology, ecology, and behavior. The results obtained in this chapter could answer the following questions: What are the effects of copper exposure on the olfactory tissue and antipredator behavioral responses of Delta Smelt? At which concentration, copper impairs olfactory ability in Delta Smelt? and, is there any difference between short- and long-term exposures to copper in the olfactory tissue and behavioral response? To answer these questions, I tested Delta Smelt exposed to several environmentally relevant concentrations of copper for 24 and 96 hours. The histopathological changes of the olfactory epithelium were evaluated using histological methods and immunohistochemistry. I characterized the tissue responses in the olfactory epithelium by developing a semi-quantitative histopathology score and immunohistochemical staining of neurons. To link morphological changes to behavioral changes, I characterized the response to

alarm cues in these groups of fish after copper exposure and compared the response among concentrations and exposure times. The results of these studies determined that Delta Smelt are highly susceptible to copper exposure, and their olfactory system is affected even at concentrations considered sublethal, highlighting the need of linking responses at different organismal levels to understand the toxic effects of copper in Delta Smelt.

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Chapter 1

Gross morphology, histology, and ultrastructure of the olfactory rosette of a critically endangered indicator species, the Delta Smelt, *Hypomesus transpacificus*

Abstract

The Delta Smelt (*Hypomesus transpacificus*) is a small, semi-anadromous fish native to the San Francisco Bay-Delta Estuary and has been declared as critically endangered. Their olfactory biology is poorly understood, and a basic description of their sensory anatomy is needed to advance our understanding of the sensory ecology of species to inform conservation efforts that manage and protect them. The aim of this study was to describe some of the essential morphological features of the olfactory organ of Delta Smelt. I provide a description of the gross morphology, histological, immunohistochemical, and ultrastructural features of the olfactory rosette in this fish and discuss some of the functional implications in relation to olfactory abilities. I show that Delta Smelt have a multilamellar olfactory rosette with allometric growth. Calretinin immunohistochemistry revealed a diffuse distribution of olfactory receptor neurons within the epithelium. Clear distinction among ciliated, microvillous and crypt neurons were achieved with morphological and immunohistochemical features. The olfactory neurons were supported by robust ciliated and secretory sustentacular cells. Although the sense of smell has been unexplored in Delta Smelt, I conclude that their olfactory epithelium has many characteristics of macrosmatic fish. With this study, I provide a foundation for future research into the sensory ecology, and the potential impact of a compromised system, in this imperiled fish.

1. **Introduction**

Comparative anatomy of fish olfactory systems has been established as a field of study since more than a century ago when one of the most thorough reviews was published by R.H Burne (Burne, 1909). The paper was the first to highlight the astonishing diversity of fish olfactory systems. Since Burne, the field has grown rapidly to elucidate the complexity and diversity of fish olfaction, and how basic design features are evolutionarily well conserved among fish and other vertebrates (Kasumyan, 2004, Hansen and Zielinski, 2005). This comparative approach led researchers to conclude that structural morphology can

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predict ecological adaptations (Schluessel et al., 2008, Kasumyan, 2004). For example, a fish species could be classified as microsmatic (highly olfactory species) or microsmatic (poorly olfactory species), depending on the morphological characteristics of their olfactory systems (Atta, 2013).

In general, the fish olfactory system is composed of a pair of peripheral multilamellar sensory organs called the olfactory rosettes. The axons of sensory neurons in the rosette make up the olfactory nerve, and form synapses in the olfactory bulbs. In many teleost species that have been studied (Zielinski and Hara, 2006, Satou, 1990), each olfactory bulb is composed of four distinctive concentric layers referred to, from periphery to center, as the olfactory nerve layer, the glomerular layer, the mitral cell layer and the granular cell layer. The olfactory nerve fascicles form the olfactory nerve layer and connect the peripheral sensory neurons to primary output neurons (*i.e.,* the mitral cells and the ruffed cells) in the olfactory bulb. The synapsis between the olfactory neurons and the primary output neurons in the bulb form spherical structures composed of neuropil called glomeruli which together form the glomerular layer. The mitral and granular cell layers are formed by mitral cells which are primary output neurons, and granule cells, which are interneurons that synapse mainly with mitral cells (Zielinski and Hara, 2006). The olfactory bulbs connect to higher brain centers via the olfactory tracts (Hamdani and Døving, 2007, Hara, 2011).

The olfactory rosette is made up of multiple leaf-like structures that contain a variety of functionally specific cell populations. The olfactory epithelium in a macrosmatic fish is composed of millions of olfactory neurons (Kreutzberg and Gross, 1977, Easton, 1971), embedded between sustentacular cells and ciliate cells that support them. A wide diversity of olfactory neurons has been described in fishes (Hansen and Zielinski, 2005), including ciliate neurons and microvillous neurons. Recently other neuronal cell types have been described, including the crypt neurons, the Kappe neurons (Ahuja et al., 2014, Hansen and Finger, 2000) and the pear-shaped neurons (Wakisaka et al., 2017). Different experimental approaches have been developed to characterize the morphology and function of these sensory neurons, ranging from fundamental histological (Ferrando et al., 2016), ultrastructural (Bannister, 1965), and

immunohistochemical methods (Ferrando et al., 2009) to whole transcriptome sequencing (Fatsini et al., 2016).

Functionally, the olfactory neurons have specific olfactory receptors that are tuned to detect specific types of odorant ligands (Bazaes et al., 2013). The ciliated olfactory neurons are characterized by the appearance of a long dendrite with an olfactory knob crowned by several sensory cilia (Bannister, 1965). These cells express olfactory receptors coupled to GTP-binding proteins. The $G_{\alpha\,olf/s}$ is a subunit of the GTP-binding protein, and is expressed only in ciliated neurons (Hansen et al., 2004). The microvillous neurons are shorter in appearance. These olfactory neurons are characterized by having multiple microvilli in the apical domain, hence the name microvillous neurons. They mainly express V1R and V2R receptors and are tuned to detect nucleotides, amino acids (Hansen et al., 2003) and sex pheromones (Zippel et al., 1996). Microvillous neurons express the $G_{\alpha O}$, $G_{\alpha q/11}$ or $G_{\alpha i3}$ subunits of the GTP-binding protein-coupled receptors and can be identified immunocytochemically (Hansen et al., 2004). Crypt cells are a less studied cell type. In zebrafish (*Danio rerio*), this type of neuron is characterized by the expression of ORA4 receptors (Oka et al., 2011). Crypt cells are morphologically characterized by their round shape and an invagination with microvilli and cilia at the apical domain. They are also supported by specialized sustentacular cells characterized by a clear electron-lucent cytoplasm and prominent mitochondria (Hansen and Finger, 2000). Pear-shaped neurons express mainly olfactory marker protein (OMP) and differentially detect ATP and related molecules with an adenosine moiety (Wakisaka et al., 2017). Kappe neurons, are characterized by the expression of G_O receptors and are similar in shape to crypt neurons, except for the characteristic "cap" structure by which they are named. The odorant specificity of Kappe neurons is unknown (Ahuja et al., 2014, Klimenkov et al., 2020a).

In most fish species, the olfactory system is crucial to a variety of behaviors. These include foraging, predator/prey interactions (Hamdani and Døving, 2007, Tierney et al., 2007), shoaling (Kasumyan, 2004, Partridge and Pitcher, 1980), homing (Ueda, 2019) and reproductive behaviors (Sorensen and Baker,

2014). To date, salmonids (Bertmar, 1973, Moran et al., 1992), lampreys (VanDenbossche et al., 1995) and cyprinids (Hansen and Zielinski, 2005, Pashchenko and Kasumyan, 2017) have been the primary fish models used to investigate cellular-level olfactory morphology. Only recently have these methods been applied to non-model species of conservation concern, including elasmobranchs (Ferrando et al., 2007, Ferrando et al., 2016).

The Delta Smelt, *Hypomesus transpacificus* (Osmeriformes, Osmeridae) is endemic to the Sacramento-San Joaquin Delta and the upper San Francisco Estuary. Since the mid-1980s, their populations have been declining rapidly and dramatically (CDFW, 2021, CDFW, 2020) such that they are now listed as critically endangered by the International Union for Conservation of Nature (IUCN) (NatureServe, 2014). Several hypotheses have been proposed to explain this decline and to find ways to recover the population (Sommer et al., 2007). The decision to list Delta Smelt as endangered has led to a plethora of studies geared towards better understanding their biology (Moyle et al., 2016, Brown et al., 2013). However, the olfactory system has been largely ignored even though climate change and anthropogenic alteration of habitats are known to disrupt the sensory behaviors of fish (Tierney et al., 2010, Lürling, 2012), including Delta Smelt (Davis et al., 2019). A basic description of the morphology of the olfactory organs of Delta Smelt is needed as a foundation for more applied studies addressing the potential that anthropogenic influences disrupt olfactory function (Tierney et al., 2010) and may contribute to declines in population health. Therefore, our aim in this work was to provide a comprehensive anatomical description of the olfactory organ of Delta Smelt, using a combination of histological, ultrastructural, and immunohistochemical approaches.

2. Materials and Methods

2.1 Animals

Delta Smelt (*Hypomesus transpacificus*) were obtained from the Fish Conservation and Culture Laboratory (FCCL) in Byron, CA, USA. The original broodstock (F0) came from the San Francisco Estuary and had been bred in captivity with annual incorporation of small amounts of wild fish for F12 generations using established breeding, genetic and rearing methods for the species (Lindberg et al., 2013, Fisch et al., 2012). All the fish welfare and experimental protocols were approved by the University of California Davis Institutional Animal Care and Use Committee [protocol no. 20052].

2.2 Gross Morphology

I used 37 (20 males and 17 females) sub-adult and adult Delta Smelt between 263 to 357 days post hatch (DPH; **See Table 1**). The fish were euthanized following the American Veterinary Medical Association (AVMA) guidelines for the euthanasia of animals (Leary et al., 2013). Briefly, each individual was placed in ice water for 10 minutes until no opercular movement nor peduncular reflex were detected. The spinal cord was then cut directly behind the head. After euthanasia, each fish was measured, weighed, and fixed in 10% neutrally buffered formalin for 48 hours.

After 48 hours of fixation, the olfactory rosettes were dissected at 7X to 70X magnification using an Olympus SZH10 (Olympus Corporation, Japan) research stereomicroscope as follows. I introduced 2 µl of Mayer's Hematoxylin in each nasal cavity to highlight the olfactory rosette. Then, the fixed fish was placed in a plastic tray containing phosphate buffered saline (PBS), and micro-scissors were used to remove the skin and surrounding tissue covering the nasal cavity to fully expose the rosette. The number of lamellae were recorded, and the diameter of each lamellae was measured using AmScope software coupled to an AmScope MU1400 camera (AmScope, Irvine, CA).

2.3 Histology and immunohistochemistry

I used 30 (19 males and 11 females) sub-adult Delta Smelt (mean ± SD, fork length (the length of the fish measured from the tip of the lower mandible to the center of the fork of the tail) of 6.96±0.5 cm and weight of 2.57±0.7 grams). The fish were euthanized at 240 DPH, fixed, and dissected as described above in the 'Gross Morphology' section. Each whole rosette was removed from the nasal cavity, placed in an individual tissue cassette, dehydrated in ascending concentrations of alcohols, cleared in xylene, and embedded in paraffin (TissuePrep 2, Fisher Scientific). The tissue blocks were sectioned at 3 µm thickness using a rotatory microtome. Sections were wet mounted on glass slides and stained with Hematoxylin and Eosin (H&E). For immunohistochemical analysis, the following primary antibodies were used (See Table 4 for details): 1) Mouse monoclonal anti $G_{\alpha S/olf}$ (C-10, 1:500 dilution sc:377435 Santa Cruz Biotechnology) against the G protein alpha olf subunit to specifically identify ciliated olfactory neurons, 2) Rabbit polyclonal anti S-100 (1:250 dilution, RB-044-A0 Thermo scientific) to identify ciliated olfactory neurons, crypt neurons and microvillous neurons and 3) Rabbit polyclonal anti Calretinin (1:2000 dilution, AB5054 EMD Millipore) to detect the sensory area within the rosette. Additionally, I used anti TRPC2, $G_{\alpha O}$, $G_{\alpha i-3}$ and $G_{\alpha q-11}$ antibodies (See Table 4) to further identify crypt cells and microvillous neurons; however, results were inconclusive and are therefore not reported.

All of these markers have been previously used to identify these cell types in fishes and have shown similar staining patterns across several model species, including zebrafish (*Danio rerio*), Goldfish (*Carassius auratus*) and catfish (*Ictalurus punctatus*) (Lazzari et al., 2017, Hansen et al., 2004, Hansen et al., 2003). The primary antibodies were detected with ImmPRESS (Peroxidase) Polymer Reagent Horse-Anti-Mouse IgG MP-7402 (Vector Labs) for monoclonal antibodies and ImmPRESS (Peroxidase) Polymer Reagent Horse-Anti-Mouse/Rabbit IgG MP-7500 (Vector Labs) for polyclonal antibodies. The omission of primary antibodies was used as a negative control. The tissue sections were cleared in Xylene substitute (Histoclear), followed by rehydration in descending concentrations of alcohol and a final rinse in deionized

water. The endogenous peroxidase was quenched with 1% hydrogen peroxide in PBS buffer for 20 minutes, followed by heat antigen retrieval in citrate buffer (Ph 6.1) (Target retrieval solution, S1 699, Dako) for 30 minutes at 92°C using a commercial vegetable steamer. The nonspecific binding was blocked with 10% normal horse serum (Vector Laboratories, Burlingame, CA, USA), plus 1% bovine serum albumin (Sigma) and 0.1% Tween^{TM} 20 (Fisher BioReagents) for 1 hour. The sections were incubated with the primary antibodies for 3 hours, and then with the secondary antibody for 30 minutes in a humidified chamber at room temperature. The antigen antibody reaction was revealed with vector Nova-Red peroxidase substrate kit (Vector laboratories, SK-4800) and counterstained with Mayer's hematoxylin. Images of representative olfactory rosette structures were taken with an Olympus BX60 microscope coupled to a DP71 camera. The pictures were then adjusted for brightness and contrast with the CellSenses software (V 1.8.1 Olympus Corporation of the Americas, Center Valley, PA). Image post processing (sizing, intensity, and labeling) was made with Adobe Photoshop (V 20.0.3).

2.4 Ultrastructural morphology

Sub-adult Delta Smelt (4 females and 1 male at 240 DPH) were used for ultrastructural characterization of the olfactory rosette. From each pair of rosettes, one was sampled and prepared for scanning electron microscopy (SEM) and the other for transmission electron microscopy (TEM).

2.5 Scanning electron microscopy (SEM)

Whole olfactory rosettes were fixed in 50% strength Karnovsky's solution (Fournie et al., 2000) for 48 hours and dehydrated in ascending concentrations of ethanol. Then, the rosettes where critically point dried with CO_2, mounted in aluminum stubs and double sticky carbon discs and received 3 cycles of gold sputtering. The tissues were examined with a Philips XL 30 scanning electron microscope at 20 Kev accelerating voltage. All the procedures were performed in the Biological Electron Microscopy Facility at the University of California, Davis. (https://bioem.ucdavis.edu/)

2.6 Transmission electron microscopy (TEM)

Whole olfactory rosettes were fixed in the same way as for SEM, washed in sodium Cacodylate buffer and post fixed in 1% osmium tetroxide. After osmium fixation (osmification), the tissue was rinsed in 0.1M sodium cacodylate, dehydrated through a graded ethanol series, transitioned through propylene oxide and infiltrated and embedded in Eponate-12 epoxy formulation (Eponate-12; Ted Pella Inc., Redding, CA). Thick sections were cut, mounted on glass slides, stained with toluidine blue O (Millipore Sigma., Darmstadt, Germany), and examined by light microscopy. Thin sections were mounted on 300-mesh copper grids, stained with 4% uranyl acetate in 75% ethanol and post stained in lead citrate. The grids were examined with a FEI Talos L120C Transmission Electron Microscope at 80 Kev accelerating voltage (Thermo Fisher Scientific, Hillsboro, OR).

2.7 Statistical analysis

The relationships between fork length and discrete variables (age and number of lamellae) were analyzed using Spearman rank correlations. Also, I analyzed the relationship between fork length and rosette diameter using a Pearson's product-moment correlation with a Bonferroni correction for multiple comparisons. Results were considered significant at P <0.005. All the analyses and graphs were done with JMP software V 15 (SAS institute, Cary, NC).

3. Results

The morphological features of the olfactory rosette of the Delta Smelt resemble what has been observed in other Teleosts that have well developed olfactory systems and behaviors (Hansen and Zielinski, 2005, Kasumyan, 2004).

3.1 Gross morphology and ultrastructure of the Delta Smelt olfactory rosette

External examination revealed that Delta Smelt have a round nasal cavity immediately in front of each eye (**Figure 1A**). Paired nasal cavities are each covered by a boat sail shaped skin flap that forms anterior and

posterior nasal openings for water circulation (**Figure 1C**). As in other fish species, the olfactory rosette in

Delta Smelt is a round, multi-lamellar sensory structure that sits in the nasal cavity (**Figure 1B and D**). The

rosette structure is supported and protected by a delicate, fibrous capsule that attaches the lamellar folds

to the base and sides of the nasal bones (**Figure 1D).**

The lamellae radiate and extend towards the periphery of the nasal cavity, and increase in size, rostral to

caudally **(Figure 1D).** A central connective tissue raphe **(Figure 1D)** provides scaffolding and nutrition to

the lamellar epithelium through an abundant network of capillary vessels (see "**Fine structure of the Delta**

Smelt olfactory epithelium"). Within the connective tissue meshwork, the axons of the olfactory neurons

coalesce and form nerve bundles which make up the olfactory nerve **(Figure 1B)**.

I found that the number of lamellae and diameter of the rosette varied with age and fork length (**Table 2;**

Figure 2). In general, smaller, and younger fish had smaller rosettes and fewer lamellae than larger and

older fish. We also found that fork length increased with age in females (Spearman's rank correlation,

P=0.7335, P=0.0008), but not males (Spearman's rank correlation, P=0.2398, P=0.3085) (**Figure 2A-C**).

However, in both sexes, the average diameter of the rosette and the number of lamellae increased with

fork length (Spearman's rank correlation, number of lamellae vs fork length, females: P=0.7969, P=0.0001,

males: P=0.7070, P=0.0005; Pearson's product-moment correlation, average rosette diameter vs fork

length, females: r=0.8264, P<0.0001; males: r=0.8333, P<0.0001) (**Figure 2D-I**), suggesting that the overall

size of the peripheral olfactory system scales with size rather than age.

Under scanning electron microscopy (SEM), I found the olfactory lamellae to lack secondary folds or

sensory islands that have been reported in other fish species (**Figure 3A**) (Theisen et al., 1991,

Thommesen, 1983). Instead, each lamella was completely covered by a thick mat of cilia on each side

(**Figure 3B**). Under SEM, the ciliated olfactory neurons were identified by the presence of olfactory knobs

(**Figure 3C**). These structures were found to protrude, forming a raised membrane cap characteristic of

ciliated olfactory neurons (**Figure 3D).** I was not able to conclusively identify microvillar neurons by SEM due to the thick mat of cilia covering the lamellar surface.

3.2 Fine structure of the Delta Smelt olfactory epithelium

I next examined the fine structure of the olfactory epithelium using light and transmission electron microscopy (TEM). I found that the whole surface of the olfactory lamellae of Delta Smelt was composed of a sensory epithelium supported by a delicate fibrovascular stroma, with abundant nerve bundles and blood vessels (**Figure 4 and 5**). The epithelium rested in a delicate thin basal lamina that separated it from connective tissue in the central raphe (**Figure 6A**). The olfactory epithelium itself was a pseudo-stratified columnar layer of 43.86±10.67 (mean ±SD) μm thick composed of bipolar neurons, sustentacular, basal (**Figure 4C and D**) and goblet cells (**Figure 8B**). The epithelial surface was mostly composed of motile cilia from sustentacular cells, which covered the sensory cilia and microvilli from ciliated and microvillous sensory neurons (**Figure 6B and C**). A prominent apical basophilic band consisting of the rootlets and basal bodies of cilia from ciliated sustentacular cells typically spanned the entire epithelial surface (**Figure 4D and Figure 6C).**

3.3 Neuronal diversity of the olfactory epithelium of the Delta Smelt

Using a variety of immunohistochemical markers (**Table 3**), I was able to identify ciliate neurons, microvillous neurons and crypt cells. These cell types appeared to be distributed in a heterogenous population throughout the epithelium. The various cell types are described below.

Ciliate Neurons: The cell bodies of ciliate neurons were elongated and irregular and were usually found in the mid and lower depths of the epithelium near the basal membrane. Ciliate neurons were characterized by a round to oval nucleus that occupied most of the neuronal soma, and by a long and slender dendrite extending to the epithelial surface (**Figure 7**). Ultra-structurally, I was also able to identify ciliate neurons by their characteristic olfactory knob, and five to six sensory cilia 2 to 3 μm in length projecting from it

(**Figure 7D).** The cell bodies and dendrites had abundant tubular mitochondria (**Figure 7D**). Their nuclei were typically round to oval and were observed in the medial to basal aspect of the sensory epithelium. The nuclei contained abundant electron lucent agranular euchromatin. I also observed small clumps of electron dense, granular heterochromatin in the nuclear periphery (**Figure 7C**).

Delta Smelt ciliate neurons were positive for $G_{\alpha s/olf}$, Calretinin and S-100 (**Figures 5, 7 and 9**). The $G_{\alpha s/olf}$ expression was confined to ciliate olfactory neurons and showed a coarse granular cytoplasmic and dendritic staining pattern. The olfactory knobs and cilia were densely stained for $G_{\alpha s/olf}$ and formed a rim on the surface of the epithelium **(Figure 7B).** Calretinin **(Figure 5)** and S-100 **(Figure 9)** were distributed within the nucleus and the cytoplasm, all along the dendritic process to the olfactory knob and olfactory cilia on the epithelial surface. The nerve bundles formed by the axons of olfactory neurons were also heavily reactive to calretinin (Figure 5, B), and $G_{\alpha s/olf}$ (Figure 7, A and B).

Microvillous Neurons: Microvillous neurons were characterized by a short pear-shaped soma, a short dendrite, and a round, superficial nucleus (**Figure 8A**). These neurons were easily distinguished from other types of neurons by a dendritic process that was short and thin (**Figure 8B**). and the presence of microvilli on the apical domain. The microvilli appeared to be thinner than sensory cilia in ciliate neurons (**Figure 8 D**). Few mitochondria were observed in microvillous cells. The cytoplasm was typically electron dense, containing abundant rough endoplasmic reticulum, well-developed Golgi complexes and vesicles. The nuclei tended to be round, euchromatic and lack significant peripheral heterochromatin (**Figure 8C**). The S-100 strongly stained nuclei, cytoplasm, and microvilli (**Figure 8A and B**), however, immunostaining for calretinin was weaker and more variable in microvillous neurons. In addition, the nuclei tended to be stained more weakly, with clearer cytoplasm.

Crypt Neurons: Crypt neurons are ovoid neurons that were found to occur in clusters of three to four cells surrounded by well-developed sustentacular cells (**Figure 9 and 10**). The apical domain of crypt neurons

had the characteristic membrane invagination from which both cilia and microvilli projected to the surface (**Figure 10C**). Nuclei of crypt neurons tended to be round to ovoid with occasional indentations, well developed euchromatin and scant peripheral heterochromatin. Small round mitochondria were observed in the apical portion of crypt cells close to the cilia. The cytoplasm was mostly electron dense and granular, with occasional electron lucent vesicles. The sustentacular cells supporting the crypt neurons tended to be rich in tubular elongated mitochondria and smooth endoplasmic reticulum, which gave their cytoplasm a clear electron-lucent appearance (**Figure 10B**). Immunohistochemically, crypt cells were easily identified by this distinctive shape and the expression of calretinin and S-100 in the cytoplasm and nuclei (**Figure 9B**). Crypt neurons stained weakly for calretinin, although the staining was denser in the nucleus than in the cytoplasm. The apical domain also occasionally stained, highlighting small microvilli.

Individual axons of sensory neurons projecting to the basal domain of the epithelium strongly stained for all the markers. They could be visualized projecting to the basal layer and forming nerve bundles (*fila olfactoria*) in the lamina propria (**Figure 5B and 7B**).

3.4 Non-neuronal cell populations in the olfactory epithelium of the Delta Smelt

Sustentacular cells: Sustentacular cells surrounded olfactory neurons and formed cytoplasmic-membrane folds resembling cytoplasmic invaginations (**Figure 10C**). I observed both ciliated and non-ciliated sustentacular cells. These cell types were characterized by an elongated cytoplasm and a sausage-shaped nucleus located primarily in the basal domain of the epithelium. Occasionally nuclei were also observed in a more intermediate position (**Figure 8A**). The nuclear chromatin was active with abundant euchromatin which gave the nucleus an electron-lucent appearance. The ciliated non-sensory sustentacular cells were characterized by a prominent ciliated apical domain with a ciliary apparatus. The cilia were observed to be anchored to the cell by prominent basal bodies that formed the rootlets of the cilia. The apical portion of these cells contained abundant mitochondria and small, electron dense

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granules (presumably glycogen) interspersed close to the mitochondria. The cilia were observed to have a 9+2 microtubular array. Measured from the epithelial surface, motile cilia had an average length of 11.56±2.69 (mean ±SD) µm and were abundant on the surface of the epithelium (Fig 6, B). The non-ciliated sustentacular cells contained prominent electron dense secretory granules in the apical domain, suggesting they functioned as secretory cells. The secretory granules were round to oval, with an average diameter of 0.57±0.09 (mean ± SD) µm. These granules had a thin membrane that enveloped the granular contents. As **Figure 6C** illustrates, the granular contents were occasionally arranged in stripes of electron dense material that gave the granule a lamellated/striped appearance. The ciliated surface intermingled with abundant electron dense filamentous material that formed strands that attached to the ciliary surface (**Figure 6B**). I also observed, several clusters of crypt neurons surrounded by prominent sustentacular cells visible in some areas of the olfactory lamellae, mainly in the folds in the junctions between contiguous lamellae. These sustentacular cells were characterized by an abundant number of mitochondria and smooth endoplasmic reticulum. The cytoplasm of sustentacular cells was electron lucent when compared with that of sensory neurons. This was mainly due to the abundance of Golgi cisternae and smooth endoplasmic reticulum. These sustentacular cells had small microvilli in the apical domain but did not have cilia (**Figure 9 and 10**).

In general, the nuclei and cytoplasm of sustentacular cells were not labeled by any of the markers used to identify sensory neurons (S-100, Calretinin, $G_{\alpha S/olf}$); nor were the cilia on the surface of the epithelium labeled (**Figures 8A and 9B**).

Basal cells: The epithelial basal layer consisted of two identifiable cell types. One type of basal cell could be distinguished as having a round, globoid shape with a round, highly basophilic nucleus, whereas the second type had an elongated shape with ovoid nucleus parallel to the basal lamina (**Figure 4D**). Occasionally I observed cells that had elongated and intensely basophilic nuclei, closer to the epithelial surface in the middle and apical portions of the epithelium. Presumably, these were basal cells in the

process of developing into mature receptor cells, however the basal cells were not positive for any of the markers used to identify sensory neurons.

3.5 Other cellular populations

Normally, a few mucous-secreting goblet cells were scattered within the olfactory epithelium (**Figure 8 B**), and they were more abundant and prominent on the surface of the capsule in which the rosette sits. The capsule epithelium showed eosinophilic bright refringent granules interspersed within the epithelial cells on the surface (**Figure 11A**). Most of the resident immune cells were found within the capsular connective tissue, and were characterized by round, highly basophilic central nuclei and small amounts of cytoplasm. Occasionally, rodlet cells were also seen in the capsular epithelium. These cells were characterized by having an ovoid, elongated, brightly eosinophilic cytoplasm containing several birefringent rod structures (**Figure 11B**).

Finally, in the olfactory epithelium, I also observed cells with a different apical modification consisting of a thick knob with a single prominent rod like structure protruding to the surface. I did not observe ciliary structures, microtubules, or centromeres on this cell type. This rod structure resembled a dendrite that was thicker than a normal cilium (**Figure 6, D**).

4. Discussion

My morphological examination suggests that the Delta Smelt has a well-developed peripheral olfactory system, that is in many ways similar to other highly olfactory fish species that have previously been described (Hansen et al., 2004, Hansen et al., 2003, Hansen and Zielinski, 2005). The overall implication is that these fish have a functional and potentially keen sense of smell that needs to be further explored. The Delta Smelt have a pair of well-developed olfactory rosettes, typical of the morphology of macrosmatic fish (Kasumyan, 2004). Within each rosette, lamellae are arranged in a radial array, with lamellae increasing in size caudally. The number of lamellae and lamellar arrangement are similar to other

fish species, including zebrafish (*Danio rerio*) (Hansen and Eckart, 1998), Goldfish (*Carassius auratus*) (Hansen et al., 2004) and Chum Salmon (*Oncorhynchus keta*) (Kudo et al., 2009). As with other species (Kudo et al., 2009, Pashchenko and Kasumyan, 2017) , I found that additional lamellae were added as fish grew, suggesting that olfactory function is adaptive from juvenile life stages into maturity (Pashchenko and Kasumyan, 2017, Kudo et al., 2009, Schluessel et al., 2008, Hara and Zielinski, 1989).

As in other fishes, most of the olfactory neurons are in the lateral parts of lamellae and embedded within a mat of cilia that are thought to aid in the sampling and movement of odorant within the sensory surfaces of the olfactory rosette (Pashchenko and Kasumyan, 2017). Ciliated sustentacular cells, in particular, have been shown to be involved in driving microcurrents over the olfactory lamellae of other fish species (Reiten et al., 2017, Cox, 2013, Cox, 2008). Together with the nasal flap, ciliary movement is presumed to generate unidirectional water currents through the nares. In my gross anatomical study, I did not find evidence of accessory sacs that might help in the movement of water into the nares, suggesting that ventilation occurs by swimming activity or opercular movement linked to gill ventilation. While accessory sacs have been described in some fish, they are more commonly present in bottom dwelling or less active fish and are thought to be an adaptation to draw water into the nasal cavity in the absence of a current (Nevitt, 1991, Burne, 1909, Døving et al., 1977).

I found that Delta Smelt are unusual in that both sides of the olfactory lamellae are completely covered with sensory epithelium interspersed with non-sensory epithelium. This is not the case for goldfish (*Carassius auratus*, (Hansen et al., 2004), zebrafish (*Danio rerio*, (Hansen and Eckart, 1998), salmonids (Thommesen, 1983), guppies (*Poecillia reticulata*) (Lazzari et al., 2007) or catfish (*Ictalurus punctatus*) (Caprio and Raderman-Little, 1978, Theisen et al., 1991), which have well differentiated regions or 'islands' of sensory and non-sensory epithelium in the olfactory lamellae. This may reflect fewer infoldings in the lamellae of Delta Smelt. However, the neuronal populations in the olfactory epithelium were similar to those of other teleosts and showed similar morphological and immunohistochemical features as well

(Hansen and Zielinski, 2005). Ciliate neurons from Delta Smelt had a long dendrite and ciliated olfactory knob, and both the number and length of cilia were similar to those reported in zebrafish (*Danio rerio*) (Hansen and Eckart, 1998), Brown trout (Salmo trutta) (Moran et al., 1992) and goldfish (*Carassius auratus*). The open and agranular euchromatin in these neurons reflected their high transcriptional activity (Kierszenbaum and Tres, 2019). Interestingly, in Delta Smelt ciliate neurons, I observed $G_{\alpha s/olf}$ immunoreactivity in the cytoplasm, dendrites and apical structures as well as in the axons originating from the *fila olfactoria* in the *lamina propria*. This distribution of the staining is not the same in other fish species that have been examined. For example in goldfish (*Carassius auratus*), $G_{\alpha s/olf}$ was limited to the ciliary surface of the olfactory epithelium, the cell membrane and the axons in the *fila olfactoria* (Hansen et al., 2004). In contrast, in catsharks (*Scyliorhinus canicula)* and sharks in general*,* the $G_{\alpha s/olf}$ immunoreactivity was absent due to the lack of ciliated neurons (Ferrando et al., 2009). I could also attribute the different staining patterns in ciliate olfactory neurons of Delta Smelt to the cross reactivity of the antibody with perhaps a different protein, or that we used formalin fixed paraffin embedded sections, which could change antigenic properties of the target protein.

In addition to ciliated neurons, I identified a variety of other cell types indicative of a functional sense of smell. These included microvillous neurons and crypt neurons. Microvillous neurons were similar to those described in other fish species, in that their microvilli are shorter than cilia and lack microtubules (Zippel et al., 1996, Hansen and Zielinski, 2005). In goldfish (*Carassius auratus*), it has been suggested that microvillous cells play a role in pheromone (Zippel et al., 1996) and amino acid detection (Speca et al., 1999), while ciliated neurons are involved in the recognition of food odors, amino acids (Zippel et al., 1996) and nucleotides (Hansen et al., 2003). Additionally, in male zebrafish (*Danio rerio*), ciliate neurons have been shown to detect prostaglandin $F_{2\alpha}$ involved in mating behavior (Yabuki et al., 2016). Whether these properties are similar in Delta Smelt is unknown and warrants further investigation.

Interestingly, I also observed populations of crypt neurons surrounded by sustentacular cells. Crypt neurons have frequently been described in zebrafish *(Danio rerio)* (Hansen and Eckart, 1998) and other fish species (Hansen and Finger, 2000). Using calcium imaging and confocal microscopy, crypt neurons have been demonstrated to respond to gonadal extracts and hormones in rainbow trout (*Oncorhynchus mykiss*) and to kin odors in zebrafish (*Danio rerio*), suggesting they are involved in mediating reproductive signaling and kin recognition (Hansen and Finger, 2000, Biechl et al., 2016, Bazaes and Schmachtenberg, 2012, Hamdani el and Doving, 2006).

Whereas in model research fish species, S-100 immunoreactivity tends to be exclusively restricted to crypt cells (Lazzari et al., 2017, Germanà et al., 2004). I observed staining in all sensory neuronal subpopulations in the Delta Smelt olfactory epithelium. In addition, the calretinin stain identified sensory neurons that ran continuously along the epithelial surface, implying that they were more widespread in Delta Smelt than has previously been reported in zebrafish (*Danio rerio*) (Bettini et al., 2016) and goldfish (*Carassius auratus*) (Hansen et al., 2004) . Finally, I observed cells with rod shaped apical modifications. This cell type was originally described by Bannister (1965) in the minnow (*Phoxinus phoxinus*) and later in the goldfish (*Carassius auratus*) (Ichikawa and Ueda, 1977) as a cell type that lacks both cilia and microvilli, but instead has a naked single rod that extends from the surface. On the other hand, Moran et al. (1992) suggested that this was a fixation artifact caused by the fusion of cilia into a single structure. Using fluorescence and electron microscopy, these cells have, however, recently been confirmed to be an actin rich cell type in larval zebrafish (*Danio rerio*) (Cheung et al., 2020). Whether or not this type of cell has a sensory function is unknown.

 Other sensory neurons described in the olfactory epithelium of fish are the Kappe neurons(Ahuja et al., 2014) and the pear shaped neurons (Wakisaka et al., 2017) These cell types have similar morphologies and distributions to crypt cells, but express different markers. The kappe neurons are G_O immunoreactive and are positioned apically in the olfactory epithelium (Ahuja et al., 2014). On the other hand, pear-shaped

neurons have been shown to detect adenosine and ATP and are morphologically similar to crypt neurons. I was not able to identify these neurons in Delta Smelt. Immunostaining for G_O was inconclusive in formalin fixed paraffin embedded sections. More targeted studies using, frozen sections, immunofluorescence, and in situ hybridization would likely be needed to definitively confirm whether these cell types occur in Delta Smelt.

In the Delta Smelt olfactory rosette, non-sensory cells were also similar to those described in other fish species. I identified morphologically distinct populations of both ciliated and secretory sustentacular cells. The nuclei of these cells were mostly located basally within the epithelium. This basal position suggests an inverted morphology, as has been demonstrated in zebrafish (*Danio rerio*) (Demirler et al., 2020). The granules in secretory sustentacular cells were ultra-structurally different from mucin vesicles in goblet cells and were more electron dense. It has been suggested that these secretory granules produce the mucopolysaccharides that form the mucinous layer that coats the olfactory epithelium (Zeiske et al., 1992, Hansen and Zielinski, 2005). These cells are thus thought to serve the same lubrication function as the Bowman's gland found in the olfactory epithelium of other vertebrates (*e.g.,* Chinese softshell turtles, *Pelodiscus sinensis*) (Nakamuta et al., 2016, Getchell and Getchell, 1992). In goldfish (*Carassius auratus*) and zebrafish (*Danio rerio*), sustentacular cells have been reported to contain clear electron lucent vesicles in the apical domain (Zippel et al., 1996, Byrd and Brunjes, 1995, Hansen and Eckart, 1998), whereas the granules in sustentacular cells from Delta Smelt were more electron dense, consistent with secretory granules.

I also observed a filamentous layer of electron dense material on the ciliary surface of the lamellae. This material likely originated from the mucin strands that formed the mucous layer on the epithelial surface when the fish was alive. This mucous layer has been described in different fish species, and is thought to play a role in concentrating odorants before they reach the olfactory neurons (Getchell and Getchell, 1992). For example, in fresh water, the amino acids glycine and alanine were more readily soluble in the

mucus phase (partition coefficient less than one than in the aqueous phase of the mucus layer, which could serve to concentrate odorants, prior to detection at the level of the epithelium (Rygg et al., 2013). Interestingly, in the Delta Smelt, I observed that sustentacular cells and olfactory neurons were evenly distributed throughout the lamellar surface. If the layer of mucus is also uniformly distributed over a diffuse population of olfactory neurons, the chance of fish detecting an odorant might be enhanced (Rygg et al., 2013).

I observed a rich network of blood vessels in the olfactory rosette *lamina propria*, which suggests that the olfactory system of Delta Smelt is functionally robust and that an ample blood supply is available to support the increased energetic and metabolic demands of olfactory neurons and sustentacular cells (Klimenkov et al., 2020a). The abundant mitochondria in sustentacular cells and olfactory neurons would require a considerable amount of oxygen to produce energy, and a well-developed capillary network would help in this function. These observations provide further evidence that Delta Smelt have a functional sense of smell rather than a vestigial structure.

Finally, the capsule in which the rosette sits was lined by an epithelium characteristic of a mucosal surface committed to immune surveillance and secretion. The abundance of goblet cells and secretory granules suggest a secretory function, and an abundant population of immune resident cells is a characteristic of fish mucosal surfaces including the sensory and non-sensory epithelium (Gomez et al., 2013). For example, Tacchi et al. (2014) found that rainbow trout (*Oncorhynchus mykiss*) have a diffuse population of lymphoid cells (predominantly B-lymphocytes) that forms the Nasopharynx-Associated Lymphoid Tissue (NALT), in the sensory and non-sensory epithelium, and in the *lamina propria* of the olfactory rosette. Together, the mucosal immune cells and the sensory neurons have been shown to trigger an effective immune response against pathogens (Sepahi et al., 2019, Das and Salinas, 2020). Cells in the capsular epithelium of Delta Smelt resembled lymphocytes in terms of their shape and nuclear morphology. However, specific immunohistochemical studies would be needed to confirm their identity. In other teleosts, rodlet cells

have been observed in various tissues and mucosal surfaces (Reite, 2005), including the olfactory epithelium (Hansen and Zielinski, 2005). The rodlet cells are thought to play a role in parasitic infections, acting mainly by secreting the products in the crystalline core of rods to the extracellular space (Reite and Evensen, 2006). These histological features suggest an immunological function of the sensory and non-sensory epithelium of Delta Smelt, leaving this question open for further study.

5. Conclusions

I demonstrated that the morphological features of the Delta Smelt olfactory rosette suggest that these fish have a well-developed sense of smell. The variety and complexity in cell structure and morphology indicates a diverse function and specialization of olfactory neurons to detect complex blends of odorants, suggesting that Delta Smelt rely on their sense of smell and that this feature of their sensory ecology has been overlooked. My morphologic examination suggests that their olfactory system is robust and should be ecologically important for their survival. We are starting to understand the olfactory biology in this endangered fish. In the Anthropocene, a changing environment has been shown to deleteriously impact fish that rely on olfaction for many aspects of their life history (Tierney et al., 2010, Cattano et al., 2019) Thus, studies on basic morphology will inform and give context to research on the pathological effects of contaminant exposure and broadens our understanding of the olfactory biology of this endangered fish.

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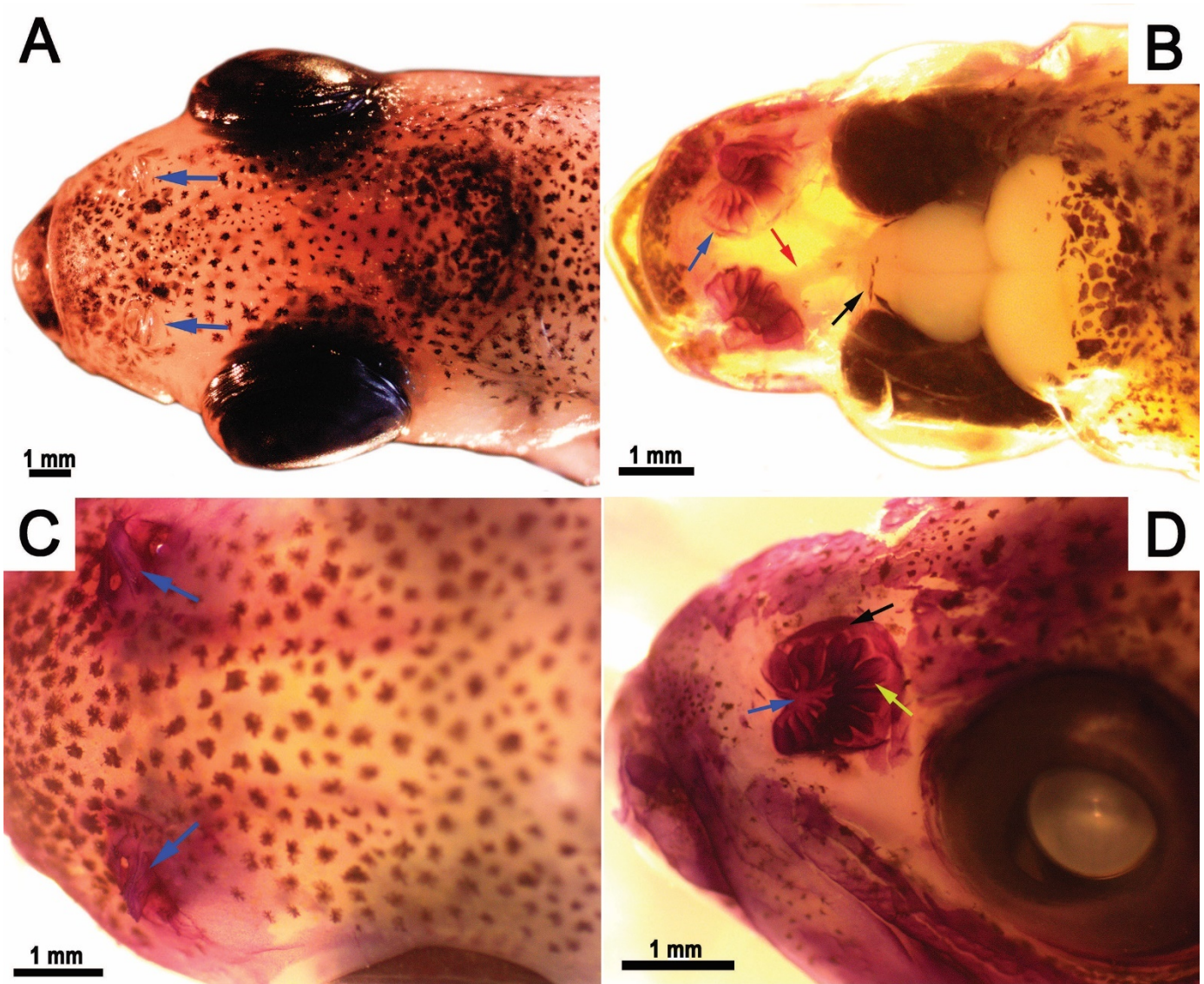
Figure 1. Gross morphology of the Delta Smelt olfactory rosette and main olfactory system structures.

A) Nasal cavities (blue arrows) of a 294 Days Post Hatch (DPH) Delta Smelt. **B)** Dorsal view of the olfactory system in a 294 DPH Delta Smelt. Arrows indicate the olfactory rosette (blue), the olfactory nerves (red) and the olfactory bulbs (black). **C)** This photograph was taken with the fish submerged in PBS to highlight the boat sail shaped flap (Blue arrows) forming of the anterior and posterior nares. **D)** Lateral view of an

olfactory rosette in a 263 DPH Delta Smelt. Arrows indicate the central raphe (blue arrow), the lamellae

(yellow arrow) and the connective tissue capsule (black arrow).
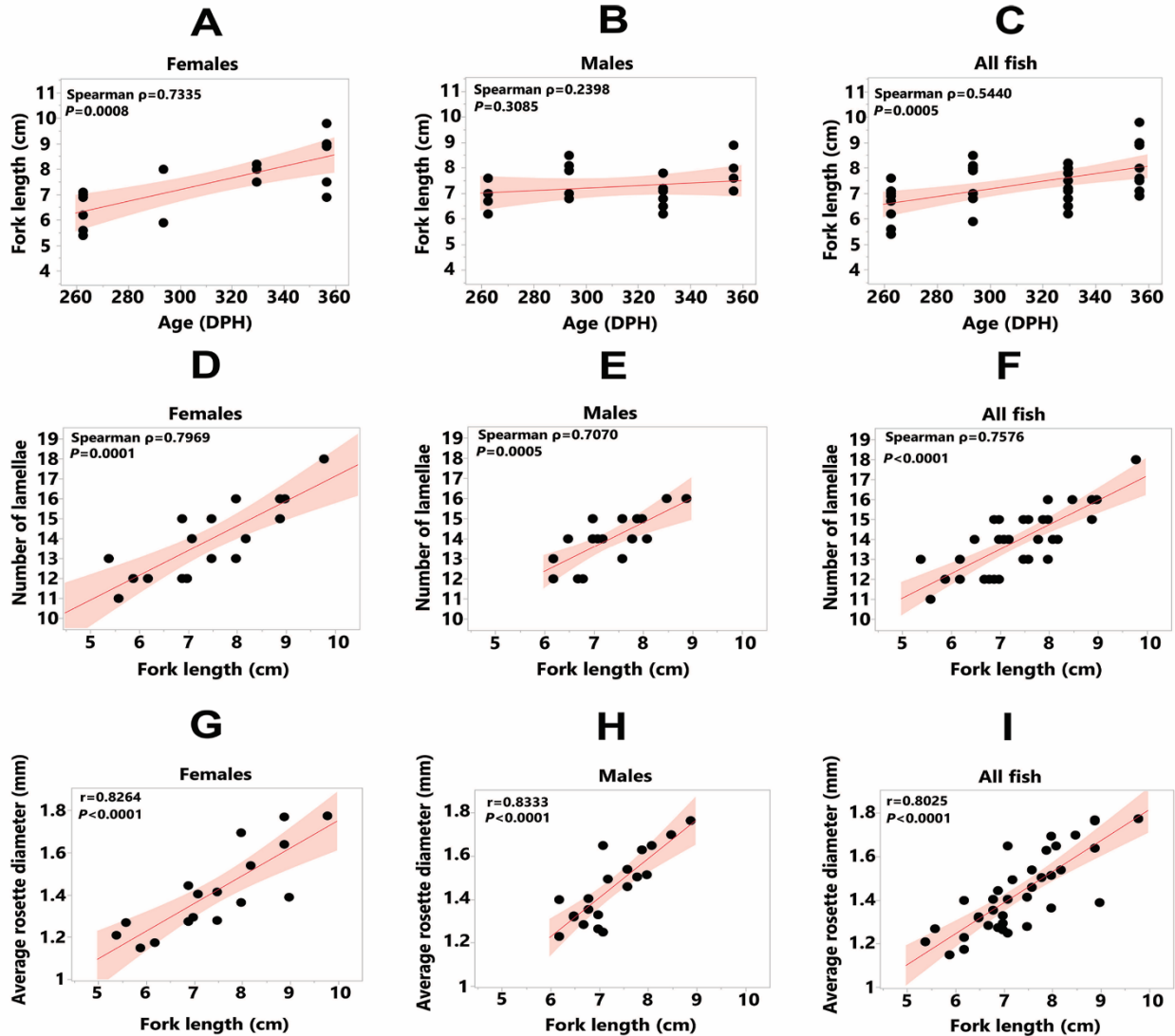
Figure 2. The olfactory rosette of Delta Smelt relative to age and size. The first rows (A-C) illustrate the relationship between age and fork length for females **(A),** males **(B),** and males and females considered together **(C).** The second row **(D-F)** illustrates the relationship between fork length and number of lamellae of one rosette for females **(D)**, males **€** and both males and females considered together **(F)**. The bottom row illustrates the relationship between fork length and average rosette diameter (n=2 rosettes per fish) for females **(G)**, males **(H)** and both males and females considered together **(I)**. In panels **A** to **F**, P values were determined using Spearman's rank correlation, and in panels **G** to **I,** P values were determined using

a Pearson's product-moment correlation. For all data Bonferroni correction for multiple comparison (P=0.005) was applied (see Methods).
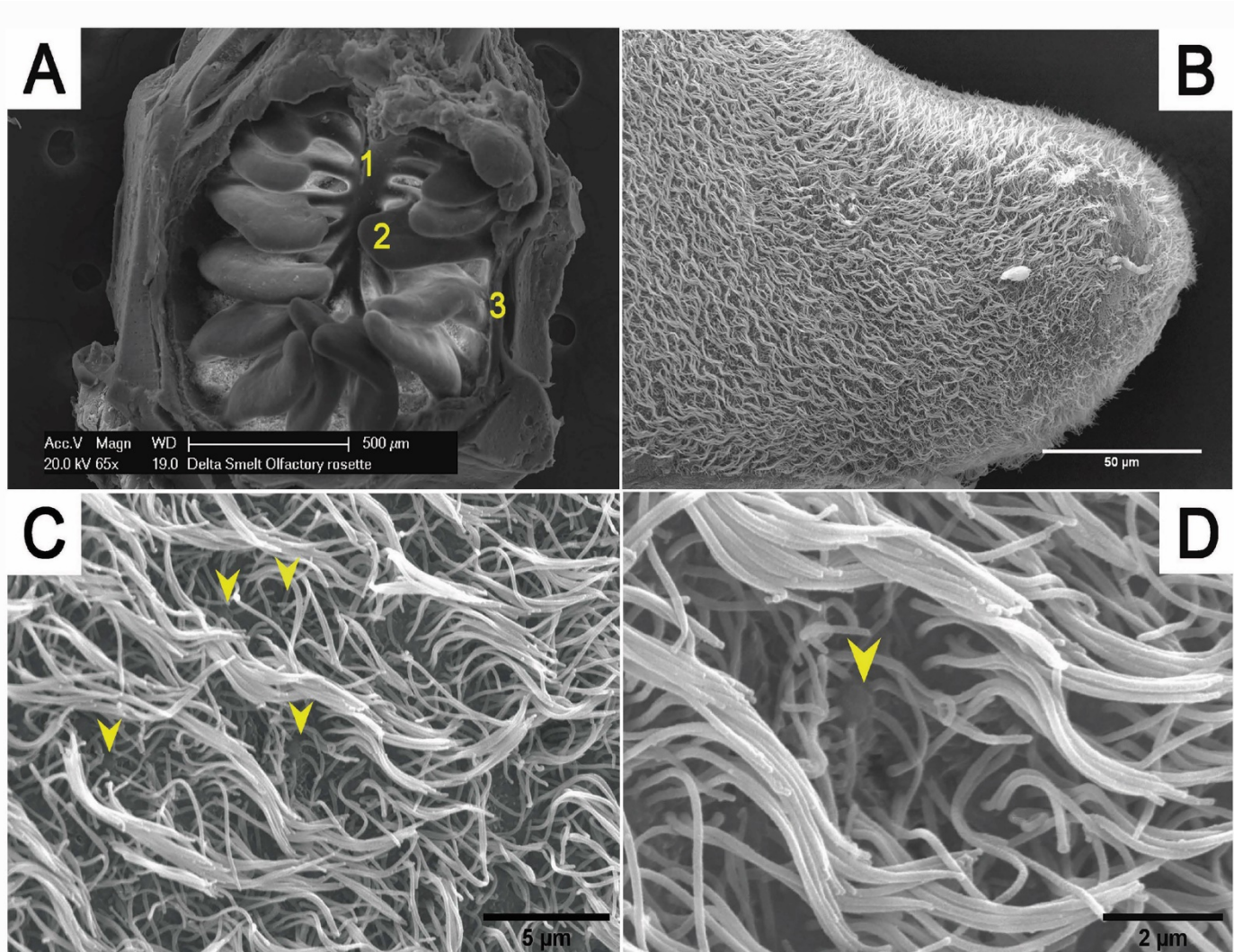
Figure 3. Ultrastructural features of the surface epithelium of a Delta Smelt olfactory rosette. A) Scanning electron micrograph of an olfactory rosette showing the lamellar array surrounded by the fibrous capsule (1= central raphe, 2= olfactory lamella, 3= capsule, 65X, SEM). **B)** Higher magnification view of the surface of a lamella, showing the dense mat of ciliary structures covering the whole lamellar surface, 650X, SEM. **C)** The olfactory knobs of several olfactory neurons (arrow heads) embedded in the dense ciliary mat of the lamellar surface are shown, 5000X, SEM. **D)** Note the sensory cilia projecting from the olfactory knob of the sensory neuron, 12000X, SEM. For all panels, the subject was a 240 DPH Female Delta Smelt.
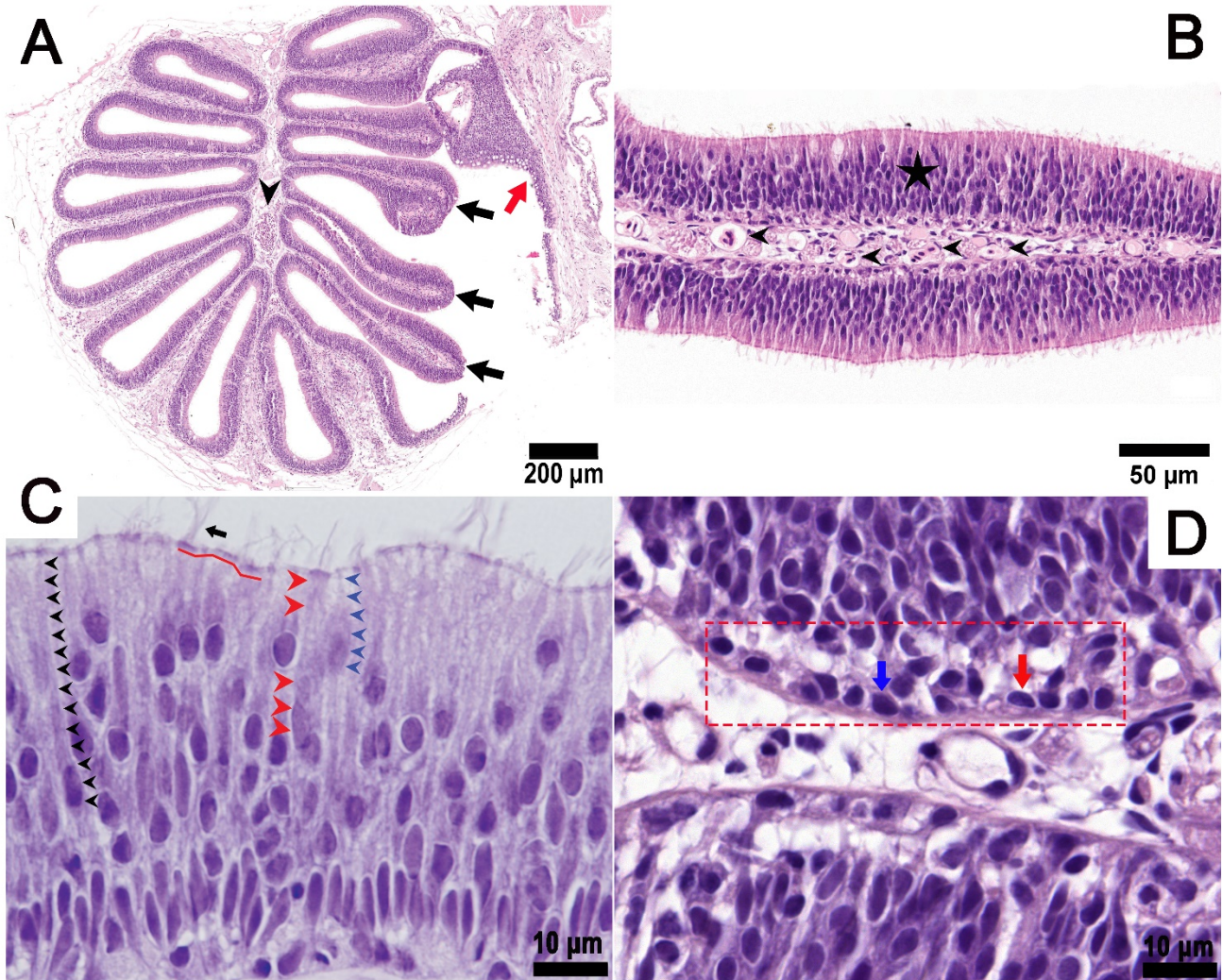
Figure 4. Olfactory rosette and Olfactory epithelium in the Delta Smelt. A) Horizontal section of the whole olfactory rosette showing multiple lamellae (arrows), that contain the sensory epithelium supported by a delicate fibrovascular stroma (lamina propria, arrowhead). The rosette capsule (red arrow, **Figure 11**) surrounds the whole organ,40X, H&E stain. **B)** Single olfactory lamella covered by olfactory epithelium, supported by a fibrovascular stroma with abundant capillary vessels (black arrowheads), ,400X, H&E stain. **C)** Detail of the olfactory epithelium showing a heterogeneous cell population. Motile cilia in the apical domain of the epithelium are clearly visible (black arrow). Arrowheads highlight the body of a ciliate neuron (black), the body of a microvillous neuron (blue), and the body of a sustentacular ciliated cell (red). A red line highlights the basophilic apical band in the epithelial apical sustentacular cell

(red), 1000X, H&E stain. **D)** Basal domain of the olfactory epithelium (red squared area), showing two main basal cell types. The horizontal basal cells (red arrow) characterized by an elongated nucleus, and the globose basal cells (blue arrow), characterized by a round nucleus, 1000X, H&E stain.
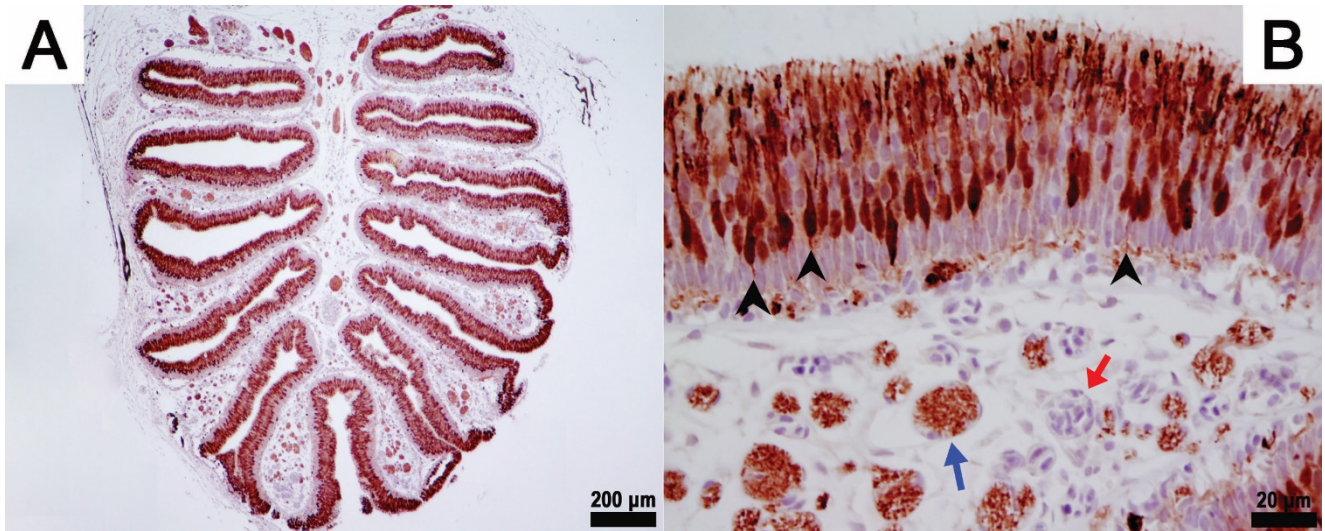
Figure 5. Distribution of sensory neurons in the olfactory lamellae of Delta Smelt. The Calretinin immunohistochemical labeling (red stain) indicates a heterogeneous population of sensory neurons in the olfactory epithelium. **A)** Whole olfactory rosette showing the distribution of sensory neurons, 40X, Light microscopy photomicrograph, Immunohistochemical stain for Calretinin **B)** Higher magnification of sensory neurons. Axons (black arrowheads) can be seen projecting from olfactory neurons towards the lamina propria. Nerve bundles (blue arrow) and capillary vessels (red arrow) are abundant in the lamina propria, 400X.Light microscopy photomicrograph, Immunohistochemical stain for Calretinin.
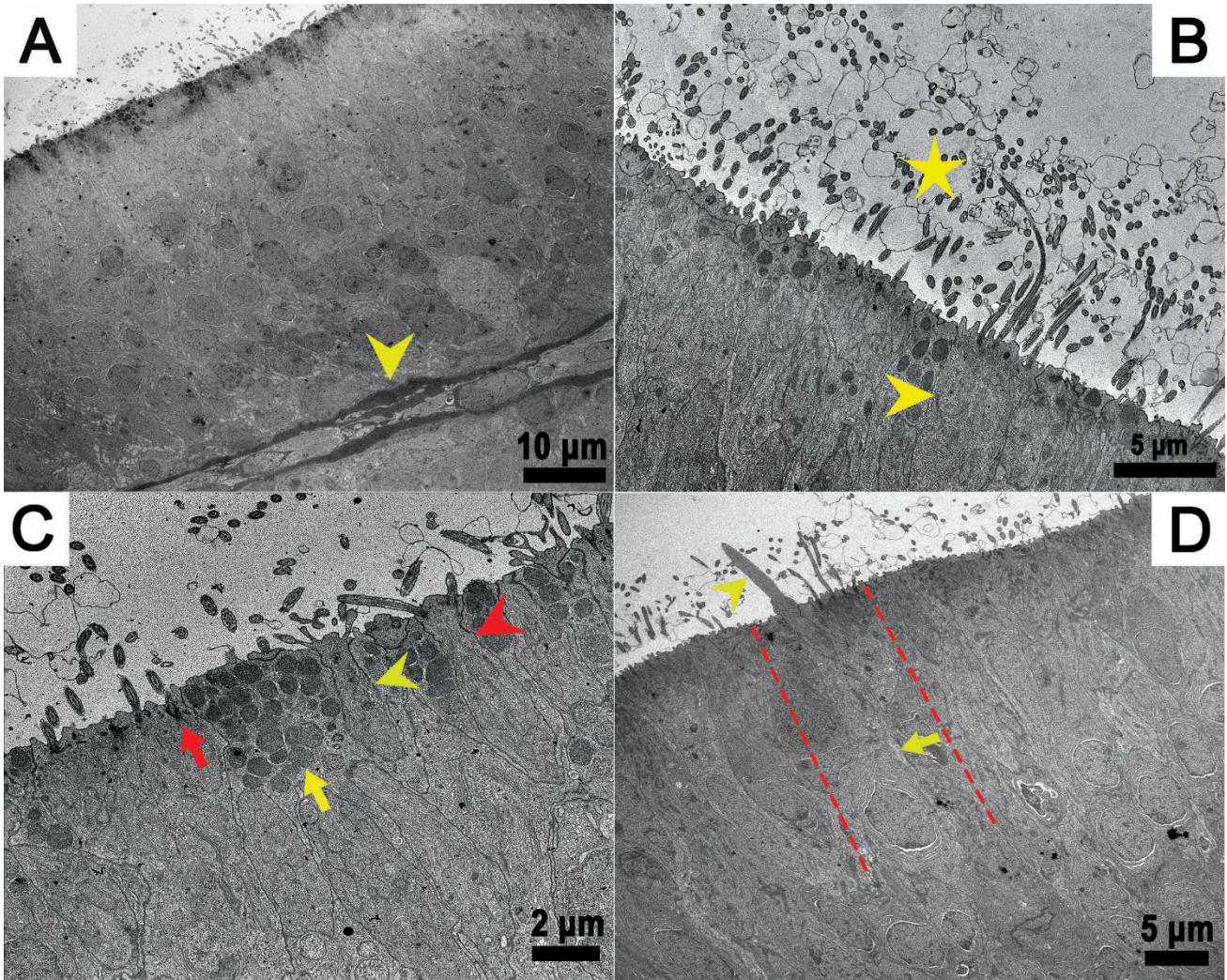
Figure 6. Diversity of apical modifications in the olfactory epithelium of Delta Smelt. A) Transmission Electron micrograph from the basal lamina to the surface of the olfactory epithelium. The arrowhead (yellow) shows the basal lamina in which the epithelium is settled, 600X, TEM. **B)** Detail view of the epithelial surface. Note the epithelium is covered by non-sensory cilia (yellow star) that project from sustentacular cells (yellow arrowhead), 1500X, TEM. **C)** Detail view of the epithelial surface showing a diversity of apical modifications of cell types. Ciliated sustentacular cell (yellow star) with prominent microtubular basal bodies and rootlets (red arrow). Secretory cells with abundant electron dense secretory granules are also indicated (yellow arrow). The microvillous neurons (yellow arrowhead) and ciliate neurons (red arrowhead), are Interspersed between the sustentacular cells, 2500X, TEM. **D)** Detail

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of a rod-shaped apical modification (yellow arrowhead) that was observed in some cells. These cells (bracketed by red dotted lines) resembled olfactory neurons in that they also had a long dendrite (arrow) and a nearly basal nucleus,1250X, TEM.
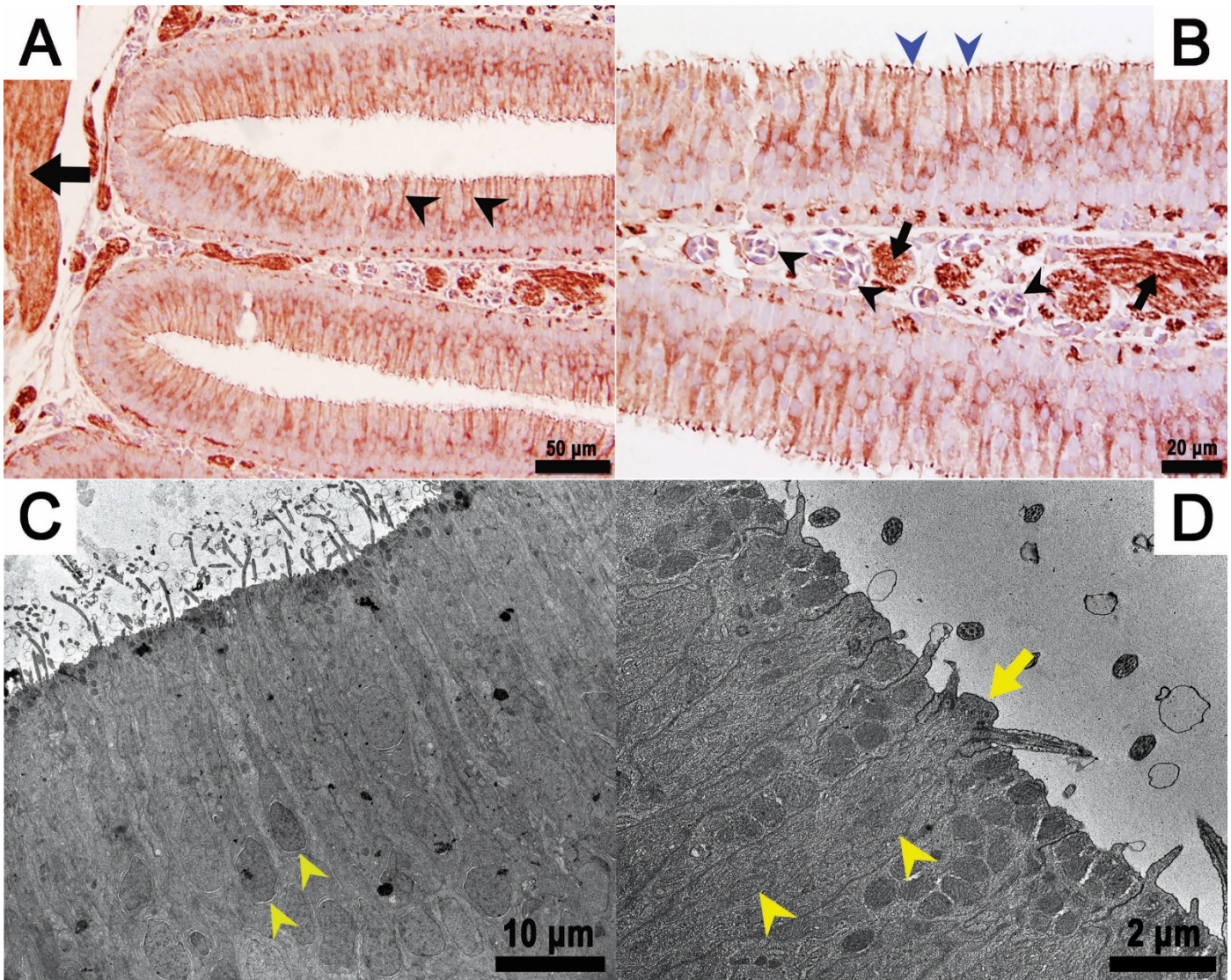
Figure 7. Immunohistochemical and ultrastructural characteristics of ciliate neurons. A) Ciliate neurons stained with anti Gαs/olf antibodies, highlighting their long, slender cell body and dendrite (black arrowheads). The prominent *fila olfactoria* in the lamina propria labeled with anti Gαs/olf antibodies is also shown (black arrow),200X, Gαs/olf Immunohistochemistry, bright field microscopy. B**)** Higher magnification of (A). The olfactory knobs are well defined (blue arrowheads) forming a linear staining pattern on the epithelial surface. The *lamina propria* contains abundant blood vessels (black arrowheads) and nerve bundles (black arrows) in cross section, 400X, Gαs/olf Immunohistochemistry, bright field microscopy. **C)** Transmission electron microscopy of Ciliate neurons. These cells project a long, slender dendrite towards the epithelial surface (yellow arrowheads), 800X, TEM. **D)** The dendrites end with an

olfactory knob (yellow arrow). Multiple tubular mitochondria are observed in the dendrite process (yellow

arrowheads), 4000X, TEM.
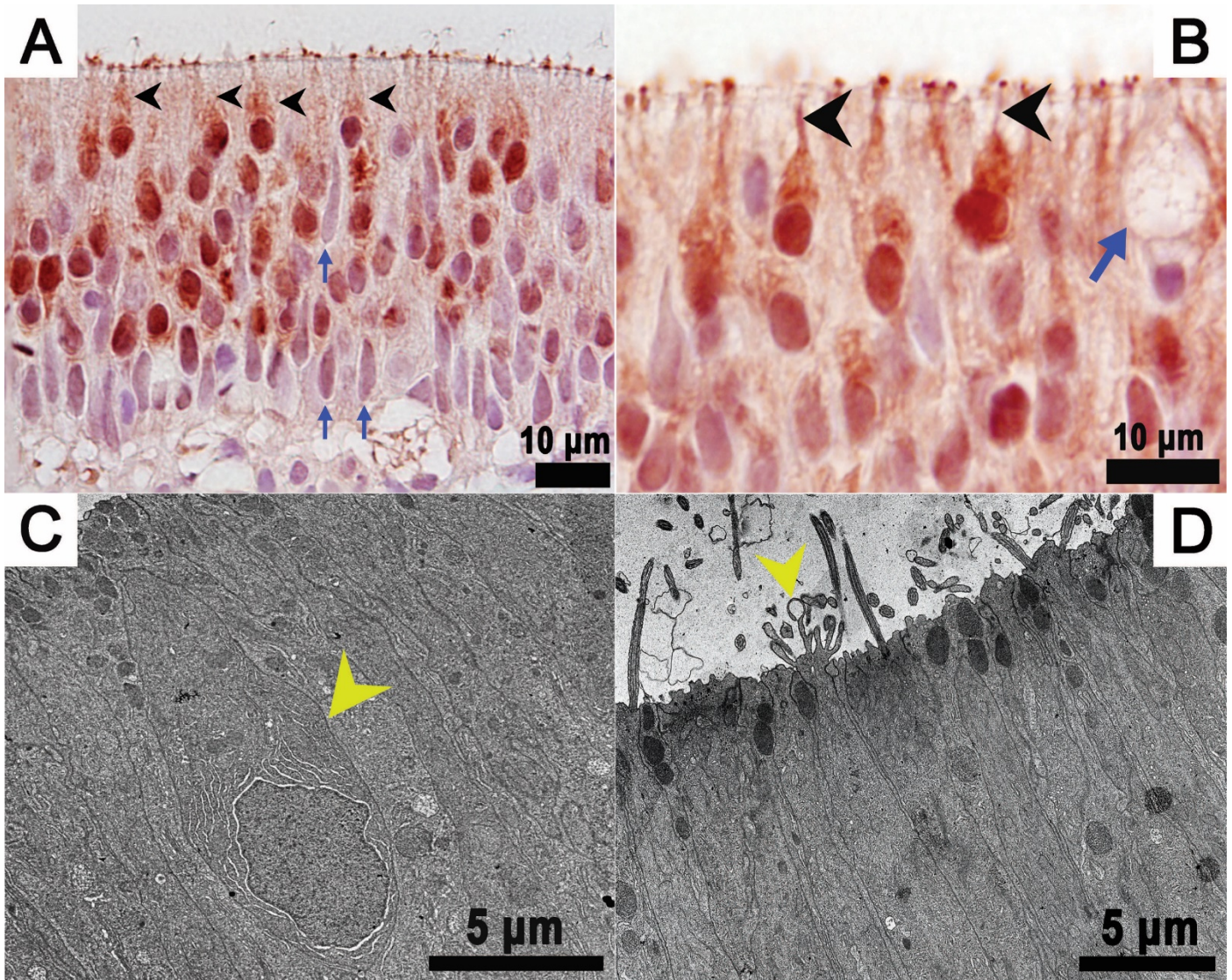
**Figure 8. Cross section of a lamella showing the microvillous neurons in the olfactory epithelium of Delta Smelt. A)** Microvillous neurons are positive for S-100 (black arrowheads). Note the short, pear-shaped body with a round to ovoid nucleus. The blue arrows point to the nucleus of sustentacular cells which are S-100 negative and intermediate-basally located, 1000X, S-100 Immunohistochemistry, bright field microscopy. **B)** Closer view microvillous neurons. The image shows microvillous neurons near the surface of the olfactory epithelium with occasional, short dendrites (black arrowheads), the blue arrow points to a goblet cell, 1000X, S-100 Immunohistochemistry, bright field microscopy. **C)** TEM photomicrograph near the epithelial surface showing the body of a microvillous neuron characterized by an electron dense cytoplasm with few mitochondria and abundant cytocavitary network (yellow arrowhead), 1200X, TEM

D) TEM photomicrograph of a microvillous neuron projections. Several short microvillous extensions are illustrated projecting from a microvillous neuron to the epithelial surface (yellow arrowhead), 2000X, TEM.
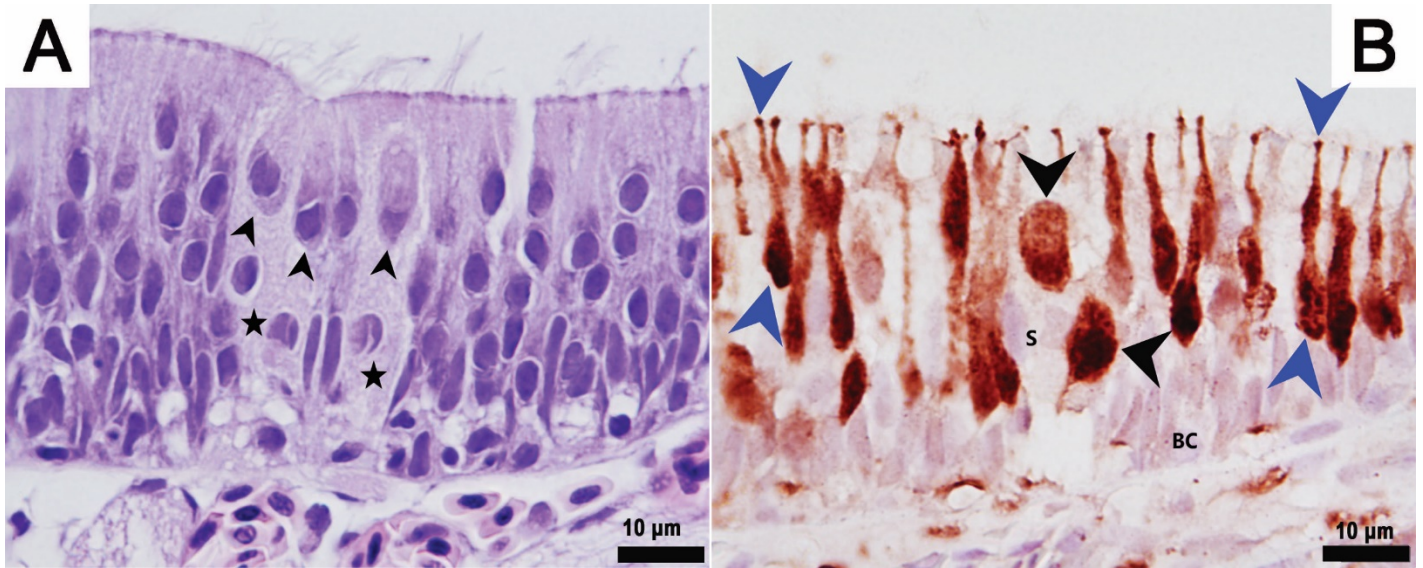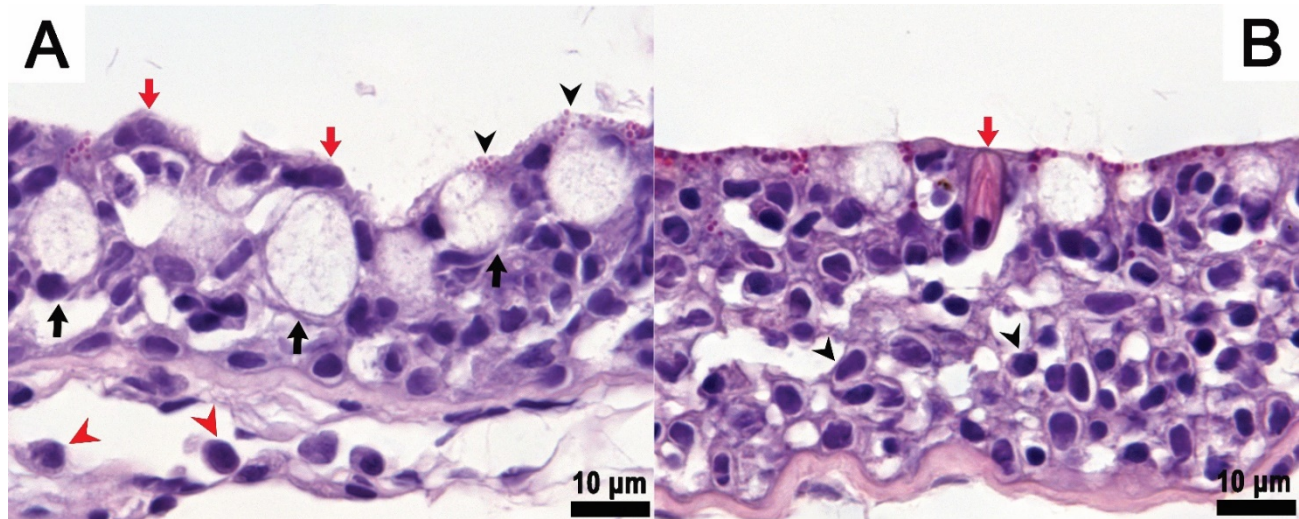
Figure 9. Histological features of the crypt neurons in an adult Female Delta Smelt. **A**) Hematoxylin and Eosin (H&E) section showing a cluster of crypt cells (black arrowheads) surrounded by several supporting cells (black stars),1000X, H&E Stain. **B)** S-100 immunostaining identifying the crypt neurons (black arrowheads) and ciliate neurons (blue arrowheads) in the olfactory epithelium, basal cells (BC) and sustentacular cells (S) were not stained, S-100 Immunohistochemistry, 1000X. Female Delta Smelt, 240 DPH.
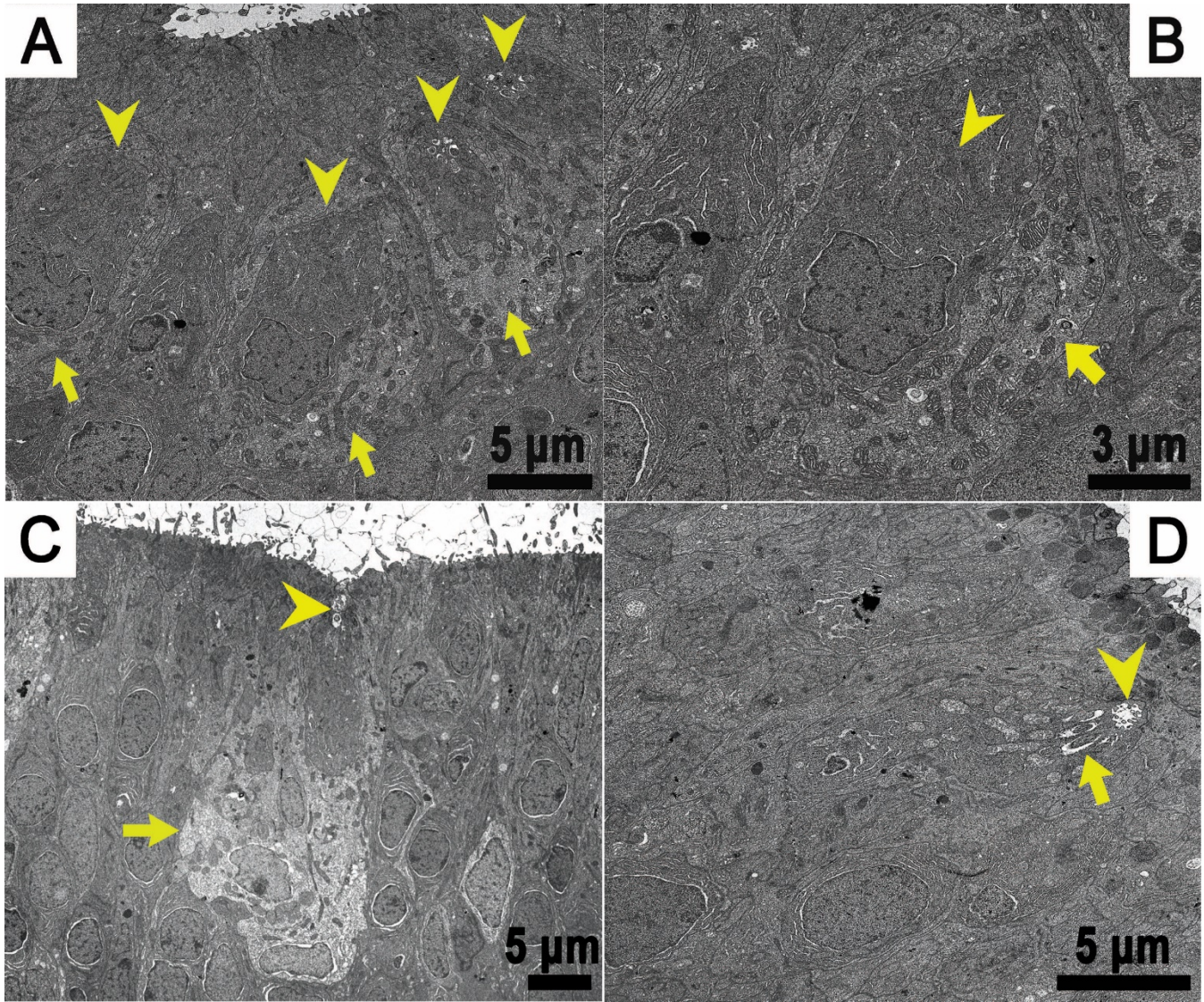
Figure 10. TEM micrographs of crypt neurons. A) Crypt neurons (yellow arrowheads) are shown associated with prominent sustentacular cells (arrows) that form clusters in the olfactory epithelium,2000X, TEM. **B and C**) Detail of crypt neurons. Note that these are round to ovoid cells (B, yellow arrowhead) with an apical invagination (C, yellow arrowhead). The sustentacular cells associated with crypt neurons have abundant smooth endoplasmic reticulum and mitochondria (C, B, yellow arrows),1250X, TEM. **D**) Microvilli and ciliary structures associated with crypt neurons. The crypt neuron invagination projects microvilli towards the lumen (yellow arrowhead). Ciliary structures are also visible (yellow arrow). Note that mitochondria are associated with the ciliary rootlets,2000X, TEM.

Figure 11. Histological features of the olfactory rosette capsule epithelium. A) The capsular epithelium (See Figure 4A, for sub-gross morphology) is composed by several cell types, including pavement cells (red arrows), abundant goblet cells (black arrows), and immune cells (red arrow heads). Abundant bright eosinophilic granules are observed in the epithelial surface (black arrowheads)**,** 1000X, H&E Stain. **B)** Abundant resident immune cells infiltrate the capsular epithelium. These cells are characterized by a round to elongated basophilic nuclei (arrow heads). Also, occasionally rodlet cells are observed residing within the epithelium (red arrow), 1000X, H&E Stain.

TABLES

Table 1. Age, life stage, fork length, and weight of Delta Smelt used for Gross Morphology. Values are given in mean ± SD.

Age (DPH)	Life stage	Sex		Fork length (cm)	Weight (grams)	Number of Individuals
		M	F			
263	Sub-Adult	4	6	6.57±0.70	2.34±0.93	10
294	Sub-Adult	5	2	7.45±0.91	2.92±1.30	7
330	Adult	7	3	7.18±0.68	3.09±0.86	10
357	Adult	4	6	8.26±0.96	5.27±2.52	10

Table 2. Size of the olfactory rosette of Delta Smelt relative to sex, days post hatch (DPH), fork length and weight. Values are given in mean ± SD. One rosette was measured (for number of lamellae) for each fish.

Sex	Age (DPH)	Number of Individuals	Number of Lamellae	Rosette Diameter (mm)	Fork length (cm)	Weight (gr)
Females	263	6	11 to 14	1.27±0.09	6.36±0.74	2.05±0.81
	294	2	12 to 13	1.42±0.31	6.95±1.48	2.18±1.83
	330	3	13 to 16	1.39±0.12	7.90±0.36	3.69±0.38
	357	6	15 to 18	1.57±0.17	8.50±1.07	6.12±2.87
Males	263	4	12 to 14	1.31±0.09	6.87±0.58	2.77±1.04
	294	5	12 to 16	1.54±0.16	7.66±0.73	3.22±1.16
	330	7	12 to 14	1.37±0.10	6.87±0.54	2.84±0.90
	357	4	14 to 16	1.62±0.11	7.90±0.76	4.00±1.32

Table 3. Summary of immunocytochemical characteristics of Delta Smelt olfactory neurons.

Target protein	Immunostaining distribution		
	Ciliate neurons	Microvillous neurons	Crypt neurons
Gαs/olf	Cytoplasm, olfactory knob, sensory cilia, olfactory nerve bundles *(fila olfactoria)*	None	None
Calretinin	Cytoplasm and nucleus	Cytoplasm and nucleus	Cytoplasm and nucleus
S-100	Cytoplasm and nucleus	Cytoplasm and nucleus	Cytoplasm and nucleus

Table 4. Primary antibodies used to detect olfactory neurons in the olfactory rosette of Delta Smelt.

Target protein	Art number	Lot number	Supplier	Species/sequence
$G_{\alpha S/olf}$	SC-377435	C2218	Santa Cruz Biotechnology	Rat 369-394
$G_{\alpha O}$	TA333538	QC2517175-90602	ORIGENE	Human
$G_{\alpha O}$	SC-13532	F2118	Santa Cruz Biotechnology	N/A
$G_{\alpha i-3}$	SC-365422	DO119	Santa Cruz Biotechnology	Rat 339-354
$G_{\alpha q-11}$	SC-365906	A2219	Santa Cruz Biotechnology	Human 60-359
TRPC2	LS-C95010	59673	LSBio	Zebrafish
S-100	RB-044-A0	044A1802J	Thermoscientific	N/A
Calretinin	AB5054-K	3088686	AMD Millipore-Sigma	N/A

Chapter 2

The olfactory mediated alarm response of Delta Smelt (*Hypomesus transpacificus*) to different

concentrations of skin extracts

Abstract

The Delta Smelt (*Hypomesus transpacificus)* is an imperiled fish native to the San Francisco Estuary in California. One of the causes for their decline is predation by nonnative invasive species. Detection of predators may be further impacted by pollutants and changes in the environment. The response of Delta Smelt to anti-predatory skin extracts at different concentrations has, to my knowledge, never been reported. I examined the behavioral response of Delta Smelt and assessed variation to different concentrations of conspecific skin extracts using video recordings and behavior tracking software. I found that Delta Smelt responded to skin extracts in a graded way. Upon detection of skin extracts, Delta Smelt responded with darting and a sudden change in speed. Also, Delta Smelt responded to skin extract of conspecifics with changes in movement. Delta Smelt demonstrated a strong alarm response at medium and high concentrations of skin extracts. This is the first work demonstrating that Delta Smelt have a graded olfactory mediated antipredator response to skin extracts.

1. Introduction

The Delta Smelt, *Hypomesus transpacificus* (Osmeriformes, Osmeridae) is a small, planktivorous osmerid that inhabits shallow open waters and is endemic to the San Francisco Estuary in California (Moyle et al., 2016, Hobbs et al., 2017, Hobbs et al., 2019). Currently Delta Smelt are critically endangered, and populations have reached historically low numbers (Teh et al., 2020). Delta Smelt have been the focus of extensive research efforts to understand the causes of their decline and to identify paths to recovery (Lessard et al., 2018). The life history, biology, physiology and behavioral research of Delta Smelt have helped to inform models to address, sum efforts and take measurements to mitigate the population decline (Hamilton and Murphy, 2018). The picture that is emerging is that predation by invasive species is a critical factor contributing to population declines, and that improving our understanding of predator-

prey interactions has the potential to impact the recovery of Delta Smelt (Schreier et al., 2016, Hobbs et al., 2017).

To date, little is known about the antipredator behaviors of Delta Smelt, or about the sensory modalities used by these fish to assess predation risk. It has long been recognized that some species of fish (i.e., cyprinids) respond to odors of freshly killed conspecifics. This substances "Schreckstoff" was first demonstrated in a classic study by Karl Von Frisch where he showed that presenting minnows with skin extract would elicit an anti-predatory escape response in conspecifics (Von Frisch, 1941). Injury-associated skin extracts have since been studied extensively in a variety of fish species including fathead minnows (*Pimephales promelas*) (Chivers and Smith, 1994, Chivers et al., 2013), zebrafish (*Danio rerio*) (Barkhymer et al., 2019, Mathuru et al., 2012) , goldfish (*Carassius auratus*) (Zhao and Chivers, 2005), and rainbow trout (*Oncorhynchus mykiss*) (Horn and Chivers, 2017), which are main models in behavioral research. Results from these various investigation concur that skin extracts are detected by olfaction and are typically released via mechanical damage to the skin as occurs during a predator attack, and therefore they represent a reliable indicator of predation risk (Chivers et al., 2012, Wisenden et al., 2004).

According to the threat-sensitive predator avoidance model, the strength of response to predation cues may covariate with the intensity of the stimulus, in which some fish will have a strong threat sensitivity with a stronger antipredator response with an increased perceived risk (Brown et al., 2006, Helfman, 1989). Cue concentration may provide prey with context regarding, for example, predator proximity or size (Ferrari et al., 2010). A low concentration of the cue might represent a lower level of risk compared to a high concentration; however, there are thresholds at which an antipredator response is most relevant, for example in the assessment of proximity and density of predators (Ferrari et al., 2006). Olfactory assessment of risk has become a topic of in deep investigation given its ecological importance to the survival of a species. Fitness and survival decrease when olfactory cues cannot be correctly

interpreted under degraded environmental conditions. Therefore, failure to react appropriately to a predator means death (Kelley and Magurran, 2003).

Antipredator behaviors displayed after detection of skin extracts are dominated by changes in swimming speed, darting, freezing and formation of a more cohesive group in schooling fish (Maximino et al., 2019). A previous study demonstrated that Delta Smelt respond to skin extracts by mainly increasing their swimming speed and decreasing their inter individual distance forming a more cohesive group. These behaviors are well studied antipredator behaviors (Davis et al., 2019); however, their response thresholds to different concentrations was not addressed.

The behavioral responses of Delta Smelt to different concentrations of skin extracts is unknown and critical for the understanding of antipredator behaviors of Delta Smelt. Studying the normal behaviors under controlled conditions is a starting point to understand the biology of this imperiled fish. The objectives of this work were to characterize the olfactory mediated alarm response of Delta Smelt and its variation under different levels of skin extract concentration in the water. Using a laboratory trial, I tested the hypothesis that Delta Smelt respond to skin extracts in a dose-dependent way. I predicted, according to the threat-sensitive predator avoidance model (Helfman, 1989, Brown et al., 2006), that Delta Smelt will respond in a dose dependent manner to skin extracts with a stronger response at high concentrations and a weak response at low concentrations (strong threat sensitivity).

2. Materials and Methods

Fish acclimation and care: Sub-adult Delta Smelt (n=120, age: 240 days post hatch (DPH), fork length: 5.95±0.36 (mean ± SD) centimeters, weight: 1.50±0.36 (mean ± SD) grams) were transported in mid-September 2019 from the Fish Conservation and Culture Laboratory (FCCL) in Byron, CA, to the Center for Aquatic Biology and Aquaculture (CABA) at the University of California. The fish were transported in 90-liter insulated carboys. The transport water was conditioned with 5 parts per thousand (ppt) of Instant

Ocean salt (Instant Ocean, Blacksburg, VA), oxygenated to a 90% saturation and kept at 12 °C. The original

population of captive bred Delta Smelt (F0) was obtained from the San Francisco Estuary and had been

bred for 12 (F12) generations in captivity using established methods for the species (Lindberg et al., 2013).

In order to prevent inbreeding and adaptation to the hatchery, generations of Delta Smelt were bred using

stablished genetic management protocols and exposed to semi-natural conditions (life stage differential

lighting, rearing in multifamily groups, family size equalizing) during their life cycles in the conservation

hatchery (Fisch et al., 2012). Before the assays, the fish were acclimated for two weeks at CABA, in three

circular tanks with a capacity of 300 liters, connected to a recirculating system supplied with well water.

The water quality parameters (mean ± SD) were as follows: temperature: 15.56±0.64 C°, pH: 8.61±0.09,

dissolved oxygen 9.16±0.5 mg/L and ammonia 0.13±0.18 mg/L. The fish were fed 3% of their total biomass

daily with a balanced ration (BioPro2-CRUM #1, Bio-Oregon®, Vancouver). Fish welfare and experimental

protocols were approved by the University of California Davis Institutional Animal Care and Use

Committee (IACUC protocol no. 20052).

2.1 Behavioral assays

Preparation of the conspecific skin extracts: A total of 18 donor fish were used for skin extract preparation.

The fish were euthanized following the American Veterinary Medical Association (AVMA) guidelines for

euthanasia of animals (Leary et al., 2013). In brief, I placed the fish in ice water for 10 minutes until

opercular movements and peduncular reflexes stopped, and then severed the spinal cord just behind the

operculum. After euthanasia, I weighed and measured each fish, removed the head, wrapped the body in

aluminum foil and flash froze it in liquid nitrogen for further dissection. The skin samples were dissected

from each side of the fish using the methodology of Wisenden (2011) with some modifications as follows:

I thawed the fish and washed any excess mucus from the skin with ultrapure water (18.2MΩ/cm ddH2O,

Milli-Q®, Millipore-Sigma, USA). After mucus removal, each fish was placed on top of a frozen cryo-gel pad

covered with aluminum foil for skin dissection. I used a razor blade to make a superficial cut along the

dorsal and the ventral surface of the fish body. The skin was gently pulled out to separate it from the muscle to yield a single skin sample. After removal, the skin samples were weighed, placed in a microcentrifuge tube and flash frozen in liquid nitrogen. The skin was then ground into a fine powder with an autoclaved pestle on the same tube. Then, 1 mL of Ultra-pure water was mixed into the powder, followed by homogenization for 1 minute with a drill and autoclaved pestle. Then, 0.5 ml of Ultra-Pure water was added to the tube and mixed by hand shaking, followed by centrifugation at 14000 rpm for 1 minute at 4°C. The supernatant was recovered, transferred to a clean 1.5 ml Eppendorf microcentrifuge tube and stored at -20°C until use.

Dilutions: Skin extract was diluted in ultrapure water at 1:50 (high), 1:500 (medium) and 1:5000 (low) ratios. A total volume of 50 ml was prepared for each concentration. The concentrations of skin per dilution dose are shown in **Table 1**

Behavioral arenas: Two circular arenas were used to evaluate responses to skin extracts. I used a shuttle box system (Loligo® Systems, https://www.loligosystems.com/shuttle-box-system) in which the two circular arenas connect through a shuttle between them (**Figure 1**). In order to have two separate arenas we sealed each opening to the shuttle in each arena to avoid water mixing and movement of fish between them. The arenas were 1 m diameter and 20 cm height with a volume of 117.8 liters. The arenas were filled with deionized water reconstituted with $CaCl_2.2H_2O$, $NaHCO_3$, KCl and $MgSO_4$, with pH of 7.5±0.44 (mean±SD), dissolved oxygen 7.38±0.51 mg/l (mean±SD) and temperature 15.22±0.26 °C (mean±SD). Water circulation was driven independently in each arena by an external pump. Each pump was connected to an external buffer tank through plastic tubing with a flow of 5 liters/minute, which returned the water by gravity to the arena, generating a flow velocity of 4.68 cm/s. The arenas were illuminated from underneath the tanks using a custom-made light box with an average lux intensity of 146±6 lumen/m^2 and surrounded with a black plastic curtain to minimize potential changes in fish behavior due to external influences.

Experimental setup: I assigned 120 fish (N=6 replicates with five fish per replicate) to one of four treatments (control (0), low (1:5000), medium (1:500) and high (1:50)) of Delta Smelt skin extract; **See Figure 1 and Table 2**). Each group of five fish was used only once on each assigned treatment so each replicate is treated as an independent group. The four treatments were randomly assigned to the two arenas using a random number generator such that each of the four treatments was run each 'day' with two concentrations tested simultaneously in the morning (8:00) or afternoon (14:00) in the same day, comprising an experimental block. Before a trial started (a trial was defined as a period of 45 minutes of behavior recording for each group tested), the fish were given 1 hour to acclimate to the arenas. Trials were performed in October 2019.

Trial recording setup: Trials were recorded from above of each tank, at a height of 1.20 m using two GoPro Hero Black 6 (GoPro, Inc. United States) cameras at 30 frames per second in linear mode. Once the acclimation period ended, the recording of a trial began. Each trial had three time intervals of 15 minutes for a total of 45 minutes. A complete trial was divided into three phases for analysis in time intervals called base line period (first 15 minutes), base line subtracted (BLS) time interval 1 (15 to 30 minutes) and BLS time interval 2 (30 to 45 minutes of recording). Three doses of 50 ml/dose of control or diluted skin extract were applied at an interval of one minute to the buffer tanks during the first 3 minutes of the BLS time interval 1 using plastic syringes (**Figure 1**). It took the skin extract around 30 minutes to disperse evenly within the test arenas during previous trials using dyes. In this way, I divided the period after addition of the cue in two equal periods of 15 minutes to account for differences in the dispersion of the cue within the test arenas. After each trial, the two arenas were thoroughly washed three times with a soap-bleach diluted solution, followed by a wash with 100% ethanol and a final rinse in deionized water. For each trial, a set of clean tubing was used to avoid any contamination with odorants from previous trials.

Video recording analysis: The video recordings were analyzed with Noldus Ethovision XT 14-tracking Software with the social interaction module (V. 14.0, Noldus Information technology, NL). High-resolution

videos were converted from mp4 to mpeg2 format using 4Media HD Video Converter (version 7.8.23.4 Media Software Studio, Xilisoft Corporation 2018). To adjust the frame rate that the videos were tracked, I used a calibration curve using "speed" as the response variable and "frame rate" as the independent variable. This avoided under- or over-estimate of the evaluated parameters and ensured consistent results over the whole analysis (Noldus Ethovision XT 14, Help manual, Sample rate). I tracked the fish at a frame rate of 3.75 frames/s.

Behavioral parameters: For each fish, I determined the number of darting episodes, the swimming speed (cm/s), maximum acceleration (cm/s^2), total distance traveled (meters) and interindividual distance (cm) using Ethovision. I counted the number of darting episodes during each time interval (**see Trial recording setup section**). The darting episodes were scored blindly from the recorded videos by the same individual for all the experiments.

Data handling: The darting episodes at time intervals 1 and 2 were counted and summed up from all the fish from each treatment to obtain the total number of darting episodes. The swimming speed, interindividual distance and total distance moved was calculated from the x, y coordinates in Ethovision on each frame for each fish. The maximum acceleration observed for each fish at each time period was used for analysis. The acceleration was calculated by dividing the difference in swimming speed of each fish during one second of recording by the time difference in any second of recording. The interindividual distance was given by the distance between a focal fish with respect to each of the other four fish. Raw data were edited and evaluated for missing frames before averaging. Missing frames occurred when the fish either was not recognized by the tracking software or overlapped with other fish track at some point. When missing frames were observed, the interpolation tool in Ethovision was used to reconstruct the missing frames in each tracked fish. The data used for analysis were obtained as follows: 1) the raw data output (frame rate: 4 observations per second) was averaged to obtain one mean observation every second (2700 data points per trial). This was done for every fish in every treatment, 2) for each parameter

analyzed, the mean value was obtained for each fish for each time interval. This was done for each treatment and 3) the baseline values were subtracted from each exposure interval (**See Figure 1**). The base line subtracted data of each fish were used for statistical analysis. A positive change implies an increase in the magnitude of the parameter and a negative change implies a decrease in the magnitude of the measured parameter. A summary of parameters used for analysis are given in **Table 2**.

2.2 Statistical analysis

The number of darting episodes was not normally distributed and were therefore evaluated for significant differences among treatments using a Kruskal Wallis ANOVA and a post hoc Dunn test for joint ranks. For the remaining variables, the data were evaluated for normality and homogeneity of variance using residual analysis. The dilutions of skin extract (Treatment) and the base line subtracted interval (time interval 1 and 2) were used as explanatory variables. The swimming speed (cm/s), maximum acceleration (cm/s^2), total distance traveled (m) and inter individual distance (cm) were used as response variables. The parameters were assessed using identical factorial ANOVAS in which the interaction was treatment by base line subtracted interval. I used 'day' and 'fish' as random effects, with 'fish' nested within 'day', and 'fish' nested within treatments to account for the multiple measurements of the same fish. The blocking variable 'day' was included as a random effect, but it was insignificant and removed from the final analysis. Also planned linear contrasts were used for each treatment to compare the base line subtracted exposure time intervals. Data are presented as mean± SE or median and Inter quartile range (IQR) for nonparametric data. All analyses were performed at α=0.05 in JMP pro software V 15.1 (SAS institute, Cary, NC).

3. Results

3.1 The alarm response of Delta Smelt

The response of Delta Smelt to skin extracts was characterized by 1) a sudden acceleration, with bending of the fish body resembling a "C", or an "S", shape followed by a sudden burst in swimming speed with wobbling and undulation of the whole body, 2) change in direction, that directs the fish away from the initial position followed by 3) a period of deceleration or immobility (**Figure 2**). This swimming pattern is consistent with an 'escape response' that has been described in other fish species, for example, the cichlid *Neolamprologus pulcher* (Fischer et al., 2017) and the grey mullet *Liza aurata* (Domenici and Hale, 2019). The sudden 1-2 second acceleration followed by a deceleration and a subsequent reduction in swimming speed **(Figure 2)** is consistent with a "freezing" behavior (Maximino et al., 2019). This behavior is characterized by slow swimming intervals, in which the fish barely moves and stays still in the water.

3.2 Darting episodes

A darting episode was a sudden increase in acceleration and swimming speed, with a change in swimming direction. In general, fish exposed to skin extracts showed an increased number of darting episodes (Kruskal Wallis ANOVA, $\chi^2_{,3}$ =9.9288, *P*=0.0192) (**Figure 3)**. The darting episodes increased significantly at low concentrations of skin extract when compared to the control group (control vs low, Dunn, Z=2.9593, *P*=0.0092). The median darting increased from 2 (IQR=3.75) in the fish from control groups to 12 (IQR=9.25) at low concentrations of skin extract. At medium and high concentrations of skin extract darting increased above the controls in half of the replicates examined, with the maximum number of darting episodes observed at the medium concentration; however, no significant difference was observed when compared to the control group (control vs medium, Dunn, Z=2.2401, *P*=0.0753).

3.3 Changes in swimming speed

I observed a decrease in swimming speed of the groups of fish that were exposed to high and medium concentrations of skin extract **(Figure 4).** There was a significant decrease in swimming speed over time with a significant interaction between the treatment and the time interval (ANOVA, treatment × time interval, $F_{3, 203}$ 3.5839, P=0.0147). The mean baseline subtracted swimming speed of the control and low skin extract concentration treatment was not significantly different (control vs low, planned linear contrast, $F_{1, 203}$ =0.0383, P=0.8451) and did not change significantly between the baseline subtracted (BLS) time intervals 1 and 2 (control, BLS time interval 1 vs BLS time interval 2, planned linear contrast, $F_{1, 203}$ =0.0502, P=0.8230; low, BLS time interval 1 vs BLS time interval 2, planned linear contrast, $F_{1, 203}$=2.9634, P=0.0867). In contrast, the mean baseline subtracted speed in the medium and high concentration groups was significantly lower than control groups (control vs medium, planned linear contrast, $F_{1, 203}$ =44.1496, P<0.0001; control vs high, planned linear contrast, $F_{1, 203}$ =36.0758, P<0.0001). Only at medium concentration, there was a greater reduction through time, in which the baseline subtracted speed was significantly different at time interval 2 (medium, BLS time interval 1 vs BLS time interval 2, planned linear contrast, $F_{1, 203}$ =22.1610, P <0.0001). Mean speed and baseline subtracted speed change were given in **Table 3**.

3.4 Changes in maximum acceleration

Overall, the mean baseline subtracted maximum acceleration decreased with an increase in the concentration of skin extract and a significant interaction between the treatments and the recording period was observed (ANOVA, treatment × time interval, $F_{3,203}$ = 3.1579, *P*=0.0258) (**Figure 5).** I observed an increase in acceleration in the groups exposed to low concentrations of skin extract and a deceleration in the groups exposed to medium and high concentrations. The largest decrease in baseline subtracted maximum acceleration was observed in the medium concentration, which showed a significant

deceleration at baseline subtracted time interval 2 (medium, BLS exposure 1 vs BLS exposure 2, planned linear contrast, F1,203 =12.0375, *P*=0.0006). Likewise, in the high concentration group, I observed a deceleration in the time intervals 1 and 2; however, this changes were not significantly different between recording periods (high, BLS time interval 1 vs BLS time interval 2, planned linear contrast, $F_{1,203}$ =0.0583, P=0.8094).

3.5 Changes in total distance traveled

In general, the fish exposed to skin extract swam less distance at medium and high concentrations when compared to the control groups. I observed a significant interaction between the treatment and the time interval (ANOVA, treatment × time interval, $F_{3,203}$ = 3.4160, *P*=0.0184) **(Figure 6)**. The total distance traveled of fish exposed to low skin extract concentration were not significantly different when compared to the control groups (control vs low, planned linear contrast, $F_{1, 203}$ =0.1814, *P*=0.6706). In contrast, at medium concentration, there was a significant reduction in the total distance traveled when compared to the control group (control vs medium, planned linear contrast, $F_{1, 203}$ =40.9527, *P*<0.0001), and I observed a larger reduction in the baseline subtracted time interval 2 within the medium concentration of skin extract (medium, BLS time interval 1 vs BLS time interval 2, planned linear contrast, $F_{1,203}$ =21.8300, *P*<0.0001). Similarly, in the high concentration group, the fish swam less distance when compared to the control group (control vs high, planned linear contrast, $F_{1, 203}$ =29.4366, *P*<0.0001), and this reduction was similar at both baseline subtracted time intervals 1 and 2, with no significant differences observed (high, BLS time interval 1 vs BLS time interval 2, planned linear contrast, $F_{1,203}$ =21.8300, *P*<0.0001).

3.6 Changes in interindividual distance

After the exposure to the cue, the fish group disaggregated, reflected by an increase in the average interindividual distance, followed by a gradual decrease in the interindividual distance with a more aggregated group (**Figure 7**). This disaggregation and increase of the interindividual distance were

concurrent with the escape response shown by the fish after the skin extract was detected. Examining the change in interindividual distance in the different groups, there was a trend towards a decrease in interindividual distance at both time intervals; however, no significant differences were found among treatments (ANOVA, treatment, $F_{3,413}$= 2.4504, *P*=0.0631). Only in the medium concentration group, I observed a significant decrease in interindividual distance after addition of the cue (medium, BLS time interval 1 vs BLS time interval 2, planned linear contrast, $F_{1,413}$ =9.2487, P=0.0025).

4. Discussion

This study indicates that Delta Smelt respond to skin extracts in a context dependent manner with a graded response to different concentrations of skin extracts. Delta Smelt responded to skin extracts with a sudden change in swimming speed and acceleration followed by episodes of darting and immobility. These behavioral changes are together defined as the escape responses (Domenici, 2010). Kinematic studies have demonstrated that escape responses in fish are highly variable among and within fish species; however, some essential behaviors are well conserved in fast starts escape responses (Ramasamy et al., 2015). In a first phase, there is a bending of the body, followed by a second phase in which there is a propulsive stroke, with an increase of acceleration that drives the fish away from its initial position, finally reaching a third phase in which the fish continues swimming or starts gliding. Phase 1 is characterized by two types of motor patterns, when the body takes the shape of a "C" or "S" (Domenici and Hale, 2019). These swimming starts are called fast starts, and usually last less than 20 milli seconds, and the "C" start is most commonly used by prey fish to avoid predation attacks (Domenici and Blake, 1997, Domenici and Hale, 2019). In Delta Smelt, I observed an initial bending of the body either into a "C" or an "S" shapes by examining individual frames during the span of the escape response; however, to discern if these are two distinct motor behaviors, a thorough kinematic analysis with electromyography would be needed. A highly accelerative motion was observed during the second phase of the escape response triggered by skin extracts in Delta Smelt. This accelerative motion is characterized by a sudden peak of acceleration and

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speed that suddenly drives the fish away from its initial position and are performed during an escape response when the fish is under a threat (Domenici, 2010). Finally, the fast swimming responses are accompanied by a fast increase in acceleration that drives the fish far from the detected stimuli rendering it less susceptible to a predation event (Domenici and Hale, 2019). The escape response is a finely coordinated reflex that involves sensory motor outputs modulated by the motor neural circuits controlled by the Mauthner cell system. This neural circuit has been described to modulate the fast response in movement to a startling stimuli that can be chemical, visual or mechanical (Domenici and Hale, 2019). The reticulospinal cell circuit has also been described as the neural substrate for the sensory motor responses mediated by olfactory stimuli in lampreys (Derjean et al., 2010). Whether any of these circuits is the responsible for the escape response in Delta Smelt is unknown.

In smelts, an alarm response and anti-predatory behaviors have been reported previously (Davis et al., 2019, Skinner et al., 1962). In the top smelt (*Atherinops affinis*), the alarm reaction consists of a rapid swimming pattern, darting to the surface and rapid swimming motion, which can lead to sinking and death. The alarm reaction in top smelt lasted 3 to 5 minutes after the fish resumed normal activity (Skinner et al., 1962)

Delta Smelt showed a transient increase in swimming speed followed by a decrease below the basal levels after skin extract detection at the medium concentration. In a recent work, Davis et al. (2019) demonstrated an increase in speed of Delta Smelt shoals after exposure to low concentrations of conspecific skin extracts, although a decrease in speed below the basal levels was not observed.

The changes in Delta Smelt behavior at different concentrations of skin extract can be interpreted as a chemosensory assessment of predation risk (Brown, 2005). As demonstrated in studies with goldfish (*Carassius auratus*) (Zhao and Chivers, 2005) and redbelly dace (*Phoxinus eos*) (Dupuch et al., 2004), there is a concentration dependent change in the magnitude of antipredator responses of fish exposed to skin

extracts. At high concentrations, goldfish (*Carassius auratus*) and redbelly dace (*Phoxinus eos*) displayed more pronounced and prolonged antipredator responses compared to those exposed to low concentrations of skin extracts. At the low concentration of skin extracts, Delta Smelt perceive predation risk as low and return to activity in a short period of time. In contrast, when exposed to medium and high concentrations of skin extracts, Delta Smelt activity decreased below basal levels implying a chemosensory assessment of high risk of predation with adjustment of behaviors accordingly.

In contrast to changes in mobility, darting was a more general antipredator behavior and observed at all the concentration tested with a stronger response at low and medium concentration.

At low concentrations, Delta Smelt were still able to detect skin extracts since we observed changes in the number of darting episodes; however, the changes in swimming speed, maximum swimming speed, maximum acceleration and total distance moved were not significant when compared to control groups. This implies that there are other context dependent cues derived from multiple sensory modalities like vision or the lateral line that can help in the assessment of risk and in the adjustments of behavior accordingly. For example, carps exposed to predator cues derived from water in which pike were feeding on carp, did not show behavioral changes in clear water; however, with the same cue exposure in turbid water there was a decrease in activity, which indicates that fish are able to assess the environment for risk even at subthreshold concentrations of odorants if one of the sensory modalities is impaired (e.g. vision) (Ranåker et al., 2012). Rapid darting has been hypothesized as an antipredator strategy against visual hunting predators and can confer advantages by confounding predators (Paglianti et al., 2010). At low concentrations, individuals exhibit a less intense antipredator response, giving benefits acquired from other activities as foraging but still with vigilance (Brown, 2005). In studies with trout (Mirza and Chivers, 2003) and Minnows (Brown and Smith, 1996), there was still risk assessment of predation under low concentrations of skin extracts, even when an overt alarm reaction was not evident. Antipredator responses are also shaped by previous experiences with predators and the background level of predation

risk. The background risk of predation for the Delta Smelt used in this study is likely low, since individuals were not exposed to the physical presence of predators in the hatchery. Taking all together, Delta Smelt exposed to a high and medium concentration of skin extracts changed their swimming patterns by an overall reduction in swimming speed over time, indicating that decreased movement is a main response to skin extracts. Decrease in swimming speed and immobility have been described as an antipredator response in a range of fish species, including Minnows (Chivers et al., 2013, Chivers et al., 2012, Miyai et al., 2016), Tilapias (Miyai et al., 2016), Rainbow Trout (Ashley et al., 2009, Mirza and Chivers, 2003) and Zebrafish (Barkhymer et al., 2019). A decrease in swimming speed suggests a response to conspecific cues to assess risk and increase the level of vigilance. Decrease in swimming speed and activity serves to avoid being detected by a predator, presumably those that mainly hunt by vision or have ambush strategies (Smith and Webster, 2015, Magellan et al., 2020, Kats and Dill, 1998) and might indicate an antipredator behavior in Delta Smelt, with increased vigilance helped by input from other sensory systems (e.g. vision). Skin extracts released into the water indicate an ongoing predation event in real time and reduction in activity would minimize the immediate risk of being captured (Lucon-Xiccato et al., 2016).

Reductions in interindividual distances have been demonstrated as an antipredator response in a variety of fish species (Brown et al., 2006, Cattano et al., 2019, Chivers et al., 2013). The change in group cohesion is related to the amount of risk perceived or experienced by individuals in a shoal. Recent studies with Delta Smelt (Davis et al., 2019) demonstrated that there is a decrease of interindividual distances of fish exposed to highly diluted concentrations of skin extracts along with an increase of the swimming speed. I observed a significant reduction in interindividual distances in the group exposed to medium concentration of skin extracts, in contrast to what was observed previously in Delta Smelt. These changes could imply chemosensory risk assessment and changes in inter individual distances reflect an antipredator response. These responses might be influenced by interindividual variation, group size and previous experiences with predators. Previous studies have demonstrated that group size and previous

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learning and experiences can determine the outcome in behavioral responses of groups of fish towards a cohesive or loose group (Mathiron et al., 2015). For example, shoals of trout exposed previously to conspecific skin extracts increased the shoal cohesions and were more successful avoiding predation events by pikes (*Exos Lucius*) than trout not previously exposed to skin extracts (Mirza and Chivers, 2003).

The maximum acceleration was used as metrics of escape responses in which there is a sudden increase in swimming speed with maximum peaks. I observed that in the low and medium concentrations, there was an increase in the maximum acceleration that agrees with the number of darting episodes. This might indicate that the escape responses displayed by the Delta Smelt at low and medium concentrations are accompanied by peaks of maximum acceleration that change from positive during detection of the cue, followed by a sudden decrease in magnitude, reflecting freezing behaviors and immobility.

Taking all these findings together, I demonstrate that Delta Smelt have a graded olfactory mediated anti predatory response. The overall implication is that this response might be important for the survival of Delta Smelt in the wild.

Due to the decrease in natural populations of Delta Smelt and the ability to breed them in captivity, currently the supplementation of natural populations with captive bred fish is a possibility and several pilot studies are currently ongoing to test the feasibility of repopulation (Lessard et al., 2018).

One of the concerns is that fish will not survive predators since they have not been exposed previously to them. The knowledge on how Delta Smelt respond to predators through different sensory modalities and knowing to which stimuli they respond can give insights in how likely is that a trained group of Delta Smelt with predator odors could survive in the wild. These experiments have not been performed and are clearly needed to advance the efforts in conservation. The knowledge of normal olfactory mediated antipredator behaviors and at what concentrations are triggered would be useful in prerelease training to increase survival rates. Skin extracts when paired with other predator odors can help naïve fish to learn and asses

risk better increasing survival rates (Olson et al., 2012, Atherton and McCormick, 2015), since a large component of antipredator behavior is learned by experience (Kelley and Magurran, 2003, Pollock et al., 2005, Lönnstedt et al., 2012, Mirza and Chivers, 2000). Also, it has been demonstrated that naïve tadpoles and fish exposed to cues of invasive predators do not respond to predator odors due to their inability to recognize them as dangerous; therefore, this might make them more susceptible by invasive predators (Hettyey et al., 2016). Moreover, in polluted environments the olfactory ability of fish to detect predators can be impaired (Dew et al., 2014, Tierney et al., 2010) and might influence the ability of Delta Smelt to detect predators in the environment.

5. Conclusions

These results suggest that Delta Smelt possess a chemically mediated alarm system that can be used to detect injured conspecifics in natural environments and thereby assess predation risk. The response to skin extracts at different concentrations by Delta Smelt might give them an advantage by responding with alertness and decreasing the susceptibility to predation and mortality, thereby increasing fitness in highly threatening environments. This research contributes to the body of knowledge on behavior and antipredator responses on Delta Smelt needed to further conservation efforts and help in reintroduction programs.

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Behavioral trials experimental design
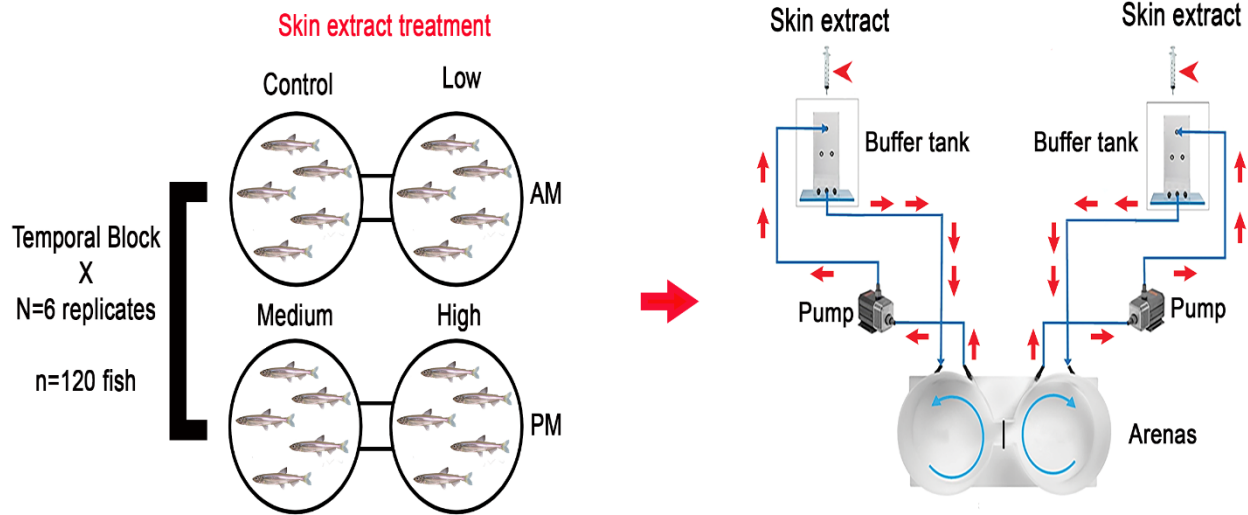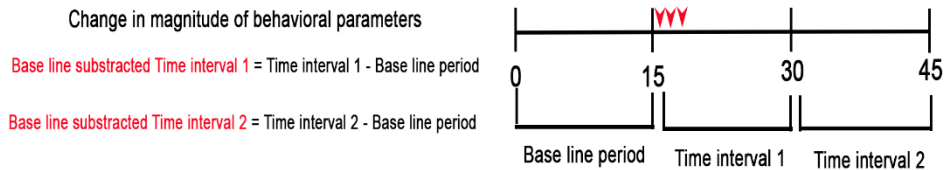
Figure 1. **Experimental design of behavioral trials used to evaluate the response of Delta Smelt to skin extract. Top panel Left** shows the concentrations of skin extract tested in a temporal block. Two treatments were tested in the morning and two treatments in the afternoon. All the treatments were tested on the same day. Each treatment had 6 replicates with a total of 30 fish in each treatment (see methods for details). **Top panel right** shows the experimental arenas setup. The skin extract was added to the buffer tanks using plastic syringes (red arrowheads).The red arrows indicate the direction of water movement within the tubing represented by blue lines. The blue arrows within the arenas indicate the direction of water flows (image modified with permission from Loligo® Systems, Shuttle Box System

Oxygen user manual, https://www.loligosystems.com/manuals). **Bottom panel** shows the timeline of

trials in which the behavior was evaluated. The change in magnitude of behavioral parameters is shown

in red (see methods for description). The red arrow heads indicate the moment in which the skin extract

was aded to the buffer tanks.

Figure 2. **The alarm behavior of Delta Smelt. A)** Shows the track of one fish in 2 seconds of recording (red arrows). The numbers indicate the frames at which the escape response was observed. The video was 26 seconds long and recorded at 30 frames per second (FPS), and then converted to individual pictures (frames), followed by merging of the frames at which the scape response was observed to render a single track. The escape response was observed at seconds 4 and 5 of recording. The tracks of the remaining fish were not included to facilitate observation of a single track. The moment before the reaction to the stimulus is depicted by frame 119, the escape response is depicted by frames 120 to 127 in which there is

sudden bending of the body into an "S" shape and wobbling that propels the fish away of the initial position. **B)** The graph shows the changes in swimming speed and acceleration of a single Delta Smelt during a complete trial in response to skin extract at medium concentration. The mean speed was calculated every second and plotted over a period of 2700 seconds. The three vertical red lines represent the time at which the skin extract was introduced into the experimental arena. The peak represented by the 'escape' response (black arrowhead) is followed by 'freezing' behavior (black arrows) and intervals of normal swimming or darting (bracketed area). **C)** From left to right individual recording frames represent the escape response (represented by the acceleration peak in B) adopted by Delta Smelt when they detect skin extract in the water. There is a sudden change in the swimming direction (frame 119 and 120), then, bending of the body ('C' or 'S' starts) (frame 121 and 122) and followed by an increase in the swimming speed (frames 123 to 127). The black line under the sequences represents 1/3 of a second approximately.
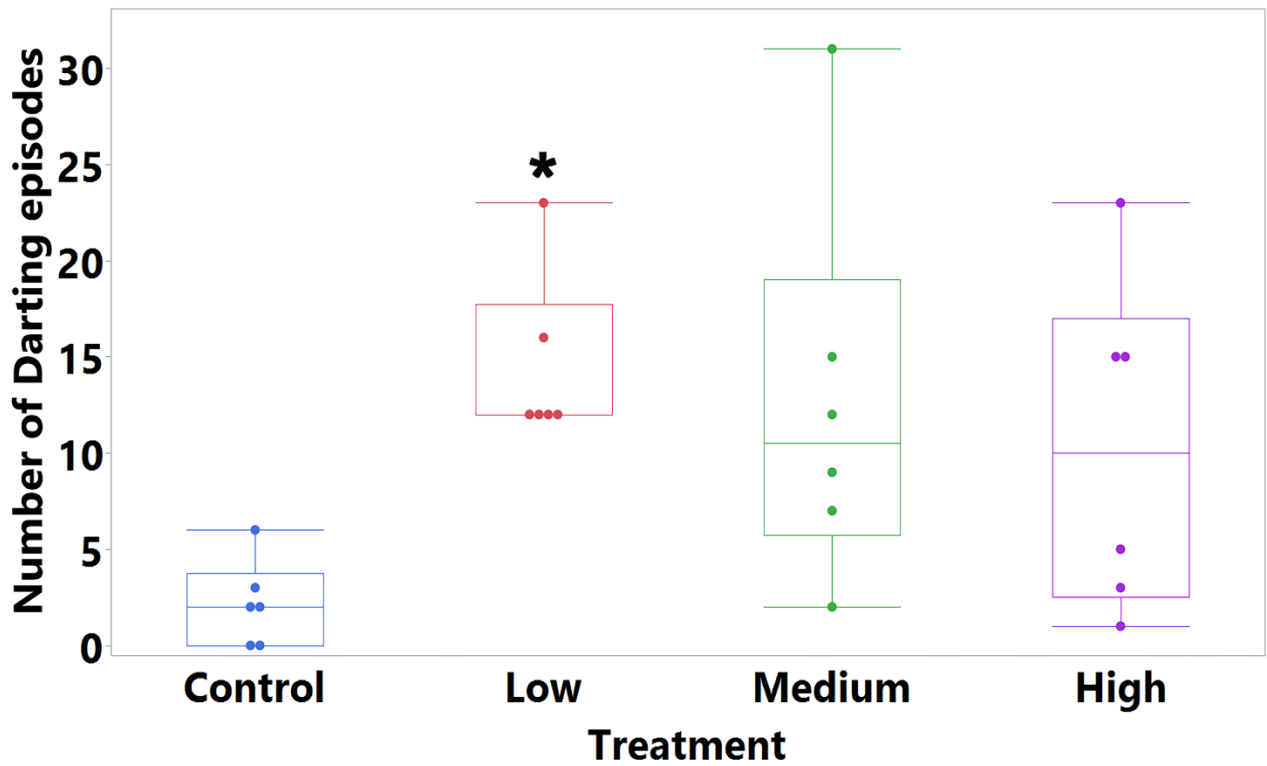
Figure 3. Darting episodes observed in groups of Delta Smelt exposed to skin extracts. The number of darting episodes was increased in all the groups exposed to skin extracts with the maximum amount observed in the low and medium concentrations of skin extracts. Each data point represents the total number of darting episodes per replicate experiment in groups of five fish after exposure to skin extracts. Asterisk represent significant differences compared to the control group.
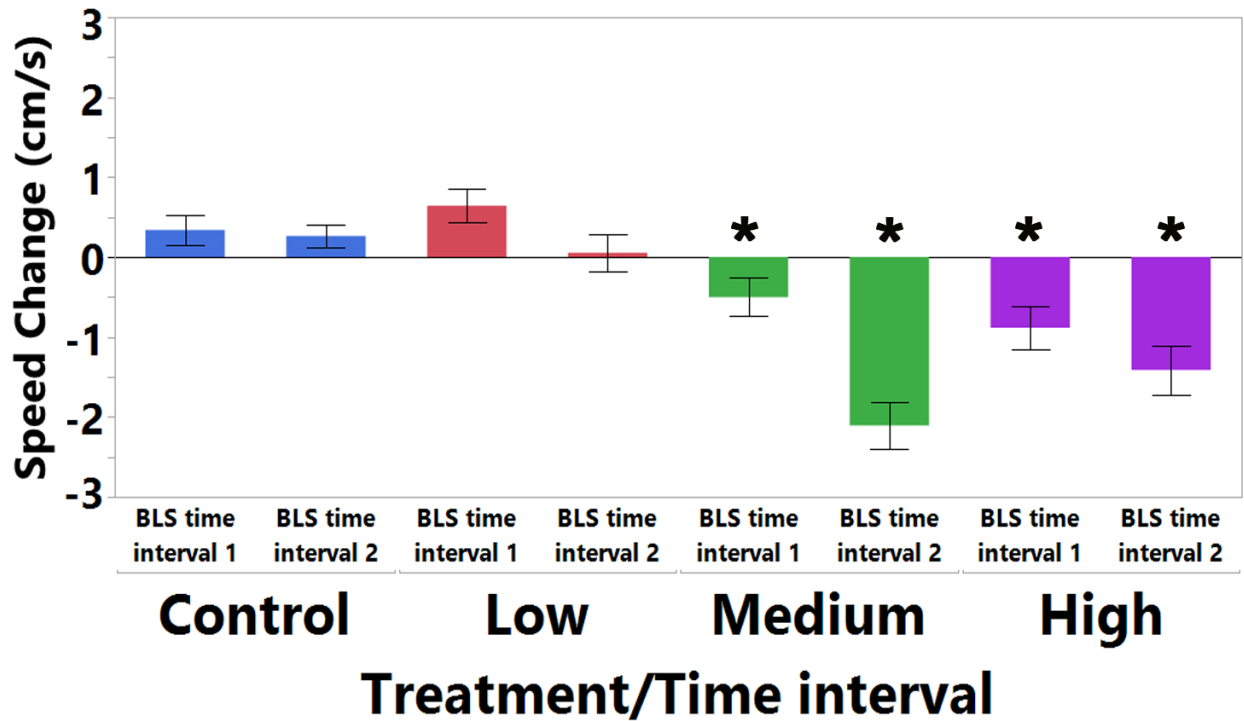
Figure 4. Swimming speed change of Delta Smelt exposed to skin extracts. The groups of fish exposed to medium (green bars) and high (purple bars) concentrations of skin extracts decreased the swimming speed during and after the exposure to skin extracts. The negative change represents a reduction in swimming speed. The star represents significant differences compared to the control group. The data is given in mean change and standard error of the mean (SE). BLS= Base line subtracted time interval.
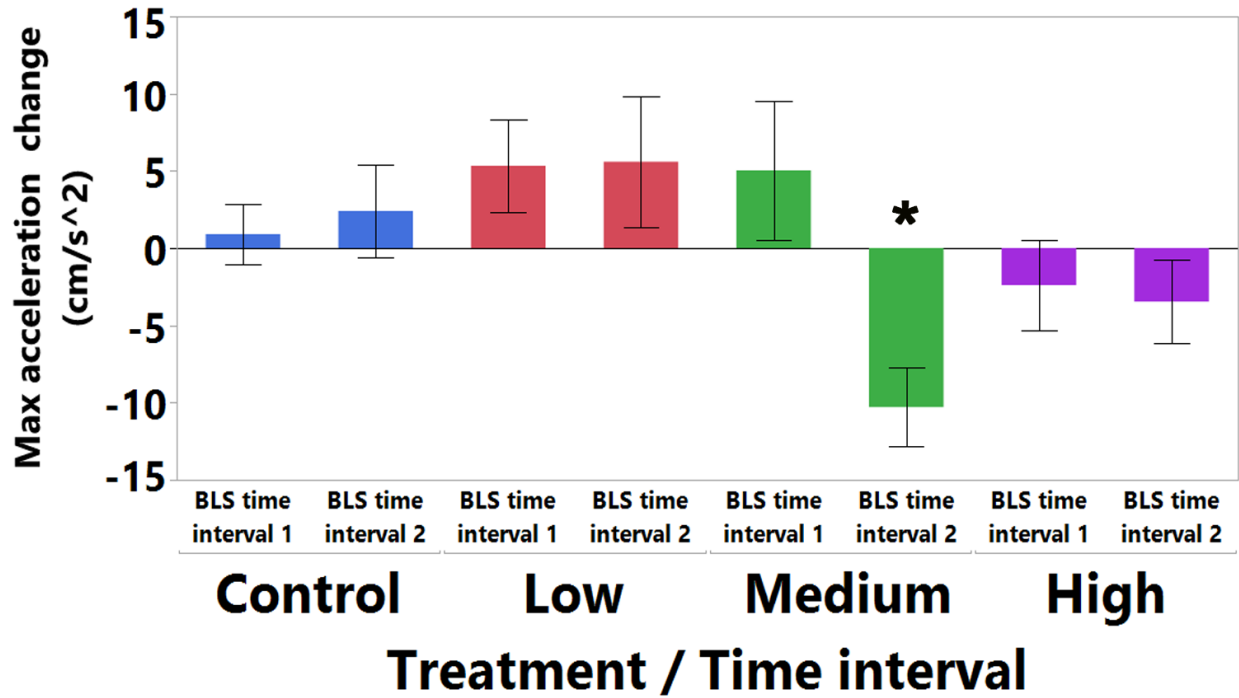
Figure 5. Swimming maximum acceleration change of Delta Smelt exposed to skin extracts. The maximum acceleration was significantly reduced after the exposure to skin extracts in the medium concentration (green Bars). The negative change represents a reduction in maximum acceleration. The star represents significant differences compared to the control group. The data is given in mean change and standard error of the mean (SE). BLS= Base line subtracted time interval.
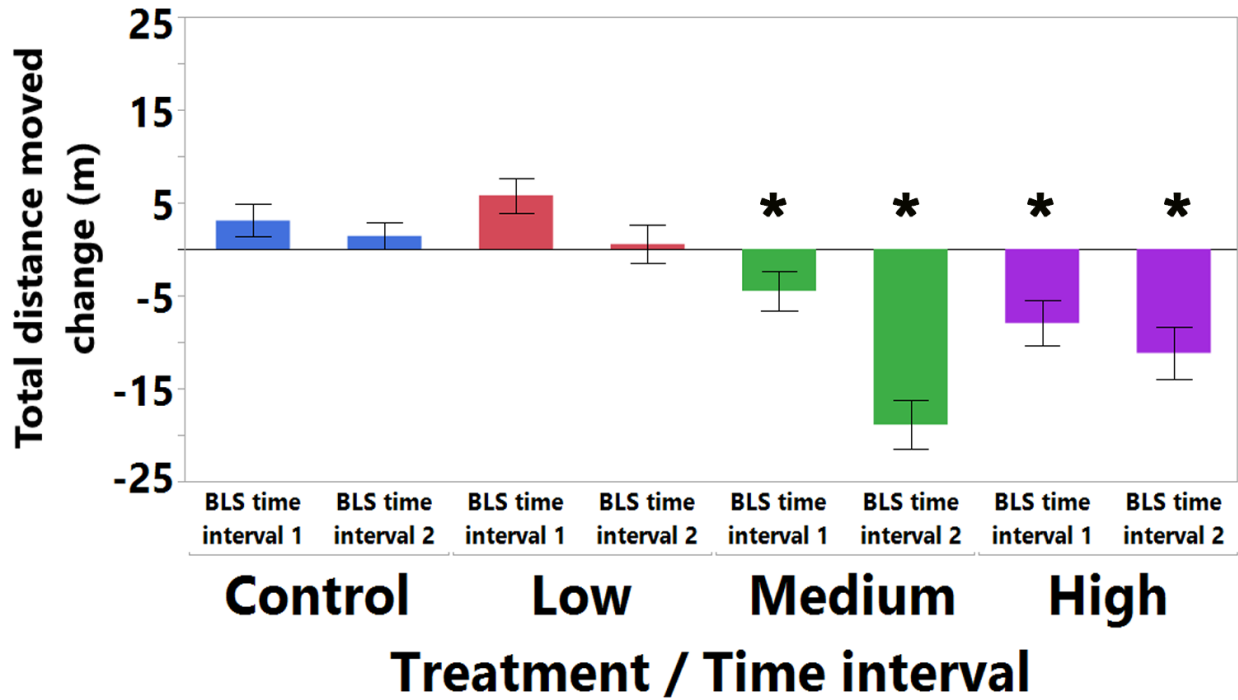
Figure 6. Total distance traveled change of groups of Delta Smelt exposed to skin extracts. The total distance traveled was significantly reduced after the exposure to medium (Green bars) and high (Purple bars) concentrations of skin extracts. The negative change represents a reduction in total distance traveled. The star represents significant differences compared to the control group. The data is given in mean change and standard error of the mean (SE). BLS= Base line subtracted time interval.
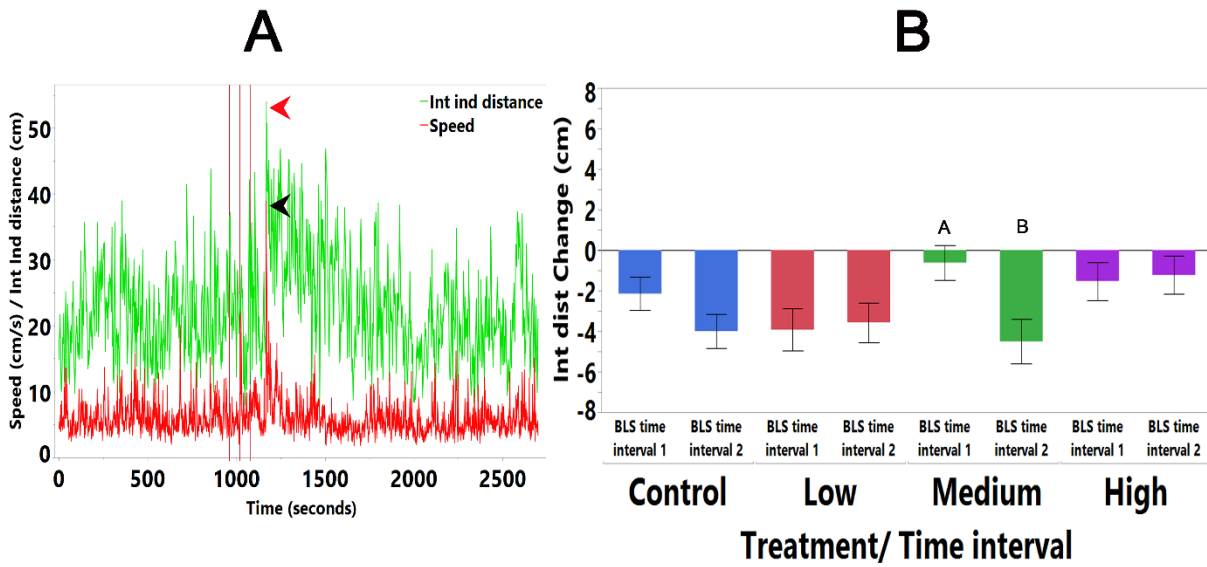
Figure 7. Changes in interindividual distance of groups of Delta Smelt exposed to skin extracts. A) represent the interindividual distance dynamics during a whole trial of a group of Delta Smelt exposed to a medium concentration of skin extracts. During detection of the skin extracts, the interindividual distance increases (red arrowhead) concurrently with an increase in the swimming speed (Black arrowhead), which represents the onset of an escape response, followed by a gradual reduction of the interindividual distance. **B)** After exposure to skin extracts, there is a reduction in the interindividual distance of fish exposed to skin extracts; however, these changes were not significant when compared to the control groups (mean change ± SE) but only significant between recording periods in the medium concentration (different letters). BLS= Base line subtracted time interval.

Table 1. Concentration of skin extract used to prepare the dilutions. Values are given in mean±SD.

Treatment	Volume of raw skin extract (µl)	Concentration of skin used per 50 ml dose (wet weight mg/ml)
Control	0	0
Low Concentration (1:5000)	10	0.0084±0.0012
Medium Concentration (1:500)	100	0.077±0.015
High Concentration (1:50)	1000	1.14±0.22

Table 2. Summary of experimental design and parameters used for analysis.

Parameters used for analysis

Parameter measured	Time intervals	Treatments	Replicates	Number of fish per replicate	Number of fish per treatment	Total number of fish
Darting episodes	Time interval 1 Time interval 2					
Swimming speed		Control Low Medium High	6	5	30	120
Maximum acceleration	BLS Time interval 1 BLS Time interval 2					
Total distance traveled						
Inter individual distance						

Table 3. Mean values of behavioral parameters evaluated in this work

Behavioral parameter	Time interval	Skin extract concentration							
		Control		Low		Medium		High	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Swimming speed (cm/s)	Base line	3.13	0.19	3.93	0.24	5.39	0.40	5.74	0.29
	Exposure 1	3.47	0.24	4.57	0.29	4.89	0.35	4.86	0.27
	Exposure 2	3.39	0.21	3.98	0.29	3.28	0.30	4.33	0.29
	Base line subtracted Exposure 1	0.33	0.19	0.67	0.20	-0.49	0.23	-0.88	0.27
	Base line subtracted Exposure 2	0.26	0.14	0.05	0.23	-2.10	0.29	-1.41	0.30
Maximum acceleration (cm/s²)	Base line	18.17	1.41	18.08	1.55	25.12	2.34	23.61	1.88
	Exposure 1	19.08	2.07	23.42	2.57	30.15	3.59	21.22	1.78
	Exposure 2	20.57	3.64	23.69	3.52	14.83	1.32	20.15	1.68
	Base line subtracted Exposure 1	0.91	1.96	5.33	2.99	5.03	4.48	-2.39	2.93
	Base line subtracted Exposure 2	2.40	2.97	5.60	4.23	-10.28	2.57	-3.46	2.71
	Base line	35.61	2.90	35.35	2.24	41.00	3.77	51.72	2.69
	Exposure 1	37.25	2.53	41.16	2.66	37.95	3.36	43.78	2.52
	Exposure 2	33.55	2.20	35.90	2.67	25.60	2.50	40.53	2.97

Total distance traveled (m)	**Base line subtracted Exposure 1**	3.08	1.72	0.55	2.07	-4.48	2.13	-7.93	2.46
	Base line subtracted Exposure 2	1.41	1.47	-4.48	2.13	-18.87	2.65	-11.18	2.80
Interindividual distance (cm)	**Base line**	31.24	1.50	39.16	0.71	30.15	0.97	37.85	0.65
	Exposure 1	28.80	1.47	35.24	0.87	29.51	0.90	36.33	0.94
	Exposure 2	28.59	1.40	35.60	1.05	25.64	1.17	36.62	0.94
	Base line subtracted Exposure 1	-2.14	0.80	-3.56	0.98	-4.50	1.08	-1.52	0.94
	Base line subtracted Exposure 2	-3.99	0.84	-0.61	0.86	-1.52	0.94	-1.22	0.94

Chapter 3

Effects of copper in the olfactory system of Delta Smelt (*Hypomesus transpacificus*): from tissues to

behavioral responses.

Abstract

The Delta Smelt is an endangered fish native to the San Francisco Estuary (SFE) in California. Currently little is known about its olfactory biology and how it is affected by contaminants. The olfactory system is critical for the survival of fish and is involved in several aspects of their life history as reproduction, kin recognition, predator detection and avoidance. Impairment of olfactory function can decrease fitness and ultimately survival of this fish species. I evaluated the morphological responses of the olfactory epithelium and behavioral olfactory mediated antipredator responses to alarm cues in Delta Smelt exposed to copper, a well-known sensory toxicant and common contaminant in the SFE. Fish were exposed to 4 copper concentrations (0, 2, 8 and 32 µg/L) in two time exposures of 24 and 96 hours and evaluated for antipredator behavioral responses (escape response, darting behavior and freezing behavior) using tracking technology. Olfactory tissues were processed for histopathological evaluation and expression of activated caspase-3 and $G_{\alpha S/olf}$ using immunohistochemistry (IHC). Delta Smelt were highly susceptible to copper exposure with high mortality at 32 µg/L at the end of 96 hours exposure. The olfactory epithelium showed acute cell swelling, degeneration and death of olfactory neurons in a concentration dependent fashion when exposed to copper independently to the exposure time. Activated caspase-3 IHC showed an increase of death neurons in fish exposed to copper, and $G_{\alpha S/olf}$ IHC demonstrated a decrease in the number of ciliate neurons, together indicating an overall toxic effect of copper exposure to olfactory sensory neurons. The behavioral antipredator responses were altered when fish exposed to 8 µg/L of copper with an overall hyperresponsive state and non-responsive state at 32 µg/L during a 24-hour exposure, while a 96-hour exposure caused hyporresponsiveness to alarm cues at 8 µg/L. This changes in behavior were in accordance with the morphological alterations in the olfactory epithelium observed at these concentrations. Overall, short exposures to environmentally relevant concentrations of copper can injure the olfactory epithelium and impair olfactory functions of Delta Smelt.

1. Introduction

The increase of human population and anthropogenic activities have changed the environment and the ways in which animals perceive and interact with it (Vitousek et al., 1997). Wild animals living in highly transformed and intervened ecosystems are challenged with a wide variety of sensory stimuli coming from anthropogenic sources that increases the signal to noise ratio of perception leading to altered behaviors and finally a decrease in survival success for a species (Dominoni et al., 2020, Saaristo et al., 2018). For example, chemical contamination of aquatic ecosystems may lead to disruption of perception in chemically mediated information, ultimately decreasing and altering pivotal behaviors as mating (Fisher et al., 2006), foraging, migration or predator avoidance (Halfwerk and Slabbekoorn, 2015, Lürling, 2012, Dominoni et al., 2020).

The chemosensory assessment of the environment is crucial for survival of many aquatic animals. Recently several studies have shown that environment degradation and climate change can impair the ability of aquatic animals to assess environmental conditions through chemical cues (Saaristo et al., 2018, Sievers et al., 2018). Ocean acidification, chemical pollutants and habitat degradation have been listed as environmental changes that can alter chemically mediated behaviors in aquatic animals (Chivers et al., 2016).

A wealth of information and research exist on the effects of pollutants at different organismal levels, but in many cases an integrated approach at different levels of organization is more relevant and needed to understand the complexities of altered environments by anthropogenic interventions. Behavioral toxicology is starting to fill these gaps, joining the research on sublethal effects at the molecular and tissue levels to the organismal and population levels on real time responses in organisms exposed to contaminants (Pyle and Ford, 2017). Water quality guidelines generally do not stablish behavioral endpoints if ecological relevance or compromise to fitness is not demonstrated. Moreover, the use of

ecotoxicology studies that include behavioral endpoints is low (Ågerstrand et al., 2020). Ecological

relevance in behavioral endpoints pertains to behaviors that are important enough to a species ecological

competitiveness (Ågerstrand et al., 2020), and this may be especially true in highly modified ecosystems.

The San Francisco Bay Delta (Bay-Delta) is one of the largest estuaries in the North America, comprising

around 1500 km^2 of aquatic habitat (Cohen and Carlton, 1998). The Bay-Delta is a largely transformed

ecosystem with most of its area changed by channels, levees, dams, and in addition to a watershed that

is highly urbanized and transformed by agricultural activities, draining a high input of anthropogenic

contaminants (Smalling et al., 2013, Weston et al., 2014). One of the iconic species in the Bay-Delta is the

Delta Smelt (*Hypomesus transpacificus*), a fish that once was abundant and ubiquitous in this ecosystem

but now has been declared as extremely endangered and facing extinction (Hobbs et al., 2017, Moyle et

al., 2016) due to several factors that changed the Bay-Delta, including water diversions, climate change

and contaminants (Moyle et al., 2018).

The effects of contaminants detected in the Bay-Delta are well studied in Delta Smelt at different

organization levels from molecular responses (Connon et al., 2009), tissue responses (Teh et al., 2020)

and behavioral responses (Segarra et al., 2021). However, just recently the effects of environment changes

in the sensory responses of Delta Smelt have been evaluated at the behavioral level (Davis et al., 2019)

and studies on the effects of common contaminants on sensory systems and sensory mediated behavioral

responses are lacking.

Copper has been widely investigated as a contaminant of concern for aquatic organisms in the San

Francisco Bay-Delta (Buck et al., 2007, Connon et al., 2011). Historically, copper became a common aquatic

contaminant since the gold rush left old mining sites from which acid drainage containing several metals

pour to natural aquatic environments (Buck et al., 2007, EPA, 2007). Following urbanization and

agricultural development, copper use became common in several agricultural and industrial formula (EPA,

2007, Sinclair-Rosselot, 2006). Several forms of inorganic copper are used as herbicides and fungicides (de Oliveira-Filho et al., 2004). Also, antifouling paints and automotive parts (Davis et al., 2001, Jabusch et al., 2018) are sources of copper discharges to aquatic environments through stormwater and urban runoff (Buck et al., 2007). The concentration of dissolved cupric ion (Cu^{2+}) can reach toxic concentrations for aquatic life at source points in which runoff and storm water reach delta tributaries, ranging from 3.63 µg/L to 52.7 µg/L of dissolved copper (Gilbreath et al., 2019). In the environment, copper is highly complexed with organic ligands, and in the Bay Delta, the total copper concentrations range from 0.43 µg/L to 3.29 µg/L (Buck et al., 2007, Buck and Bruland, 2005), although recently, copper concentrations in the Sacramento and San Joaquin rivers, ranged from 0.83 µg/L to 4.37 µg/L (Jabusch et al., 2018). Even at low concentrations, copper is highly toxic for aquatic life, with effects ranging from mortality to sublethal effects involving sensory behavioral info disruption (Tierney et al., 2010, Grosell, 2011).

The effects of copper in the olfactory tissue or on the olfactory mediated antipredator response has not been evaluated so far in an endangered indicator species such as Delta Smelt. A better understanding of the effects of common contaminants such as copper and its effects in essential behaviors as the anti-predatory response can provide information critical for habitat conservation and restoration therefore helping in decision making for a rational use and management of water for habitat preservation and economics in California.

I aimed to evaluate the effects of a short (24 hour) and long (96 hour) pulses of copper exposure under concentrations that are environmentally relevant on the olfactory mediated anti predatory behavioral responses and the morphology of the olfactory epithelium of Delta Smelt. The susceptibility of the Delta Smelt olfactory system to dissolved copper has not been assessed and at what concentrations copper alters normal olfactory mediated behaviors is unknown. My study was designed to investigate the effects of different levels of copper and exposure time, testing the hypothesis that the effect of copper varies with concentration and exposure time. I predicted that the functional impairment and morphological

alterations will be more severe with higher concentrations and longer exposures. I addressed three main questions: 1) Does copper at these concentrations induce evident and significant changes in the morphology of the olfactory epithelium? 2) Does copper at environmentally relevant concentrations found in the Bay-Delta affect the antipredator behavior of Delta Smelt? and 3) Is there a relationship between exposure time and concentration on the effects of copper on morphology and behavior?

2. Materials and Methods

Fish acclimation and care: adult Delta Smelt (n=224, age: 280 days post hatch (DPH), fork length: 7.45±0.63 (mean±SD) centimeters, weight: 3.30±1.04 (mean±SD) grams) were transported in 90-liter insulated carboys in late-October 2019 from the Fish Conservation and Culture Laboratory (FCCL) in Byron, CA, to the Center for Aquatic Biology and Aquaculture (CABA) at the University of California. The transport water was conditioned with five parts per thousand of (ppt) Instant Ocean salt, oxygenated to 90% saturation and kept at 12° C. Fish originated from a population of captive bred Delta Smelt (F0) that came from the San Francisco Estuary and was then bred for 12 (F12) generations in captivity using established methods for the species (Lindberg et al., 2013). In order to prevent inbreeding and adaptation to the hatchery, generations of Delta Smelt were bred using established genetic management protocols and exposed to semi-natural conditions (life stage differential lighting, rearing in multifamily groups, family size equalizing) (Lindberg et al., 2013), during their life cycles in the conservation hatchery (Fisch et al., 2012).

Fish were acclimated for two weeks at CABA in four 300-liter circular tanks, which were connected to a recirculating system supplied with well water. The water quality parameters (mean±SD) were as follows: temperature: 15.57±0.62°C, pH: 8.65±0.09, dissolved oxygen: 8.91±0.46 mg/L and ammonia: 0.10±0.18 mg/L. The fish were fed 3% of their total biomass daily with a balanced ration (BioPro2-CRUM #1, Bio-Oregon®, Vancouver). Fish welfare and experimental protocols were approved by the University of

California Davis Institutional Animal Care and Use Committee (protocol no. 20052). Experiments were performed in November and December 2019.

Experimental Copper solutions: To prepare standard synthetic fresh water (EPA, 2002), salts (CaCl$_2$.2H$_2$O, NaHCO$_3$, KCl and MgSO$_4$) were added to deionized water in a 1000-L Nalgene® high-density polyethylene cylindrical tank, that was coupled to a recirculating pump. The synthetic water was then recirculated and aerated for at least two days. Two days before the trials, a copper stock solution (nominal 400 µg/ml) was prepared by dissolving copper (II) chloride dihydrate (American chemical society reagent, purity ≥99.0%, CAS 10125-13-0, Millipore Sigma) in ultrapure water (18.2MΩ/cm ddH2O, Milli-Q®, Millipore-Sigma, USA) and stored this solution at 4°C. The exposure copper solutions (hereafter exposure water) were prepared in 22-L plastic Cubitainers® by diluting the copper stock solution with synthetic water. The exposure solutions were then stored at room temperature (16 °C) for two days until the beginning of the exposure experiments.

Dissolved ion measurements: Concentration of dissolved ions (Cu^{2+}, Cl$^-$, Na$^+$, Ca^{2+}, Mg^{2+}and K$^+$) were measured at the beginning and end of each experiment (**Table 1**). In brief, 50ml of exposure water was sampled using polypropylene centrifuge tubes that had been washed three times with the exposure water. The water samples were then acidified with trace metal nitric acid (67 to 70% HNO$_3$, Fisher scientific) to a concentration of 3% percent volume and stored at 4°C prior to analysis. The ion concentrations were measured by Inductively coupled plasma (quadrupole) mass spectrometry (ICP-MS) at the interdisciplinary center for plasma mass spectrometry at the University of California, Davis, United states (http://icpms.ucdavis.edu/).

Copper exposure: Exposure times were 24 and 96 hours (**See Figure 1**). Four copper concentrations (0 µg/L (control), 2 µg/L, 8 µg/L and 32 µg/L, N= 4 replicates, 7 fish per replicate; 28 fish per concentration, 112 fish per exposure time (224 fish total), see **Table 2** for measured concentrations of copper at each

exposure time. The exposure water was transferred to black polyethylene 16-liter plastic buckets. Buckets were then placed in a water bath with controlled temperature for 24 hours before exposure started. Then the fish, were allocated randomly from one of the rearing tanks to each concentration. Feeding was suspended for 24 hours prior to and during exposures. Each bucket was covered, and a glass pipet attached to tubing was inserted through the lid to provide constant aeration. The fish were exposed as follows: two replicates of each treatment (*i.e.*, control, 2 µg/L, 8 µg/L or 32 µg/L) were started at 7:00 (start time 1), and the next two replicates were started at 12:00 (start time 2) on the same day. All concentrations for one replicate were tested on the same day (comprising a temporal block), and the order was randomized by pulling numbers out of a bag. This procedure was done every day for the 24-hour exposures and every four days for the 96-hour exposure. We performed the 96-hour exposures in a semi-static way, where half the exposure water was changed every 24 hours. Fish mortality was recorded every 24 hours. The water quality parameters were as follows: 24-hour exposure (mean±SD) temperature 15±0.28C°, dissolved oxygen 8.24±0.65 mg/L, nitrite 0.0032±0.0009 mg/L, nitrate 0.0014±0.0037 mg/L, pH 7.51±0.03, hardness 81.71±3.14 mg/L $CaCO_3$, alkalinity 56.85±1.06 mg/L and ammonia 0.022±0.056 mg/L and 96-hour exposure temperature 15.3±0.82C°, dissolved oxygen 8.49±0.53 mg/L, nitrite 0.003±0.001 mg/L, nitrate 0.017±0.0262 mg/L, pH 7.57±0.14, hardness 82.85±1.95 mg/L $CaCO_3$, alkalinity 53.14±6.81 mg/L and ammonia 0.02±0.052 mg/L.

Histopathology and Immunohistochemistry: Immediately after the behavioral trials (**see next section on behavioral trials**), the fish were euthanized following the American Veterinary Medical Association (AVMA) guidelines for euthanasia of animals (Leary et al., 2013) and sub-sampled for histopathological and immunohistochemical analysis. I placed the fish in ice water for 10 minutes until opercular movements and peduncular reflexes stopped. I then severed the spinal cord just behind the operculum. In total, ten fish (two to three per replicate) for each copper exposure concentration were used for this purpose. After euthanasia, each fish was measured, weighed and fixed in 10% neutral buffered formalin

for 48 hours. After fixation, the olfactory rosettes were dissected at 7X to 70X magnification using an Olympus SZH10 (Olympus Corporation, Japan) research stereomicroscope as follows. I placed the fixed fish in a plastic tray containing phosphate buffered saline (PBS) and used micro-scissors to remove the skin and surrounding tissue covering the nasal cavity to fully expose the rosette. Then, the nasal bones containing the whole rosette were removed using a razor blade. Each whole rosette was placed in an individual tissue cassette, dehydrated in ascending concentrations of alcohols, cleared in xylene and embedded in paraffin (TissuePrep 2, Fisher Scientific). The tissue blocks were sectioned at 3 μm thickness using a rotatory microtome. Sections were wet mounted on glass slides and stained with Hematoxylin and Eosin (H&E, Millipore sigma, Darmstadt, Germany).

For immunohistochemical analysis, the following primary antibodies were used: 1) Mouse monoclonal anti G $_{\alpha \, S/olf}$ (C-10, 1:500 dilution sc:377435 Santa Cruz Biotechnology) against the G protein alpha olf subunit specifically to identify ciliated olfactory neurons and 2) Rabbit polyclonal anti-cleaved caspase-3 (1:4000 dilution Cat# G7481, Promega, Madison, WI, USA). The primary antibodies were detected with ImmPRESS (Peroxidase) Polymer Reagent Horse-Anti-Mouse IgG MP-7402 (Vector Labs) for monoclonal antibodies and ImmPRESS (Peroxidase) Polymer Reagent Horse-Anti-Mouse/Rabbit IgG MP-7500 (Vector Labs) for polyclonal antibodies. Omissions of primary antibodies were used as a negative control. Tissue sections were cleared in Xylene substitute (Histoclear), followed by rehydration in descending concentrations of alcohol and a final rinse in deionized water. The endogenous peroxidase was quenched with hydrogen peroxide (1% in PBS buffer) for 20 minutes, followed by heat antigen retrieval in citrate buffer (pH 6.1) (Target retrieval solution, S1 699, Dako) for 30 minutes at 92°C using a commercial vegetable steamer (BLACK + DECKER, USA). The nonspecific binding was blocked with 10% normal horse serum (Vector Laboratories, Burlingame, CA, USA), plus 1% bovine serum albumin (Sigma) and 0.1% Tween^{TM} 20 (Fisher BioReagents) for one hour. The sections were incubated with the primary antibodies for three hours and then with the secondary antibody for 30 minutes in a humidified chamber at room

temperature. The antigen-antibody reaction was revealed with vector Nova-Red peroxidase substrate kit (Vector laboratories, SK-4800) counterstained with Mayer's hematoxylin.

Histopathological assessment: The morphologic changes in the olfactory epithelium were evaluated by an observer blind to treatment using a semi-quantitative score that included five classes of lesions or abnormal changes based on initial observations of control and copper exposed fish (**See Table 3**). These five classes were: 1) indications of cell death (nuclear pyknosis or fragmentation, karyorrhexis), shrunken or rounded hyper-eosinophilic cytoplasm and cellular isolation), 2) lack of surface structures (cilia), 3) disruption to the overall organization of the epithelium, 4) inflammatory infiltrates and 5) degenerative changes including cytoplasmic vacuolation and cell swelling. Each of these categories was then assigned a quantitative score between zero and three (0: unremarkable, or normal structure; 1: mild; 2: moderate; and 3: severe). The scores for each category were summed to obtain a composite score for each fish.

Assessment of sensory epithelial thickness: To indirectly evaluate loss of cells in the sensory epithelium, I evaluated the lamellar epithelial thickness. The epithelial thickness is defined as the distance (in microns) from the apical portion of the epithelium excluding ciliary structures, to the basal domain of the epithelium. From each rosette, three representative lamellae were evaluated using an Olympus BX60 microscope coupled to a DP71 camera. The thickness was measured at three positions along the length of the lamellae: near the central raphe, the middle of the lamella and the periphery of the lamella. The measurements were taken with the CellSens software (V 1.8.1 Olympus Corporation of the Americas, Center Valley, PA) using built-in measurement tools.

Immunohistochemical assessment

$G_{\alpha S/olf}$ *staining assessment:* To further evaluate whether there was any loss of sensory neurons in response to copper exposure, I used an antibody against the $G_{\alpha S/olf}$ subunit of the GTP binding protein coupled olfactory receptors. This subunit is a known marker for ciliate neurons in fish (Hansen et al., 2004). Ciliate

neurons are one of the most abundant olfactory neurons in the sensory epithelium (Hansen and Zielinski, 2005) and have been shown to be susceptible to copper exposure in other species (Lazzari et al., 2017, Ma et al., 2018). In Delta Smelt (**described in chapter 1**), $G_{\alpha\,S/olf}$ is mostly localized to the surface structures (including the sensory cilia and the olfactory knob), the cell membrane and the cytoplasm of the ciliate neurons. Because ciliated neurons are surrounded by sustentacular cells that do not stain with this antibody, the $G_{\alpha\,S/olf}$ staining gives a somewhat orderly striped pattern to the epithelium (**Table 4, $G_{\alpha\,S/olf}$ Normal (0)**). Based on this normal pattern and the variations observed in the fish exposed to copper, I designed a semi-quantitative score which included the following characteristics (**see Table 4 for examples**): 1) disruption of the striped pattern, 2) missing surface features (cilia and olfactory knobs) and 3) reduced staining intensity. For each characteristic, I assigned a score from 0 to 3, in which 0 indicated normal histology, 1 indicated mild changes, 2 moderate changes, and 3 severe changes.

Apoptotic index: To evaluate cell death, I used a previously established apoptotic index with some modifications (Guedes et al., 2017).This method is based on counting the number of apoptotic cells vs non apoptotic cells and adding up in quadrants to get an apoptotic index for the tissue. For each fish, I counted cells from three different lamellae using image J software. Each image (400X) was scaled to microns, and then a grid with an area of 6000 μm^2 per quadrant was overlayed on the image. Within four central quadrants, I counted the number of apoptotic fragment profiles positive for anti-cleaved caspase 3 and divided it by the total number of non-apoptotic nuclei and multiplied this fraction by a 100. Results from three lamellae were averaged to get an apoptotic index for each fish. The number of positive apoptotic fragments profiles were counted with the cell counter plugging in image J. The total number of cells was obtained by counting the total number of nuclei within the selected area. If a cell was touching any of the grid lines, it was not counted. When several small fragments were clustered near a larger fragment, I counted this as a single apoptotic cluster.

Preparation of the conspecific skin extracts (conspecific alarm cue): I used the same method as described in Chapter 2. The fish were euthanized as described before (**See histopathology and immunohistochemistry section**). A total of 30 donor fish were used for skin extract preparation. After euthanasia, I weighed and measured each fish, wrapped them in aluminum foil and flash froze them in liquid nitrogen for further dissection. The skin samples were dissected from each side of the fish using the methodology of Wisenden (2011) with some modifications as follows: I thawed the fish and washed any excess mucus from the skin with ultrapure water (18.2MΩ/cm ddH2O, Milli-Q®, Millipore-Sigma, USA). After mucus removal, each fish was placed on top of a frozen cryo-gel pad covered with aluminum foil for skin dissection. I used a razor blade to make a superficial cut along the dorsal and the ventral surface of the fish's body. The skin was gently pulled out to separate it from the muscle to yield a single skin sample. After removal, the skin samples were weighed, placed in a microcentrifuge tube and flash frozen in liquid nitrogen. The skin was then ground into a fine powder with an autoclaved pestle on the same tube. Then, 1 mL of Ultra-pure water was mixed into the powder, followed by homogenization for 1 minute with a drill and autoclaved pestle. Then, 0.5 ml of Ultra-Pure water was added to the tube and mixed by hand shaking, followed by centrifugation at 14000 rpm for 1 minute at 4°C. The supernatant was recovered, transferred to a clean 1.5 ml Eppendorf microcentrifuge tube, and stored at -20°C until use.

Dilutions: Skin extract was diluted in ultrapure water (100 µl of raw skin extract diluted to a final volume of 50 ml, dilution 1:500). I previously demonstrated that this concentration elicits an escape response in Delta Smelt (see chapter 2 and Davis et al. (2019)).

Behavioral arenas: Two circular arenas were used to evaluate behavioral responses to skin extracts. I used a shuttle box system (Loligo® Systems, https://www.loligosystems.com/shuttle-box-system), where typically the arenas connect through a shuttle (**Figure 1, B**). I modified this system to have two independent arenas by sealing the opening to the shuttle on each side. Each arena was 1 m diameter and 20 cm high, with a volume of 117.8 liters. The arenas were filled with synthetic standard fresh water.

Water circulation was driven independently in each arena by an external pump (**Figure 1, B**). Each pump pumped water from the arena into and external buffer tank through plastic tubing at 5 liter/minute. Water from the buffer tank was fed by gravity into the arena to generate a unidirectional water current of 4.68 cm/s. The arenas were illuminated from underneath using a custom-made light box with an average lux intensity of 146 ± 6 lumen/m^2 (mean$\pm$SD) and surrounded with a black plastic curtain to minimize external influences on fish behavior.

Behavioral testing: For each exposure time (24 and 96 hours), I performed 4 replicates per concentration with 5 fish per replicate (although 7 fish were exposed, two were used for other purposes.) Each group of five fish was used only once so each replicate was treated as an independent group. The four concentrations were randomly assigned to the two arenas using a random number generator such that each of the four concentrations was run each day after the copper exposure start time finished, and the fish were acclimated for one hour in the arena, with two concentrations tested simultaneously in the morning (8:00) or afternoon (13:00) on the same day. Thus, each day comprised an experimental block (**Figure 1, A, C**). Each group of five fish was allowed to acclimate in the arena for one hour before the trial began.

Trial recording setup: Trials were recorded using two GoPro Hero Black 6 cameras (30 Frames per second in linear mode) positioned above each arena, at a height of 1.20 m. The recording of a trial lasted 45 minutes and began following an acclimation period of one hour. For the first 15 minutes, baseline behavior (baseline period) was recorded. Then an alarm cue was added, and behavioral responses were recorded for over 30 minutes. At the start of this 30-minute period, three doses of alarm cue (50 ml diluted skin extract per dose, 1:500 dilution, this dose was used based on a strong response in previous behavioral trials; **see chapter 2**) were applied to the buffer tanks at an interval of one minute using plastic syringes. At the conclusion of the trial, fish were euthanized for histology. The two arenas were then washed three times with a soap-bleach diluted solution, followed by a wash with 100% ethanol and a final rinse in

deionized water. For each trial, all tubing was replaced with clean tubing to avoid any contamination with odorants from previous trials.

Video recording analysis: The video recordings were analyzed with Noldus Ethovision XT 14-tracking Software with the social interaction module (V. 14.0, Noldus Information technology, NL). High-resolution videos were converted from mp4 to mpeg-2 format using 4Media HD Video Converter (version 7.8.23.4 Media Software Studio, Xilisoft Corporation 2018). To adjust the frame rate at which the videos were tracked, we used a calibration curve assigning "speed" as the response variable and "frame rate" as the independent variable. This avoided under- or over-estimate of the evaluated parameters and ensured consistent results over the whole analysis (Noldus Ethovision XT 14, Help manual, Sample rate). We tracked the fish at a frame rate of 3.75 frame/s.

In preliminary trials, it took approximately 30 minutes for dye to disperse throughout the test arena, and I could not accurately predict when a fish would encounter the odor filament. I therefore analyzed data in three 15-minute time blocks: the 15-minute baseline period, time interval 1 (15 to 30 minutes) and time interval 2 (30 to 45 minutes).

Behavioral parameters: For each fish, I determined the number of darting episodes, the swimming speed (cm/s), maximum acceleration (cm/s^2), total distance traveled (meters) and interindividual distance (cm) using Ethovision. Baseline subtracted data of each fish were used for statistical analyses.

Data handling: For darting episodes, I used the total number observed for the group of fish in the arena 30 minutes following the presentation of the cue for each trial. Swimming speed, interindividual distance and total distance moved was calculated from the x, y coordinates in Ethovision on each frame for each fish. The maximum acceleration observed for each fish at each time interval was used for analysis. The acceleration was calculated by dividing the difference in swimming speed of each fish during one second of recording by the time difference in any second of recording. The interindividual distance was given by

the distance between a focal fish with respect to each of the other four fish. Raw data were edited and evaluated for missing frames before averaging. Missing frames occurred when a fish either was not recognized by the tracking software or overlapped with other fish tracks at some point. When missing frames were observed, the interpolation tool in Ethovision was used to reconstruct them in each tracked fish. The data used for analysis were obtained as follows: 1) the raw data output (frame rate: 4 observations per second) was averaged to obtain one mean observation every second (2700 data points per trial). This was done for every fish in every trial. 2) For each parameter analyzed, the mean value was obtained for each fish for each time interval. This was done for each trial. 3) The 15-minute baseline values were subtracted from each 15 minute time interval for each fish and used for statistical analysis. A positive change implies increase in the magnitude and a negative change implies a decrease in the magnitude of the measured parameter with respect to the baseline.

3. Statistical analysis

All the parameters were evaluated using an $\alpha=0.05$, presented as mean ± standard deviation (SD) for parametric data and median interquartile range (IQR) for non-parametric data. All analyses were performed using JMPpro software V 15.1 (SAS institute, Cary, NC).

Survival analysis: Mortality was recorded for each treatment every 24 hours. For the 96-hour exposure, I fitted a Kaplan-Meier curve to compare mortality rates among groups. A log rank (Mantel-Cox) test was used to determine significant differences in the survival distribution among treatments. I then fitted a logistic regression to estimate the concentration of copper at which the expected survival is 95, 75, 50, 25 and 5%.

Histopathological analysis: I analyzed the relationship between the discrete variables (treatment and histopathology score) and (treatment and immunostaining score) using Spearman's rank correlations. To evaluate differences in histopathology and $G_{\alpha S/olf}$ immunostaining scores among treatments, I used a non-

parametric Kruskal-Wallis analysis of variance and a post-hoc Dunn test for joint ranks. I evaluated the effects of copper concentration, exposure time and interactions with epithelial thickness and apoptotic index using a factorial ANOVA. All data were evaluated for normality assumptions using Shapiro-wilk test and Levene's test for homogeneity of variance. The apoptotic index was Log 10 (n+1) transformed to meet the normality assumptions.

Behavioral assays: The numbers of darting episodes were not normally distributed and were therefore evaluated for significant differences among treatments using a Kruskal Wallis ANOVA and a post hoc Dunn test for joint ranks. For the remaining variables, the data were evaluated for normality and homogeneity of variance using residual analysis. The copper concentrations and the baseline-subtracted time interval (BLS time interval 1 and 2) were used as explanatory variables. The response variables were swimming speed (cm/s), maximum acceleration (cm/s^2), total distance traveled (m) and interindividual distance (cm). These parameters were assessed using identical factorial ANOVAS in which the interaction was treatment by baseline-subtracted time interval. I used 'day' and 'fish' as random effects, with 'fish' nested within 'day', and 'fish' nested within treatments (copper exposure concentration) to account for multiple measurements of the same fish. The blocking variable 'day' was included as a random effect, but it was insignificant and removed from the final analysis. Also, planned linear contrasts were used for each concentration to compare the baseline-subtracted time intervals with each other and among treatments. Data are presented as mean ± SE or median and Inter quartile range (IQR) for nonparametric data.

4. Results

4.1 Susceptibility of adult Delta Smelt to copper exposure

Survival at 24 hours copper exposure: Delta Smelt generally survived the short-term copper exposures, except at highest copper concentrations where some mortality and toxicity impacts were noted. The survival rate was 100% for all the control, 2 and 8 µg/L copper concentrations. In the 32 µg/L treatment,

I observed four dead fish in one of the replicates at the end of the 24 hours exposure (85.71% survival rate, overall). The fish exposed to 32 µg/L of copper also showed signs of toxicity by aberrant swimming patterns lacking a swimming axis, where fish either swim laterally on their side, upside down or vertical.

Survival at 96-hour copper exposure: Delta Smelt were more susceptible to long-term copper exposure, especially at the highest concentration. The survival was 100% for the control, 2 and 8 µg/L of copper, except for 1 fish that died in the copper 2 µg/L concentration after 48 hours. In the 32 µg/L treatment, four fish survived in the four replicates during 96 hours of exposure. In this treatment, three were found dead during the first 24 hours of exposure, 6 fish died and 3 were euthanized showing signs of toxicity at 48 hours of exposure and 11 fish died and 1 was euthanized at 72 hours of exposure. The survival distributions between treatments were statistically different (log rank test, χ^2_3=106.7886, *P* < 0.001). The difference in survival became significant at 48 hours of exposure in the treatment of 32 µg/L of copper. The fish in the 32 µg/L treatment had a median time to mortality of 72 hours, 95% CI (48-72), while in the control, 2 and 8 µg/L copper treatments, the survival was 100% at 96 hours (**Figure 2, A**). Based on my prediction from mortality using the measured concentrations of copper (**see Table 2)**, the survival of adult Delta Smelt was within 95% in a predicted concentration of 9.60 µg/L (95% CI 0.63-14.40), 75% at 16.97 µg/L (95% CI 11.38-20.71), 50 % at a concentration of 21.35 µg/L (95% CI 17.14-25.12), 25 % at 25.74 µg/L (95% CI 22.07-30.35) and 5 % survival with a concentration of 33.11 µg/L (95% CI 28.82-40.66) of copper (**Figure 2, B**).

4.2 Histopathological alterations

24-hour copper exposure

Overall, I found that a 24-hour copper exposure impacted olfactory tissue morphology at concentrations found to be sublethal to Delta Smelt. I observed significant differences in the histopathological scores of fish exposed to copper compared to non-exposed control groups (Kruskal Wallis ANOVA, $\chi^2_{,3}$ =34.9241,

P<0.0001) (**Figure 3A**). Compared to controls, the histopathology score was significantly higher in the fish exposed to 8 µg/L (Dunn, Z=3.54, *P*=0.0012) and 32 µg/L of copper (Dunn, Z= 5.55, *P*<0.0001), with a median score of 9 (IQR=2.25) and 15.5 (IQR=1), respectively. However, scores were similar to controls at the lowest concentration tested (2 µg/L of copper and the control group; Dunn, Z= 1.60, p=0.3253). I observed a strong association between the histopathology score and the concentration of copper (**Figure 3B**) with an increase in severity of the lesions following an increase in the copper concentration (Spearman's rank correlation, P=0.9452, *P*<0.0001).

The morphology of the olfactory epithelium in fish from control groups (**Figure 4A and B**), resembled that of healthy, adult Delta Smelt that have been previously described (**see chapter 1 for description**).

Figure 4C and D, shows the olfactory epithelium of fish exposed to 2 µg/L of copper. At this concentration, the cytoplasm of ciliated neurons looked diffusely micro-vacuolated, with a clearer cytoplasm and expanded dendritic process. In some of these neurons, there is a clear halo surrounding the nucleus (endoplasmic reticulum swelling). Dead cells were frequently observed and characterized by rounding of the cytoplasm and pyknotic small nuclei. The epithelium looks mildly disorganized, and cells are more separated from each other.

In fish exposed to 8 µg/L (**Figure 4E and F**), I observed what I concluded to be olfactory receptor neurons (by its nuclear morphology and position: round, mid-basally location within the epithelium and position in between two sustentacular ciliated cells with an elongated sausage shaped nucleus) in several stages of degeneration. Degenerated neurons were characterized by swollen and fibrillar cytoplasm, rounding and separation from neighboring cells. The cytoplasm became more eosinophilic and the nuclei pyknotic, followed by condensation of the cytoplasm and fragmentation of the nuclei, which left a clear space with small fragments or remnants of dead cells. The clusters of vacuolated or degenerated neurons were more frequent and the disorganization of the epithelium was moderate in numerous sustentacular cells with

more elongated nuclei. Dead cells were also more abundant and scattered basally and within the mid-level of the epithelium.

In the fish exposed to 32 µg/L of copper (**Figure 4G and H**), the epithelium was disorganized, lacking apical structures and olfactory knobs; cilia were lost or shortened in some lamellae. The neurons looked swollen with pale cytoplasm. I also noted an abundance of dead and fragmented cells throughout the entire epithelium, along with large gaps between neighboring sustentacular cells. These dead neurons were lysed forming small lacunae with clear spaces and fragments of dead cells. The "normal" look of the epithelium was replaced with sustentacular cells showing abnormally elongated nuclei spanning the entire cell cytoplasm. The bodies, nuclei and cytoplasm of sustentacular cells look more prominent and are more visible due to the loss of olfactory neurons. There was also a marked increase of round cells with round nuclei in the upper superficial region of the epithelium, with gaps between cells. There was mild infiltration of inflammatory cells in the lamina propria. The wedge shape of the sustentacular cells was more visible with apical degeneration and a loss of cilia on the apical domain. These features are characteristic of both necrotic and apoptotic cell death

96-hour copper exposure

The histopathological score was significantly higher (**Figure 5, A**) in the groups of fish exposed to copper compared to the control groups (Kruskal Wallis ANOVA, $\chi^2_{,3}$ =34.0886, *P*<0.0001). Similar to what was observed in the 24-hours exposure, the histopathology score was significantly higher in the fish exposed to 8 µg/L (Dunn, Z= 3.88, *P*= 0.0003) and 32 µg/L of copper (Dunn, Z= 5.44, *P*<0.0001) when compared to the control group, with a median score of 9 (IQR= 6.25) and 17 (IQR=1), respectively. In contrast, no significant differences were observed for the groups of fish exposed to 2 µg/L of copper when compared to the control group (Dunn, Z= 1.87, *P*=0.1843). I observed a strong association between the histopathology score and the concentration of copper (**Figure 5, B**) with an increase in severity of the

lesions following an increase in the copper concentration (Spearman's rank correlation, P=0.9768, *P*<0.0001)

The appearance of the olfactory epithelium from the control groups was in accordance with the characteristics of normal olfactory epithelium described previously (**see chapter 1**). The olfactory epithelium looked mildly disorganized in some focal small areas of the lamellae; however, the nuclei and cytoplasm were well defined without remarkable changes. The apical structures were well defined. Occasional dead cells were observed randomly throughout the epithelium (**Figure 6A and B**).

In the groups exposed to 2 µg/L at 96 hours, the epithelium looked diffusely micro vacuolated. Both sensory neurons and sustentacular cells were affected; however, sensory neurons were more evidently affected than sustentacular cells, characterized by a more clear and swollen cytoplasm. Separation of sensory neurons from neighboring sustentacular cells with formation of a clear space around them is already noticeable at this concentration in the 96-hour exposures. The surface structures are still conserved, and few dead cells were observed (**Figure 6C and D**).

In the 8 µg/L treatment, the sensory neurons were moderately to severely swollen with abundant clear fibrillar cytoplasm, and separation from sustentacular cells was noticeable. In this treatment, abundant basal swollen cells were visible characterized by a lightly eosinophilic (clear pink) poorly defined and vacuolated cytoplasm, with peripheralized highly basophilic nuclei. These cells were surrounded by clear spaces and separated from neighboring cells. The epithelium looked highly disorganized with proliferation of cells with elongated nuclei compatible with sustentacular cells. There were neurons with early changes like clear halos around the nuclei still visible in small areas of the epithelium but just rarely seen. Dead cells with pyknotic nuclei and condensed cytoplasm were frequently observed. Additionally, increase infiltration of inflammatory cells were observed within the lamina propria and epithelium. Prominent cells infiltrated and formed a clear lacunae within the epithelium, and these cells were characterized by having

a clear vesiculated cytoplasm and a round to oval central to peripheral nucleus with open and abundant euchromatin. Several clusters of these cells infiltrated the epithelium. All of these features are compatible with activated foamy macrophages (**Figure 6, E and F**).

In the 32 μg/L treatment, the epithelium was completely disorganized and disrupted with abundant dead cells, lack of surface structures and attenuation of the epithelial thickness. The epithelium was mostly composed by sustentacular cells, with elongated nuclei giving the epithelium a striped appearance. The nuclei of sensory neurons had a perinuclear round halo. The cells were rounded, smaller and swollen with clear spaces around them and evident separation from neighboring cells (**Figure 6, G and H**).

4.2 Apoptotic index

In general, I observed a significant effect of copper treatment in the apoptotic index (two-way ANOVA, $F_{3, 63.46}$= 49.25, *P <0.0001)* regardless of the exposure time (two-way ANOVA, $F_{1, 60.62}$=1.40, *P=0.2412*). The apoptotic index increased significantly 2- to 3-fold in all the copper treatments when compared to the control group, with an increase of apoptotic index in the highest concentration at both exposure times. In controls, few apoptotic cell profiles can be found in the olfactory epithelium. Most of these cells are in basal-middle-portions. At higher concentrations (8 and 32 μg/L), activated caspase 3 positive cells were observed at basal, middle and upper portions of the olfactory epithelium. I observed mainly at the middle portions immunoreactive cells with the characteristic morphology of olfactory neurons mid-level in the epithelium. As they die, these neurons lost the olfactory knob, and the dendrite retracted from the apical surface, while the cell fragmented and disintegrated into small apoptotic bodies that can be identified at the basal and mid-levels of the epithelium (**Figure 8**).

4.3 $G_{\alpha S/olf}$ Immunohistochemistry and epithelial thickness assessment

Normally, $G_{\alpha S/olf}$ staining is localized to the cytoplasm, olfactory knob and cilia of sensory neurons. The staining is homogeneous, finely granular and clearly delineates the profiles of ciliate neurons from the

surface of the olfactory epithelium to basal portion. The ciliate neurons are intercalated within sustentacular cells which normally do not express $G_{\alpha\,S/olf}$ giving a striped pattern to the epithelium. The localization of $G_{\alpha\,S/olf}$ to olfactory knobs and sensory cilia on the epithelial surface delineates with a continuous rimmed staining the epithelial surface.

I observed a positive association between the concentration of copper and the severity of the score in the different staining patterns analyzed. In general, there is an increase in the severity of changes in the total staining score with a severe loss of normal pattern and staining intensity with an increase in the concentration of dissolved copper at 24 hours of exposure (Spearman's correlation, P= 0.9358, *P<0.0001*) and 96 hours of exposure (Spearman's correlation, P = 0.8493, *P<0.0001*). The concentration of copper exposure did not affect the thickness of the olfactory epithelium (two-way ANOVA, treatment, $F_{3,65.7}$=1.2458, *P*=0.3003). However, I observed a significant effect of the exposure time in the reduction of epithelial cell thickness (Two-way ANOVA, Time, $F_{1,63.43}$=5.3309, *P*=0.0242).

24-hour copper exposure

A significant effect of copper treatment on the immunostaining score was observed at 24 hours of exposure (Kruskal Wallis ANOVA, $\chi^2_{,3}$ =34.9203, *P<0.0001*) (**Figure 9, 24 hours exposure**). The immunostaining score increased significantly in the fish exposed to 8 µg/L (median score of 6.5 (IQR= 1), Dunn, Z= 3.55, *P= 0.0012*) and 32 µg/L of copper (median score of 9 (IQR= 0), Dunn, Z= 5.29, *P<0.0001*), when compared to the control group (median score of 0 (IQR=0)). In contrast, no significant differences were observed for the groups of fish exposed to 2 µg/L of copper when compared to the control group (median score of 1 (IQR= 1), Dunn, Z= 1.02, *P=0.9230*).

Striped staining pattern: In fish from the control groups, the staining patterns were conserved without significant changes. There were 10% of fish (one out of ten) with mild changes in the distribution and organization of the stripped pattern within the epithelium. Similarly, at 2 µg/L of copper, 10% of the fish

(one out of ten) had mild changes in the distribution of the stripped pattern. In contrast, at higher concentrations of copper, the severe changes in staining were 40% of the fish (four out of ten) exposed in 8 µg/L treatments and 100% (ten out of ten) in 32 µg/L treatments.

Surface staining pattern: In the control groups, 10% of the fish (one out of ten) presented mild changes in the distribution of the surface staining. At 2 µg/L, mild and moderate changes in this pattern started to be noticeable, with 50% of the fish (five out of ten fish) affected. A bigger proportion of fish (80%, eight out of ten) had moderate changes in the distribution of the surface staining patterns at 8 µg/L with a 100% severe change (ten out of ten), a complete loss of staining patterns at 32 µg/L.

Stain intensity: Similarly, the stain intensity score increased with the increase in concentration of copper at 24 hours of exposure, with a severe loss of staining intensity in 90% (nine out of ten) of the fish exposed to 32 µg/L.

Epithelial thickness: A 24-hour copper exposure reduced epithelial cell thickness by about 4 µm (3.92 µm) in the group exposed to 32 µg/L of copper (39.94±1.50 µm, mean ± SE) compared to the control group (43.86±1.97 µm, mean ± SE).

96-hour copper exposure

At 96 hours of exposure, we observed a significant effect of copper treatment on the immunostaining score (Kruskal Wallis ANOVA, $\chi^2_{,3}$ =29.7110, *P<0.0001*) (**Figure 9, 96 hours exposure**). The immunostaining score increased significantly in the fish exposed to 8 µg/L (median score of 9 (IQR= 1), Dunn, Z= 4.13, *P= 0.0001*) and 32 µg/L of copper (median score of 9 (IQR= 0.75), Dunn, Z= 4.19, *P<0.0001*) compared to the control group (median score of 1.5 (IQR=1.25)). The scores were not significant different for the groups of fish exposed to 2 µg/L of copper when compared to the control group (median score of 2 (IQR= 2.25), Dunn, Z= 0.74, *P=1.0000*).

Stripped staining pattern: At 96 hours of exposure, 90% (nine out of ten) of the control fish had mild changes in the distribution of the staining pattern, and 10% (one out of ten) had moderate changes. At 2 µg/L, 40% (four out of ten) of the fish had mild changes and 60% (six out of ten) moderate changes in the staining pattern. At high copper concentrations, 100% (ten out of ten) of the fish had severe changes in the staining pattern with lack of stain at 8 µg/L and 100% (eight out of eight) of the fish at 32 µg/L.

Surface staining pattern: The surface staining pattern is characterized by a well delineated rim of positive staining. In control groups, 30% (three out of ten) had a mild loss of the surface staining. At 2 µg/L, 40% (four out of ten) of the fish had a mild loss of the surface staining. The staining pattern is mostly lost with 100% (eight out of eight) of the fish showing severe changes at 8 and 32 µg/L.

Stain intensity: In the control groups, the staining intensity was mildly reduced in 40% (four out of ten) of the fish evaluated. Similarly, at 2 µg/L of copper, the staining intensity was mildly reduced in 20% (two out of ten) of the cases. *A*t the 96 hours of exposure, 60 % (six out of ten) and 75% (six out of eight) of the fish had a severe attenuation of the staining in the 8 and 32 µg/L copper concentration, respectively.

Epithelial thickness: In the 96-hour exposure experiment, an overall reduction in epithelial thickness was observed in all the treatments including the control groups when compared to the 24-hour exposure time. After 96 hours of exposure, the groups exposed to 8 and 32 µg/L of copper presented a reduction in the epithelial thickness of 1.72 µm and 1.51 µm (38.65±2.72 µm, mean ± SE and 38.86±5.60 µm, mean ± SE, respectively) when compared to the control group (40.37±3.00 µm, mean ± SE) (**Figure 7**).

4.5 Antipredator response to skin extracts (conspecific alarm cues) in Delta Smelt exposed to copper

Changes in darting episodes: A darting episode was characterized as a sudden increase in acceleration and swimming speed, with change in swimming direction. In general, I observed that fish darted more in response to skin extract in the control groups and the 8 µg/L copper concentration; however, no

significant differences were observed in the number of darting episodes among the copper exposed and control treatments (Kruskal Wallis ANOVA, $\chi^2_{,3}$ =3.7946, *P=0.2845*) (**Figure 10**).

Changes in swimming speed: In general, copper exposure affected the swimming velocity of Delta Smelt after presentation of alarm cues. There was a significant effect of copper exposure in the swimming speed of Delta Smelt (ANOVA, Concentration, $F_{(3, 133)}$ =9.4944, *P<0.0001*). The groups of fish exposed to 8 µg/L of copper were swimming at faster speed than the control group (control vs 8 µg/L, planned linear contrast, $F_{(1, 133)}$ = 13.7455, *P=0.0003*) (**See table 5**) and significantly increased their swimming speed after addition of skin extracts (control, BLS time interval 2 vs 8 µg/L, BLS time interval 2, planned linear contrast, $F_{(1, 133)}$ = 13.9594, *P=0.0003*). There was a slight reduction in swimming speed in the group exposed to 2 µg/L of copper; however, this change was not significant when compared to the control group (control vs 2 µg/L, planned linear contrast, $F_{(1, 133)}$ = 2.1636, *P=0.1437*). Similarly, at 32 µg/L, no significant changes in the swimming speed were observed when compared to the control groups (control vs 32 µg/L, planned linear contrast, $F_{(1, 133)}$ = 0.5562, *P=0.4571*) (**Figure 11**).

Maximum acceleration: I observed a significant effect of copper treatment in the acceleration of Delta Smelt (ANOVA, concentration, $F_{(3, 133)}$ =6.8438, *P=0.0003*). The groups of fish exposed to 8 µg/L of copper reached higher maximum accelerations when compared to the control groups (control vs 8 µg/L, planned linear contrast, $F_{(1, 133)}$ = 7.4734, *P=0.0071*) (**see table 6**), with a significant increase in maximum acceleration after exposure to skin extracts (control, BLS time interval 2 vs 8 µg/L, BLS time interval 2, planned linear contrast, $F_{(1, 133)}$ = 9.4344, *P=0.0026*). The groups of fish exposed to 2 µg/L of copper showed a reduced maximum acceleration; however, no significant differences were observed when compared to the control group (control vs 2 µg/L, planned linear contrast, $F_{(1, 133)}$ = 2.7044, *P=0.1024*). Similarly, at 32 µg/L of copper, no significant differences in the maximum acceleration were observed when compared to the control groups (control vs 32 µg/L, planned linear contrast, $F_{(1, 133)}$ = 0.1934, *P=0.6608*) (**Figure 12**).

Changes in total distance moved: In general, I observed a significant effect of copper exposure in the total distance moved of Delta Smelt (ANOVA, concentration, $F_{(3, 133)}$ =9.4870, *P<0.0001*). A significant increase in the total distance moved in the groups of fish exposed to 8 µg/L of copper was observed when compared to the control groups (control vs 8 µg/L, planned linear contrast, $F_{(1, 133)}$ = 13.6873, *P=0.0003*; **see Table 7**), with a significant increase in total distance moved after exposure to skin extracts (control, BLS exposure 2 vs 8 µg/L, BLS time interval 2, planned linear contrast, $F_{(1, 133)}$ = 13.7942, *P=0.0003*). The groups exposed to 2 µg/L showed a reduction in the total distance moved; however, this reduction was not significant (control vs 2 µg/L, planned linear contrast, $F_{(1, 133)}$ = 2.1871, *P=0.1415*). At 32 µg/L, the total distance moved did not change significantly when compared to control groups (control vs 32 µg/L, planned linear contrast, $F_{(1, 133)}$ = 0.5462, *P=0.4612*) (**Figure 13**).

Changes in interindividual distance: 24-hour copper exposure did not impact how fish shoaled together in the tanks. In general, I did not observe a significant effect of either copper concentration (ANOVA, concentration, $F_{(3, 267.4)}$ =0.9047, *P=0.4393*) or the time interval following skin-extract exposure (ANOVA, time interval, $F_{(1, 264.6)}$ =0.0566, *P=0.8121*) on the interindividual distance of Delta Smelt exposed to skin extracts (**Figure 14**, **Table 8**).

96-hour copper exposure

Most of the fish did not survived for behavioral trials in 32 µg/L; therefore, only the results from control, 2 and 8 µg/L of copper were described in the behavioral analysis.

Changes in darting episodes: I observed an increased number of darting episodes after addition of the skin extract in all the treatments. The median number of darting episodes was higher in control conditions and in the fish exposed to 8 µg/L of copper; however, this trend was not statistically significant (Kruskal Wallis ANOVA, $\chi^2_{,2}$ =2.4739, *P=0.2903)* (**Figure 15**).

Changes in swimming speed: I found that fish tended to swim more slowly over the course of the trial, with a significant effect of the time interval (ANOVA, time interval, $F_{(2, 95)}$ =8.9411 *P=0.0036*), independently of the concentration of copper used (ANOVA, concentration, $F_{(2, 95)}$ =0.8621, *P=0.4256*). The fish in the concentration of 8 µg/L of copper became less active over time after exposure to alarm cues (8 µg/L, BLS time interval 1 vs BLS time interval 2, planned linear contrast, $F_{(1, 95)}$ = 4.8520, *P=0.003*) (**Figure 16, Table 9**).

Maximum acceleration: I observed that fish tended to slow down over the course of the trial, but there was no differences among concentrations (ANOVA, concentration, $F_{(2, 95)}$ =0.7459 *P=0.4770*). I observed a general reduction in the maximum acceleration on the second time interval after presentation of the alarm cue when contrasted to the first time interval; however, this difference was not significant (ANOVA, time interval, $F_{(1, 95)}$ =3.4925 *P=0.0647*) (**Figure 17, Table 10**).

Changes in total distance moved: In general, over the time course of a trial, the fish tend to move less in all the copper concentrations; however, I did not observe significant differences in the total distance moved among treatments (ANOVA, concentration, $F_{(2, 95)}$ =0.6974 *P=0.5004*). On the other hand, I observed a significant effect of the time interval (ANOVA, time interval, $F_{(1, 95)}$ =7.9705 *P=0.0058*). The fish exposed to 8 µg/L showed a significant reduction in total distance moved at BLS time interval 2 when compared to BLS time interval 1 in the same concentration (Copper 8 µg/L, BLS time interval 1vs BLS time interval 2, planned linear contrast, $F_{(1, 95)}$ = 8.1505, *P=0.0053*) (**Figure 18, Table 11).**

Changes in interindividual distance: In general, fish from control groups and 8 µg/L of copper were more closer to each other after the presence of alarm cue. I observed a significant effect of the copper concentration (ANOVA, concentration, $F_{(2, 195)}$ =5.8805 *P=0.0033*) in the average interindividual distance change. The reduction in interindividual distance was significantly less in the concentration of 2 µg/L of

copper when compared to the control groups (Control vs 2 µg/L, planned linear contrast, $F_{(1, 195)}$ = 9.4310, *P=0.0024*) (**Figure 19, Table 12**).

5. Discussion

5.1 Copper toxicity

I demonstrated that copper exposure has a significant effect on survival, olfactory epithelium morphology and antipredator behavioral responses in adult Delta Smelt. The histopathological alterations on the olfactory epithelium and the effects of copper on olfactory mediated behavioral responses in Delta Smelt have not been investigated before. Delta Smelt were highly susceptible to copper exposure at concentrations considered sublethal to other fish species (Malhotra et al., 2020). Previous reports in juvenile Delta Smelt (90 DPH) showed a 7-day median lethal concentration (LC50) of 17.8 µg/L; in contrast, larvae Delta Smelt showed an LC50 of 80.4 µg/L in a 4-day copper exposure, indicating that juveniles were more susceptible to copper toxicity (Connon et al., 2011). Adult Delta Smelt, which had a median survival time of 72 hours at 28 µg/L (highest measured concentration used in this work), were more resistant than juveniles but less resistant than larvae (Connon et al., 2011).

When compared to other fish (Tierney et al., 2010, Shekh et al., 2020, Malhotra et al., 2020), Delta Smelt were more susceptible to long-term copper exposures. For instance, zebrafish (*Danio rerio*) survival was 100% in a 10-day exposure to 50 µg/L of copper (Pilehvar et al., 2020), and in juvenile Coho salmon (*Oncorhynchus kisutch*), the 96-hour LC50 was 192 µg/L of copper (Hedtke et al., 1982). Copper toxicity can vary with water quality parameters (Grosell, 2011). In fish, copper is a systemic toxicant whose main and consistent effect is a profound impairment in osmoregulation due to inhibition of the Na^+/K^+ ATPase pump activity and impairment of ammonia excretion inducing hyperammonemia, alterations in plasma osmolality, increased blood viscosity due to swelling of red blood cells and splenic release, leading to a cascade of events that cause cardiovascular collapse and dead (Grosell, 2011, Chowdhury et al., 2016).

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Although speculative, this is the most likely cause of dead in the Delta Smelt exposed to 32 µg/L in the 24- and 96-hour exposures. Water chemistry can influence copper toxicity to sensory cells. In a study with zebrafish larvae, Linbo et al. (2009) found that increasing the pH, sodium ions and dissolved organic carbon (DOC) together resulted in a reduction of copper sensory toxicity. In fresh water, the toxicity of copper increases and mortality of fish exposed to copper tends to be higher. For example, the euryhaline killifish (*Fundulus heteroclitus*) exposed to concentrations as high as 150 µg/L showed ionoregulatory impairment and high mortality in fresh water; however, in salt water (11 and 20 ppt), a higher concentration of copper did not cause mortality nor ionoregulatory impairment (Blanchard and Grosell, 2006).

Additionally, the dissolved organic carbon concentration (DOC) predicts copper olfactory toxicity in salmonids, with an increase in humic acids decreasing the concentration of copper needed to impair olfactory detection of L-Histidine (Kennedy et al., 2012). Even at short-term exposures, Delta Smelt showed signs of toxicity and mortality at measured copper concentrations of 28 µg/L. In the exposure conditions used in this study, mortality is most likely due to ionoregulatory impairment induced by the inhibition of the Na^+/K^+ ATPase proton pump, which has been reported in different fish species (Grosell, 2011, Chowdhury et al., 2016). Delta Smelt have been classified as estuarine species with localized hot spots of abundance that include fresh, brackish water and semi-anadromous (Hobbs et al., 2019). In low salinity habitats (1-6 PSU) and freshwater, copper pollution might thus represent an additional stressor (Connon et al., 2011).

The north Sacramento-San Joaquin delta is characterized by being a low salinity fresh water modified habitat composed by shallow tidal waters (Lund et al., 2010). In this habitat, several of the native fish species including Delta Smelt, migrate and spawn; therefore the freshwater of the delta is critical for native fish survival (Moyle et al., 2018, Lund et al., 2010). Previous studies have demonstrated histological lesions consistent with contaminant exposures in Delta Smelt sampled from several regions of the San

Francisco Delta including freshwater and brackish water (Hammock et al., 2015). Adult Delta Smelt are more likely to be susceptible to waterborne copper in fresh water with low amounts of dissolved carbon and organic matter conditions that they would encounter during spawning migrations in the upper Sacramento River. Copper exposure may therefore compromise the survival of Delta Smelt in freshwater even at short-term exposures and at concentrations considered to be sublethal for other fish species.

5.2 Histopathologic changes in the olfactory epithelium

The olfactory epithelium is an out pocketing of neural tissue that directly synapses in the olfactory bulb of the brain. My data suggest that the exposure to copper may disrupt the olfactory epithelium morphology and function causing cell death followed by a regenerative response. In Delta Smelt, the first response of the olfactory epithelium to copper exposure is formation of intracytoplasmic vacuoles followed by cell swelling. This occurred at both 24- and 96-hours exposure times. When exposed to copper, olfactory neurons usually reacted with initial acute cell swelling, a typical morphological characteristic of acute cell injury prior to cell death by necrosis or apoptosis (Julliard et al., 1993). There is ultrastructural evidence indicating the damage to cell membranes and acute cell swelling characterized by swollen mitochondria and electron lucent cytoplasm (Hansen et al., 1999, Julliard et al., 1993, Julliard et al., 1996). Systemic effects of copper, might affect olfactory neurons indirectly through hypoperfusion, hypoxia and changes in osmolality of interstitial fluids derived from Na^+/K^+ ATPase pump inhibition and accumulation of excretion products such ammonia which could induce intracellular edema and cell death (Grosell, 2011). I demonstrated that there is an increase in apoptotic cells labeled with anti-cleaved-caspase 3 antibodies in fish exposed to copper. However, not all the neurons with characteristics of apoptotic cells in H&E were labeled for cleaved-caspase 3, suggesting a different pathway for cell death. Abundant number of cells were swollen, rounded and with pyknotic or fragmented nuclei, indicating necrotic changes. These findings suggest that copper induced both necrosis and apoptosis of olfactory neurons in Delta Smelt.

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Damage to neurons is also highly dependent on the copper concentration, as demonstrated by the loss of staining for $G_{\alpha S/olf}$ at copper concentrations of 8 and 32 µg/L. At 96 h of 8 ug/L treatment, sustentacular cells are more prominent and cover the entire thickness of the epithelium. There is complete absence of ciliated neurons and loss of $G_{\alpha S/olf}$ staining. In the 32 µg/L treatment, ciliate neurons retracted, and the olfactory knobs became attenuated and embedded within neighboring sustentacular cells. The neuronal cytoplasm was swollen or fragmented and stained faintly for $G_{\alpha S/olf}$.

Since ciliate neurons are directly contact and exposed to waterborne contaminants, copper induced degradation of ciliary structures were observed, and it was supported by the loss of cilia and olfactory knobs in the olfactory epithelium and decrease in $G_{\alpha S/olf}$ immunostaining intensity for ciliate neurons in this study. At the low concentrations of copper, the neurons start to loss their olfactory knobs, as seen in some patchy areas with lack of surface staining with $G_{\alpha S/olf,}$ in which the neuronal dendrite and body conserve its linear staining pattern, but lack of staining of the surface structures. In contrast, at 32 µg/L copper concentrations, the surface is completely devoided of cilia and olfactory knobs. In zebrafish, an exposure of 30 µg/L (considered sublethal to this species) induced a decrease in the expression of $G_{\alpha S/olf}$, indicating loss of cilia only or whole olfactory ciliated neurons, but with no disruption or morphological alterations observed (Lazzari et al., 2017) suggesting an initial effect on cilia only in zebrafish. Also, a short-term exposure to copper (3 h) can induce toxicity in sensory cell of lateral line (Linbo et al., 2009) and olfactory neurons (Ma et al., 2018), even at sublethal concentrations (11 to 20 µg/L). Similarly, in Fathead Minnows and Yellow Perch, copper targeted specifically on ciliate neurons at low concentrations (5 and 10 µg/L). However, at a higher concentration (20 µg/L), microvillous and ciliated neurons were affected equally (Dew et al., 2014). Other studies have suggested that ciliated olfactory neurons are more sensitive than microvillous neurons to copper toxicity (Ashley et al., 2009). The fact that these lesions appeared after short-term exposures at low concentrations in this study demonstrate the extremely susceptible

nature of Delta Smelt to copper exposures and raises concerns on how environmental exposures might affect the olfactory system and therefore survival of this fish.

5.3 Concentration-time dependent responses of the olfactory epithelium to copper

I hypothesized that longer exposure time would cause more severe damage in a dose-dependent relationship and also predicted to see more damage in the fish exposed for 96 hours compared to 24 hours for a given concentration. Interestingly, we did not find any interactions in the apoptotic index between the concentration and exposure time, and the severity of the lesions were fairly similar at 24- and 96-hour exposures in the medium and high concentrations. In Delta Smelt, a 24-hour exposure of copper at 8 and 32 µg/L, induced severe degeneration and damage to the olfactory epithelium, with severe vacuolation and death and loss of sensory neurons. Similarly, in a long-term exposure of 96 hours, these concentrations also induced severe degeneration of the olfactory epithelium, but also compromising the survival of the fish at 32 µg/L.

The progression of lesions and susceptibility of the olfactory epithelium varies among fish species. In rainbow trout, exposed to copper for 24 hours, degeneration and death of sensory neurons were observed at concentrations of 50, 75 and 100 µg/L. Surprisingly, at the concentrations of 50 µg/L, considered as low concentrations in this study, only moderate changes were reported, including vacuolation and loss of cilia (Klima and Applehans, 1990). In another study with Rainbow Trout yearlings, the amount of degenerated and apoptotic neurons increased after 24 hours of exposure to 20 µg/L of copper when compared to non-exposed groups and peaked at day 5 of exposure at the same concentration, suggesting an effect of exposure time at concentrations deemed to be sublethal (Julliard et al., 1996, Saucier et al., 1991a, Saucier et al., 1991b, Saucier and Astic, 1995). Consistent with my findings, in the African Cichlid (*Tilapia mariae*), copper effects were concentration dependent, with a severe damage and degeneration of the olfactory epithelium at 100 µg/L of copper in a 96-hour exposure.

At this concentration, the sensory epithelium showed wide spaces in the whole thickness of the epithelial surface. The apical surface was seriously injured with indentations and loss of cilia and microvilli; however, the survival of the fish was not compromised at this concentration. At 40 and 20 µg/L, the severity of damage was less; however, swollen and degenerated neurons were still evident with more selective effects on olfactory neurons (Bettini et al., 2006b).

Other studies have demonstrated that even short-term exposures (hours) can impact and damaged the olfactory epithelium. For example, the number of sensory neurons decreased in Chinook Salmon and Rainbow Trout in a short-term (4 hour) exposure to copper. This decrease was concentration-dependent (25 to 200 µg/L), with a maximum at 200 µg/L of copper. Damage to ciliated neurons was observed within 4 hours of exposure to 50 µg/L of copper. The changes reported were loss of cilia and microvilli, swollen mitochondria, and ruptured plasma membranes (Hansen et al., 1999). Also, cell condensation and fragmentation and epithelial disorganization have been identified in the olfactory epithelium of zebrafish exposed to copper (Ma et al., 2018). In the study, zebrafish larvae were exposed to a range of copper concentrations (16 to 635 µg/L) for a short period of time (3 and 24 hour). Degeneration and olfactory neurons death were observed even at short-term exposures (3 hours) with the lowest dose used (16 µg/L). This is evident that olfactory neuron degeneration occurs early after exposure. Similarly, changes in the olfactory epithelium were observed in rainbow trout exposed to 50 µg/L of copper for 12 hours, with loss of ciliary structures in the epithelial surface (Sovova et al., 2014).

I did not find significant differences in epithelial thickness among treatments. Similarly, Saucier et al. (1991b) did not find significant changes in the thickness of the olfactory epithelium of rainbow trout exposed to 22 µg/L of copper, although in their study, the fish were exposed for a longer time (37 weeks), suggesting that sustentacular cells are still conserved and are more resistant and not impacted by copper at this concentration. In the olfactory epithelium of rainbow trout, sustentacular and supporting cells have been reported to hypertrophy following exposure to 20 µg/L of copper (Julliard et al., 1993). I observed

somewhat similar changes in Delta Smelt exposed to 32 µg/L of copper for 96 hours, with more prominent sustentacular cells and abundant ovoid elongated sausage shaped nuclei that spanned almost half of the width of the epithelium. The changes in epithelial thickness observed in the 96-hour experiment, might represent the unspecific and general toxicity of copper in both sensory neurons and sustentacular cells at high concentrations and long exposure times (Julliard et al., 1993, Lazzari et al., 2017). Klimenkov et al. (2020b) found that a long-term exposure to naturally occurring odorants (15 days to an amino acid mixture of 6.60 X 10^{-7} to 1.02 X 10^{-5} M) can cause a compensatory response and loss of functional activity in the olfactory system. When this happens, some olfactory neurons changed from sensory phenotype to a secretory phenotype and also some underwent cell death. Metals can be sensed by fish and act as odorants, too, and the changes in hypertrophy and more prominence in sustentacular cells could be a response to chronic stimulation with copper, inducing changes in cell phenotype, together with loss of neurons (Klimenkov et al., 2020a, Klimenkov et al., 2020b). Copper alone can be detected by olfaction and avoided at concentrations as low as 1 µg/L; however, at the concentrations that impair olfaction and are toxic to chemosensory systems, the ability of fish to detect contaminants might be impaired, and therefore, avoidance of polluted areas might be compromised (Tierney, 2016, Scherer and McNicol, 1998). More research is needed to confirm or discard this hypothesis.

In Delta Smelt, after 96 hours of exposure at nominal copper concentrations of 8 and 32 µg/L, the olfactory epithelium basal layer became packed with basophilic and elongated nuclei that occupied most of the basal layer, moving into the mid-layer of the epithelium (**see Figure 6, H**). This finding indicates increased proliferative activity to replace death and degenerated neurons in the epithelium, as has been demonstrated previously (Bettini et al., 2006a). After 96 hours of 30 µg/L copper exposure, the olfactory epithelium of guppies showed a similar pattern of proliferative activity in the basal layer that progressively moved upward towards superficial layers in the epithelium. This indicates a high proliferative activity and regeneration in the olfactory epithelium.

There is evidence that bony fish possess a functional NALT (nasopharynx associated lymphoid tissue), that is mainly scattered in the lamina propria of the olfactory lamellae (Das and Salinas, 2020). An increase in inflammatory cells infiltration of the lamina propria could indicate an acute reaction to epithelial and neuronal injury, helping in clearing of dead, injured cells and stimulating regeneration. It has been demonstrated in mice that an acute inflammatory reaction induces differentiation and is crucial for neurogenesis in the olfactory epithelium immediately after injury. Moreover, macrophages were consistently found in mice and rats with olfactory mucosa injury (Chen et al., 2017, Suzuki et al., 1995). These reports are consistent with my findings that inflammatory cells (presumably macrophages) were found to infiltrating the lamina propria of the fish exposed to 32 µg/L during a short-term exposure of 24 hours and 8 and 32 µg/L at 96 hours of exposure.

An increase of inflammatory cells (presumably macrophages) in the lamina propria could be implicated in the removal of basal necrotic and apoptotic cells. At the first 24 hours, I noted more inflammatory cell infiltration in the lamina propria, compared to fish exposed to 96 hours. It could be possible that degeneration of olfactory neurons starts within hours after exposure, and within 24-hours of copper exposure, this process is already well underway, and in contrast, following our 96-hour exposure time, most of the neurons could already be removed and some of the infiltrated cells traffic out of the olfactory rosette. The infiltration of macrophages might be implicated in the repairing and healing mechanism of the sensory epithelium after exposure, and the mechanisms involved in this process are worth further investigation. In the ultrastructural studies, sustentacular cells were observed to have intracytoplasmic remnants of apoptotic cells, suggesting a mechanism of clearing and repairing of damaged epithelial cells, which could explain, also the proliferation and prominence of this cells during cell injury to the olfactory neuron population (Saucier et al., 1991a, Saucier et al., 1991b). Moreover, we observed at 8 and 32 µg/L (See **Figure 6, F**), clusters of prominent cells infiltrating the sensory epithelium. These cells were characterized by having a clear foamy cytoplasm with discrete vacuoles, round and pale, sometimes

central, or peripheral nuclei with a prominent single nucleolus. These cells were surrounded by a clear space, that sometimes, contained debris and fragments of dead cells. The nature of these cells is not clear; however, based on the morphological features, we suggest that these cells could be activated macrophages in response to epithelial cell injury.

5.4 Mechanisms of copper toxicity in the olfactory epithelium

One of the early responses of the olfactory neurons to copper exposure was acute cell swelling. The acute cell swelling can be explained by an inhibition of the Na^+/K^+ ATPase proton pump. In this scenario, the proton pump would not be able to excrete sodium out of the cells. This would increase the osmotic potential and draw water inside cells, since in freshwater the cell cytoplasm would be more concentrated (Castaldo et al., 2020). In addition, copper might damage cell membranes by lipid peroxidation. An alteration in membrane permeability might enhance uptake of extracellular fluids into sensory neurons. In sea lampreys (*Petromyzon marinus*), a 24-hour exposure to copper (5 to 30 µg/L) induced a dose-dependent transcriptional upregulation in genes related to cell death, oxidative stress, neurogenesis and repair mechanisms (Jones et al., 2019). In zebrafish, copper (24 hours exposure, 6.3, 16 and 40 µg/L copper) caused a deregulation in ion homeostasis, related to ion channel closing. Also, at 16 and 40 µg/L, copper induced perturbations in genes involved in catabolic processes and regeneration, presumably induced by copper toxicity. The main down regulated genes were parvalbumin 8, calbindin and intracellular chloride channel 4, involved in calcium and chloride ions regulations (Tilton et al., 2008). Additionally, copper (16 and 40 µg/L, 24 hours exposure) downregulated the expression of genes related to olfactory receptors, olfactory signal transduction and G-proteins, and thereby rendering zebrafish less sensitive to odorants (Tilton et al., 2008). Alterations in ionic balance might explain the initial cell swelling observed in the olfactory neurons of Delta Smelt exposed to low and medium concentrations (2 and 8 µg/L) of copper, followed by induction of cell death from calcium overload due to inhibition and down regulation of parvalbumin (Tilton et al., 2008, Tilton et al., 2011). Also, copper has been shown to decrease

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the expression of $G_{\alpha S/olf}$ in zebrafish olfactory tissue (Tilton et al., 2008, Lazzari et al., 2017), at high concentrations (30 and 40 µg/L, 24- and 96-hour exposure), which agrees with my results of decreased $G_{\alpha S/olf}$ staining intensity by immunohistochemistry at medium and high concentrations of copper in the olfactory epithelium of Delta Smelt. This result suggest that the lack of staining is caused by direct inhibition of $G_{\alpha S/olf}$ expression and by death of ciliated neurons. The direct targeting of $G_{\alpha S/olf}$ may render ciliate neurons more susceptible by impairment of odorant signal transduction and triggering of cell death mechanisms. These hypotheses have not been tested, and the highest susceptibility of ciliate neurons to copper is not well understood.

5.5 Antipredator responses of Delta Smelt exposed to copper

Under normal conditions, the antipredator response of Delta Smelt to skin extracts, consists of an initial escape response with a sudden increase in speed followed by intervals of darting and freezing, follow by an overall decrease in activity. In the 24- and 96-hour exposure experiments, except for an increase in darting behavior, I did not observe significant changes in the speed, maximum acceleration, total distance moved and interindividual distance of the control groups, which means an overall hyporresponsiveness to the skin extracts.

An absence or decrease in response to skin extracts may be related to the hunger level of fish (Brown, 2005). For example, the Fine Scale Dace (*Chrosomus neogaeu*) did not displayed an antipredator response to skin extract or predator odor after being food deprived for 24 to 48 hours (Brown and Cowan, 2000). It is possible that food deprivation during my assays with Delta Smelt might also have influenced the antipredator response to skin extracts. This observation should be further investigated, since in the wild, Delta Smelt have shown signs of food limitation and starvation, which might limit or change the responsiveness of fish to predators making them more susceptible to predation (Hammock et al., 2015, Hammock et al., 2020, Hung et al., 2019).

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In this study, Delta Smelt post exposure to 2 µg/L copper for 24 and 96 hours showed a slight decrease in swimming speed and movement after the addition of skin extract to the arenas. Although the fish had been exposed to copper darted less than the control fish in response to the skin extract, Delta Smelt were still able to respond to skin extracts in a manner consistent with antipredatory behavior.

After 24 hours of 8 µg/L copper exposure, significant changes in the behavior of Delta Smelt were observed. These fish swam faster, reached higher maximum accelerations, and moved greater distances in response to skin extracts. These exacerbated responses may be due to toxic effects of copper. For instance, zebrafish larvae moved twice as far and two and a half times faster than controls after experiencing copper exposure levels of 7.25 µg/L for 96 hours (Acosta et al., 2016). Similarly in larval zebrafish, the total distance moved increased in fish exposed to 125 µg/L when compared to unexposed controls (Santos et al., 2020). The potential hyperexcitability of fish exposed to 8 µg/L could be associated with an imbalance in ionic regulation, and neurotransmission leading to an hyperresponsive state and altered behavior.

Razmara et al. (2020) showed an increase in the response to taurocholic acid in rainbow trout exposed to low copper concentrations and hypothesized that calcium dysregulation may contribute to hyperexcitability in olfactory neurons. These results are in line with the evidence showed by Tilton et al. (2008) and Tilton et al. (2011) in which the genes related to calcium homeostasis are downregulated in zebrafish olfactory tissue exposed to copper. I suggest that calcium dysregulation may explain in part the damage observed in the olfactory epithelium and the behavioral hyperexcitability of Delta Smelt after 24 hours of exposure to 8 µg/L of copper. An additional mechanism in which potential hyperexcitability can occur is the impairment of ammonia excretion caused by copper (Grosell, 2011). Ammonia can be converted in excitatory neurotransmitters, and impairment in excretion can cause hyperresponsiveness (Grosell, 2011, Ip et al., 2001). Another proposed mechanism is based on the finding that acute copper exposure can impair partially acetylcholinesterase (AChE) activity.

Copper exposure has been shown to impair AChE activity in zebrafish and the Streaked prochilod (*Prochilodus lineatus*). This was in line with alteration in swimming a locomotory behavior towards hyperexcitability (Santos et al., 2020, Haverroth et al., 2015, Tilton et al., 2011, Roda et al., 2020). Also, copper exposure increased the levels of dopamine and serotonin in brain tissue, which may lead to hyperexcitability (De León et al., 2020). A heightened response to skin extracts in the Delta Smelt post 8 µg/L of copper exposure suggests that fish are able to detect this cue to a certain degree, and that their hyper-responsiveness is due to the effects of copper on the function of the central nervous system and the neuromuscular system (Haverroth et al., 2015), in conjunction with the metabolic effects that lead to hyperammonemia. Also, alterations in swimming behavior and hyperresponsiveness may be related to lateral line damage impacting rheotaxis in fish exposed to metals (Ma et al., 2018).

In contrast to 24-hour exposures, following a 96-hour exposure to 8 µg/L copper, fish became noticeably lethargic as evidenced by slower swimming, reduced acceleration and tended not to discover as much distance. Similarly at a copper concentration of 32 µg/L in a 24-hour exposure, the response to alarm cues did not change suggesting an impairment on the detection upon exposure. At these concentrations and exposure times, the damage to the epithelium was more severe, and the lack of olfactory knobs was extensive, which can explain the unresponsiveness of this fish to the skin extracts.

Changes in behavior have been associated with copper exposure in other fish species, but effects vary among species, exposure time and concentration. For example, Pilehvar et al. (2020) reported a decrease in the total distance traveled in zebrafish exposed to 100 µg/L copper for 10 days. By contrast, zebrafish exposed to 50 µg/L had no effect on these parameters. Still other researchers have reported that exposure to just 6 µg/L of copper over 24 hours, significantly decreased the total distance traveled of zebrafish (Haverroth et al., 2015). In terms of olfactory mediated behaviors, low levels of dissolved copper (5 to 20 µg/L) inhibited the antipredator alarm response of Coho salmon (*Oncorhynchus kisutch*)(McIntyre et al., 2012). Similarly, a 3-hour exposure to 5 µg/L of copper impaired the recognition of predator odors and

made the Spiny Loach (*Lepidocephalichthys thermalis*) more susceptible to predation (Gosavi et al., 2020). Exposure to copper for 48 hours with concentrations considered to be sublethal (20µg/L) impaired the alarm responses in behavioral assays of Fathead Minnows, and also, impaired the function of ciliate neurons measured in electro-olfactograms (Dew et al., 2014). In Rainbow Trout, exposure to copper at 50 µg/L of free copper ion or nanoparticles for 12 hours decreased their response to skin extracts, but this effect was reduced in groups exposed to free copper ions as $CuSO_4$ compared to those exposed to nanoparticles, suggesting that fish retained some ability to detect alarm cues despite copper exposure (Sovova et al., 2014).

The ability to detect odorants is also inhibited by copper in different ways. For example, in Rainbow Trout, (*Salmo gairdneri*) a 2-hour exposure to 5 µg/L was required to suppress olfactory response to L-serine using electro-olfactograms. Recovery of sensitivity was rapid once the toxicant was rinsed out of the nares, suggesting that copper ions interfered with odorant detection at the level of the olfactory receptor, possibly by binding to sulfhydryl groups in proteins (Hara et al., 1976). Exposure to 5 µg/L of copper for 1 hour did not impair the ability of juvenile white perch (*Morone americana*) to detect conspecific odors in behavioral preference/avoidance assays (Ward et al., 2013). In Chinook Salmon (*Oncorhynchus tshawytscha*) and Rainbow Trout (*Oncorhynchus mykiss*), a 1-hour copper exposure to 200 and 300 µg/L followed by electrophysiological testing to amino acids showed impairment of the olfactory response to L-serine (Hansen et al., 1999). Results in Rainbow Trout exposed to copper for long periods of time (40 weeks to 20 µg/L) showed that even at long exposures some fish were still able to recognize odorants (Saucier and Astic, 1995, Saucier et al., 1991a, Saucier et al., 1991b). These findings suggest that at a short-term or even a long-term exposure, the detection of an odorant and an olfactory mediated behavioral response is not completely impaired, with some individuals responding to stimulus, evidence of the robust mechanism of detection in peripheral neurons and central processing in high olfactory centers.

6. Conclusions

Delta Smelt were highly susceptible to copper exposure at concentrations deemed to be sublethal in other fish species. At 32 μg/L, copper was considered highly toxic in 24- and 96-hours exposures by causing high mortality in prolonged exposures. My results demonstrate that the olfactory epithelium of Delta Smelt is highly sensitive to copper exposure showing morphological changes at even low concentrations. The morphological changes were more severe with an increase of copper concentration but not affected by the exposure time, indicating that short exposures are equally harmful to the olfactory system as long exposures. Overall, this study suggests that at low concentrations (2μg/L) of 24 and 96 hours exposure time, copper does not impact the ability of Delta Smelt to detect odorants. On the other hand, at medium and high concentrations of copper, the perception of odorants was significantly altered, although not completely impaired at 8 μg/L. The response was anomalous dominated by an hyperexcitability at 24-hour exposures and hypo responsiveness at 96-hour exposures. Delta Smelt are still able to recognize the skin extract even after longer exposures or due to systemic toxicity to copper since the fish were depressed and unable to respond. My data supports the first theory in which I observed a significant change in speed, acceleration and total distance moved after skin extract addition meaning that Delta Smelt were still able to detect the cue, even when there is evident damage to the olfactory epithelium.

Acknowledgements

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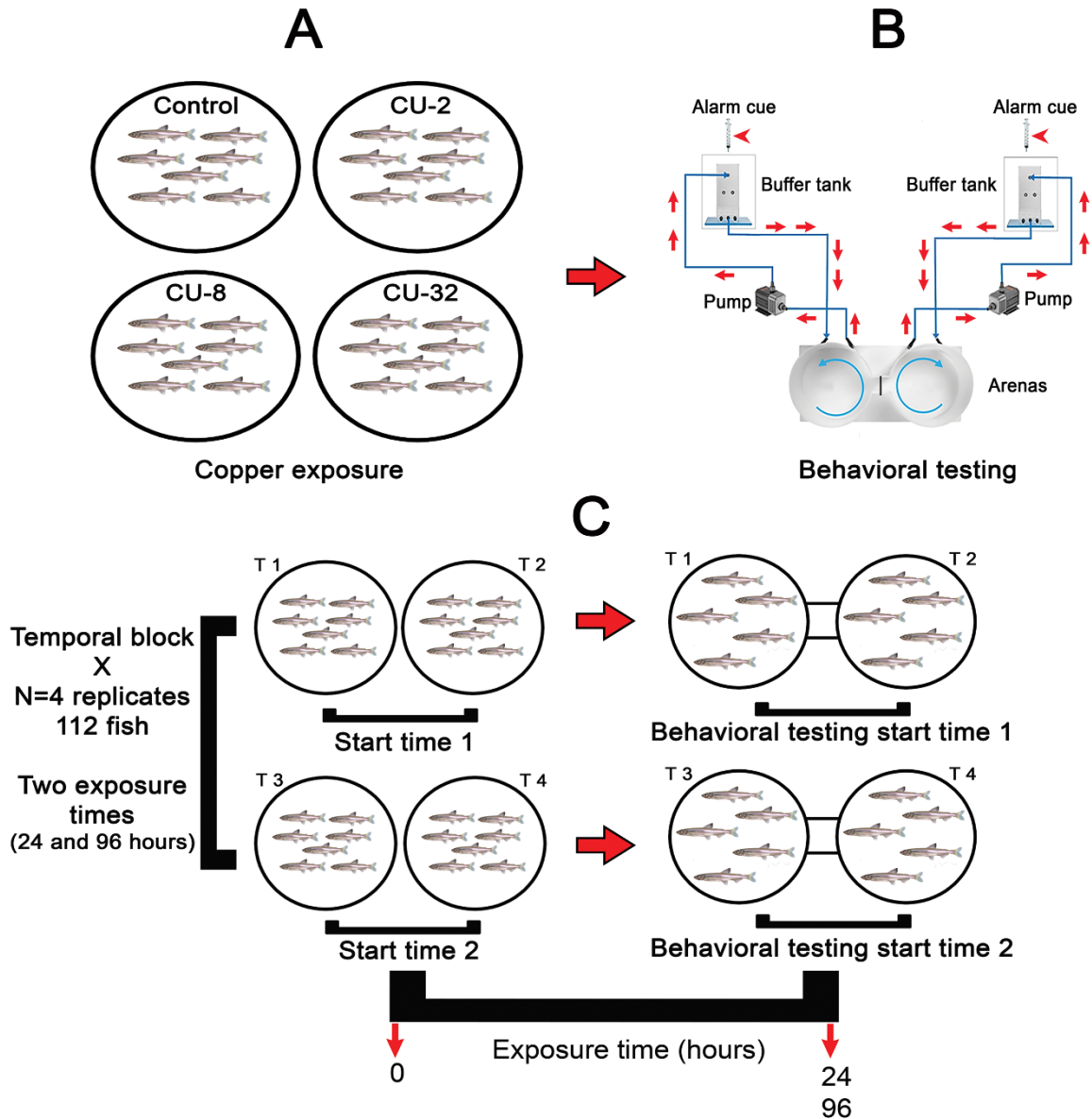
Figure 1. Summary of the experimental design for this work. A) Groups of 7 fish were exposed to 4 concentrations of copper (treatments) for 24 or 96 hours. Each circle represents 1 bucket (experimental unit), B) after copper exposures, 5 fish were chosen for each concentration and tested for alarm behavior in two independent arenas coupled to a recirculation system. The red arrows indicate the path of water movement (Image modified from Loligo® Systems, Shuttle Box System Oxygen user manual,

) and C) indicates the scheme for behavioral testing in temporal

blocks. Each of the four treatments was tested each day (behavioral recording exposure time 1 and 2)

after two exposure times (exposure time 1 and 2) to copper. This was done four times until completing

four replicates for each treatment for each experiment (24 and 96 hours).
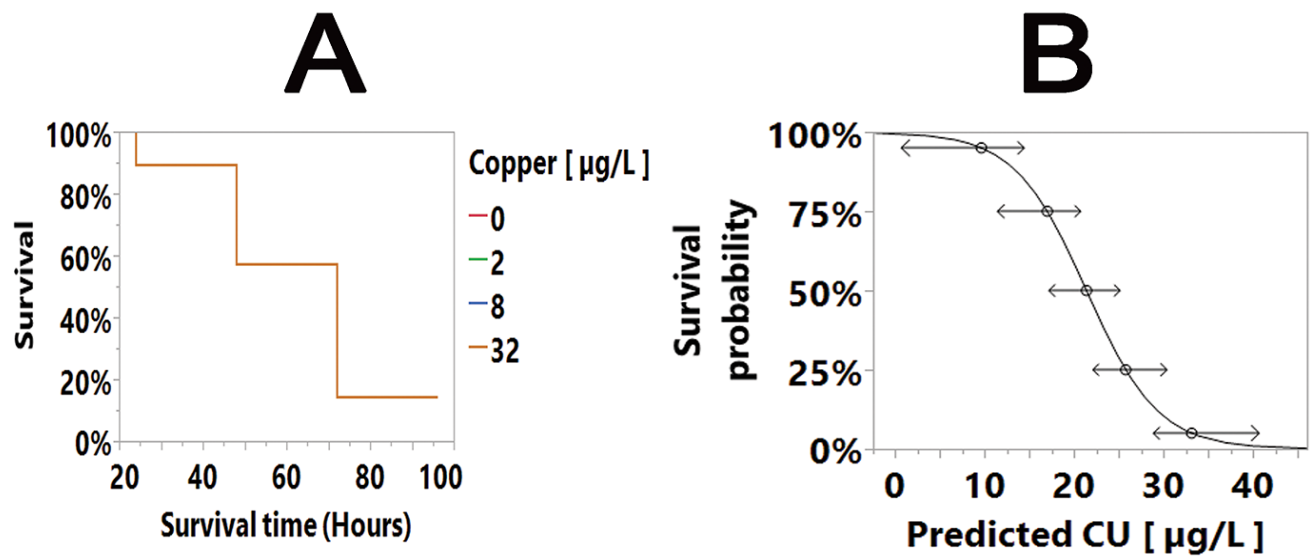
Figure 2. Percentage survival of adult Delta Smelt exposed to copper. A) survival plot indicates the percentage survival of Delta Smelt exposed to copper in a 96-hour exposure. Survival at concentrations of 0 to 8 µg/L were 100% therefore not shown in the graph. B) Predicted survival probability of Delta Smelt exposed to copper. The points indicate survival probabilities of 95%, 75%, 50%, 25% and 5 %. The arrows indicate the 95% intervals (logistic regression).
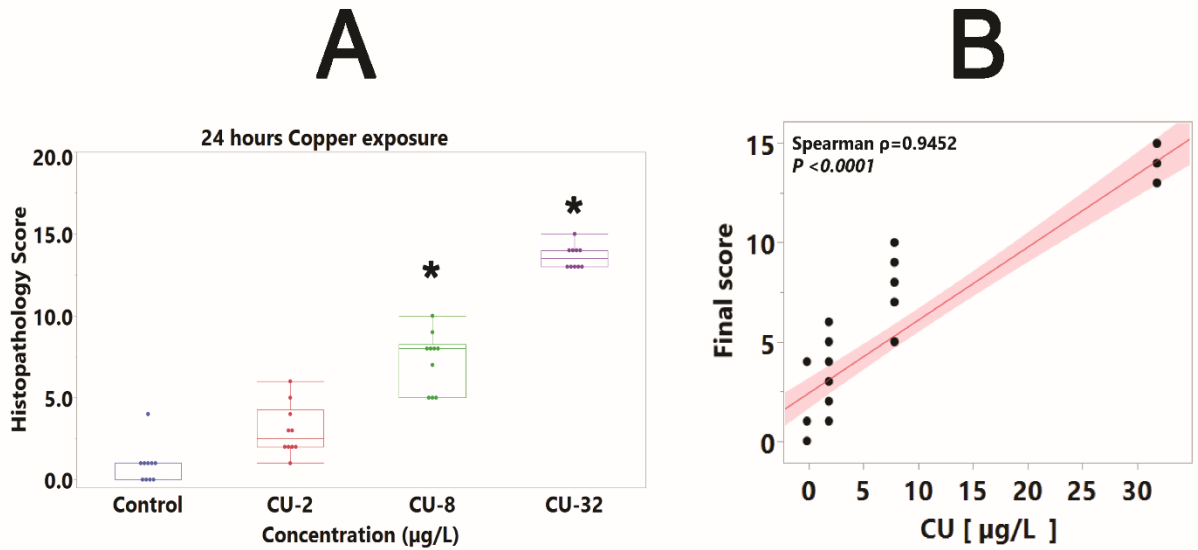
Figure 3. Severity of lesions of the olfactory epithelium of Delta Smelt exposed to increasing concentrations of copper for 24 hours. A) The severity of lesions was significantly higher in the olfactory epithelium of fish exposed to 8 and 32 µg/L. Each point represents one individual. The asterisks indicate significant differences with respect to the control at *P< 0.05*. B) A strong association is shown between the severity of lesions and the dose of copper. Each point represents a different score value in histopathology.
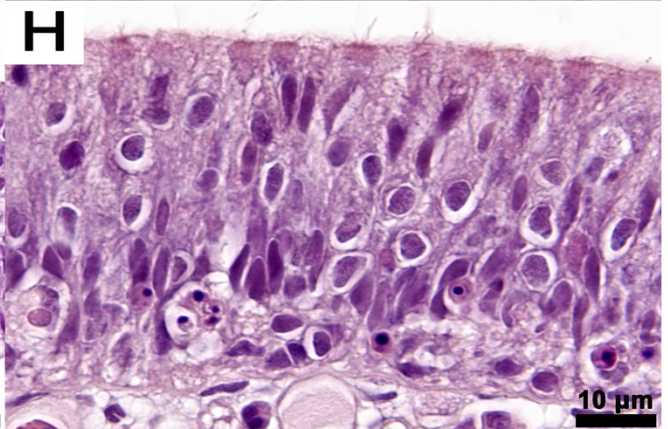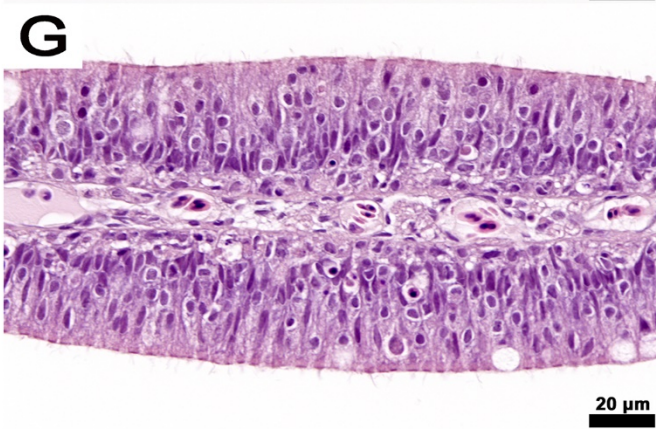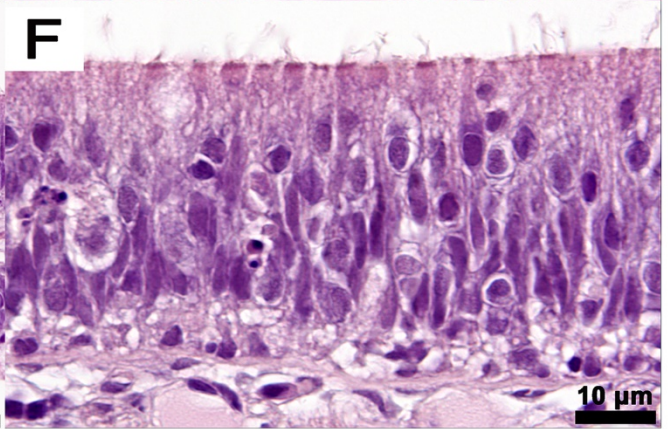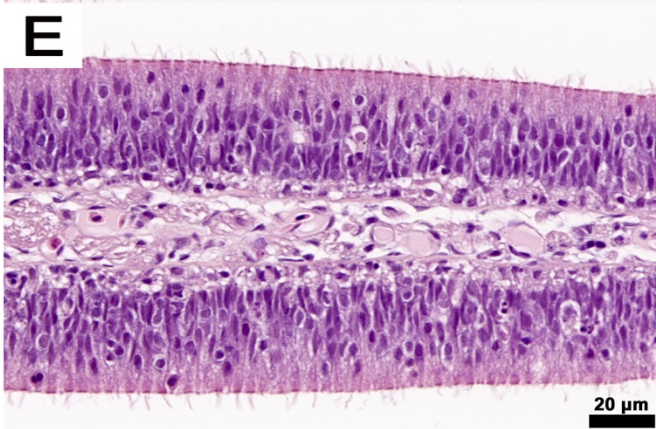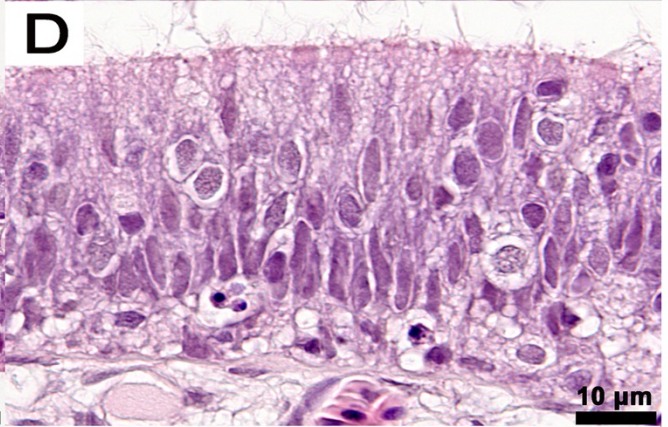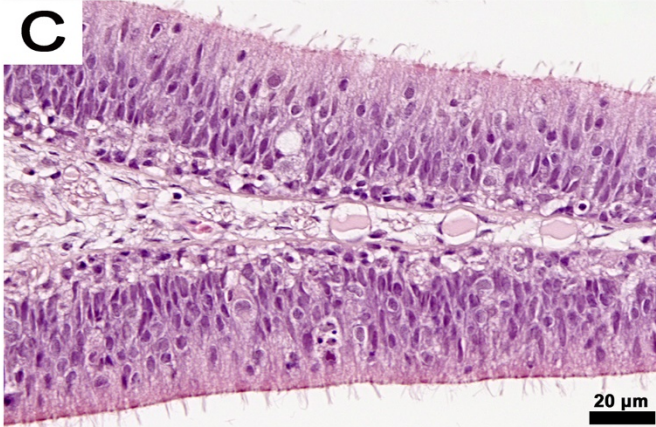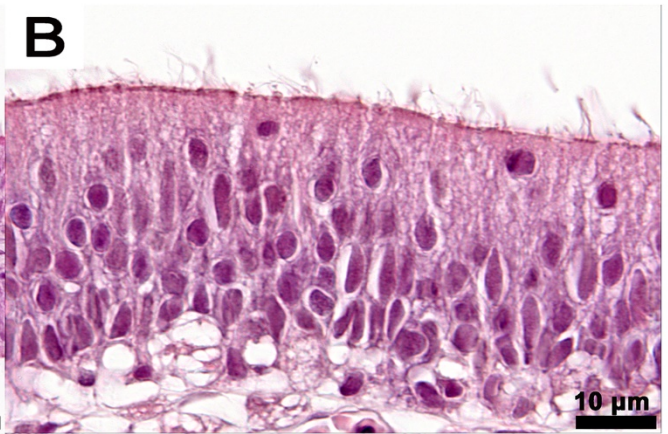
Figure 4. Histopathological changes in the olfactory epithelium of Delta Smelt exposed to copper for 24 hours. A) and B) Olfactory epithelium from a fish in the control group. C) and D) olfactory epithelium from a fish exposed to 2 µg/L of copper. E) and F) olfactory epithelium from a fish exposed to 8 µg/L of copper. G) and H) olfactory epithelium from a fish exposed to 32 µg/L of copper. All sections were stained with H&E stain. The left and rigth panels show a single lamellae. Left panel was taken at 400X and the right panel at 1000X. 3 µm thick sections.
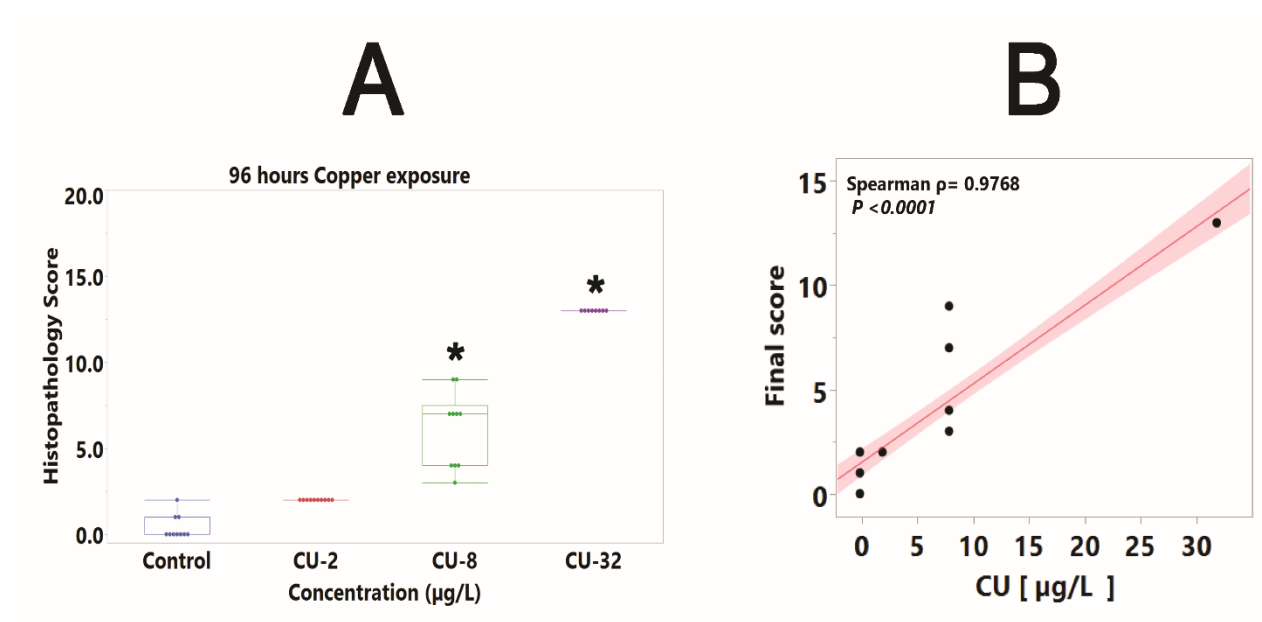
Figure 5. Severity of lesions of the olfactory epithelium of Delta Smelt exposed to increasing concentrations of copper for 96 hours. A) The severity of lesions was significantly higher in the olfactory epithelium of fish exposed to 8 and 32 µg/L. At 32 µg/L, only two fish were sampled at 96 hours of exposure, the remaining fish analyzed for this exposure time were sampled at 24, 48 and 72 hours due to signs of toxicity before conclusion of the experiment. Each point represents one individual. The asterisks indicate significant differences with respect to the control at *P ≤ 0.05*. B) A strong association is shown between the severity of lesions and the dose of copper. Each point represents a different score value in histopathology.
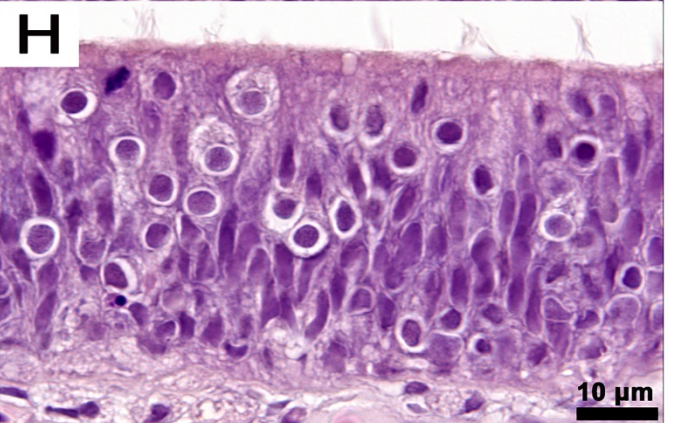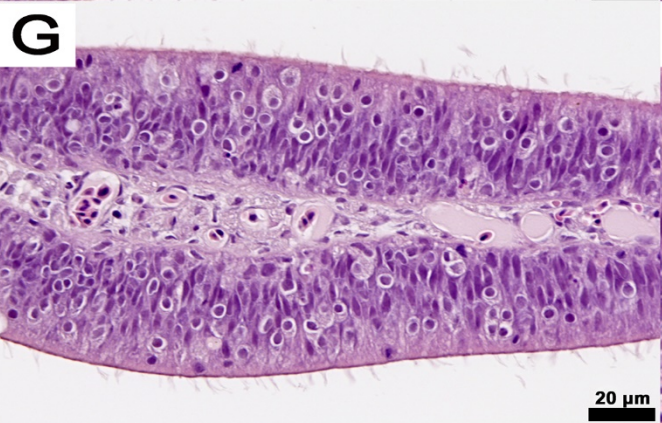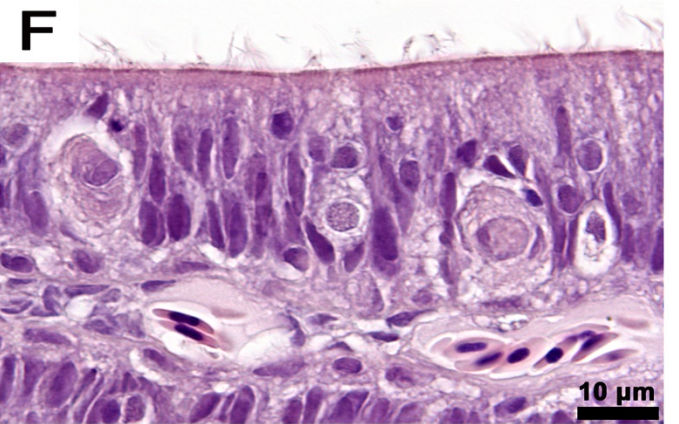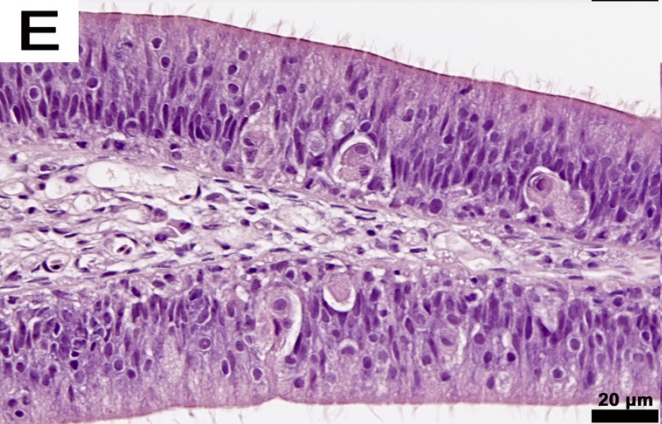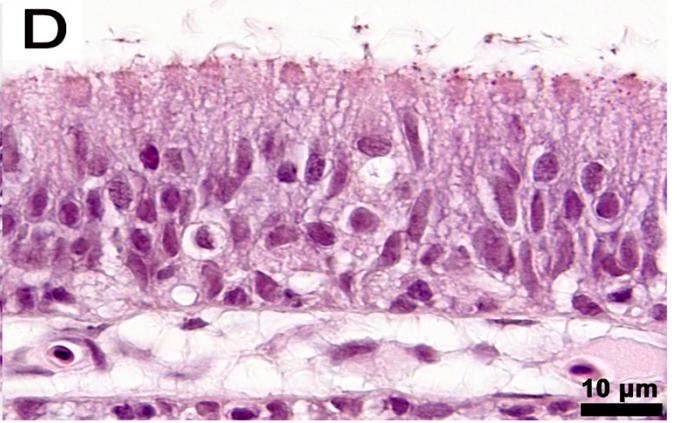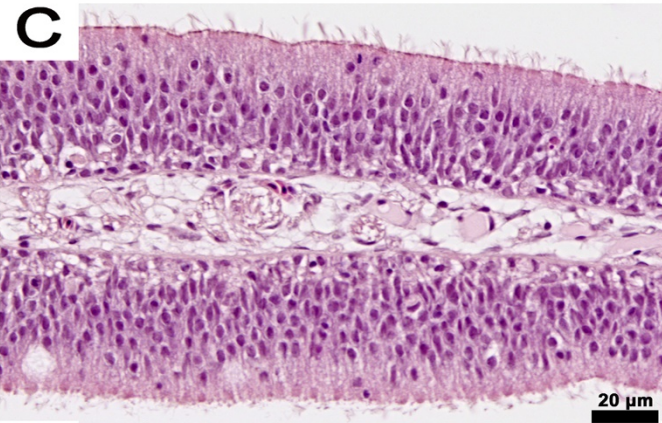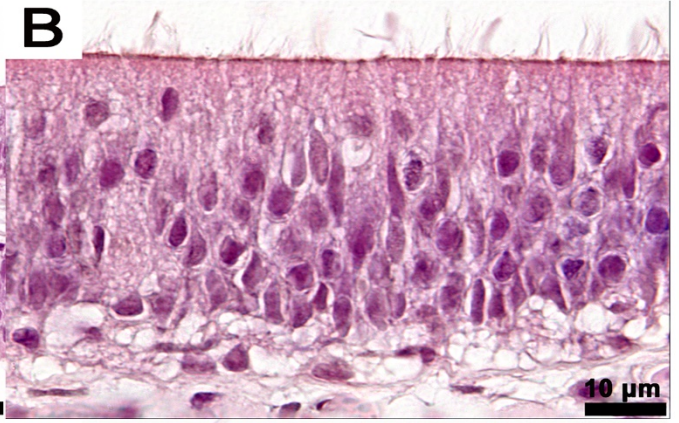
Figure 6. Histopathological changes in the olfactory epithelium of Delta Smelt exposed to copper for 96 hours. A) and B) Olfactory epithelium from a fish in the control group. C) and D) olfactory epithelium from a fish exposed to 2 µg/L of copper. E) and F) olfactory epithelium from a fish exposed to 8 µg/L of copper. G) and H) olfactory epithelium from a fish exposed to 32 µg/L of copper. All sections were stained with H&E stain. The left and rigth panels show a single lamellae. Left panel was taken at 400X and the right panel at 1000X. 3 µm thick sections.
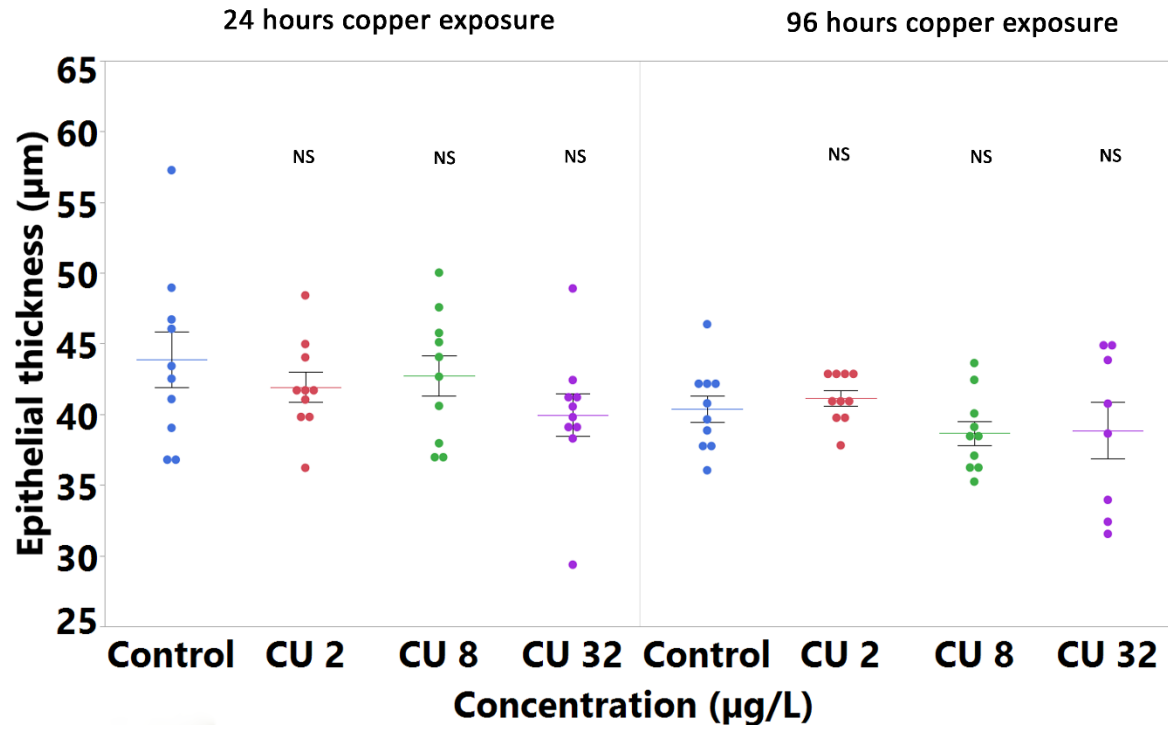
Figure 7. Thickness of the olfactory epithelium of Delta Smelt exposed to copper during 24 and 96 hours.

Data is given in mean ± SE, and each dot represents one individual. NS= non-significant differences when compared to the control groups.
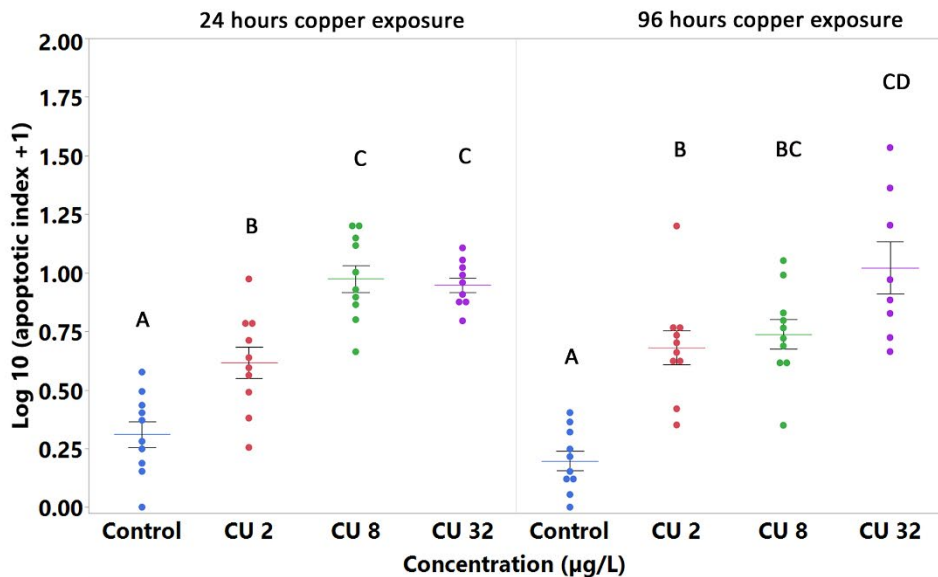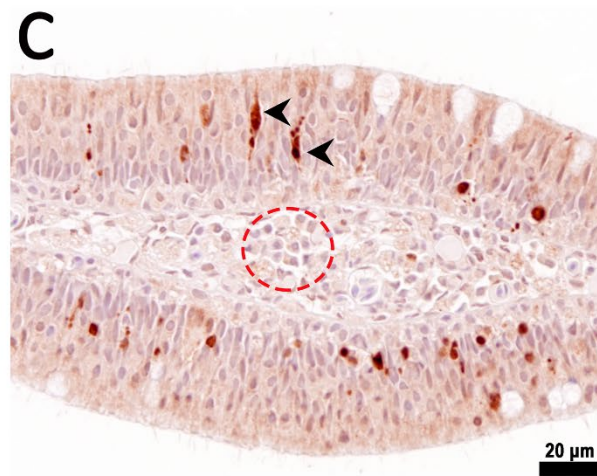
Figure 8. Apoptotic cell death in the olfactory epithelium of Delta Smelt increase with higher doses of copper. A) The apoptotic index is higher in the olfactory epithelium at 8 and 32 µg/L of copper in 24 hour and 96-hour exposures. Each data point represents one individual. The transverse central line in each distribution is the mean, and transverse lines above and below the mean line represent standard error of the mean. Different letters represent significant differences at $P < 0.05$. B) Olfactory lamella of a Delta Smelt from a control group. The arrow heads indicate some basal cells positive for activated caspase 3

(arrowheads). Cleaved caspase 3 immunohistochemistry, 400X. C) Olfactory lamella of a Delta Smelt exposed to 32 µg/L of copper. The arrowheads point to two olfactory neurons undergoing apoptosis. The apoptotic cells are observed at different levels of the sensory epithelium. The dotted circle indicates abundant inflammatory cells infiltrating the lamina propria. Cleaved caspase-3 immunohistochemistry, 400X.
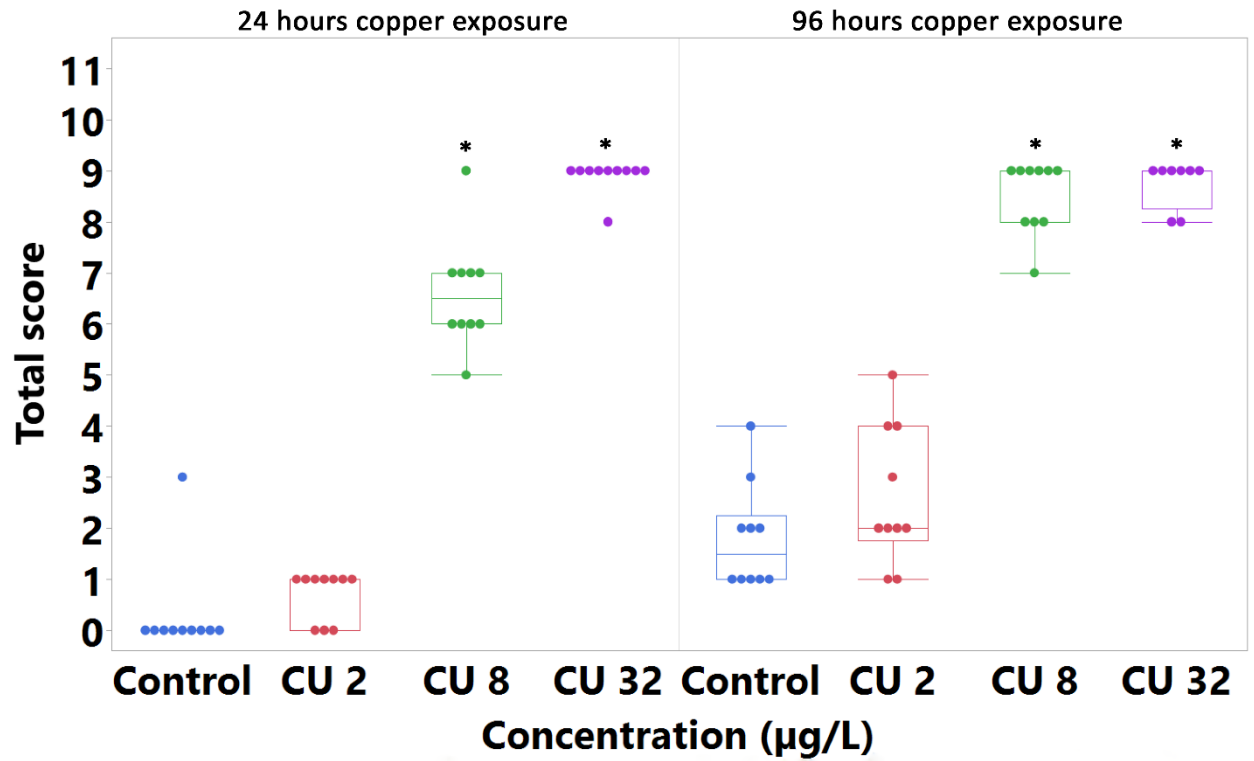
Figure 9. $G_{\alpha S/olf}$ immunostaining score in the olfactory epithelium of Delta Smelt exposed to copper. The final composite score is the sum of the immunostaining sores in three categories (staining of ciliate neurons (stripped pattern), staining of surface structures (rimmed pattern) and staining intensity). Each point represents one individual. The asterisks indicate significant differences when compared to the control group.
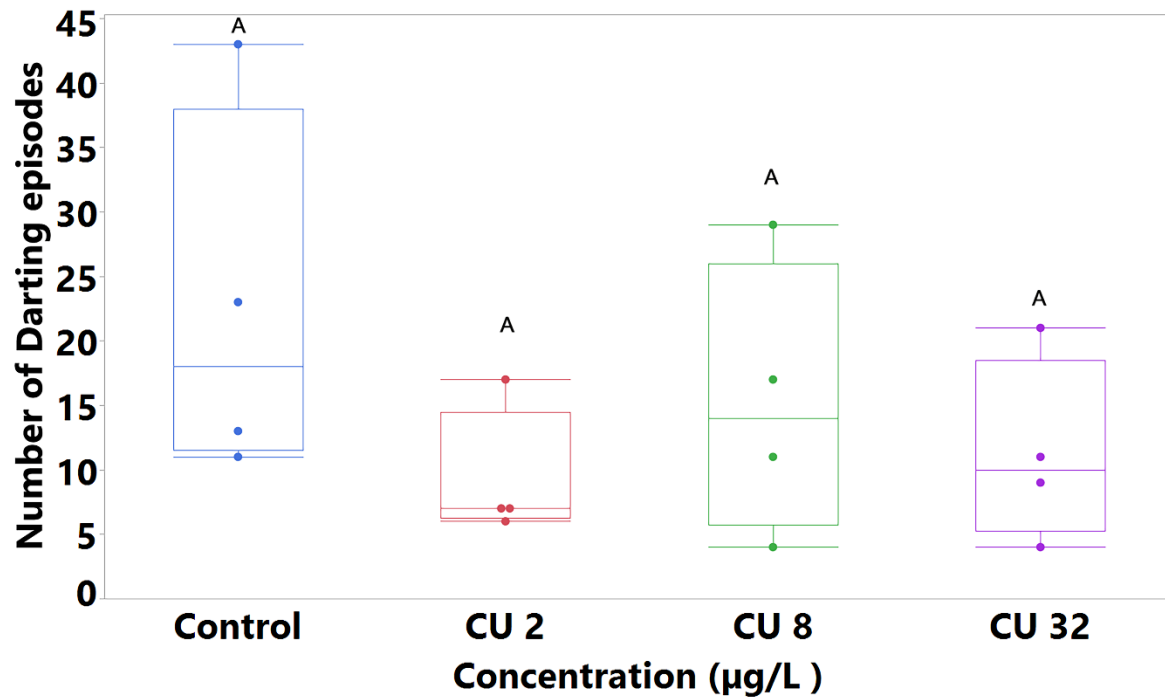
Figure 10. Number of darting episodes of Delta Smelt after exposure to skin extracts among groups previously exposed to copper for 24 hours. Each data point represents the total number of darting episodes for the group of fish per replicate on each concentration. Different letters denote statistically significant differences.
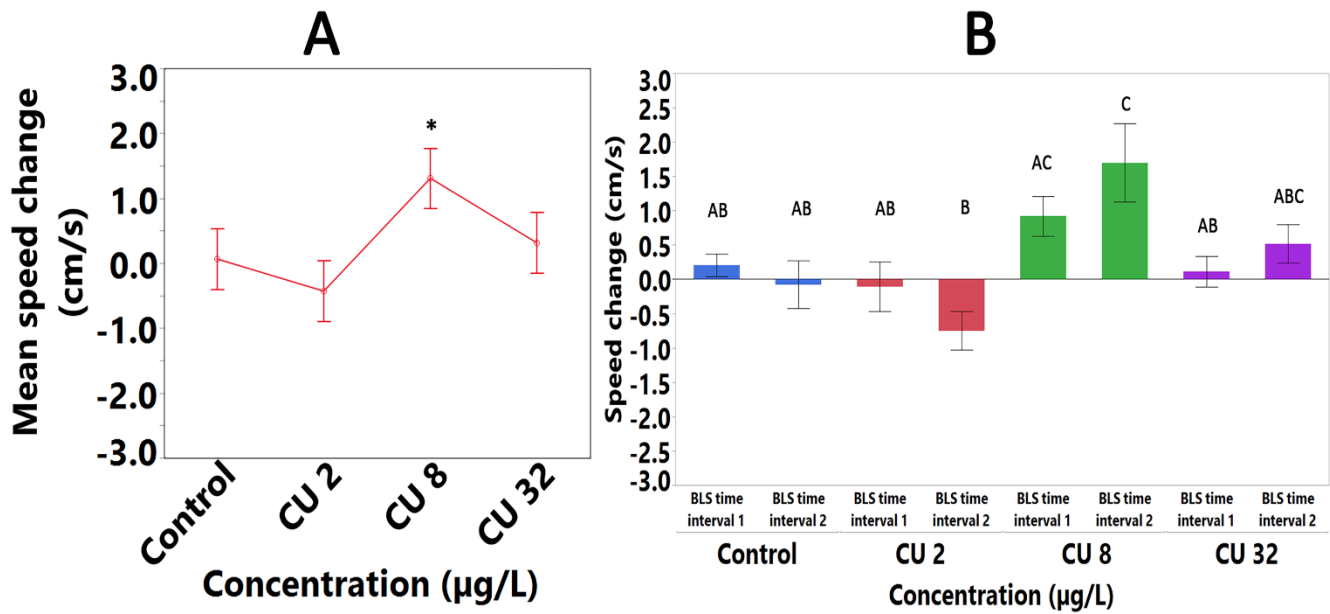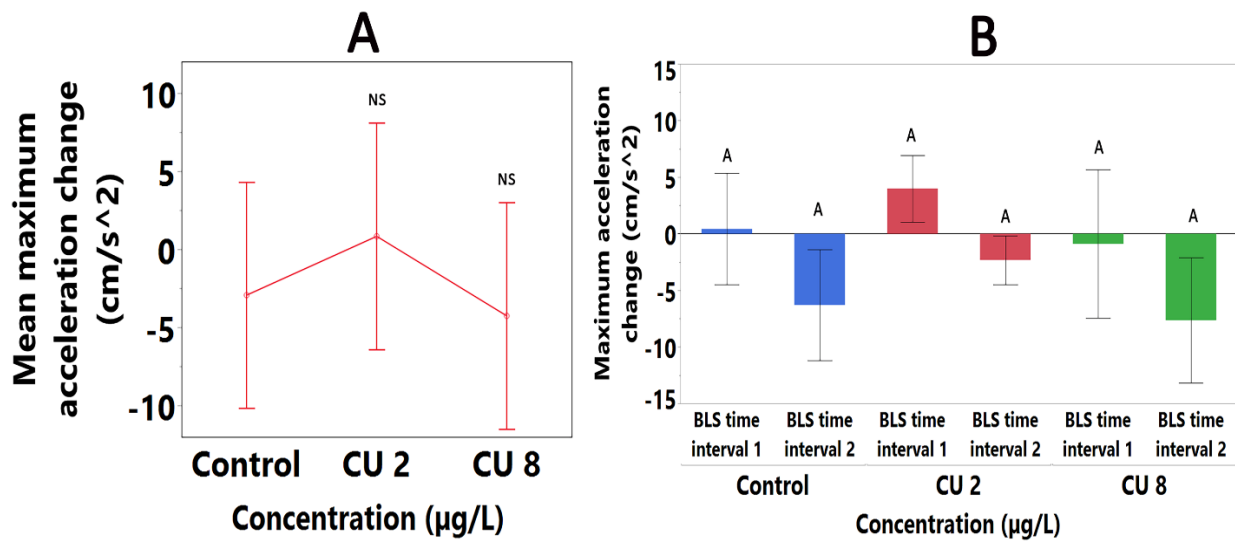
Figure 11. Mean speed change of Delta Smelt after exposure to skin extract. The fish were previously exposed to copper for 24 hours. A) Mean speed change on each concentration. The asterisk represents significant differences to the control group. **B)** Mean speed change by concentration and time interval (BLS= Base line subtracted time intervals 1 and 2). Data is given as mean±SE. The data represent mean change of four behavioral trials. Different letters represent significant differences among concentrations.
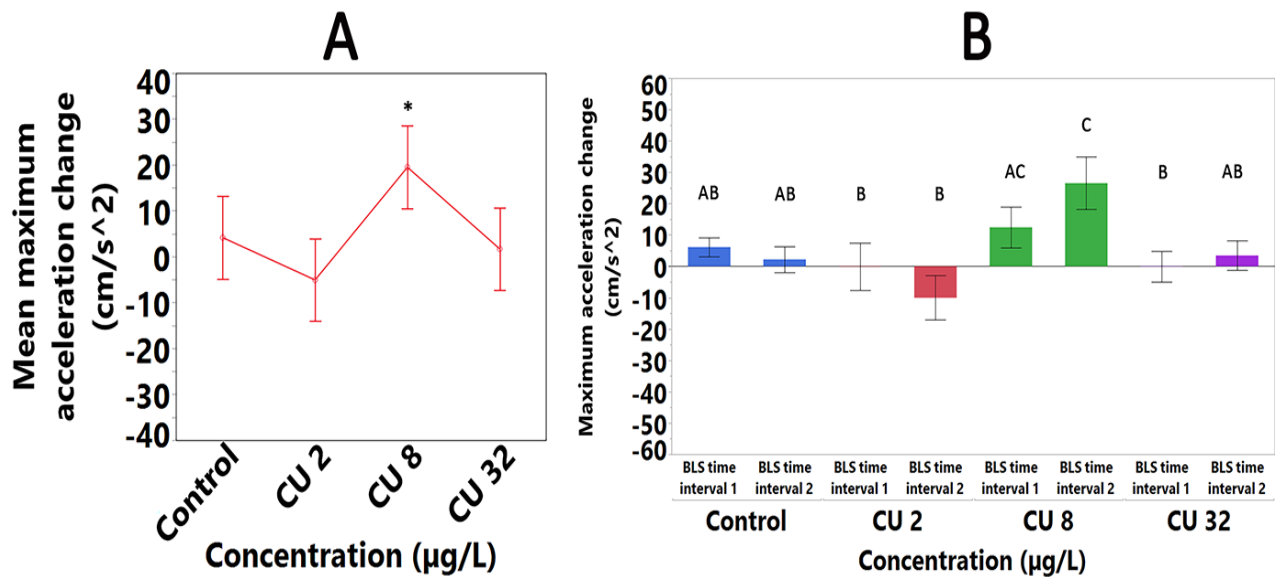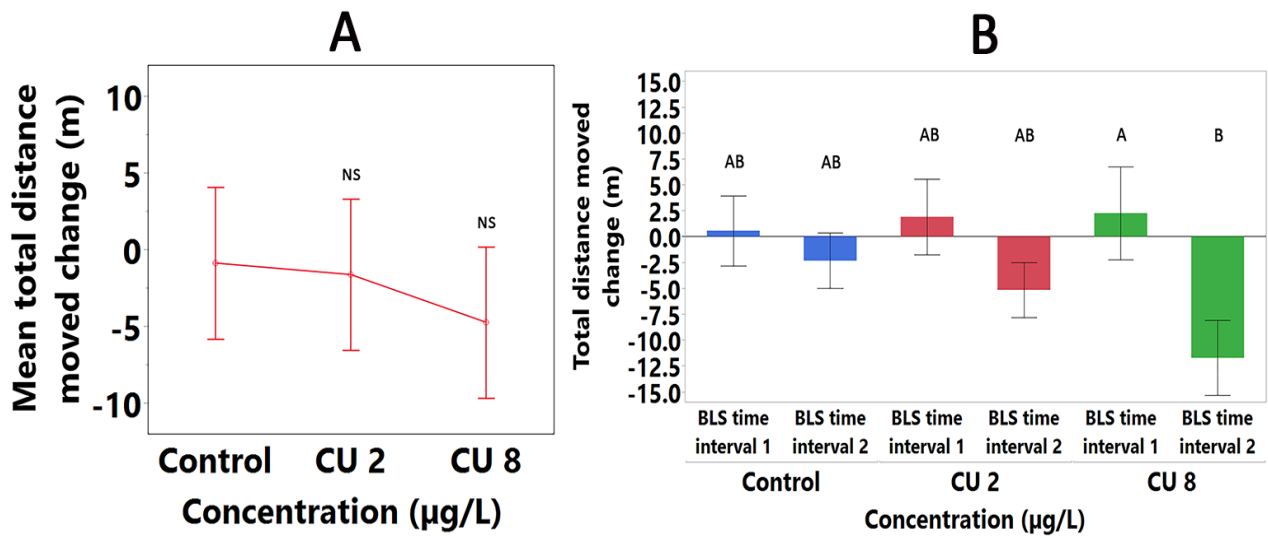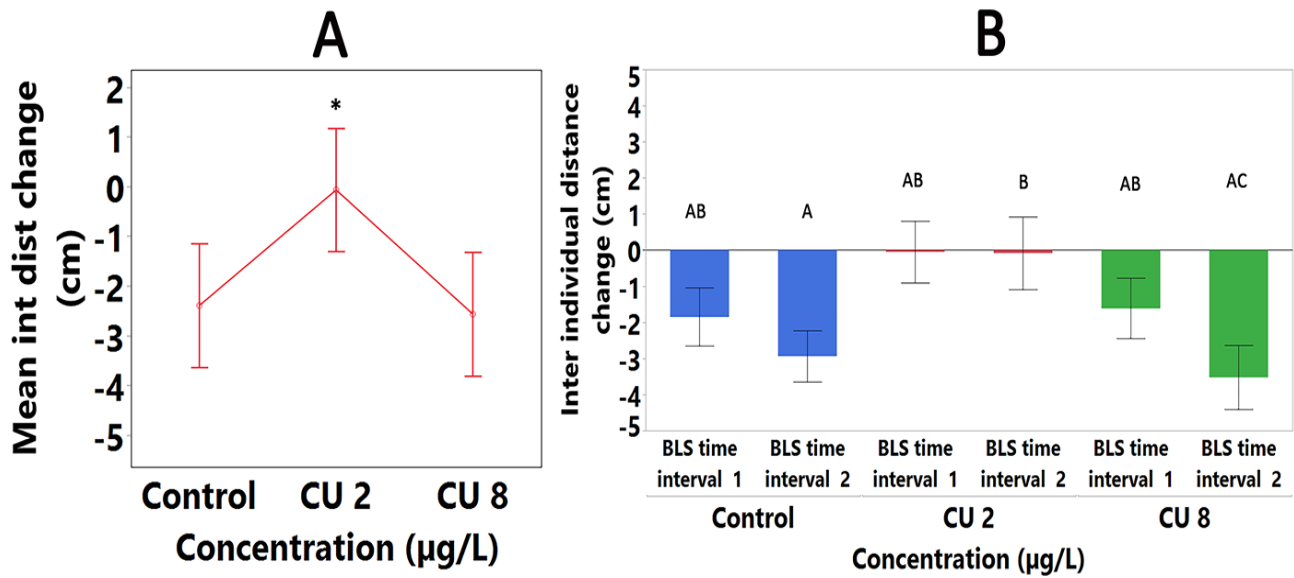
Figure 12. Mean maximum acceleration change of Delta Smelt after exposure to skin extract. The fish were previously exposed to copper for 24 hours. A) Mean maximum acceleration change on each treatment. The asterisk represents significant differences to the control group. **B)** Mean maximum acceleration change by concentration and time interval (BLS= Base line subtracted time intervals 1 and 2). Data is given as mean±SE. The data represent mean change of four behavioral trials. Different letters represent significant differences among concentrations.
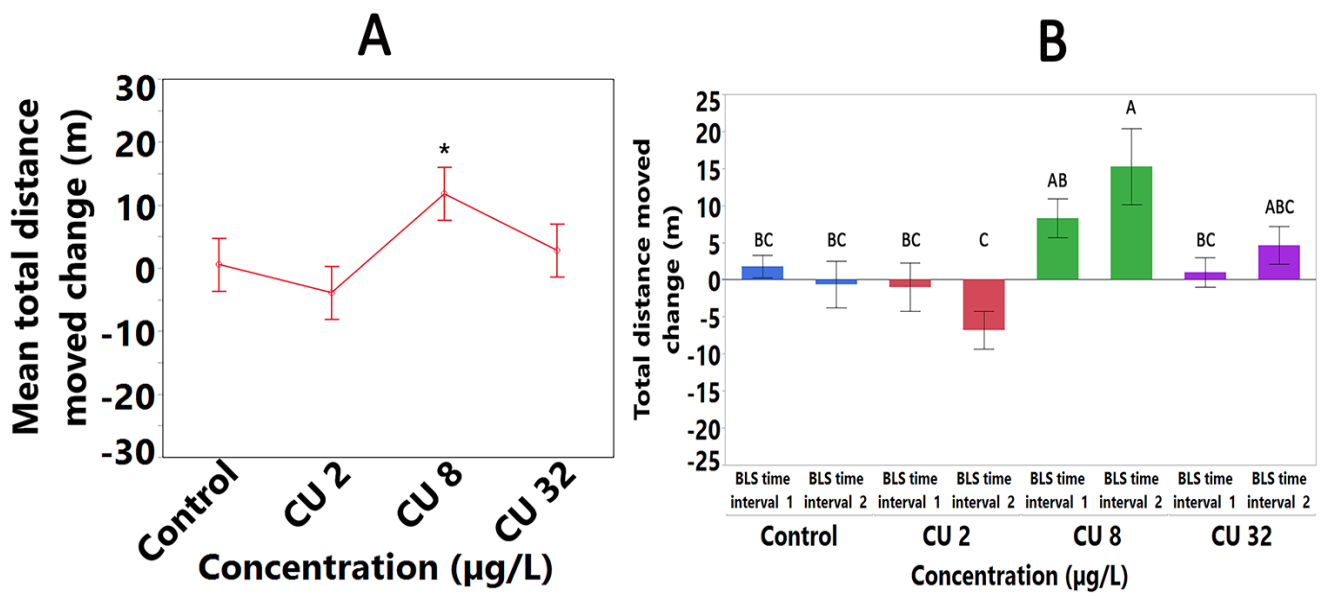
Figure 13. Mean total distance moved change of Delta Smelt after exposure to skin extract. The fish were previously exposed to copper for 24 hours. A) Mean total distance moved change on each treatment. The asterisk represents significant differences to the control group. **B)** Mean total distance moved change by concentration and time interval (BLS=Baseline subtracted time intervals 1 and 2). Different letters represent significant differences among treatments. Data is given as mean±SE. The data represent mean change of four behavioral trials. Different letters represent significant differences among concentrations.
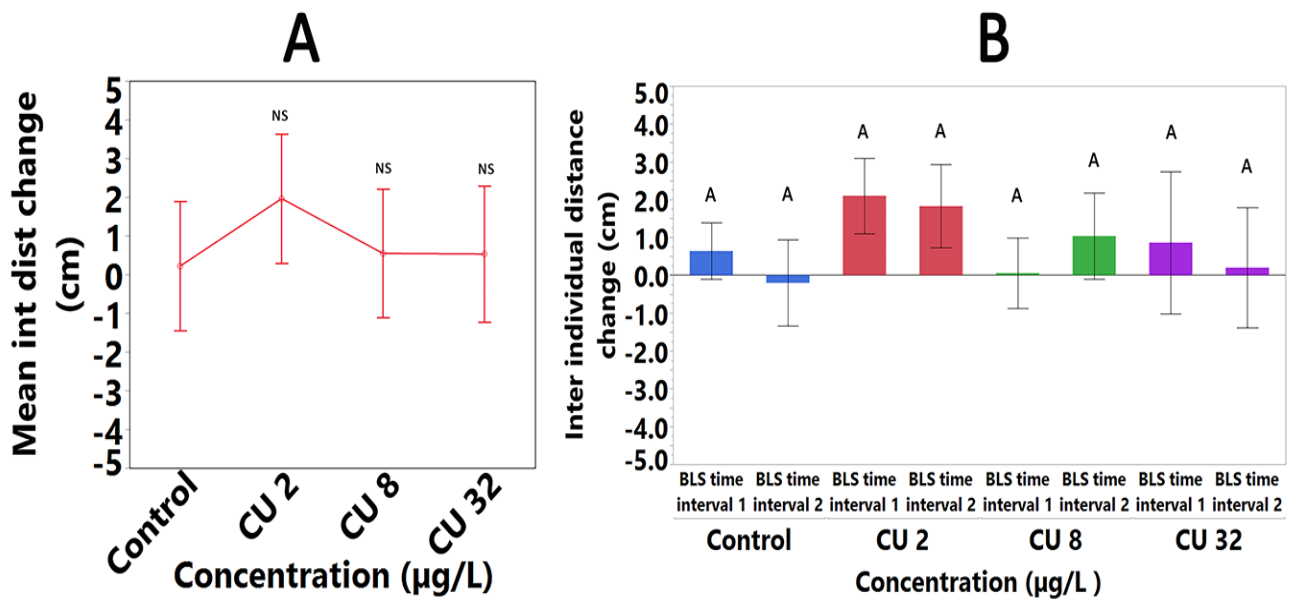
Figure 14. Mean interindividual distance change of Delta Smelt after exposure to skin extract. The fish were previously exposed to copper for 24 hours. A) Mean interindividual distance change on each concentration. NS=nonsignificant differences to the control group. **B)** Mean interindividual distance change on each concentration and time interval (BLS= Baseline subtracted time intervals 1 and 2). Data is given as mean±SE. The data represent mean change of four behavioral trials. Different letters represent significant differences among concentrations.
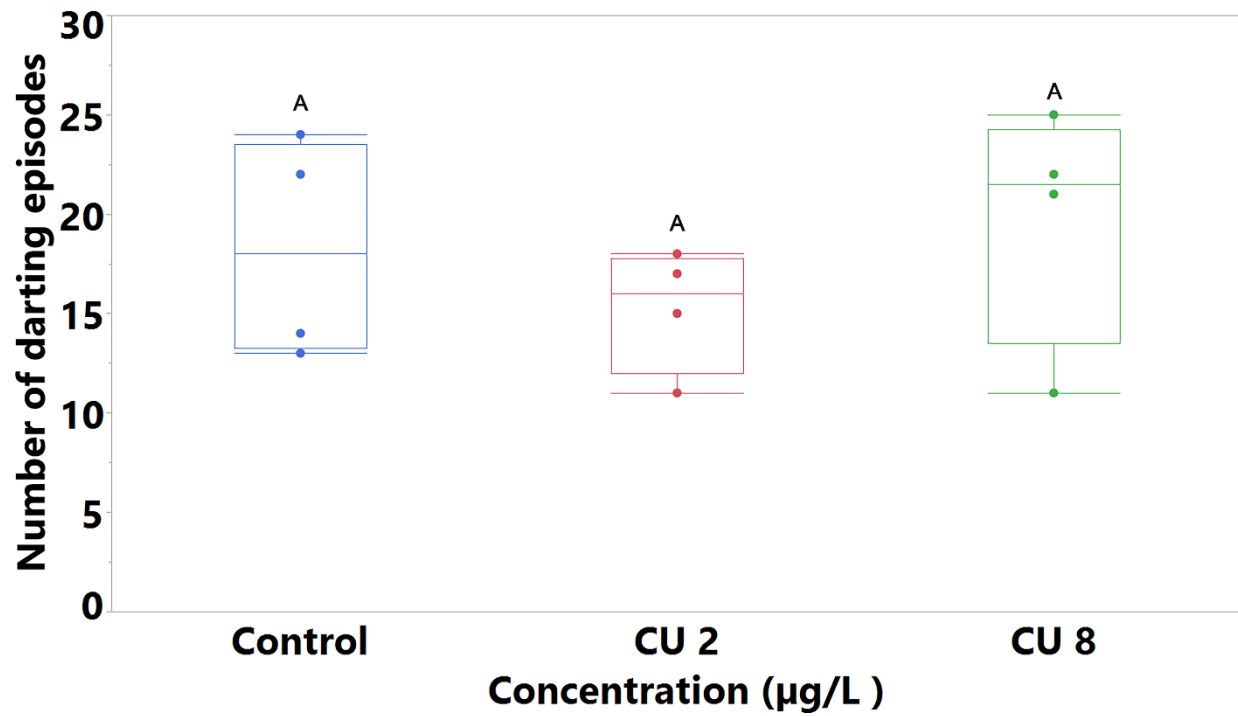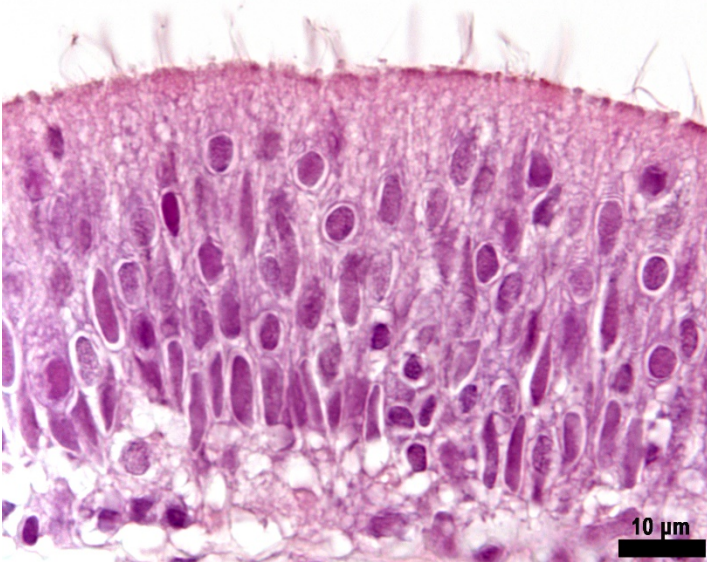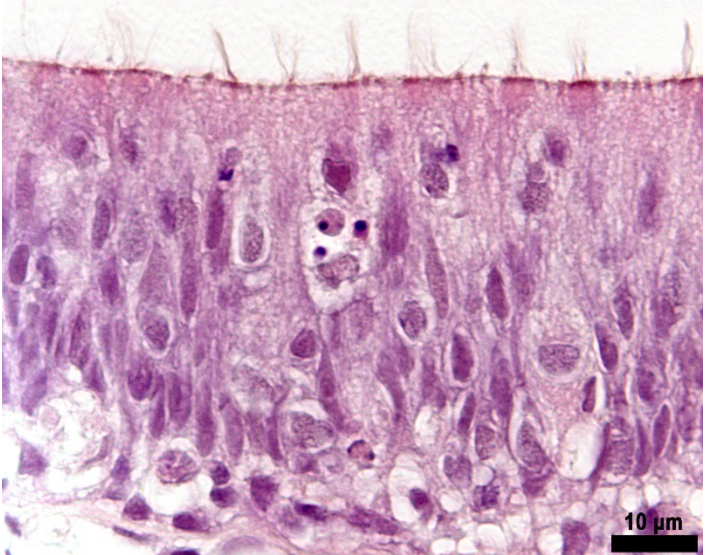
Figure 15. Number of darting episodes of Delta Smelt after exposure to skin extracts among groups previously exposed to copper for 96 hours. Each data point represents the total number of darting episodes for the group of fish per replicate on each concentration. Different letters denote statistically significant differences.
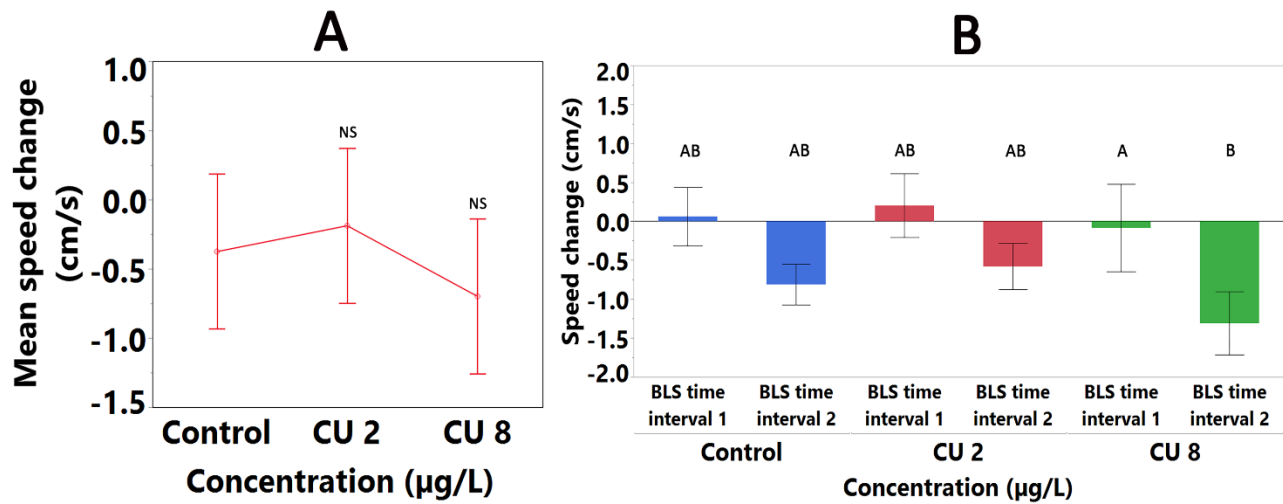
Figure 16. Mean speed change of Delta Smelt after exposure to skin extract. The fish were previously exposed to copper for 96 hours. A) Mean speed change on each concentration. NS=nonsignificant differences to the control group. **B)** Mean speed change by concentration and time interval (BLS= Baseline subtracted time intervals 1 and 2). Data is given as mean±SE. The data represent mean change of four behavioral trials. Different letters represent significant differences among concentrations.

Figure 17. Mean maximum acceleration change of Delta Smelt after exposure to skin extract. The fish were previously exposed to copper for 96 hours. A) Mean maximum acceleration change on each concentration. NS=nonsignificant differences to the control group. **B)** Mean maximum acceleration change by concentration and time interval (BLS= Baseline subtracted time interval 1 and 2). Data is given as mean±SE. The data represent mean change of four behavioral trials. Different letters represent significant differences among concentrations.

Figure 18. Mean total distance moved change of Delta Smelt after exposure to skin extract. The fish were previously exposed to copper for 96 hours. A) Mean total distance moved change on each treatment. NS= nonsignificant differences to the control group. **B)** Mean total distance moved change by treatment and time interval (BLS= Baseline subtracted time intervals 1 and 2). Data is given as mean±SE. The data represent mean change of four behavioral trials. Different letters represent significant differences among concentrations.

Figure 19. Mean interindividual distance change of Delta Smelt after exposure to skin extract. The fish were previously exposed to copper for 96 hours. A) Mean interindividual distance change on each copper concentration. The asterisk represents significant differences to the control group. **B**) Mean interindividual distance change on each copper concentration and time interval (BLS= Baseline subtracted time intervals 1 and 2). Data is given as mean±SE. The data represent mean change of four behavioral trials. Different letters represent significant differences among concentrations.

TABLES

Table 1. Total measured concentration of ions in the experimental solutions used in this work. The concentrations are given in mg/L and mean±standard deviation. Ion concentrations were measured by ICP/MS (see Methods section).

Ion [] mg/L	Experiment			
	24 hours		96 hours	
	Mean	SD	Mean	SD
Na$^+$	24.56	0.34	24.70	0.31
Mg^{2+}	11.31	0.19	11.31	0.16
Cl$^-$	24.86	0.40	25.14	0.48
K$^+$	2.04	0.01	2.07	0.02
Ca^{2+}	13.23	0.11	13.39	0.21

Table 2. Nominal and measured concentrations of copper in exposure solutions used in this study. The concentrations are given in µg/L and mean±standard deviation. Cu^{2+} concentration was measured by ICP/MS (see Methods section).

Nominal [ ] of Copper (Cu^{+2} µg/L)	Experiment			
	Measured [ ] of Copper		Measured [ ] of Copper	
	24 hours		96 hours	
	Mean	SD	Mean	SD
0 (control)	0.66	0.45	0.25	0.06
2	1.82	0.13	1.95	0.04
8	7.03	0.19	7.25	0.26
32	28.41	0.63	28.34	0.16

Table 3. Scoring system of histopathological lesions observed in the olfactory epithelium of Delta Smelt exposed to copper.

Characteristic	Score description	Example
Cell Death	**Normal (0):** None to few dead cells (0 to less than 3) in a 400X high power field. Dead cells are in basal regions of olfactory epithelium, isolated and do not form clusters or big areas of dead cells.	
	Mild (1): Occasional (more than 4 but less than 6) dead cells with small clusters formation, more frequently localized to basal and medium portion of the epithelium.	

Moderate (2): Frequent (greater than 7 but less than 10) dead cells with cluster formation, localized to basal and medium portion of the epithelium.
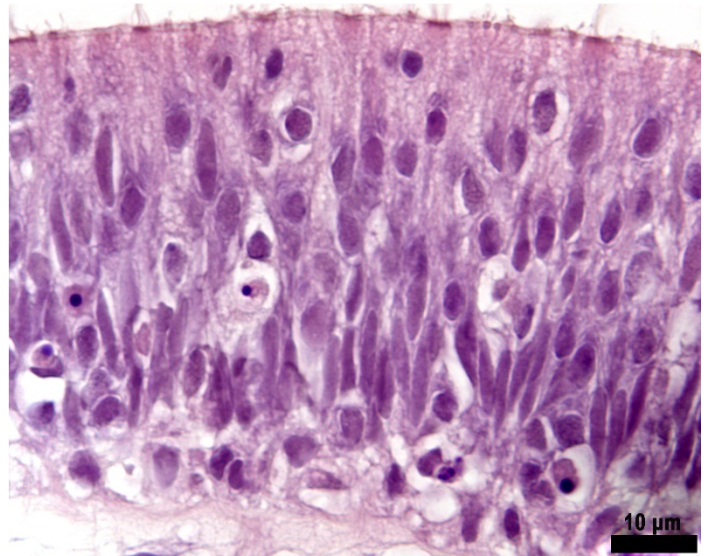
Severe (3): Abundant (>10) dead cells, formation of big clusters of several dead cells, distributed along the whole sensory epithelium.
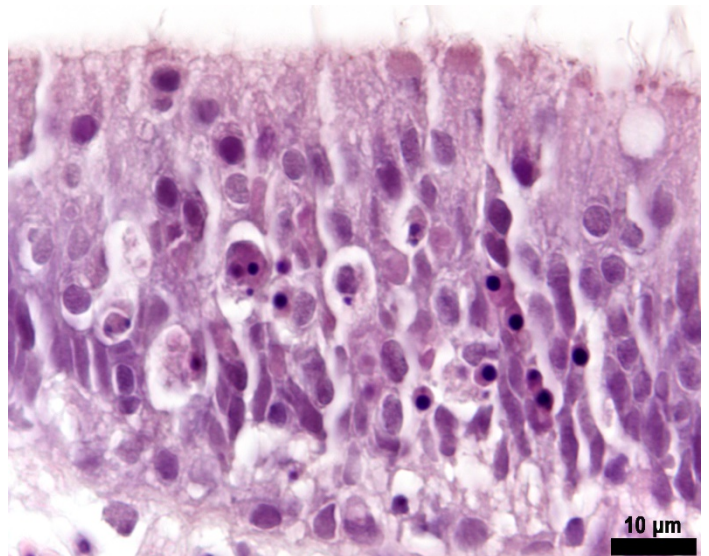
Lack of surface structures

Normal (0): Ciliated epithelial surface looks homogeneous in the whole lamella with well-developed intact cilia.
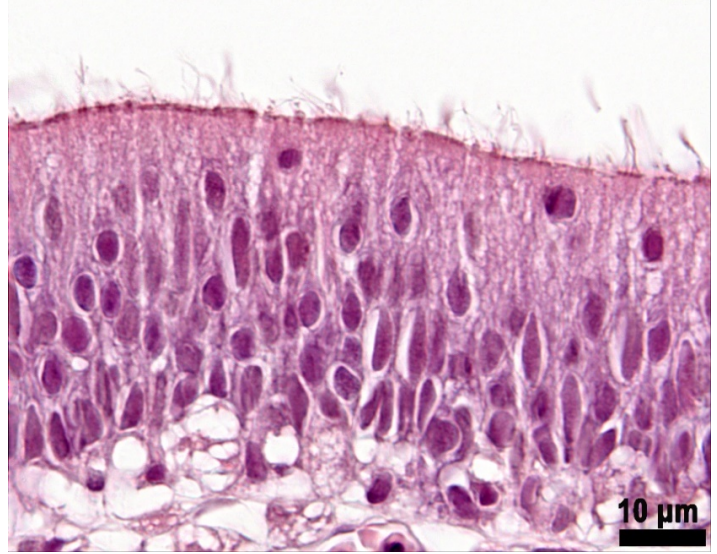
Mild (1): Small focal areas of cilia shortening and clumping, less than 10% of lamellar surface affected
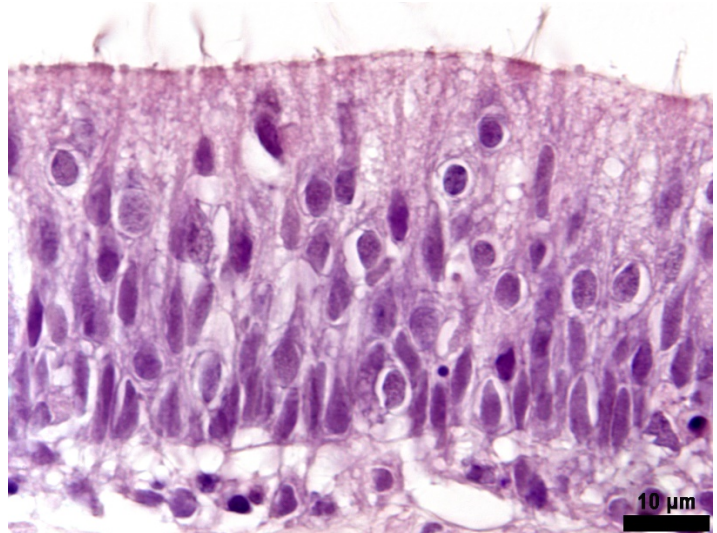
Moderate (2): Cilia are shortened, difficult to identify in focally extensive areas of lamellae.
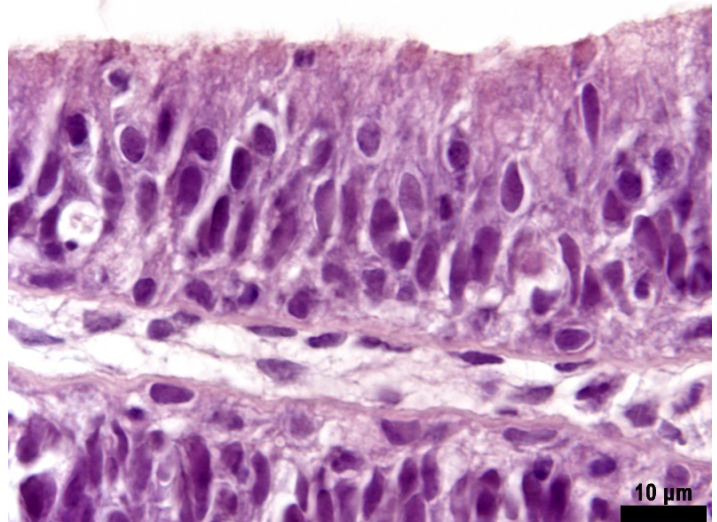
Severe (3): Cilia are shortened, clumped or absent in whole lamellar surface.
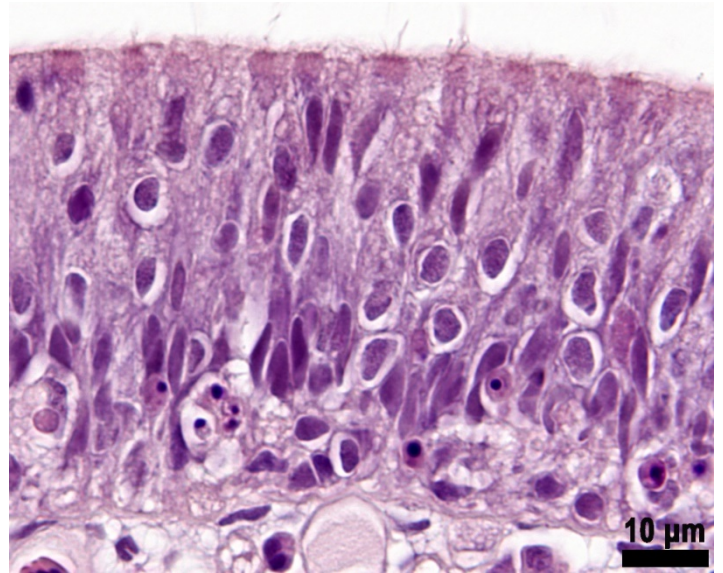
Disorganization of epithelium

Normal (0): The epithelium has abundant nuclei profiles at different levels of epithelial thickness, staining is homogeneous and basophilic, and basal cells look continuous to each other without any spaces or interruption between them.
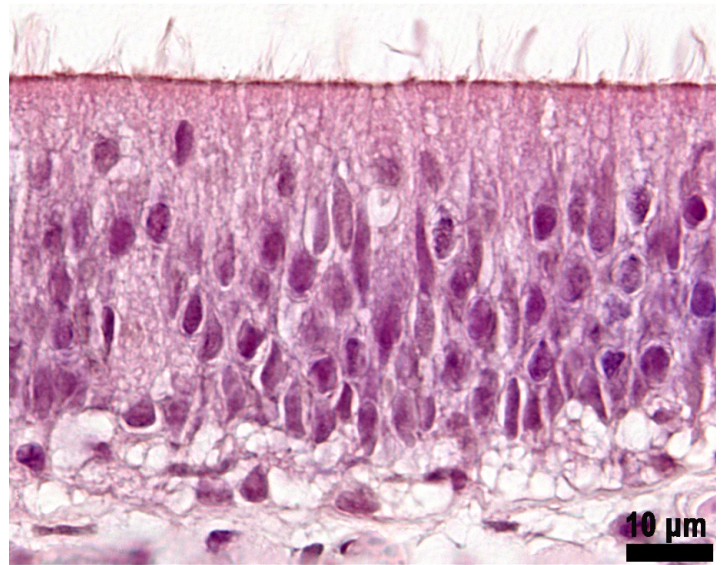
Mild (1): Spaces between cells start to be visible occasionally, with a clear hallo around the nucleus and separation from neighboring cells, there is slight clearing of cytoplasm of neurons and evident linear intercalated staining pattern of epithelia.
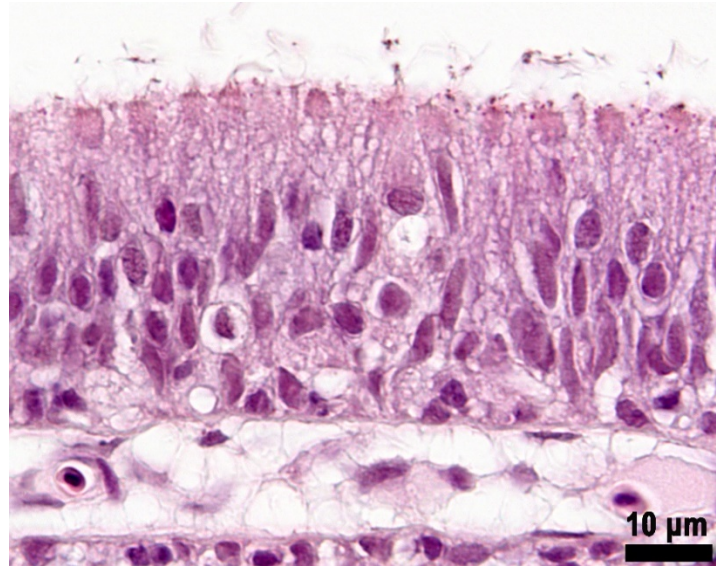
Moderate (2): There is evident clearing of the cell cytoplasm, with clear spaces between cells and loss of continuity between neighboring cells. Basal cells are discontinuous, and separated by death cells or vacuolated cells.
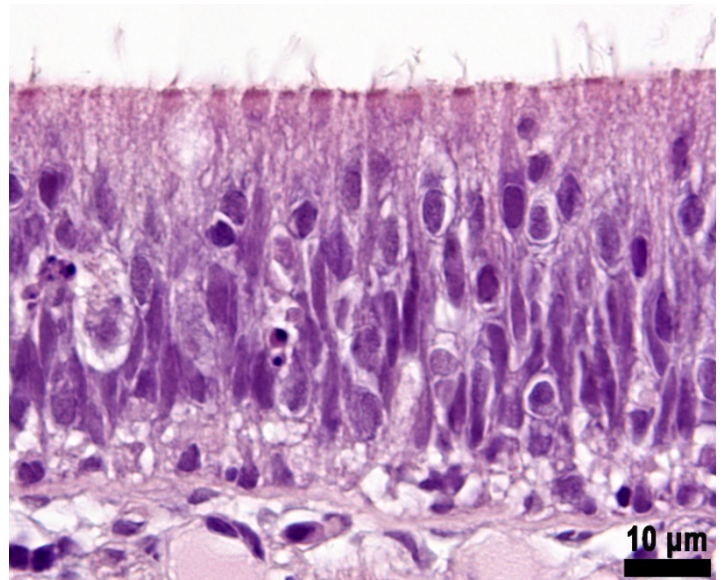
Severe (3): There is clearing of the epithelium and hypocellularity, the basal cells are mostly loss and disorganized. The nuclei and bodies of sustentacular cells are highlighted by the clear background giving the epithelium a stripped intercalated pattern.
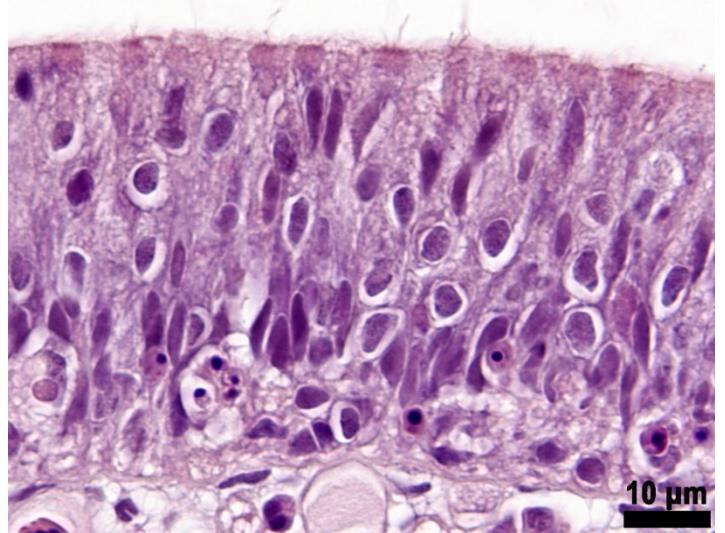
Inflammatory cell infiltrates

Normal (0): Few resident immune cells infiltrate the lamina propria, mainly lymphocytes and macrophages.
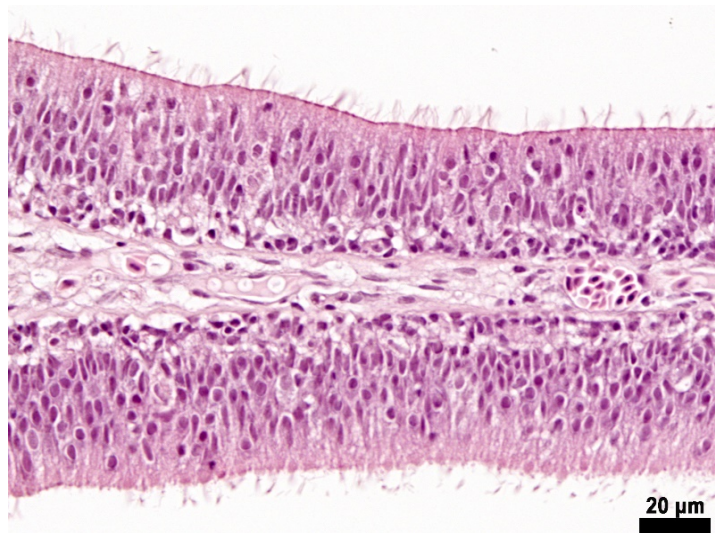
Mild (1): Small clusters of immune cells in lamina propria and basal portion of epithelium, mainly macrophages.
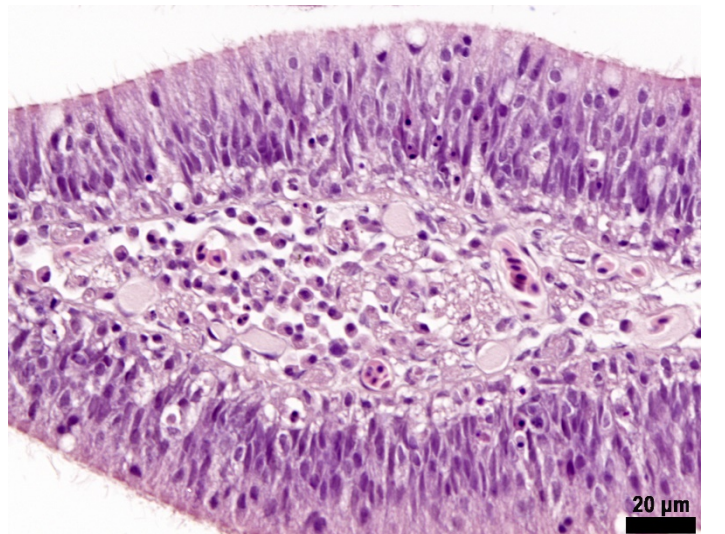
Moderate (2): Macrophages and few neutrophils diffusely infiltrate the lamina propria and basal portions of epithelium accompanied by dead cells in basal portion.
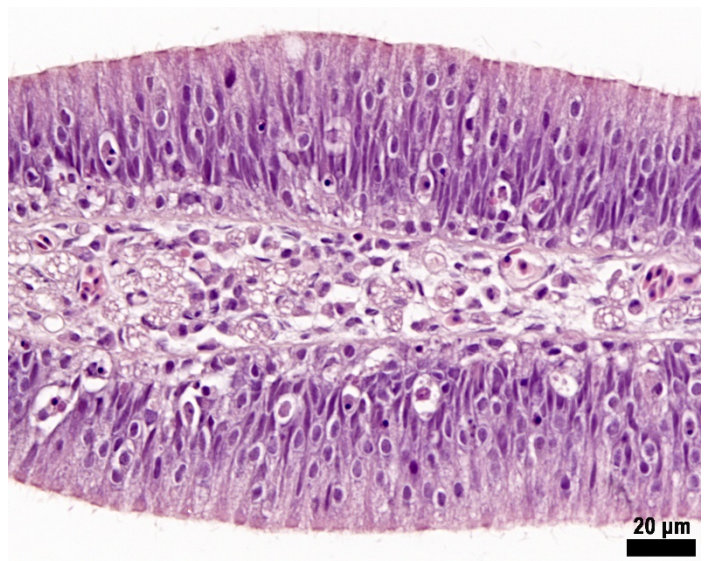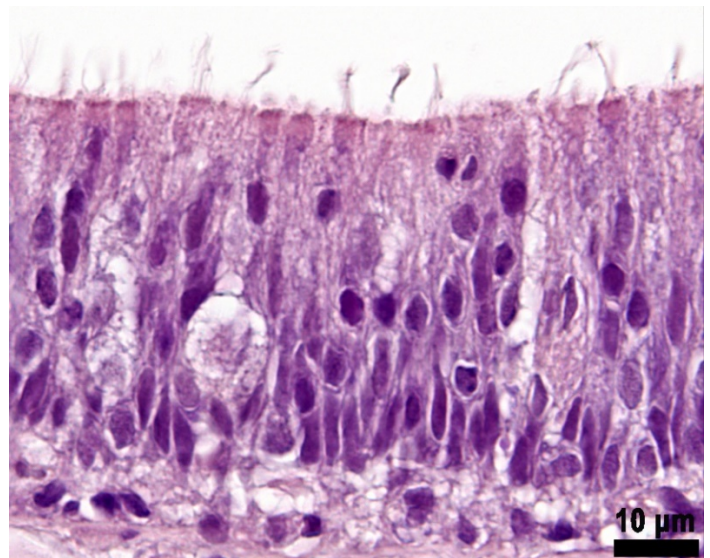

Cytoplasmic vacuolation

Normal (0): Homogeneous, moderately basophilic cytoplasmic staining with no clear spaces in between cells, around nucleus or intracytoplasmic.
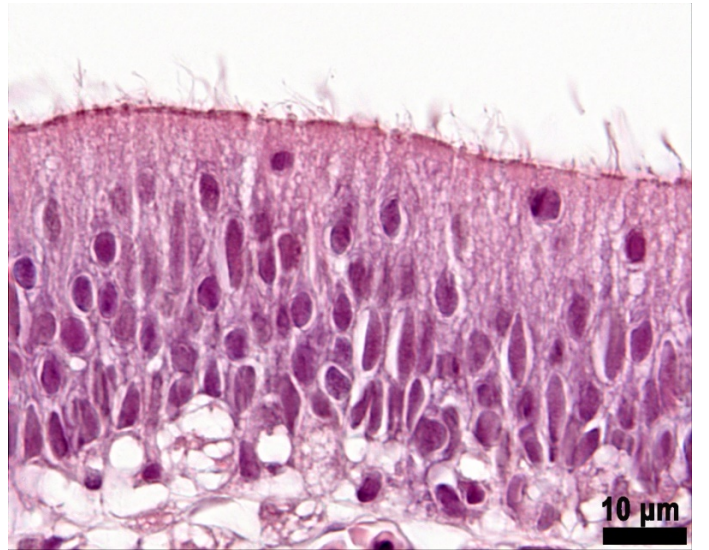
Mild (1): Cytoplasm is slightly micro vacuolated and clear. There is evident separation and clustering of mildly swollen cells, with a clear perinuclear hallo. These areas are admixed with wide areas of normal epithelium.

Moderate (2): Multiple clusters of swollen rounded cells are evident, surrounded by micro vacuolated clear cytoplasm of sustentacular and sensory cells. The clearing of the cytoplasm is prominent giving the epithelium a clear appearance, the epithelial cells are more prominently separated from neighboring ones with clear spaces. Frequent lysed cells are evident.
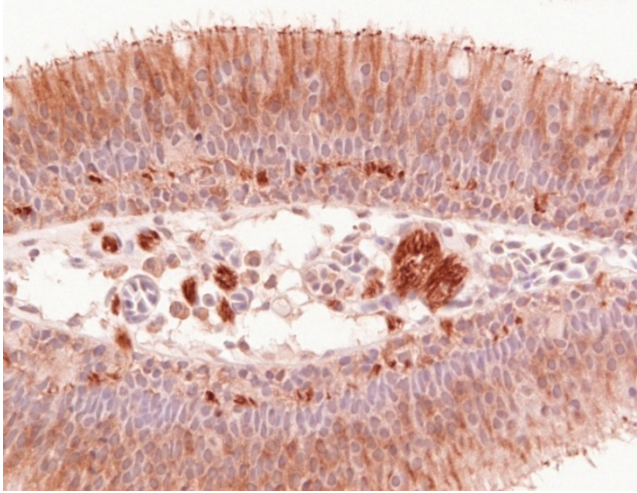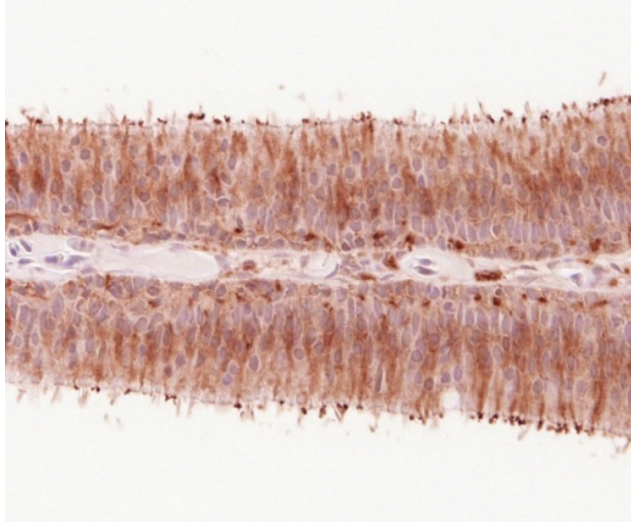
Severe (3): The vacuolar changes are highly diffuse affecting the whole epithelial surface. There are numerous clear swollen cells with abundant intracytoplasmic vacuoles. Cells are separated from neighboring ones by clear spaces. Frequently the cells individualize, round and become lysed with abundant clear cytoplasm. At this point, the cytoplasm, and nuclei of sustentacular cells is easily identified, highlighted by the clear background of swollen neuronal cells, giving the epithelium a striped appearance.

10 µm
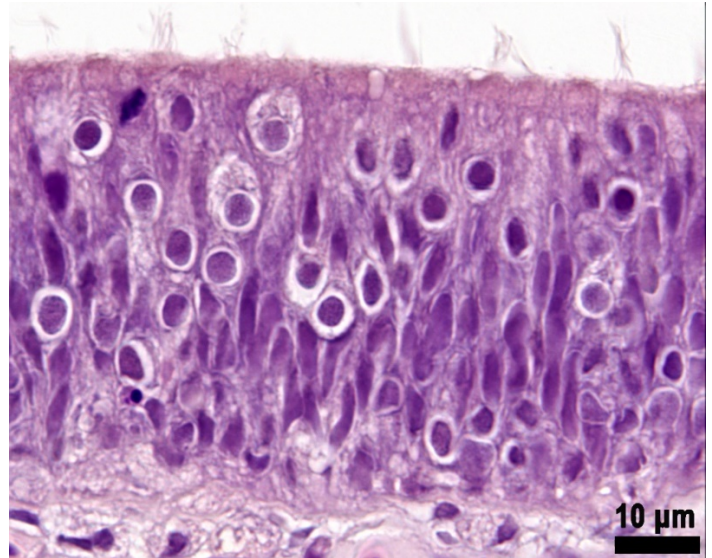
Table 4. $G_{\alpha S/olf}$ immunohistochemical staining score system evaluating the distribution of ciliate neurons in the olfactory epithelium of Delta Smelt exposed to copper.

| Characteristic	Score description	Example
striped-intercalated linear pattern distribution	**Normal (0):** A clear linear stripped-intercalated pattern is visible with delineation of the ciliate neurons and intercalation between positive neurons and negative sustentacular cells.	
	Mild (1): The stripped pattern is still evident, the stain in the neurons is slightly patchy and more scattered within the cytoplasm of the neurons.	

Moderate (2): The stripped pattern is attenuated in about 50% of lamellar epithelium, with focally extensive areas in which there is lack of staining of neurons and stripped pattern.
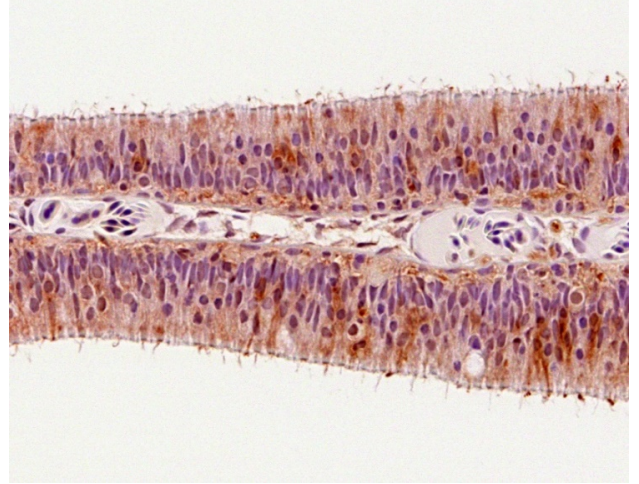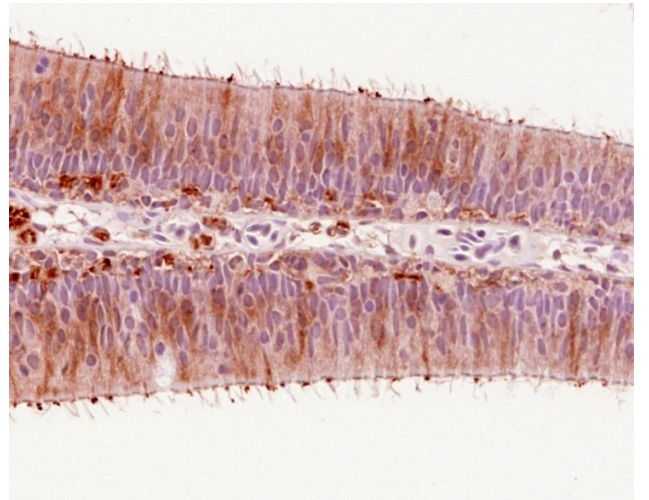

Severe (3): The stripped pattern is completely loss in the whole epithelium with formation of small clusters of disorganized irregular positive cells that form small patches that gives the epithelium a clumped appearance.
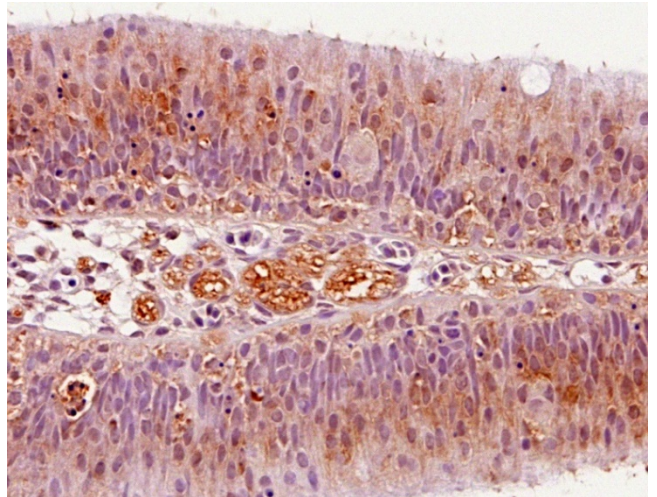
surface delineation

pattern distribution

Normal (0): a clear well-defined rim of apical structures is visible with a fine granular pattern distributed along the whole surface of the epithelium.
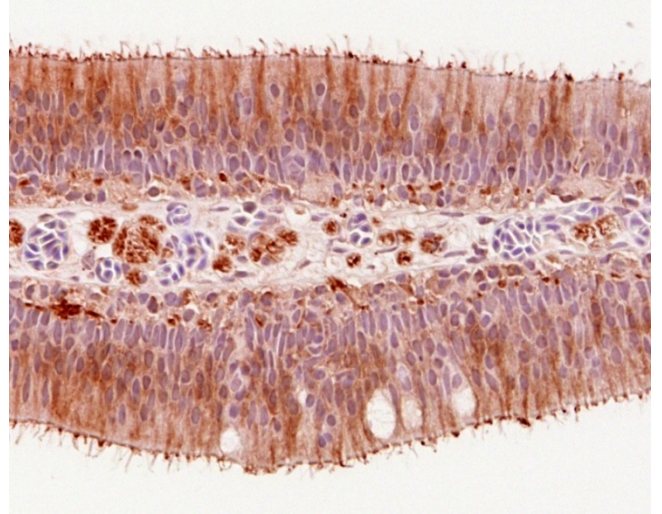

Mild (1): The rimmed pattern is slightly discontinuous and some of the surface structures are lost in between sustentacular cells.

Moderate (2): The surface structures and rimmed pattern is lost in about 50 % of the lamellar surface, there is discontinuity of the dotted pattern with occasional clusters of dots on the surface
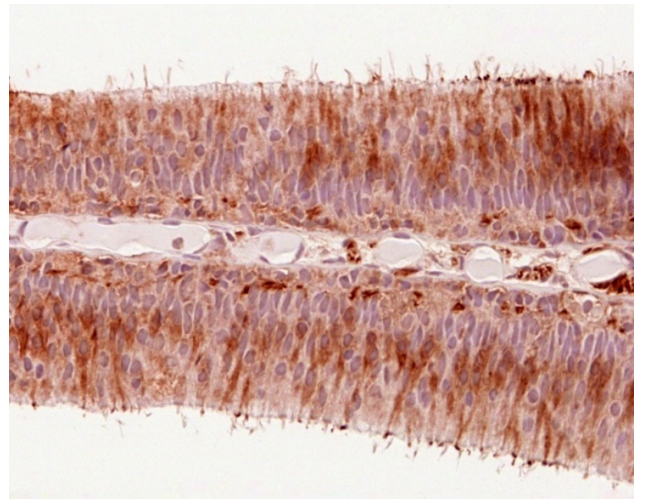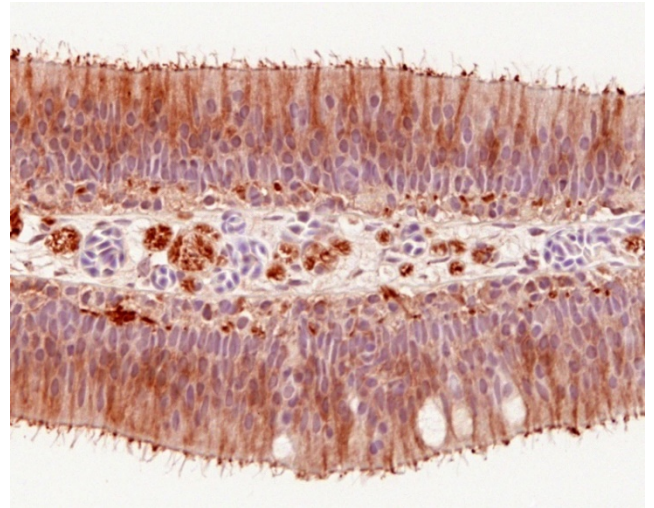
Severe (3): The rimmed pattern is completely lost on the surface of the epithelium. The dots on the surface formed by the olfactory knobs are absent.
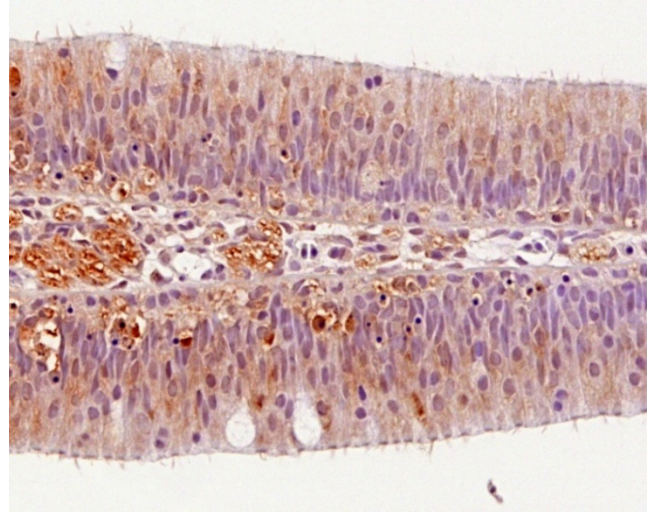
Stain attenuation

Normal, no attenuation (0): The staining is continuous in the whole lamellar epithelium and the whole neuron with a solid and even staining.

Mild attenuation (1): There is discontinuity in the staining patterns in small patches of the lamellar epithelium, the neurons show a slightly clear staining that gives a more spread and less homogeneous appearance of the cytoplasmic staining.
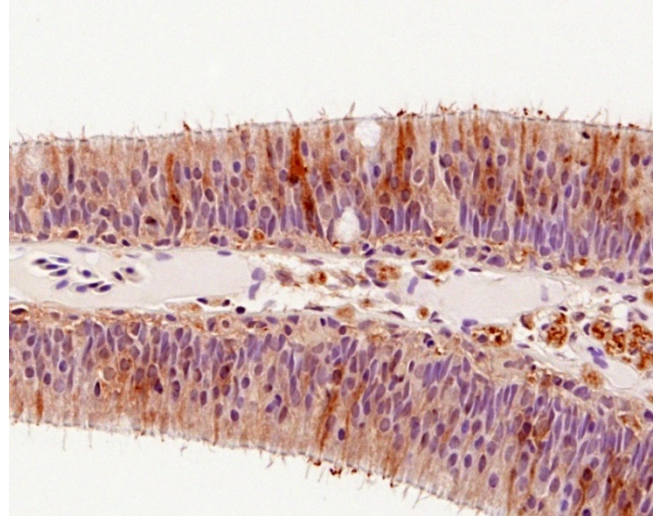
Moderate (2): The cytoplasmic staining is clumpy and discontinuous and gives a less homogeneous, spread and clearer appearance to the neurons and lamellar epithelium in general.
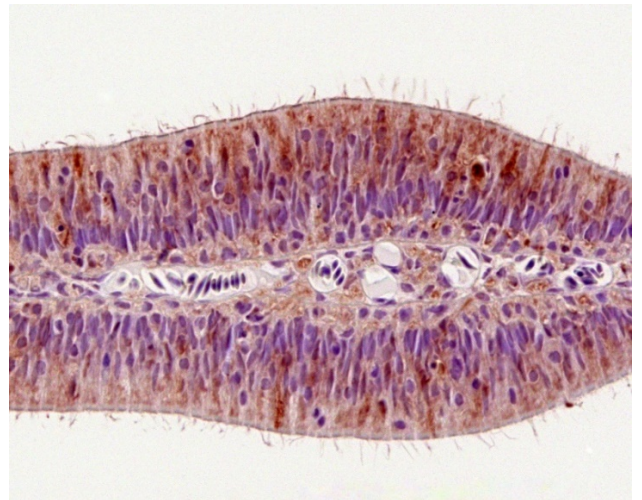
Severe (3): There is a complete loss of staining with just a faint remnant of granular staining in random small patches of the epithelium, giving a clear and less homogeneous and dense staining to the epithelium.
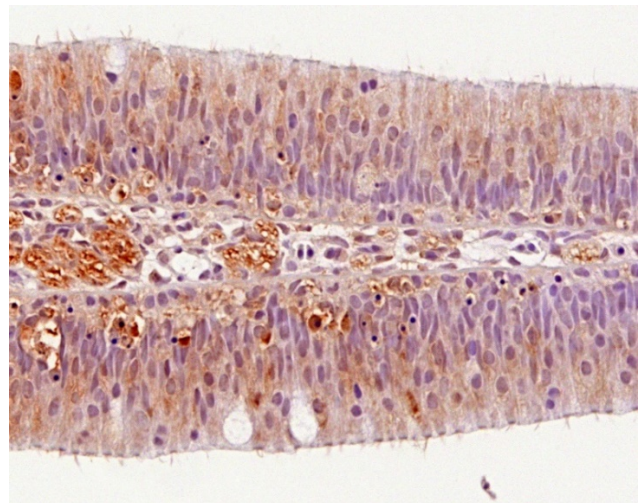
Table 5. Mean swimming speed and mean swimming speed change of Delta Smelt exposed to copper for 24 hours followed by exposure to alarm cues.

Treatment (µg/L)	24-hour copper exposure									
	Swimming speed (cm/s)						Swimming speed change (cm/s)			
	Baseline		Time interval 1		Time interval 2		BLS Time interval 1		BLS Time interval 2	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	2.58	0.18	2.78	0.16	2.5	0.33	0.2	0.16	-0.07	0.34
Copper-2	3.59	0.34	3.48	0.32	2.84	0.3	-0.1	0.35	-0.75	0.28
Copper-8	2.99	0.35	3.92	0.36	4.69	0.73	0.92	0.28	1.69	0.57
Copper-32	2.28	0.22	2.41	0.34	2.89	0.34	0.11	0.22	0.51	0.28

Table 6. Mean maximum acceleration and mean maximum acceleration change of Delta Smelt exposed to copper for 24 hours followed by exposure to alarm cues.

Treatment (μg/L)	24-hour copper exposure									
	Maximum acceleration (cm/s^2)						Maximum acceleration change (cm/s^2)			
	Baseline		Time interval 1		Time interval 2		BLS Time interval 1		BLS Time interval 2	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	19.48	1.51	25.63	2.52	21.67	3.54	6.14	2.99	2.18	4.17
Copper-2	34.04	5.89	33.93	7.18	24.04	3.62	-0.1	7.55	-10	6.99
Copper-8	25.90	4.89	38.35	6.84	52.45	10.14	12.45	6.44	26.55	8.31
Copper-32	19.44	3.90	19.35	4.02	23.53	3.37	-0.07	4.86	3.47	4.71

Table 7. Mean total distance moved and mean total distance change of Delta Smelt exposed to copper for 24 hours followed by exposure to alarm cues.

Treatment (µg/L)	24-hour copper exposure									
	Total distance moved (meters)						Total distance moved change (meters)			
	Baseline		Time interval 1		Time interval 2		BLS Time interval 1		BLS Time interval 2	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	23.17	1.65	24.97	1.45	22.53	3.05	1.80	1.49	-0.63	3.13
Copper-2	32.35	3.14	31.36	2.94	25.57	2.71	-0.99	3.23	-6.78	2.53
Copper-8	27	3.16	35.31	3.25	42.27	6.62	8.30	2.61	15.26	5.15
Copper-32	17.49	2.42	18.49	3.19	22.14	3.40	1	1.99	4.64	2.55

Table 8. Mean interindividual distance and mean interindividual distance change of Delta Smelt exposed to copper for 24 hours followed by exposure to alarm cues.

Treatment (µg/L)	24-hour copper exposure									
	Interindividual distance (cm)						Interindividual distance change (cm)			
	Baseline		Time interval 1		Time interval 2		BLS Time interval 1		BLS Time interval 2	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	25.01	1.72	25.65	1.72	24.80	1.90	0.64	0.74	-0.20	1.14
Copper-2	33.56	1.03	35.65	1.03	35.38	1.39	2.09	0.99	1.82	1.09
Copper-8	32.39	1.24	32.44	1.31	33.42	1.32	0.053	0.93	1.03	1.13
Copper-32	44.12	1.50	45.19	2.49	44.37	1.64	0.85	1.88	0.19	1.58

Table 9. Mean swimming speed and mean swimming speed change of Delta Smelt exposed to copper for 96 hours followed by exposure to alarm cues.

Treatment (µg/L)	96-hour copper exposure									
	Swimming speed (cm/s)						Swimming speed change (cm/s)			
	Baseline		Time interval 1		Time interval 2		BLS Time interval 1		BLS Time interval 2	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	5.49	0.30	5.55	0.46	4.68	0.36	0.062	0.37	-0.81	0.25
Copper-2	5.71	0.45	5.92	0.45	5.10	0.44	0.20	0.40	-0.57	0.29
Copper-8	5.30	0.34	5.22	0.45	3.99	0.23	-0.08	0.55	-1.31	0.40

Table 10. Mean maximum acceleration and mean maximum acceleration change of Delta Smelt exposed to copper for 96 hours followed by exposure to alarm cues.

Treatment (µg/L)	96-hour copper exposure									
	Maximum acceleration (cm/s^2)						Maximum acceleration change (cm/s^2)			
	Baseline		Time interval 1		Time interval 2		BLS Time interval 1		BLS Time interval 2	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	28.66	4.18	29.09	3.07	23.37	1.61	0.43	4.92	-6.29	4.88
Copper-2	28.40	2.07	32.59	2.64	25.95	2.10	3.98	2.92	-2.32	2.16
Copper-8	31.76	5.11	30.88	4.17	24.13	3.37	-0.87	6.54	-7.63	5.51

Table 11. Mean total distance moved and mean total distance change of Delta Smelt exposed to copper for 96 hours followed by exposure to alarm cues.

Treatment (µg/L)	96-hour copper exposure									
	Total distance moved (meters)						Total distance moved change (meters)			
	Baseline		Time interval 1		Time interval 2		BLS Time interval 1		BLS Time interval 2	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	49.48	2.71	50.05	4.16	47.15	3.94	0.56	3.38	-2.32	2.67
Copper-2	51.38	4.09	53.38	4.07	45.94	4.02	1.89	3.67	-5.16	2.67
Copper-8	47.66	3.10	49.91	3.69	35.93	2.11	2.24	4.50	-11.72	3.64

Table 12. Mean interindividual distance and mean interindividual distance change of Delta Smelt exposed to copper for 96 hours followed by exposure to alarm cues.

Treatment (μg/L)	96-hour copper exposure									
	Interindividual distance (cm)						Interindividual distance change (cm)			
	Baseline		Time interval 1		Time interval 2		BLS Time interval 1		BLS Time interval 2	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	27.91	1.03	26.06	1.31	24.97	1.18	-1.84	0.80	-2.93	0.70
Copper-2	33.19	0.85	33.14	0.83	33.10	1.13	-0.04	0.85	-0.08	1
Copper-8	34.16	0.67	32.55	0.89	30.64	0.84	-2.32	0.69	-3.48	0.84

Dissertation Conclusions

Delta Smelt have a well-developed and highly sensitive olfactory system. Its structure resembles in many aspects to that of fish denominated macrosmatic which use olfaction in many aspects of their life history and might be important for survival. Moreover, Delta Smelt use olfaction to assess predation risk using alarm cues, a feature that is well conserved among aquatic species and has been regarded as key for fitness and survival. Additionally, in the context of anthropogenic disturbances to the environment and effects on the sensory ecology of this fish species, Delta Smelt were highly susceptible to copper exposure with a concentration dependent response of the olfactory epithelium to injury and alterations in antipredator behaviors, demonstrating that copper can be a contaminant of concern causing info disruption and alteration of the olfactory ecology of this fish even at concentrations considered sublethal. My work gives a starting point to evaluate behaviors that are important for survival in the environment and to use the results derived from these studies to select and protect habitats for conservation priority of Delta Smelt.