Changing the nature of olfaction throughout life in a model vertebrate, *Danio rerio*

by

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Abstract

Animals use olfaction to receive invaluable information about their chemical environment. Chemical cues, also referred to as odourants, are detected by the olfactory system, which begins at the olfactory epithelium. Within this olfactory epithelium are olfactory sensory neurons (OSNs) that generate signals in response to odourants. Olfactory signals are transmitted to higher-order processing centers, which mediate behaviour. Olfaction develops early and changes throughout an animal's lifespan as it further develops into adulthood until after a certain point when olfaction deteriorates due to aging-related processes. For my thesis, I used zebrafish to examine how olfaction changes throughout life. I also considered the complications of a more natural setting where multiple odourants are present.

Animals encounter multiple odourants simultaneously. Often, these odourants evoke opposite responses when detected on their own. I used zebrafish to determine how animals may respond to a mixture containing an attracting and a repelling odourant. L-alanine was an attracting odourant while L-cysteine was a repelling odourant. When zebrafish detected L-alanine and L-cysteine together, they evoked an avoidance response. When the repelling odourant's concentration decreased, the avoidance response towards the mixture became attraction.

Animals imprint to odourants during early development. Correspondingly, long-term memories of odourants are formed and responses to these odourants change when re-exposed later in life. Olfactory imprinting may be disrupted by the addition of an odourant either during imprinting or during behavioural testing. I tested if zebrafish could imprint to single amino acids and to a binary amino acid mixture. Fish imprinted to L-leucine and L-lysine, but not to L-valine.

However, fish that were exposed to L-leucine and L-lysine together did not imprint to either odourants separately or as a mixture, demonstrating that the complexity of the olfactory environment should be considered when examining imprinting.

While imprinting shapes future olfactory-mediated behaviours, aging often causes hyposmia, which is a weakened olfactory sense. Hyposmia is associated with a thinning of the olfactory epithelium and a reduced number of OSNs, of which there are multiple classes. Each class is sensitive to a different subset of odourants. Two classes found across vertebrates are ciliated OSNs and microvillus OSNs, which in fish detect bile acids and amino acids, respectively. Aging studies have not yet examined if all OSN classes decrease in number equally. I examined if age has class-specific effects on OSN density with impacts on response to associated odourants. Even though olfactory epithelium thickness was unchanged, overall cell density decreased as fish aged. Class-specific changes to OSN density were observed as ciliated OSN density was the same across age, but microvillus density decreased with age. As zebrafish aged, only middle-aged fish demonstrated an increase in neuronal activation and elicited a behavioural response to a bile acid or an amino acid. Young- or old-aged fish displayed no response to the odourants. Therefore, olfaction may be most functional at middle age.

Throughout an animal's lifespan, olfaction faces chemical pressure from the environment such as from toxicant exposures. The olfactory epithelium is especially vulnerable to damage by toxicants as it directly interfaces the environment. Copper is a well-known neurotoxicant that enters the hydrosphere and induces apoptosis in the olfactory epithelium of fishes. Also, copper differentially affects ciliated and microvillus OSN density, as ciliated OSNs are more sensitive to the metal's toxic effects than microvillus OSNs. Regardless, olfaction has remarkable resistance to toxicants from high biotransformation enzyme activity and OSN turnover rates. As animals

age, resistance to olfactory toxicity may weaken as biotransformation activity and OSN turnover rates decreases. I examined how age affects olfactory toxicity from copper exposures with a focus on OSN class-specific effects. Age did not change how copper affected the anatomy of the zebrafish olfactory epithelium as its thickness decreased while ciliated and microvillus OSN density was unchanged after copper exposures regardless of age. Differential toxicity was seen in aging fish as copper downregulated expression of genes involved in the signal-transduction pathway of ciliated OSNs only. Responses to an amino acid and a bile acid were seen in middle-aged fish only. Therefore, copper exposures only affected middle-aged fish responses to the odourants. While olfaction may be most functional at middle age, it is also at this age group when olfaction may be most vulnerable to disruption by toxicants.

Preface

This thesis is an original work of Arash Shahriari. Research ethics approval was given for all research under this thesis by the University of Alberta Animal Care and Use Committee under the animal use protocol of AUP00052 – Chemicals, effluents, and fishes.

Chapter two of my thesis is a published review article in *Aquaculture and Fisheries*.

The published citation is:

Shahriari, A., Aoudi, B., & Tierney, K. B. (2023). The signal-transduction pathways of the peripheral olfactory organ and their impairment in vertebrates. *Aquaculture and Fisheries*.

I was responsible for writing this manuscript. Bouthaina Aoudi created the figures in this manuscript using BioRender.

Chapter three of my thesis is a published journal article in *Behaviour*.

The published citation is:

Shahriari, A., Khara, L. S., Allison, W. T., & Tierney, K. B. (2021). Zebrafish (Danio rerio) behavioural response to an odorant mixture containing attracting and repelling odorants. *Behaviour*, *158*(5), 355-375

I was responsible for writing this manuscript. I collected all behavioural data with the help of Lakhan S. Khara, who under my supervision, gathered data on fish response to a binary amino acid mixture. I analyzed all data.

I was responsible for manuscript composition of chapter four of my thesis. I collected all behavioural data with the help of Dustin Doty, Nojan Mannani and Patricia Ann Villarama, who were under my supervision. I analyzed all data.

I was responsible for manuscript composition of chapter five of my thesis. I collected all behavioural and anatomical data. Dustin Doty collected qPCR data under my supervision. Bouthaina Aoudi created Figure 5.1 using BioRender. I analyzed all data.

I was responsible for manuscript composition of chapter six of my thesis. I collected all behavioural and immunohistochemistry data. Hudson Bovee collected olfactory epithelium thickness data under my supervision. Dustin Doty collected qPCR data under my supervision. I analyzed all data.

Dedication

"So, fish smell the ocean?"

- A friend

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It is without saying that I could not have gone to the finishing line without a special group of people to me: my family! Even though I moved one province away, it never felt like it. Thank you for everything!

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Chapter 1: General Introduction

Olfaction, also referred to as the sense of smell, facilitates interactions between animals and the chemical environment. This chemical environment is comprised of odourant molecules that are released by a plethora of sources including animals, vegetation, microbial activity, and the physical environment. Odourants are detected by an olfactory system, which transduces the chemical stimuli into an electrical signal that leads to the animal sensing the odourant and having a change in behaviour (Manzini et al. 2022). Fundamental olfactory-mediated behaviours include foraging, avoiding harm, and finding kin or mates (Petranka et al., 1987; Nevitt, 1999; Barata et al., 2007; Roberts et al., 2018). The importance of olfaction can be realized by the fact that olfactory-mediated behaviours are seen early in development and persist through to adulthood (Filogamo & Robecchi, 1969; Norgren & Lehman, 1991; Hansen & Zeiske, 1993; Vitebsky et al., 2005). After a certain point in an animal's lifespan, age-dependent degradation of olfactory functionality occurs. Altogether, olfactory functionality changes throughout an animal's lifespan.

An overview of the olfactory system

In vertebrates, the olfactory system begins at the peripheral olfactory organ, which contains an olfactory epithelium. This olfactory epithelium is located within the nasal cavity and consists of olfactory sensory neurons (OSNs), supporting cells and basal stem cells (Morrison & Costanzo, 1990, 1992). Odourants are detected by G-protein coupled receptors that are located on OSNs, of which multiple classes exist (Buck & Axel, 1991; Dulac & Axel, 1995). Different OSN classes detect different odourants. For example, two OSN classes consistently found across vertebrates are ciliated and microvillus OSNs, which in fish, detect bile acids and amino acids, respectively (Lipschitz, 2002; Sato et al., 2005). In terrestrial vertebrates, ciliated and microvillus OSNs detect volatile compounds and water-soluble compounds, respectively (Monti-Bloch & Grosser, 1991; Trinh & Storm, 2003; Kimchi et al., 2007). With the transition from an aquatic to terrestrial lifestyle came a spatial segregation between OSN classes as amphibians, reptiles and mammals have an accessory olfactory chamber known as the vomeronasal organ (VNO) that also contains OSNs. While ciliated and microvillus OSNs, among other classes, are located within a single olfactory epithelium in fish, these two OSNs classes in terrestrial vertebrates are

restricted to the olfactory epithelium and VNO, respectively (Graziadei & Tucker, 1970; Vaccarezza et al., 1981; Moran et al., 1982; Oikawa et al., 1998) .

During odourant detection, transduction pathways generate signals at the cilia or microvilli of OSN dendritic ends (Pace et al., 1985; Sklar et al., 1986; Berghard & Buck, 1996). Ciliated OSNs use an adenylyl cyclase pathway while microvillus OSNs use a phospholipase-C pathway. The signals propagate along the neurons towards the axonal terminals and are relayed to the olfactory bulb, which is the first processing center of the olfactory system (Ahn et al., 2011; Bolz et al., 2017). As different OSN classes project their axons to distinct regions of the olfactory bulb, olfactory processing is based on a spatial pattern of activation that is referred to as chemotopy (Johnson et al., 1999; Friedrich & Korsching, 1998; Nikonov & Caprio, 2001). For example, amino acids and bile acids activate the ventrolateral and dorsomedial region of the zebrafish olfactory bulb, respectively (Friedrich & Korsching, 1998). Signals from the olfactory bulb are relayed to the forebrain for higher-order processing that mediates physiology and behaviour (Pace et al., 1985; Levy et al., 1991; Xu et al., 2005).

Discrimination of odourant mixtures

Animals may be continuously surrounded by a ‘bouquet’ of odourants and therefore, at any given moment may detect multiple odourants simultaneously. Interactions between odourants during their simultaneous detection by OSNs may lead to synergistic or suppressive effects on the generation of signals within the neurons (Kang and Caprio 1997; Ishii et al. 2008; Miyazawa et al. 2008; Chaput et al. 2012; McClintock et al. 2020). Odourants at lower concentrations tend to have synergistic interactions, such as in the case of septic odour being potentiated by the addition of low concentrations of 2- methylisoborneol, which on its own gives a musty smell (Guo et al., 2019). As for suppressive interactions between odourants, the inclusion of an aversive odourant may dampen the signal generated from an attracting one as aversive odours evoke stronger responses than attracting odours (Ehrlichman et al., 1995; Boesveldt et al., 2010). Furthermore, odourants may mask responses normally seen towards other odourants present, such as in the case of mice not responding to the aversive odourant dimethyl sulfide in the presence of citrous odourants (Cain, 1975; Osada et al., 2013).

When animals detect odourants in mixture, the mixture is perceived as its individual components or as a new odour that is independent from its constituents. These two means of odour processing are termed as *elemental* and *configurative*, respectively (Laska & Freyer, 1997; Kay et al., 2005; Coureaud et al., 2009). The former is seen when fewer odourants are present, in which case the more stimulatory component drives the response. For example, catfish responded to a ternary amino acid mixture, in a similar manner as they did with L-cysteine, the most stimulatory constituent of the mixture (Valentinčič et al., 2000). Configural processing is seen in mixtures that have a higher number of constituents as animals have a limited capacity on the number of odourants they can discriminate between, with higher detection limits being three to four odourants simultaneously (Livermore & Laing, 1996; Valentinčič et al., 2000). As animals in nature are exposed to a 'bouquet' of odourants, configurative over elemental processing is more likely to occur.

Olfactory imprinting

How animals respond to olfactory stimuli is often shaped by their environment during early development. During a brief window in early development, animals imprint to odours that they encounter forming long-lasting memories that shape future olfactory-mediated behaviours (Marr & Gardner, 1965; Fillion & Blass, 1986). In an ecological context, olfactory imprinting facilitates homing behaviour. For example, salmon imprint to a bouquet of amino acids in their natal stream and sense these odours from afar after seaward migration to guide their return to their natal stream for spawning (Shoji et al. 2000; Yamamoto, et al. 2010; Yamamoto et al. 2013). Larval coral-reef fish imprint to odours that guide them back to their home coral reef after being dispersed by ocean currents (Atema et al., 2002; Jones et al., 2005; Gerlach et al., 2007). Besides facilitating homing behaviour, olfactory imprinting prevents inbreeding across vertebrates, including in fish, birds and mammals (Roberts & Gosling, 2003; Gerlach & Lysiak, 2006; Gerlach et al., 2008; Bonadonna & Sanz-Aguilar, 2012). Imprinting also has biological importance in the sense of being an underlying mechanism for kin recognition, which improves survivorship of young (Carter & Marr, 1970; Griffiths et al., 2004; Caspers et al., 2013; Atherton & McCormick, 2017). Furthermore, infants imprint to odours of mother's milk, thereby forming maternal associations (Russell, 1976).

Odourant detection during a sensitive period has been proposed as necessary for successful imprinting. Studies demonstrated that zebrafish imprint to the synthetic chemical, β-phenylethyl alcohol, at 2 to 3 days post fertilization (dpf) and to kin odour at 6 dpf (Harden et al., 2006; Gerlach et al., 2008). While the notion of a sensitive period being necessary for successful imprinting is unlikely, it is possible that during this sensitive period, the potential for long-term potentiation under hormonal control is at its highest. For example, salmon in their parr-smolt transformation have elevated thyroid hormones levels, which increase neuronal progenitor proliferation at the olfactory epithelium (Lema & Nevitt, 2004). This increase in OSNs may explain why electrophysiological signals at the salmon olfactory epithelium in response to amino acids increase dramatically during a two week period in their parr-smolt transformation (Yamamoto et al. 2010). Furthermore, a recent study demonstrated that N-methyl-D-aspartate receptors (NMDARs) at the olfactory bulb were upregulated by thyrotropin releasing hormone immediately before downstream migration (Ueda et al., 2016). These receptors were again upregulated during homing, but by gonadotropin releasing hormone, demonstrating hormonal control for memory retrieval as well. In addition to hormonal control, olfactory imprinting may upregulate the transcription factor *otx2*, which downregulates multiple unrelated olfactory receptors, thereby increasing the signal-to-noise ratio (Harden et al., 2006; Calfún et al., 2016). Overall, the insight of mechanisms underlying imprinting from laboratory-based studies have been thorough. One limitation often seen in these studies is that they expose animals to a single odourant, even though odourants in a natural setting rarely, if ever, occur in isolation (Harden et al., 2006; Yamamoto et al., 2010; Inoue et al., 2021; Armstrong et al., 2022).

Aging effects on olfaction

Olfaction develops early in animals and continues to develop through to adulthood. However, after a certain point in an animal's lifespan, age-dependent degradation in olfactory functionality occurs, in which a weakened sense of smell is also referred to as hyposmia (Patel & Larson, 2009; Rawson et al., 2012; Suzuki et al., 2021). For example, aging in mice reduced signalling sensitivity and weakened behavioural responses towards vanillin (Nakayasu et al., 2000). In humans, aging delayed responses to olfactory-mediated tasks (Morgan & Murphy, 2010). Anatomically, aging-associated hyposmia is associated with a thinning of the olfactory epithelium and reduced OSN density, which may derive from reduced proliferation of progenitor

cells (Jia & Hegg, 2015; Ueha et al., 2018; Zhang et al., 2018). As signals are generated within OSNs, reduced OSN density often correlates with decreased signalling during odourant detection, but a few studies have also demonstrated that signalling sensitivity can be maintained (Thesen & Murphy, 2001; Lee et al., 2009; Zhang et al., 2017; Kass et al., 2018; Sabiniewicz et al., 2023). A reason for these opposing results may be the lack of consideration for differential sensitivity towards odourants between different OSN classes (Cao et al., 1998; Lipschitz & Michel, 2002; Trinh & Storm, 2004; Benzekri & Reiss, 2012; Omura & Mombaerts, 2014). While no published studies have yet examined how age may affect different OSN classes differentially, predictions can be made from olfactory toxicology studies that examined OSN class-specific toxicity. For example, studies using zebrafish demonstrated that copper differentially affected ciliated and microvillus OSN density, and following contaminant exposure, both classes had different regenerative capacity due to differences in precursor proliferation rates (Lazzari et al., 2017; Heffern et al., 2018; Ma et al., 2018). This difference in ciliated and microvillus OSN regeneration may suggest that the two classes may be affected differently from physiological aging processes.

As animals age, it is expected that the olfactory system will receive constant chemical pressure from the environment such as by accumulating damage from contaminants, especially since the olfactory epithelium is in direct contact with the environment. For example, copper readily enters the hydrosphere through urban runoffs and induces apoptosis at the olfactory epithelium, which correspondingly decreases in thickness (Julliard et al., 1996; Wang et al., 2013; Lazzari et al., 2017). In regard to OSN density, copper differentially affects ciliated and microvillus OSNs classes (Lazzari et al., 2017; Ma et al., 2018). Copper was shown to differentially affect fish responses to bile acids and amino acids, which are detected by ciliated and microvillus OSNs, respectively (Kolmakov et al., 2009; Razmara et al., 2019). However, one study demonstrated that copper affected the generation of an olfactory signal in salmon exposed to amino acids and bile acids (Baldwin et al., 2003). In addition to affecting anatomy, contaminants disrupt olfactory signals by targeting transduction pathways, in which differential toxicity towards different transduction pathways at the olfactory epithelium is also present (Shahriari et al., 2023). With acute contaminant exposures, damage to the olfactory epithelium is reversible from proliferation of progenitor cells that lead to the renewal of OSNs (Lazzari et al., 2017; Ma et al., 2018; Lazzari et al., 2019). In the aging population, olfactory toxicity may be

more difficult to recover from as a reduced proliferation of progenitor cells from aging may lead to a neuronal turnover rate that is insufficient for adequate OSN renewal with contaminant-induced apoptosis (Julliard et al., 1996; Ueha et al., 2018). Furthermore, aging animals have lowered resistance towards contaminants such as by having reduced numbers of biotransformation enzymes, which detoxify contaminants (Getchell et al., 1993; Krishna et al., 1995; Kennedy & Tierney, 2013).

Research Objectives

The main objective of my thesis is to examine how olfaction changes at different ages in vertebrates in a laboratory setting that also considers the complications of a more natural olfactory environment, in which multiple odourants are present. This objective is spread across two research questions: 1) how does the number of odourants present in the environment during early development affect olfactory-mediated responses in adulthood, and 2) how does age affect olfaction? For my research questions, I used the popular vertebrate model, the zebrafish (*Danio rerio*), but my findings may have implications across vertebrates due to the conserved anatomy of the olfactory system.

For my first research question, I built and validated an avoidance-attraction trough to observe adult zebrafish response to an attracting and a repelling odourant separately and in mixture. After I confirmed that this behavioural assay captured fish response to single odourants and a binary odourant mixture, I determined if zebrafish could imprint to single amino acids and to a binary amino acid mixture. **I expected that our assay would less likely show that fish could imprint to multiple amino acids simultaneously over single amino acids**.

For my second research question, I first determined how olfaction changes in zebrafish as they age. Specifically, I examined if age affects ciliated and microvillus OSNs differently with impacts on hyposmia in response to their associated odourants. **I hypothesized that age would have OSN class-specific effects because of differences in neuronal precursor proliferation rates as observed in olfactory toxicology studies**. I then determined how age affects olfactory toxicity from copper exposures. I focused on examining differential toxicity to ciliated and microvillus OSNs in the context of their specific responses to associated odourants. **I hypothesized that the effects copper may have on zebrafish olfactory anatomy and**

functionality are age dependent, because the aging population has reduced resistance to contaminant toxicity from decreased neuronal regenerative capacity and biotransformation activity.

Chapter 2: REVIEW The signal-transduction pathways of the peripheral olfactory organ and their impairment in vertebrates

Abstract

Animals rely on olfaction to detect and process invaluable chemical information about their environment. For olfaction to function, chemicals must first be detected, which leads to the activation of signal-transduction pathways at the peripheral olfactory organ. As the olfactory system is in direct contact with the environment, the system is constantly vulnerable to damage by contaminants entering the atmosphere or hydrosphere. Contaminants may have a variety of effects, including disrupting olfactory signals generated during chemical detection, or altering numerous targets along the signal transduction pathway. With any impairment of chemical detection, animals may be unable to rely on olfaction to make correct decisions about their environment and thus their fitness. While other reviews have focussed on olfactory toxicology in general, here we specifically explore how contaminants may affect the signal-transduction pathways at various points and link those changes to olfactory functionality across vertebrates with a focus on fishes.

Introduction

Animals are surrounded by chemicals that provide invaluable information about the environment. The detection and perception of these chemical cues are facilitated by sensory neurons, including those of the olfactory system. The olfactory sense is critical for life, considering that it allows animals to forage, avoid predators, and help fine tune kin recognition and mate selection (Porter and Moore 1981; Gerlach and Lysiak 2006; Ferrari et al. 2010; Krause et al. 2012; Konishi et al. 2022). For olfaction to function, animals must perceive chemical olfactory cues, also known as odourants, and an olfactory signal must be generated, which begins at the peripheral olfactory organ. From the initial step of odourant detection, signal-transduction pathways at the peripheral olfactory organ translate chemical input into electrical information, which is relayed to the forebrain for processing (Manzini et al. 2022). At the forebrain, olfactory signals integrate with input provided by other sensory systems to elicit changes in physiology and behaviour.

Contaminants are omniprevalent, whether it be from chemical emissions to the atmosphere or chemical spills into the hydrosphere. As the olfactory system interfaces directly with the environment, it is susceptible to damage by toxic contaminants. The peripheral olfactory organ is the first site where contaminants would reach and potentially cause olfactory toxicity. If contaminants were to disturb the signal-transduction pathways at the peripheral olfactory organ, odourant-induced signals may not be generated. As a result, animals would be unable to benefit from the wealth of information provided by chemical odourants, and as such would be at risk of ecological death, i.e., death by natural processes such as predation. In this review, we focus our discussion on how contaminants may disrupt the main signal-transduction pathways at the peripheral olfactory organ and link changes in olfactory signalling to changes in olfactory functionality across vertebrates, with an emphasis on fishes, upon which much of the work on olfactory toxicology is focussed.

Olfactory transduction pathway at the peripheral organ

Form and function of the peripheral olfactory organ

Olfaction begins with the detection of odourants at the peripheral olfactory organ. The olfactory epithelium of this tissue is located within the nasal cavity and consists of olfactory sensory neurons (OSNs), supporting cells, and basal stem cells (Morrison and Costanzo 1990; Mackay-Sim and Kittel 1991; Hansen and Eckart 1998; Mandal et al. 2005). Odourants are received by OSNs, which is where olfactory signals to the central nervous system are generated. Anterior to the olfactory epithelium, there is an accessory olfactory chamber in amphibians, reptiles, and mammals known as the vomeronasal organ (VNO), which also contains OSNs. The dendritic end of OSNs within the olfactory epithelium are ciliated while those in the VNO are microvillar (Graziadei and Tucker 1970; Vaccarezza et al. 1981; Moran et al. 1982; Oikawa et al. 1998). While these two morphologically distinct ciliated and microvillus OSNs are spatially segregated in terrestrial vertebrates, all OSN classes in fishes are located within a single olfactory epithelium as no VNO exists in these vertebrates. OSN classes in fishes include ciliated OSNs, microvillus OSNs, crypt cells, kappe neurons and pear-shaped neurons (Muller and Marc 1984; Zielinski and Hara 1988; Byrd and Brunjes 1995; Hansen and Finger 2000; Ahuja et al. 2014; Wakisaka et al. 2017).

Different OSN classes found in vertebrates detect different odourants. In terrestrial vertebrates, ciliated OSNs of the olfactory epithelium detect volatile compounds and microvillus OSNs of the VNO detect water-soluble compounds including pheromones (Monti-Bloch and Grosser 1991; Dulac and Torello 2003; Trinh and Storm 2004; Kimchi et al. 2007). In fishes, ciliated OSNs respond most strongly to bile acids, microvillus OSNs respond most strongly to amino acids, crypt cells detect pheromones or amino acids depending on the species, and pear-shaped neurons detect nucleotides (Lipschitz and Michel 2002; Sato et al. 2005; Vielma et al. 2008; Bazáes and Schmachtenberg 2012; Wakisaka et al. 2017; Kawamura and Nikaido 2021). Odourants associated with kappe neurons have not yet been identified.

The arrangement and functionality of OSN classes within the olfactory epithelium and VNO of amphibians exemplify an intermediate reorganization of the peripheral olfactory structure that accommodates the transition from an aquatic to a terrestrial lifestyle. Early studies demonstrated that in larval anurans, ciliated and microvillus OSNs detect water-soluble compounds despite their segregation into the olfactory epithelium and VNO, respectively (Hansen et al., 1998; Oikawa et al., 1998). As anurans transition from inhabiting aquatic environments to terrestrial environments, ciliated OSNs in the olfactory epithelium detect volatile compounds, and an additional middle cavity epithelium appears, which contains ciliated and microvillus OSNs that detect water-soluble compounds. More recent studies demonstrated partial segregation of ciliated and microvillus OSNs in larval anurans, with the two classes residing in separate regions within the olfactory epithelium (Gliem et al. 2013; Sansone et al. 2014).

Olfactory signal transduction

The signal generated from odourant detection begins with the binding of odourants to G-protein-coupled receptors (GPCRs) that are located on the cilia or microvilli of OSN dendritic ends. Multiple olfactory receptor types have been identified, mainly olfactory receptors (ORs), trace amine-associated receptors (TAARs), and vomeronasal receptor type 1 and type 2 (V1Rs and V2Rs, respectively) (Buck & Axel, 1991; Dulac & Axel, 1995; Hashiguchi & Nishida, 2006, 2007; Ryba & Tirindelli, 1997; Syed et al., 2013). ORs and TAARs are expressed in ciliated OSNs, while V1Rs and V2Rs are expressed in microvillus OSNs. In fishes, crypt cells express V1Rs (Oka et al. 2012). Additional receptor types have been identified, including the

mammalian formyl peptide receptor-like receptor that detects immune-related ligands, and non-GPCR Membrane-Spanning 4A and Guanylyl Cyclase-D olfactory receptors (Leinders-Zufall et al. 2007; Rivière et al. 2009; Munger et al. 2010; Greer et al. 2016). OSNs predominantly follow a *one-neuron-one-receptor* rule, which refers to a neuron expressing a single olfactory receptor gene only (Ishii et al., 2001; Ngai et al., 1993; Saraiva et al., 2015; Serizawa et al., 2003). Exceptions to this *one-neuron-one-receptor* rule occur, but the expression of multiple olfactory receptor genes is a characteristic of immature OSNs, which once developed select for a single olfactory receptor (Hanchate et al. 2015; Tan et al. 2015). As olfactory receptors are often broadly tuned to odourants, a single olfactory receptor interacts with multiple odourants and a single odourant interacts with different olfactory receptors of the same type (Malnic et al. 1999).

The G-protein coupled to olfactory receptors consists of three subunits: G_{α}, G_{β} and G_{γ}. The three subunits form a complex at an inactive state, with G_{α} also bound to a guanosine diphosphate (GDP) molecule (Pace et al., 1985). When odourants interact with olfactory receptors, the receptor and the G-protein undergo a conformational change, which leads to the exchange of GDP for higher energy guanosine triphosphate (GTP) at the alpha subunit (Jones and Reed 1989; Vetter and Wittinghofer 2001). GTP-bound G_{α} dissociates from the $G_{\beta\gamma}$ complex and initiates the conversion of extracellular chemical signals into intracellular signals that are mediated by the signal-transduction pathway of the OSNs.

The G_{α} subunit after separating from the G-coupled protein complex activates one of two main signal-transduction pathways: adenylyl cyclase (AC) or phospholipase-C (PLC) (Figure 2.1). Signal transduction pathways may be characterized by the specific alpha subunit that is coupled to the olfactory receptor. ORs and TAARs on ciliated OSNs across vertebrates are coupled to $G_{\alpha olf}$, which activates adenylyl cyclase III (ACIII) (Pace et al. 1985; Sklar et al. 1986; Jones and Reed 1989; Bakalyar and Reed 1990; Menco et al. 1992). Activated ACIII converts ATP into cyclic adenosine monophosphate (cAMP), which binds to cyclic nucleotide gated (CNG) channels and thus initiates neuronal depolarization (Nakamura and Gold 1987; Firestein et al. 1991; Liman and Buck 1994). In a subset of OSNs, guanylyl cyclase replaces ACIII to convert GTP in cyclic guanosine monophosphate (cGMP), which also activates CNG channels (Juilfs et al., 1997; Meyer et al., 2000; Nache et al., 2012). These non-selective cation channels consist of four subunits: two CNG-α2 subunits, one CNG-α4 subunit, and one CNG-β1b subunit

(Bönigk et al. 1999; Zheng and Zagotta 2004). Even though all subunits bind to cAMP, there are conflicting reports on whether CNG-α2 or CNG-β1b are most sensitive to the cyclic nucleotide (Liman & Buck, 1994; Nache et al., 2012, 2016). However, CNG-α2 was deemed necessary for CNG channels to open (Dhallan et al., 1990; Waldeck et al., 2009). The binding of cAMP to the CNG channel leads to the channel opening, which causes an influx of Ca^{2+} and to a lesser extent, Na^{+}, into the neuron (Leinders-Zufall et al. 1997). The Ca^{2+} influx activates calcium-activated Anoctamin-2 (ANO2) channels, which cause a chloride efflux that can greatly depolarize OSNs to a membrane potential threshold that triggers the generation and propagation of action potentials along the OSN axon (Kleene and Gesteland 1991; Kurahashi and Yau 1993; Lowe and Gold 1993; Boccaccio and Menini 2007; Li et al. 2018). As a single ANO2 channel is often activated from the Ca^{2+} current generated through the opening of multiple CNG channels, a high signal-to-noise ratio is generated (Kleene 1997; Reisert et al. 2003). This high signal-to-noise ratio may allow animals to detect weak olfactory stimuli. In conjunction with ANO2 channels, ANO9 cation channels were recently discovered in mice to amplify an odourant-induced depolarizing current (Kim et al. 2022).

The PLC signal-transduction pathway is mainly associated with microvillus OSNs and is coupled to $G_{\alpha q/11}$, $G_{\alpha i2}$, or $G_{\alpha o}$ (Halpern et al. 1995; Berghard and Buck 1996; Matsunami and Buck 1997; Andreini et al. 1997; Wekesa et al. 2003; Chamero et al. 2011). In fishes, crypt cells may similarly be associated with $G_{\alpha q}$, $G_{\alpha i}$ or $G_{\alpha o}$ (Hansen et al. 2003; Belanger et al. 2003; Oka et al. 2012; Bazáes and Schmachtenberg 2012). With this second main transduction pathway, the dissociation of $G_{\beta\gamma}$ from GTP-bound G_{α} leads to the activation of PLC-β, which in turn breaks down phosphatidylinositol 4,5-biphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG) (Wekesa and Anholt 1997; Taniguchi et al. 2000; Rünnenburger et al. 2002; Wekesa et al. 2003; Sansone et al. 2014). DAG activates transient receptor potential (TRP)-C2 channels, which cause a depolarizing Ca^{2+} current when opened (Zhang et al. 2010). DAG can be hydrolyzed by DAG lipase into arachidonic acid (AA), which activates a cation channel that also causes a Ca^{2+} influx. IP_3 interacts with IP_3-receptors to release Ca^{2+} from internal stores. IP_3-receptors also co-localize with TRPC2 channels, but they may not be necessary for activating the cation channel (Brann et al. 2002; Chamero et al. 2017). The Ca^{2+} influx from internal stores, TRPC2 and other cation channels, will, as noted above, activate

ANO2 channels, which cause a Cl^- efflux that may enhance the likelihood of an action potential occurring (Dibattista et al., 2012).

While the ACIII and PLC transduction pathways are often considered segregated into ciliated and microvillus OSNs, respectively, exceptions to this organization were identified in mice. For example, a subpopulation of ciliated OSNs that detected pheromones and were associated with components from both ACIII and PLC signalling pathways, and expressed TRPM5 instead of TRPC2 channels, was found (Lin et al. 2007; López et al. 2014). Similarly, TRPC2 was co-expressed with genes that encoded for $G_{\alpha olf}$ or $G_{\alpha o}$, ACIII, and CNG-α2 in OSNs located within the olfactory epithelium (Omura & Mombaerts, 2015). This means that the ACIII and PLC signal-transduction pathway may co-exist in a subpopulation of OSNs. Microvillus sensory neurons that express TRPC6, IP_3, and PLC, but not CNG or ACIII were also identified within the olfactory epithelium (Elsaesser et al., 2005). Overall, certain odours may generate olfactory signals within ciliated and microvillus OSNs that are not restricted to the ACIII or PLC signal-transduction pathway.

When the depolarization is sufficient, action potentials are conducted from the dendritic end of OSNs along the neuron toward the axonal terminal and therefore, olfactory information is transmitted to the forebrain for processing. Studies using rodents have demonstrated that action potentials are transmitted along the axons by voltage-gated $Na_v1.7$ channels, which are expressed in OSNs from the olfactory epithelium and VNO (Ahn et al., 2011; Bolz et al., 2017; Weiss et al., 2011). Voltage-gated $Na_v1.3$ channels are also present in OSN axons, but their involvement in the signal-transduction pathway has not yet been determined.

There are at least two ways that olfactory signal transduction can be modulated. Specifically, olfactory marker proteins (OMP) tune OSNs to have higher sensitivity and selectivity, as well as faster response kinetics towards odourants (Buiakova et al. 1996; Reisert et al. 2007; Lee et al. 2011). The mechanisms underlying this relationship remain to be discovered, but recent studies suggest that OMP prevents basal and odour-induced cAMP from activating CNG channels by acting as a buffer to the secondary messenger (Dibattista & Reisert, 2016; Nakashima et al., 2020). OMP is expressed in the olfactory epithelium and VNO in mammals but restricted to ciliated OSNs in zebrafish (*Danio rerio*), and therefore likely limited to ciliated OSNs in other fishes as well (Monti et al. 1979; Farbman and Margolis 1980; Rössler et al. 1998;

Weiler et al. 1999; Sato et al. 2005; Suzuki et al. 2015). Also present are non-neuronal microvillus cells that release acetylcholine, which interacts with muscarinic acetylcholine receptors type 4 (m_4AChR) on neighbouring OSNs to suppress the transient Ca^{2+}current that is induced by odours (Ogura et al., 2011).

Termination of the olfactory signal and desensitization

Terminating an olfactory signal is equally important as generating an olfactory signal to animals, because it allows subsequent odours to elicit their own responses. Termination of an olfactory signal is also necessary for desensitization to prolonged or repeated odour stimuli that otherwise would have OSNs in a constant depolarized state (Leinders-Zufall et al. 1999; Cygnar and Zhao 2009; Stephan et al. 2012). This desensitization towards sustained olfactory stimuli allows animals to detect relevant odours that stand out from continuous background noise. Termination of an olfactory signal stems in part from the Ca^{2+} influx through CNG and TRPC2 channels during odour stimulation. Calcium ions cooperatively bind to calmodulin (CaM), which is a regulatory protein that deactivates CNG and TRPC2 channels, leading to neuronal repolarization (Olwin and Storm 1985; Liu et al. 1994; Peterson et al. 1999; Yildirim et al. 2003; Spehr et al. 2009). With CNG channels, CaM-binding domains were reported on the CNG-α4 and CNG-β1b subunits (Munger et al. 2001; Bradley et al. 2004). In addition to deactivating CNG channels within the ACIII transduction pathway, Ca^{2+}-CaM activates CaM kinase II (CaMKII), which phosphorylates ACIII, thereby also becoming deactivated (Wayman et al. 1995; Wei et al. 1996; Wei et al. 1998). Furthermore, Ca^{2+}- CaM increases phosphodiesterase-1C (PDE1C) activity, which breaks down cAMP into AMP (Borisy et al. 1992; Yan et al. 1995; Yan et al. 1996; Cygnar and Zhao 2009).

In tandem with CaM-dependent olfactory desensitization, olfactory receptors undergo clathrin-dependent internalization (Mashukova et al., 2006; Rankin et al., 2002). The clathrin-dependent pathway is initiated when GPCR kinase 3 (GRK3) or cAMP-dependent protein kinase A (PKA) selectively phosphorylate olfactory receptors (Dawson et al., 1993; Kato et al., 2014; Laporte et al., 2002; Mashukova et al., 2006; Rankin et al., 2002). The phosphorylation of olfactory receptors recruits β-arrestin-2 to dock to the receptor, which is then stored in recycling endosomes (Mashukova et al., 2006). The recruitment of β-arrestin-2 to olfactory receptors can

be inhibited by muscarinic 3-acetylcholine receptors, which interact with olfactory receptors to potentiate odourant-induced signals (Li and Matsunami 2011; Ohkuma et al. 2013; Jiang et al. 2015)

Excess Ca^{2+} arising from its influx through CNG or TRPC2 channels will eventually be expelled as OSNs maintain ionic homeostasis. Calcium ions leave the neurons through K^+-dependent and K^+-independent Na^+/Ca^{2+} exchangers (NCKX and NCX, respectively) (Jung et al. 1994; Noé et al. 1997; Pyrski et al. 2007; Stephan et al. 2012). Studies indicate that NCX1 was most abundant in mice olfactory epithelium and that NCKX4 was involved in the termination of olfactory signals and desensitization towards repeated odour stimulation (Pyrski et al. 2007; Stephan et al. 2012). OMP increases NCX1 activity, which would increase the rate of signal termination and therefore shorten the time span in which subsequent odourants evoke their own signal (Kwon et al., 2009). While plasma membrane calcium ATPase (PMCA) also removes Ca^{2+} out of OSNs, its involvement in signalling termination has been questioned (Castillo et al. 2007; Kleene 2009; Saidu et al. 2009; Castillo et al. 2010; Griff et al. 2012). The removal of excess Ca^{2+} from OSNs deactivates ANO2 channels, which in turn prevents intracellular Cl^- from being depleted (Vocke et al., 2013). Chloride depletion through ANO2 channels is also avoided by its reuptake through Na^+-K^+-Cl^- contransporter-1 (NKCC1) and Cl^-/HCO_3^- exchanger (Kaneko et al. 2004; Nickell et al. 2007). Chloride ions taken back up and Ca^{2+} ions expelled out of OSNs lead to neuronal repolarization and termination of the olfactory signal-transduction pathways, which would then be ready for re-activation from subsequent olfactory stimuli.

Olfactory toxicity at the peripheral olfactory organ

By necessity, the peripheral olfactory organ is relatively unprotected from the environment and therefore is vulnerable to damage by contaminants. The cellular cascades generated by odorant detection can be impaired at any point along the ACIII or PLC signal-transduction pathways, whether toxins or toxicants disrupt the initial interaction of odourants with olfactory receptors or prevent the final triggering and propagation of action potentials (Table 2.1, Figure 2.2). By hampering the generation of olfactory signals at the peripheral organ, animals may not detect and therefore process chemical information from their environment. The costs of such interference are obvious and relate to fitness costs of the trivial to the absolute.

Toxicity on olfactory receptors

Toxic contaminants disrupt the activation of the olfactory signal-transduction pathways by either modulating olfactory receptor expression or by preventing odourants from binding to olfactory receptors. Recent studies demonstrated that mice exposed to urban particulate matter (UPM) or dexamethasone had reduced OR and $G_{\alpha olf}$ expression, which may have dampened olfactory signalling input received by the animals as they performed poorly in foraging tasks (Kim et al. 2019; 2022). Fetal mice exposed to ethanol through maternal alcohol consumption had downregulated OR genes (Mandal et al. 2015). This observation may provide a possible explanation for why children exposed to ethanol during prenatal development have difficulty identifying household odours (Bower et al., 2013).

Most reports on changes to the olfactory receptor and G-protein subunit gene expression from contaminant exposures have been based on work with fishes. Effects from copper and the pesticide chlorpyrifos (CPF) have received considerable attention. Zebrafish and rainbow trout (*Oncorhynchus mykiss*) exposed to copper had decreased expression of multiple OR and G-protein subunit genes (Tilton et al. 2008; Razmara et al. 2021). Prolonging the duration over which rainbow trout were exposed to copper further decreased olfactory receptor expression, indicating time and dose are important factors in olfactory toxicity, as in other biological systems. Recent work has shown that copper nanoparticles also downregulate OR genes in rainbow trout, but at higher concentrations compared to that of copper ions (Razmara et al. 2021). Downregulation of olfactory receptor expression is not always the outcome with toxic chemical exposures. For example, with CPF exposures, numerous OR genes were upregulated in zebrafish (Tilton et al. 2011). However, when zebrafish were exposed to CPF and copper, simultaneously, most OR genes were downregulated, suggesting toxicity can be additive with contaminant mixture exposures (Tilton et al. 2011). Toxicants that induce minimal change to olfactory receptor gene expression may have amplified toxic effects in unfavourable environmental conditions. Consider that medaka (*Oryzias latipes*) exposed to perfluorobutane sulfonate, a surfactant of recent ecological concern, had reduced OR expression, but only when exposed in a hypoxic environment (Tang et al., 2020). Changes to VR expression in fishes from toxin or toxicant exposures are also frequently reported (Andreini et al. 1997; Williams and Gallagher 2013; Shi et al. 2021; Huang et al. 2023). Contaminants that reduce both OR and VR

expression may disrupt sensitivity towards a wider range of odourants. For example, goldfish (*Carassius auratus*) exposed to microplastics had reduced OR and V2R expression, as well as disrupted behavioural responses towards a bile acid odourant and an amino acid odourant (Shi et al., 2021).

Contaminants may prevent odourants from binding to olfactory receptors. Olfactory receptors contain a metal binding site that has a high affinity for copper and zinc ions (Wang et al. 2003). This metal binding site may provide a mechanism underlying copper and zinc-induced olfactory toxicity, one that exists alongside the initial hypothesis that copper and zinc induces structural changes to the receptor-odourant complex (Burd 1993; Kolmakov et al. 2009; Green et al. 2010; Baldwin et al. 2011; Frontera et al. 2016). Olfactory signalling may also be disrupted by antagonistic interactions between odourants as they compete to bind to olfactory receptors (Araneda et al. 2000; Oka et al. 2004; Sanz et al. 2005; Peterlin et al. 2008; Pfister et al. 2020). As contaminants can elicit their own olfactory signal, it is expected that they compete with other odourants to bind to olfactory receptors (Tierney et al. 2007; Lari and Pyle 2017; Lari et al. 2018; Razmara et al. 2020; Volz et al. 2020; Könemann et al. 2021). Olfactory signals may be dampened when odourants compete with contaminants that elicit a weaker signal (Araneda et al. 2000). Odourants with larger surface areas had increased antagonistic potency, which suggests that contaminants that contain cyclic functional groups may bind to olfactory receptors and be potent inhibitors (Peterlin et al., 2008). However, as olfactory receptors are often generalists, there may be multiple receptors that a given odourant can bind to and so contaminants that compete for olfactory receptors may have little effect (Malnic et al. 1999).

Toxicity on the ACIII and PLC transduction pathway

Activation of the ACIII or PLC signal-transduction pathway leads to the production of secondary messengers, which initiate OSN depolarization. With the ACIII signal-transduction pathway, odourant-induced cAMP production is obviously dampened by toxicants or pharmacological agents that inhibit ACIII activity (Chen et al. 2000). Additionally, toxic contaminants may also decrease ACIII expression, such as is the case for salmon and mice exposed to copper and UPM, respectively (Wang et al. 2013; Kim et al. 2022). However, salmon exposed to copper had a constant cAMP concentration despite the decreased ACIII expression (Wang et al. 2013). Instead, copper exposure reduced cGMP production, which may disrupt

olfactory imprinting, a mechanism underlying salmon homing behaviour (Dittman et al. 1997; Wang et al. 2013). ACIII disruption may also occur from contaminants that agonize dopamine-2 receptors, which are expressed in the olfactory epithelium and inhibit ACIII activity once activated (Mania-Farnell et al. 1993; Coronas et al. 1997; Maj et al. 1997; O'Neill et al. 1998; Kihara et al. 2002). Reduced cAMP production from contaminant exposures may lessen downstream cAMP-dependent activation of CNG channels, thereby preventing depolarization (Nakamura and Gold 1987; Firestein et al. 1991; Liman and Buck 1994). ACIII knockout mice demonstrate the potential consequences from toxicant-induced changes to ACIII activity, which include an absence in producing a generator potential and knock on effects of failed aversive olfactory conditioning and impaired airflow sensitivity (Wong et al. 2000; Chen et al. 2012).

Even if the ACIII pathway is working properly, contaminants can prevent neuronal depolarization by directly impairing CNG channel functionality. For example, pseudechetoxins from snake venom interact with the CNG-α2 subunit to inhibit the Ca^{2+} influx through CNG channels (Yamazaki et al. 2002; Brown et al. 2003). Also, divalent cations other than Ca^{2+} can block CNG channels (Frings et al., 1995). Besides contaminants blocking CNG channels, it was recently found that copper nanoparticles decreased CNG expression in rainbow trout (Razmara et al. 2021). CNG-α2, CNG-α4, or CNG-β1b knockout mice confirm the potential adverse effects of impairing CNG subunits. For example, knockout of CNG-α2 prevented the depolarizing Ca^{2+} influx, likely because the channel remained closed after stimulation of odourants that activated the ACIII pathway (Brunet et al. 1996; Zheng et al. 2000; Lin et al. 2004). CNG-α4 and CNG-β1b knockout mice had inhibited signalling termination and disrupted desensitization to olfactory stimuli, which may explain why mice with CNG-α4 knockouts were unable to discriminate odours from background noise (Munger et al. 2001; Kelliher et al. 2003; Song et al. 2008).

In addition to impairing the ACIII signal-transduction pathway, toxic contaminants also disrupt the PLC signal-transduction pathway. Multiple studies have demonstrated differential toxicity towards this pathway because odourants that only acted via the PLC signal-transduction pathway were disrupted by PLC inhibitors (Ma and Michel 1998; Delay and Dionne 2002; Dew et al. 2014; Sansone et al. 2014). However, a PLC inhibitor dampened the generator potential generated by amino acids and bile acids in yellow croaker (*Larimichthys polyactis*) (Zhu et al.

2023). Another PLC effect may be stimulation, which can occur from exposures to antidepressants, such as imipramine (Cadiou & Molle, 2003). Overstimulated PLC may cause DAG to accumulate to cytotoxic levels, which then in turn may induce lipid peroxidation when DAG lipase converts DAG to AA (Farooqui et al., 2001). In contrast, organophosphates and carbamates inhibit DAG lipase, which would reduce AA production (Bomser et al. 2002). Low concentrations of AA or DAG limit the activation of TRPC2 and other cation channels, thereby inhibiting OSN depolarization (Zhang et al. 2010). Neuronal depolarization may also be dampened by IP3-receptor inhibitors, which would prevent a Ca^{2+} influx from internal stores or TRPC2 channels (Brann et al., 2002; L. Ma & Michel, 1998). However, multiple studies have reported odour-induced neuronal depolarization in the presence of IP3-receptor inhibitors (Taniguchi et al. 2000; Spehr et al. 2002; Sansone et al. 2014; Chamero et al. 2017). As both IP3 and DAG production lead to a Ca^{2+} influx into OSNs, toxicants may need to disrupt both DAG and IP3 activity to have meaningful effects on olfactory signalling.

Contaminants can disrupt OSN depolarization by inhibiting TRPC2 channels or by modifying TRPC2 expression. A calcium current induced by l-arginine was reduced by 73% in frogs exposed to the TRPC2 channel inhibitor 2-aminotheoxydiphenyl borate (Sansone et al. 2014). Studies that exposed zebrafish to cadmium have shown conflicting findings with either decreased or increased TRPC2 expression (Wang and Gallagher 2013; Volz et al. 2020). Toxicity to TRPC2 channels may have negligible effects however, as one research team noted that the channel's contribution to the overall olfactory response to pheromones was not appreciably altered in TRPC2 knockout mice (Kim et al. 2011). In contrast, other studies have shown the importance of TRPC2 channels as TRPC2 knockout mice failed to demonstrate olfactory-mediated sexual behaviours (Stowers et al. 2002; Kimchi et al. 2007).

Calcium-dependent ANO2 channels may demonstrate a means of tolerance to toxicity towards the ACIII and PLC signal-transduction pathways. Unless entirely abolished, inhibition of the transient Ca^{2+} current from internal stores or through CNG, TRPC2 or other cation channels can still lead to activation of ANO2 channels (Kleene 1993). Therefore, a majority of the olfactory signal can be generated even at a low Ca^{2+} influx. For example, pheromone stimulation still activated ANO2 channels in TRPC2 knockout mice (Kim et al. 2011). While signalling amplification from ANO2 channels may minimize upstream inhibitory effects,

inhibition of Cl^- efflux may prevent the triggering of an action potential. Inhibitors of the ANO2 channels reduced a depolarizing current by 90% in frog OSNs and by 77% in mice OSNs (Kleene and Gesteland 1991; Stephan et al. 2009). However, the significance of ANO2 channels to the signal-transduction pathway has previously been questioned, as ANO2 knockout mice were still able to perform olfactory-mediated behaviours (Billig et al., 2011). ANO2 channel inhibition would nevertheless decrease the likelihood of a high signal-to noise ratio during transduction and likely prevent animals from detecting weaker olfactory stimuli (Kleene 1997; Reisert et al. 2003).

The propagation of action potentials along OSN axons is necessary for animals to perceive odours. Therefore, toxic chemicals that block voltage-gated $Na_v1.7$ channels, such as copper and zinc, may disrupt olfactory detection even in the presence of generator potentials (Horning & Trombley, 2001). One study demonstrated that mice with non-functional voltage-gated $Na_v1.7$ channels were unable to perform olfactory-mediated tasks (Weiss et al., 2011). Even though odour-induced action potentials were still triggered in these mice, signals relayed to the forebrain for processing were absent. Similarly, activity was reduced in the olfactory processing center of rhesus monkeys exposed to various drugs that block voltage-gated $Na_v1.7$ channels (Ballard et al., 2020; Roecker et al., 2021). While drugs such as fluoxetine are used for medical purposes due to their ability to inhibit voltage-gated $Na_v1.7$ channels, they also enter the hydrosphere and are taken up by aquatic animals, thereby potentially causing olfactory disruption (Paterson & Metcalfe, 2008;Bringolf et al., 2010; Izadyar et al., 2016).

The modulation of olfactory signal transduction is vulnerable to toxicity. Studies have often reported decreased OMP expression in the presence of various toxins and toxicants, including for fishes exposed to copper ions (Tilton et al. 2008; Wang et al. 2013), copper nanoparticles (Razmara et al. 2021), diesel particulate matter (Song et al. 2022) or fluoxetine (Huang et al. 2022). In mice, cholera toxin and 3-methylindole exposures also reduced OMP expression (Kim et al. 2010; Fukuyama et al. 2015). In mice exposed to 3-methylindone, decreased OMP expression was correlated with impaired olfactory-mediated foraging tasks. OMP knockouts in mice exemplify a slower increase to peak depolarization and therefore a delayed firing of action potentials after olfactory stimulation (Reisert et al. 2007). OMP knockouts also caused delayed signalling termination, which could be derived from either

reduced buffered cAMP levels or reduced NCX1 activity (Kwon et al., 2009; Nakashima et al., 2020). In sum, subsequent odours may be unable to generate their own responses if neuronal repolarization occurs slowly.

Peripheral olfactory dysfunction may also occur at the level of paracrine signal inhibition. Cholinergic microvillus cells can suppress signals that are generated in neighbouring OSNs, thus lowering sensitivity towards odourants and perhaps preventing excitotoxicity (Ogura et al., 2011). However, the acetylcholine-induced modulatory effects on an olfactory signal can be blocked by toxicants that antagonize m_4AChR, such as atropine (Lind et al. 1998). In contrast, overstimulation of m_4AChR might constantly suppress olfactory signals. Acetylcholinesterase prevents the accumulation of acetylcholine in the olfactory epithelium (Hedlund & Shepherd, 1983). Therefore, acetylcholinesterase inhibitors would prevent the breakdown of acetylcholine and in turn cause OSNs to be less responsive towards odourants from m_4AChR overstimulation (Tierney et al., 2010; Colovic et al., 2013).

Toxicity to signal termination and desensitization

While activation of the ACIII or PLC pathway is required for olfactory detection, their termination is crucial for desensitization and for maintaining ion homeostasis. Signal termination involves Ca^{2+}-CaM inhibiting ACIII, CNG and TRPC2 activity, as well as activation of PDE to breakdown cAMP (Borisy et al. 1992; Liu et al. 1994; Wayman et al. 1995; Yan et al. 1995; Wei et al. 1996; Wei et al. 1998; Yildirim et al. 2003; Cygnar and Zhao 2009; Spehr et al. 2009). Therefore, toxic contaminants that potentiate CaM or PDE activity, such as CPF, copper, fluoxetine, and manganese, are likely to dampen neuronal depolarization during olfactory stimulation (Prasad 1974; Tilton et al. 2008; Tilton et al. 2011; Maryoung et al. 2015; Huang et al. 2022). Contaminants may also decrease CaM or PDE activity, which could prolong the decay of an olfactory signal and disrupt desensitization (Leinders-Zufall et al. 1999; Cygnar and Zhao 2009; Gudziol et al. 2010; Antunes et al. 2014; Hosseinzade et al. 2021). Decreased CaM or PDE activity may correlate with persistent ACIII activity, which could lead to cytotoxic levels of cyclic nucleotides (Farber & Lolley, 1974; Lolley et al., 1977). Cytotoxicity from high cyclic nucleotide production may also occur when contaminants deactivate CNG or TRPC2 channels as CaM activity would be reduced (Frings et al. 1995; Yamazaki et al. 2002; Brown et al. 2003; Xu et al. 2013).

Another route of toxicity may be via disruption in internalization of olfactory receptors, which involves GRK3 or PKA recruiting β-arrestin-2 to olfactory receptors (Dawson et al. 1993, Laporte et al. 2002, Rankin et al. 2002, Mashukova et al. 2006, Kato et al. 2013). For example, zebrafish exposed to copper had decreased PKA and β-arrestin-2 expression (Tilton et al. 2008). While no toxicants that impact GRK3 activity have been identified yet, GRK3 inhibition would likely reduce recruitment of β-arrestin-2 (Møller et al., 2020). Contaminants may need to disrupt both GRK3 and PKA activity to effectively impact desensitization as GRK3 and PKA selectively phosphorylate olfactory receptors (Kato et al., 2014). Furthermore, muscarinic-3 acetylcholine receptor antagonists could promote β-arrestin-2 recruitment to olfactory receptors and dampen olfactory signalling (Ohkuma et al. 2013; Jiang et al. 2015).

Olfactory signal termination includes the efflux of calcium ions out of OSNs through NCX1 and NCKX4 channels (Jung et al. 1994; Noé et al. 1997; Pyrski et al. 2007; Stephan et al. 2012). However, multiple toxicants decrease NCX1 or NCKX4 expression and therefore likely dampen the calcium efflux (Kwon et al. 2009; Sørhus et al. 2016; Tang et al. 2020; Yoo et al. 2020; Razmara et al. 2021; Huang et al. 2023; Jeong et al. 2023). NCKX4 knockout mice reveal how reduced Ca^{2+} efflux can affect olfactory functionality. One study demonstrated lowered action potential frequency in NCKX4 knockout mice, which were also unable to locate buried food (Stephan et al. 2012). A more recent study demonstrated that NCKX4 knockout mice spent more time sniffing to effectively perform behavioural tasks, suggesting that animals may adjust their behavioural strategies to maintain olfactory functionality in the presence of disrupted olfactory signalling (Reisert et al., 2021). Toxic contaminants can also disrupt calcium efflux by inhibiting Ca^{2+}-ATPase, including PMCA activity (Kwon et al. 2009; Castillo et al. 2010; Griff et al. 2012; Huang et al. 2022). However, one study demonstrated that a PMCA inhibitor had no effect on the termination of a response after olfactory stimulation, and so, PMCA channels may not be involved in signalling termination (Griff et al. 2012). Other than affecting Ca^{2+} efflux during neuronal repolarization, contaminants may delay the termination of an olfactory signal by preventing the reuptake of chloride ions through NKCC1 channel inhibition (Kaneko et al. 2004; Nickell et al. 2007).

When contaminants have the largest impact on olfactory signalling

In this review, we discussed how toxic contaminants affect olfactory signalling when targeting the different steps of the main signal-transduction pathways, whether it be disruption in odourants interacting with olfactory receptors or preventing the triggering, propagation, and termination of action potentials. With no odour-induced signals relayed from OSNs to the forebrain, animals cannot process olfactory stimuli from their environment. However, multiple studies have demonstrated that toxicity towards a particular step of the signal-transduction pathway induces minimal change to olfactory signalling or functionality, which indicates the complexity and resilience of this system to perturbation (Taniguchi et al. 2000; Spehr et al. 2002; Kim et al. 2011; Chamero et al. 2017). As well, differential toxicity towards the ACIII and PLC pathway limits what odours animals are insensitive towards (Ma & Michel, 1998). In the face of signalling disruption, animals may alter their strategy to perform olfactory-mediated behaviours (Reisert et al., 2021). Nevertheless, toxic contaminants often do the biggest harm to olfactory signals and olfactory functionality when they target multiple steps of the signal-transduction pathways. For example, copper is considered a potent neurotoxin that disrupts olfactory-mediated behaviours as it targets multiple components of OSNs including altering the expression of genes encoding for olfactory receptors, ACIII, CNG channels, CaM, PKA and β-arrestin-2 (Tilton et al. 2008; Tilton et al. 2011; Pfister et al. 2020). While disruption of each step on its own may minimally impact olfactory signalling, it is the combined disruption of multiple targets of the signal-transduction pathway that would prevent animals from “smelling” their environment.

Conclusion

The signal-transduction pathways responsible for generating and terminating olfactory signals at the peripheral olfactory organ have been thoroughly described in vertebrates. Therefore, the foundation exists to build an understanding of how different contaminants might disrupt the various intricate processes involved with olfactory signalling. Over the past decade, changes in the expression of genes involved in the olfactory signal-transduction pathways due to contaminant exposures have been frequently reported. These studies have found that contaminants often decrease gene expression and that this downregulation of genes may correlate

with dampened signal transduction and delayed signal termination. A few studies have also shown that contaminants may induce toxicity by increasing expression of genes, especially those pertaining to signal termination. Knockout experiments have allowed for speculation regarding the relationship between disruption in olfactory signalling and olfactory functionality. While the implications of impairing specific targets in signal transduction can be modelled in a straightforward manner, some contaminants may target multiple sites along the signal-transduction pathways, thus limiting our ability to predict adverse olfactory effects.

Ultimately, disturbances to the generation, termination, or modulation of olfactory signals may have a consequential impact on downstream physiological and behavioural processes. Thus, future investigations should aim to evolve outcome pathways that relate changes in the functionality of olfactory machinery to critically important behaviours of fitness relevance, such as foraging, avoiding predators, mating, and migrating. In modelling such relationships, we must consider that animals may utilize behavioural strategies to maintain olfactory functionality despite alterations to the olfactory signal-transduction pathways, and that animals may detect contaminants from afar and avoid encountering the contaminants altogether.

Acknowledgements

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Tables

Table 2.1. The different target sites of the main olfactory signal transduction pathways in which toxic chemicals can disrupt.

Olfactory signal transduction pathway target sites	Toxic chemicals	Species	References
Olfactory receptors			
	2,4,6-trichloroanisole	Human	Tempere et al. 2017[Ψ]
	Benthiocarb	Common carp	Ishida and Kobayashi 1995[Ψ]
	Buspirone	Zebrafish	Abreu et al., 2016
Multiple ORs, TAARs, V1Rs, V2Rs	Cadmium	Coho salmon	Williams and Gallagher 2013, Williams et al. 2016
		Rainbow trout	Lari et al. 2018[Ψ]
Multiple ORs, TAAR340, V1R320	Copper	Sea lamprey	Jones et al. 2019
		Rainbow trout	Lari et al. 2018[Ψ], Razmara et al. 2021
Multiple ORs		Zebrafish	Tilton et al. 2008
OR2T11		Human	Li et al. 2016[Ψ]

Multiple ORs	Copper nanoparticle	Rainbow trout	Razmara et al. 2020, Razmara et al. 2021
Multiple ORs	Chlorpyrifos	Zebrafish	Tilton et al. 2011
OR1507	Dexamethasone	C57BL6 mice	Kim et al. 2019
	Diazepam	Zebrafish	Abreu et al., 2016
	Diazinon	Zebrafish	Könemann et al. 2021Ψ
Multiple ORs	Ethanol	C57BL6 mice	Mandal et al. 2015
	Fluoxetine	Zebrafish	Abreu et al., 2016
	Imidacloprid	Zebrafish	Könemann et al. 2021Ψ
	Isoprothiolane	Common carp	Ishida and Kobayashi 1995Ψ
OR23	Lyral	BALB mice	Touhara et al. 1999Ψ
OR23		Mice	Grosmaitre et al. 2006Ψ
	Nickel	Rainbow trout	Lari et al. 2018Ψ
	Oil sands process-affected water	Rainbow trout	Lari and Pyle 2017Ψ
Multiple ORs	Perfluorobutane sulfonate + hypoxia	Marine medaka	Tang et al. 2020
	Permethrin	Zebrafish	Volz et al. 2020Ψ

GFA2, GFA28, GFB1, GFB8	Polystyrene microplastics	Goldfish	Shi et al. 2021
	Propanol	Land phase salamander	Firestein and Shepherd 1992
	Risperidone	Zebrafish	(Abreu et al., 2016)
	Roundup	Rainbow trout	Tierney et al. 2007[Ψ]
OR2T11	Silver	Human	Li et al. 2016[Ψ]
	Sodium lauryl sulfate	Common carp	Ishida and Kobayashi 1995[Ψ]
GFA2, GFA28, GFB1, GFB8	Triclosan/ Triclocarban	Goldfish	Huang et al. 2023
OR1507	Urban particulate matter	C57BL6 mice	Kim et al. 2022
Adenylyl Cyclase III			
	Amitriptyline	Sprague-Dawley rats	Mania-Farnell et al. 1993
	Bromocriptine	Wister rats	Coronas et al. 1999
		Sprague-Dawley rats	Mania-Farnell et al. 1993
	Cadmium	Coho salmon	Williams et al. 2016
	Copper	Zebrafish	Tilton et al. 2008
		Coho salmon	Wang et al. 2013

Forskolin	Zebrafish	Michel 1999, Michel et al. 2003
	Goldfish	Rolen et al. 2003
	Pacific Jack Mackerel	Vielma et al. 2008
	American bullfrog	Sklar et al. 1986, Lowe et al. 1989
	Clawed frogs	Gliem et al. 2009, Brinkmann and Schild 2016
	Marsh frogs	Pace et al. 1985
	C57BL6 mice	Chen et al. 2012
	Sprague-Dawley rats	Chen et al. 2012
	Wistar rats	Asanuma and Nomura 1991, Sasaki et al. 1999, Otsuguro et al. 2005
MDL12330A	Common mudpuppy	Delay and Dionne 2002
	Goldfish	Sorensen and Sato 2005
	Tiger salamander	Chen et al. 2000
	Grass frog	Pun and Kleene 2003

		CD-1 mice	Chen et al. 2000
		Sprague-Dawley rats	Ukhanov et al. 2011
		Wistar rats	Spehr et al. 2002
	Quinpirole	Tiger salamanders	Chen et al. 2000
		CD-1 mice	
		Simonsen albino rats	Vargas and Lucero 1999
		Wister rats	Coronas et al. 1999
	SQ22536	Tiger salamander	Chen et al. 2000
		C57BL6 mice	Lin et al. 2007
		CD-1 mice	Frenz et al. 2014
		Sprague-Dawley rats	Ukhanov et al. 2011
	Urban particulate matter	C57BL6 mice	Kim et al. 2022
Phospholipase-C			
	Imipramine	Common carp	Cadiou and Molle 2003
		Goldfish	Sato and Sorensen 2018
	Neomycin	Common mudpuppy	Delay and Dionne 2002

		Goldfish	Sorensen and Sato 2005
		Zebrafish	Ma and Michel 1998
		Wistar rats	Inamura et at al. 1997
	U-73122	Goldfish	Sorensen and Sato 2005, Sato and Sorensen 2018
		Fathead minnows	Dew et al. 2014
		Yellow croaker	Zhu et al. 2023
		Zebrafish	Kaniganti et al. 2021
		Clawed frogs	Sansone et al. 2014
		Common musk turtle	Brann and Fadool 2006
		C57BL6 mice	Zhang et al. 2010
		Wistar rats	Inamura et at al. 1997, Spehr et al. 2002
		Sprague-Dawley rats	Vogl et al. 2000
Inositol triphosphate receptor			
	Ruthenium red	Zebrafish	Ma and Michel 1998

		Channel catfish	Restrepo et al. 1990, Miyamoto et al. 1992
		Clawed frogs	Lischka and Schild 1993
		Chinese pond turtles	Taniguchi et al. 1995
		Garter snakes	Taniguchi et al. 2000
		Wistar rats	Inamura et at al. 1997
	Xestospongin C	Clawed frogs	Sansone et al. 2014
DAG lipase			
	Chlorpyrifos oxon	Chinese hamster	Bomser et al. 2002
	RHC-80267	Chinese hamster	Bomser et al. 2002
		Wistar rats	Spehr et al. 2002
Cyclic nucleotide gated channels			
	2,4,6-Trichloro anisole	Japanese fire-bellied newts	Takeuchi et al. 2013
	8-Br-cAMP	BALB/c mice	Boccaccio et al. 2006
		Sprague-Dawley rats	Zhainazarov et al. 2004
		Human	Hagen et al. 1996

	Copper nanoparticle	Rainbow trout	Razmara et al. 2021
	LY83583	Tiger salamanders	Leinders-Zufall and Zufall 1995
		Chilean toads	Madrid et al. 2005
	Magnesium	Clawed frogs	Frings et al. 1995
	Nitric oxides	Tiger salamanders	Broillet and Firestein 1996
		Wistar rats	Lynch 1998
	Pseudechetoxins	Clawed frogs	Yamazaki et al. 2002, Brown et al. 2003
	Trifluoperazine	Northern grass frog	Kleene 1994
	W-7	Northern grass frog	Kleene 1994
Transient receptor potential C2 channel			
	2- APB	Zebrafish	Kaniganti et al. 2021
		Clawed frogs	Sansone et al. 2014
		C57BL6 mice	Zhang et al. 2010
	Cadmium	Zebrafish	Williams and Gallagher 2013, Volz et al. 2020
	SKF-96365	Clawed frogs	Sansone et al. 2014

Anoctamin-2 channel			
	3',5-dichloro diphenylamine-2-carboxylate	Norther grass frog	Kleene and Gesteland 1991
	DIDS	C57BL6 mice	Nickell et al. 2007, Dibattista et al. 2012
	Niflumic acid	Northern grass frog	Kleene 1993
		Mice	Stephan et al. 2009, Haering et al. 2015
		C57BL6 mice	Dibattista et al. 2012
	SITS	Japanese fire-bellied newts	Kurahashi and Yau 1993
		Chinese pond turtle	Kashiwayanagi et al. 1996
$Na_V1.7$ channels			
	Aryl sulfonamides	Rhesus macaques	Roecker et al. 2021
	Copper	Rat	Horning and Trombley 2001
	Fluoxetine	Swiss outbred mice	Igelström and Heyward 2012
	Zinc	Rat	Horning and Trombley 2001
Olfactory marker protein			

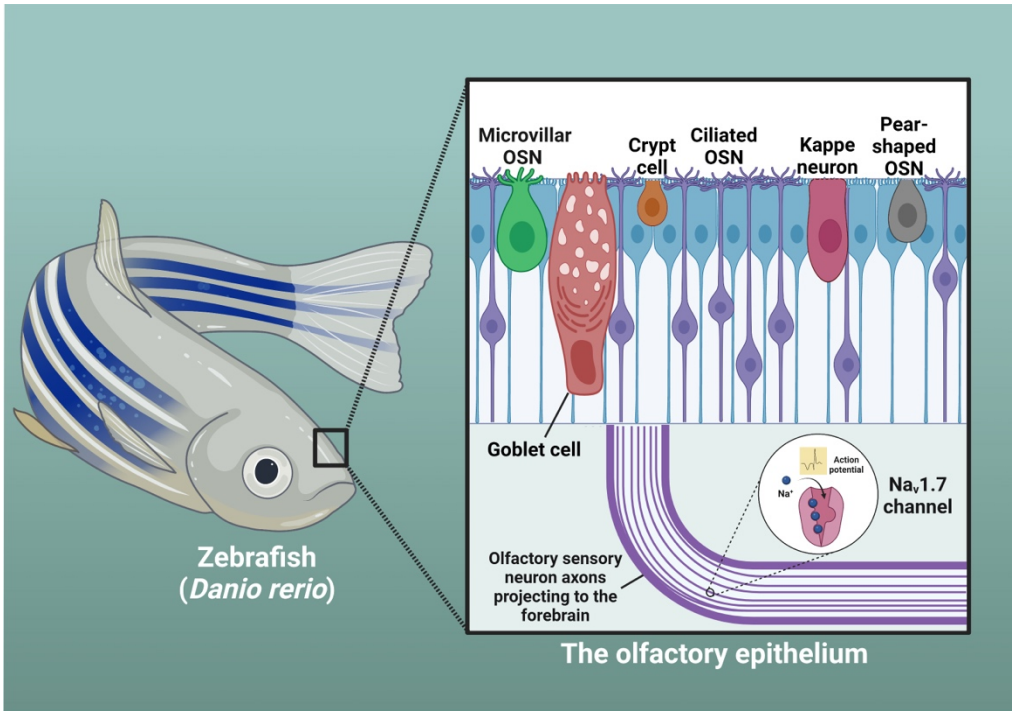
	3-methylindole	C57BL6 mice	Kim et al. 2010
	Cholera toxin	BALB/c mice	Fukuyama et al. 2015
	Copper	Zebrafish	Tilton et al. 2008
		Coho salmon	Wang et al. 2013
	Copper nanoparticle	Rainbow trout	Razmara et al. 2021
	Diesel particulate matter	Zebrafish	Song et al. 2022
	Fluoxetine	Goldfish	Huang et al. 2022
Calmodulin/Calmodulin kinase II			
	Autocamtide-2-related inhibitory peptide	C57BL6 mice	Wei et al. 1998
		Tiger salamanders	Leinders-Zufall et al. 1999
	Chlorpyrifos	Rainbow trout	Maryoung et al. 2015
		Zebrafish	Tilton et al. 2011
	Copper	Zebrafish	Tilton et al. 2008
	Diazinon	Caspian roach	Hosseinzade et al. 2021
	Fluoxetine	Goldfish	Huang et al. 2022

	KN-62	C57BL6 mice	Wei et al. 1998
	Mastoparan	Sprague-Dawley rats	Chen and Yau 1994
Phosphodiesterase			
	3-isobutyl-1-methylxanthin	Tiger salamanders	Firestein et al. 1991
		Northern leopard frogs	Antolin and Matthews 2007
		Mice	Cygnar and Zhao 2009
		BALB/c mice	Boccaccio et al. 2006
		C57BL6 mice	Wei et al. 1998
		Wistar rats	Antunes et al. 2014
	Theophylline	NMRI mice	Gudziol et al. 2010
β-arrestin-2			
	Atropine	Japanese fire-bellied newt	Ohkuma et al. 2013
		C57BL6 mice	Jiang et al. 2015
	Carbachol	C57BL6 mice	Jiang et al. 2015
	Copper	Zebrafish	Tilton et al. 2008
NCX1/NCKX4			

	3,4-dichlorobenzamil hydrochloride	Mice	Kwon et al. 2009
	Copper nanoparticle	Rainbow trout	Razmara et al. 2021
	Perfluorobutane sulfonate + hypoxia	Marine medaka	Tang et al. 2020
	Triclosan/ Triclocarban	Goldfish	Huang et al. 2023
Plasma Ca^{2+}-ATPase			
	Carboxyeosin	Northern leopard frogs	Castillo et al. 2010
		Mice	Kwon et al. 2009
	Fluoxetine	Goldfish	Huang et al. 2022
Na-K-Cl cotransporter-1			
	Bumetanide	Northern leopard frogs	Jaén et al. 2011
		Mice	Kaneko et al. 2004, Reisert et al. 2005
		C57BL6 mice	Nickell et al. 2007
		Rats	Kaneko et al. 2004

[Ψ] Studies that observed contaminants that generated an olfactory signal or evoked an olfactory-mediated response in animals, and therefore are likely to interact with olfactory receptors

Figures



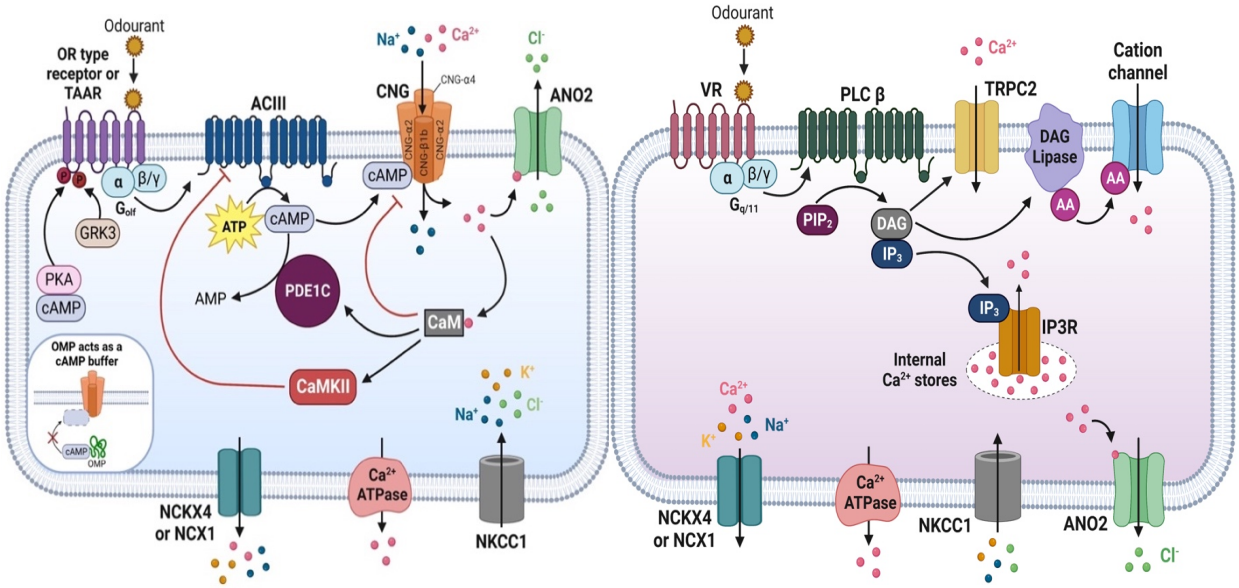


Figure 2.1. The ACIII and PLC signal transduction pathway at the zebrafish olfactory epithelium. Created with BioRender.com

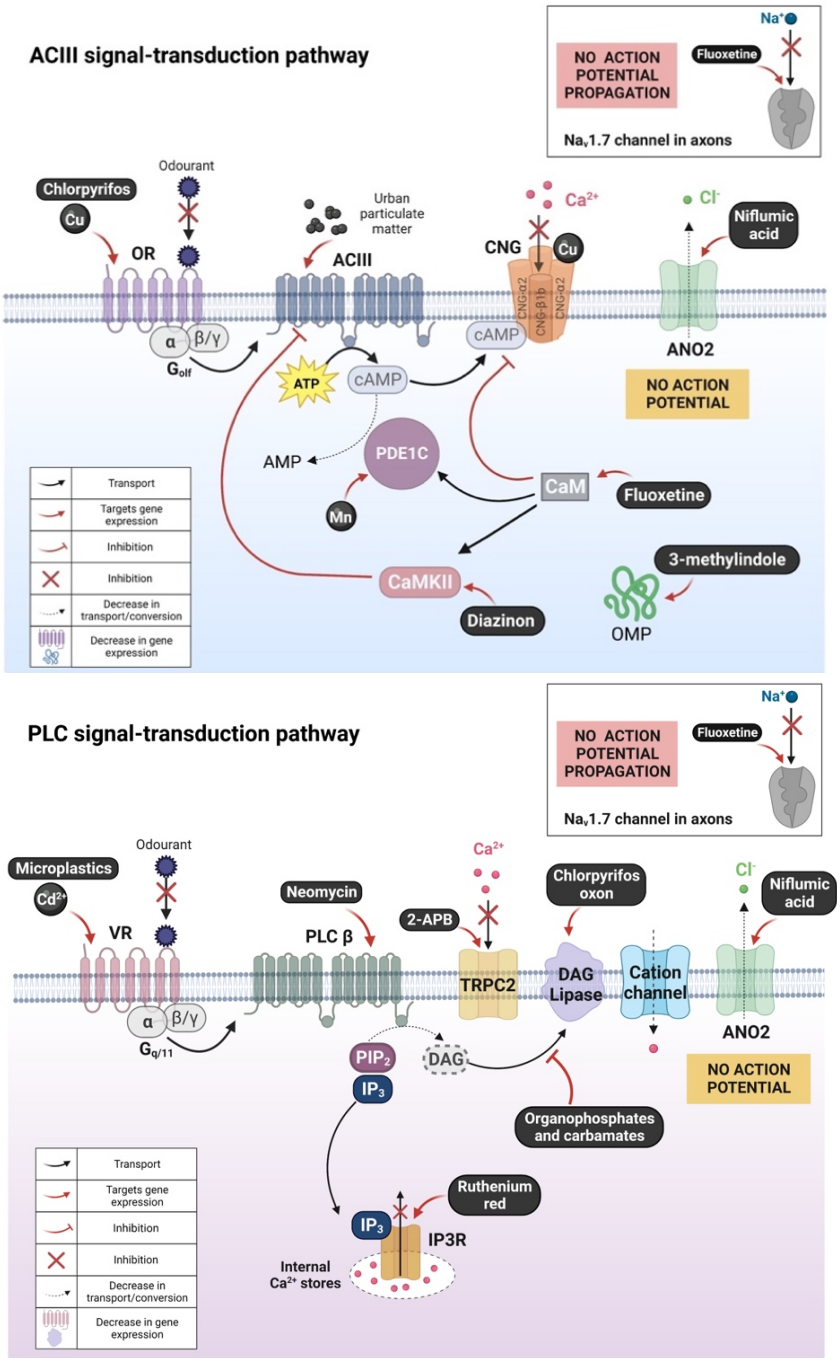


Figure 2.2. Contaminant disrupting different sites of the ACIII and the PLC signal-transduction pathway. Created with BioRender.com

Chapter 3: Zebrafish (*Danio rerio*) behavioural response to an odourant mixture containing attracting and repelling odourants

Abstract

Odourants guide essential activities such as foraging and fleeing predators. Studies usually examine behavioural and physiological effects of individual odourants, while animals in the environment are exposed to multiple stimuli simultaneously. In this study, we exposed zebrafish to a mixture of attraction-evoking and aversion-evoking amino acids, and behavioural responses were observed**.** Attraction to L-alanine and avoidance to L-cysteine were observed, and so these amino acids were used to make the mixture (zebrafish also avoided L-serine, but this was weaker than with L-cysteine exposures). When exposed to the mixture, fish responded with avoidance, which suggests that aversion-evoking stimuli outweigh attraction-evoking stimuli. Attraction towards the mixture was seen only when the concentration of L-cysteine was decreased from 0.1 to 0.001 mM. Olfactory ablation surgery confirmed that the behaviours were olfactory-mediated. Overall, this study demonstrated that odourant stimuli that repel outweigh stimuli that attract until their concentration decreases by as much as 100-fold.

Introduction

Animals live by responding to internal and external cues. Of the external cues, odourants such as amino acids drive numerous behaviours including foraging, avoiding predators and mating (Atema et al., 1980; Marvin & Hutchison, 1994; Bloss et al., 2002). For example, aquatic animals often display foraging behaviours when encountering L-alanine (Steele et al., 1990; Carr et al., 1996) and show avoidance responses when encountering L-serine or L-cysteine (Idler et al., 1956; Rehnberg & Schreck, 1987; Vitebsky et al., 2005). Such behavioural responses towards odourants are also seen in amphibians, reptiles, birds and mammals, demonstrating that oduorants responses are likely highly conserved for survival across vertebrates (Vitt & Cooper, 1986; Marvin & Hutchison, 1994; Pastro & Banks, 2006; Roth et al., 2008).

The general mechanisms of the olfactory system are the same across vertebrates (Eisthen, 2008). Odourants are detected by olfactory receptors on sensory neurons, which are located within an olfactory epithelium (Buck & Axel, 1991; Ngai et al., 1993). It is here where the

chemical input is converted to electrical signals, which are relayed to the olfactory bulb and eventually the forebrain for higher order processing (Mombaerts et al., 1996; Nikonov et al., 2005). Even though many studies have looked at the physiological and behavioural responses of individual odourants to animals, this does not necessarily represent the natural environment, because animals live within a multitude of odourants.

In a natural setting, animals are likely to encounter sensory input such as food and predator odourants simultaneously. Correspondingly, a conflict may arise in animals in making a decision regarding attraction or avoidance. Therefore, observing their attraction/avoidance to mixtures in which concentrations are varied could provide valuable information on which type of stimuli 'outcompetes' the other.

Of the studies that have exposed animals to odourant mixtures have demonstrated that either individual odourants are dominant or that the mixture acts as a new odourant (Tabor et al., 2004; Valentincic et al., 2011; Thomas-Danguin et al., 2014). The former is observed when there are fewer odourants, in which the more stimulatory one often drives an animal's response. The latter is usually observed when there are four or more odourants, including in humans (Livermore & Laing, 1996). The importance of odourants in a mixture is dependent on an individual odourant's physical properties, as was demonstrated by carpenter ants exposed to binary mixtures of alcohols and aldehydes (Perez et al., 2015).

The objective of the present study was to investigate how a model animal behaviourally responded to an odourant mixture, in which the constitutive odourants would elicit opposing behavioural responses. This was carried out through exposing adult zebrafish, a popular animal model, to a mixture made of an attraction-evoking amino acid (L-alanine) and an avoidance-evoking amino acid (L-serine or L-cysteine). These three odourants were selected because they have previously been shown to act as attraction or avoidance cues, respectively (for L-alanine attraction: Steele et al., 1990, L-serine avoidance: Idler et al., 1956; Rehnberg & Shreck, 1987, L-cysteine avoidance: Vitebsky et al., 2005). Food and predator skin extract were not used as attractive and aversive odours, respectively, since they are difficult to characterize and describe, and thus use in mechanistic examinations of behaviour (Haynes & Millar, 2012). By observing movement to combined attractive and aversive stimuli, zebrafish behavioural response towards

the odourant mixture was determined. Furthermore, the strength of the dominant stimulus was characterized by increasing or decreasing the concentrations of the individual odourants.

Methods

Fish husbandry

Adult tupfel long-fin (TL) strain zebrafish were housed at the University of Alberta (Edmont, AB, Canada) in a recirculating aquatics rack system (Aquaneering, San Diego, CA, USA) consisting of 28 ± 0.5°C reverse osmo- sis (RO) water. Fish were subjected to a 14:10 h light:dark photoperiod and were fed twice daily of a mixture containing TetraMin flakes (Tetra Holding, Blackburg, VA, USA), Cobalt Aquatics spirulina flakes (Cobalt, Rock Hill, SC, USA) and Omega OneTM freeze-dried blood worms (Omegasea, Sitka, SK, Canada). Prior to experimentation, zebrafish underwent a one day fasting period. Treatment of all zebrafish was under accordance with University of Alberta's Animal Care and Use Committee (AUP No. 052).

Chemicals used

L-alanine was a potential attraction-evoking odourant while L-serine and L-cysteine were used as avoidance-evoking odourants. A subset of zebrafish underwent nasal cavity ablation surgery, in which they were first anesthetized with tricaine methansefulfonate (TMS; Syndel, Nanaimo, BC, Canada) buffered with bicarbonate (pH 7.2). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Avoidance-attraction trough

Adult zebrafish behaviour was observed in a 50 × 10 × 10 cm (L × W × H) plexiglass avoidance-attraction trough similar to that in Saglio et al. 2001. In brief, the apparatus contained two 10-l polypropylene carboys, in which each was situated at opposite ends of the trough and provided a continuous inflow (0.7 l/min) of 28°C RO water. Water from the two inflows moved towards and drained through a central plexiglass-made pipe with an inner diameter of 7 mm. As a result, two contiguous water bodies were made. Therefore, when odourants were introduced to the trough through one of the two inflows, zebrafish could decide whether to spend more or less time in the zone containing the odourant. A water column depth of at least 5 cm was kept;

anything shallower may have induced anxiety (Córdova et al., 2016). A black opaque curtain enclosed the apparatus to minimize external influences. Overhead dome colour IR cameras (SAV-CD120; Matco, Stow, OH, USA), which were placed beneath the curtain, were used to record zebrafish swimming. Based on preliminary observations, adult fish that were introduced to the trough swam freely and lacked any preference biases to either side of the trough during a 10-min basal activity period following a 20-min acclimation period. For all experiments, trials were considered valid only when zebrafish swam freely across both sides of the trough during a 10-min basal activity period.

Red dye was used to model the movement of odourants within the trough as well as approximate dilute rates (Figure 3.1). The dye increased in concentration quickly within 1 min and peaked at 3 min before gradually declining, until being undetectable after 15 min. Since the dye was present for 10 min ($p < 0.001$ between $t = 10$ min and $t = 0$ min; Holm–Sidak one-way ANOVA), odourants were introduced to the trough for 10 min.

Zebrafish responses towards odourants and odourant mixtures

All behavioural experiments had the same procedure where one adult TL zebrafish of either sex, was randomly placed in an avoidance-attraction trough and given a 20-min acclimation period, followed by the recording of a 10-min basal activity period and a subsequent 10 min odorant(s) exposure period. The odourants were introduced to the trough through one of the inflows, which was chosen at random. The trough was rinsed with RO water between trials for 30 min, which allowed for amino acids to be removed. L-alanine (0.01, 0.05, 0.1, 0.5 mM), L-serine (0.01, 0.1, 0.5 mM), or L-cysteine (0.01, 0.1, 0.5 mM) was introduced to observe potential attraction and avoidance responses. The concentrations chosen for each amino acid were based on the reasoning that they were above zebrafish olfactory system detection threshold, according to earlier electrophysiological studies (Michel & Lubomudrov, 1995; Friedrich & Korsching, 1998). Fish were given a minimum of one week between tests to reduce stress from frequent handling and allow their behaviour to return to normal in case the amino acids changed odorant receptor expression levels in the olfactory epithelium (Wang et al., 1993). The concentration of amino acids that caused the greatest attraction or avoidance response were used to create the mixture that zebrafish were exposed to. If the mixture evoked attraction or avoidance, the dominant cue's concentration was decreased, or the weaker cue's concentration

increased, until the opposite response was seen. All experiments were performed between 9 a.m. and 6 p.m. since this is when they are considered active and not resting (Reed & Jennings, 2011). Each treatment used 10–11 fish.

Olfactory involvement in zebrafish response towards odourants

The olfactory system, gustatory system and solitary chemosensory cells (SCCs) can detect amino acids to mediate behaviours such as foraging and avoiding predators (Lindsay & Vogt, 2004). Therefore, olfactory involvement of the amino acids that caused maximal attraction and avoidance in the current study were determined by nasal cavity ablation. Using TMS buffered with bicarbonate (1:1, pH 7.2) to induce and maintain stage III anaesthesia (induction dose: 200 mg/l, maintenance dose: 120 mg/l; visualized by absent opercular movement), fish were held on a bench-top-v-shaped perfusion apparatus. A fine-tip cautery pen (Bovie Medical AA01; FL, USA) was used on both nasal cavities to render fish anosmic. Following surgery, zebrafish were given a one-week recovery period and were observed to ensure they behaved normally. Neither disorientation nor lethargy (including a lack of drive for food) were observed following surgery. Unilateral cauterization was also performed as a procedural control.

Data and statistical analysis

Zebrafish responses towards amino acids were manually scored as the average time they spent on either end of the avoidance-attraction trough. The central area of the trough where some mixing occurred, visualized by dye trials, was not included (5 cm in length). Manual scoring was binned into 1 or 10 min intervals of the exposure period and compared to the basal activity period in which the average time fish spent on either end of the trough was determined. The recordings from basal activity were pooled across treatments for a given amino acid or amino acid mixture when the values did not deviate from one another (one-way analysis of variance, $\alpha = 0.05$). EthoVision XT10 (Noldus, Wageningen, The Netherlands) was used to also analyse changes in swim speed for the amino acids at the concentrations that elicited behavioural responses. Here, swim speed (cm/s) was determined in the basal activity period and compared to that of each minute during the exposure period.

Statistical analysis for comparing percent of time zebrafish spent in zone of odourant(s) inflow between the exposure and basal period was via a two-way (concentration × time) analysis

of variance (ANOVA), followed by a Holm–Sidak post hoc test. Swimming activity was analysed by a one- way ANOVA or a two-way ANOVA. The former was used if only one concentration for a given amino acid induced a preference response in fish. The latter was used if more than one concentration for a given amino acid induced a response. For all analyses, when the assumptions of normality and equal variance failed, arcsine transformations were done, but, if this did not resolve the assumptions, a one-way ANOVA or two-way ANOVA on rank-transformed data were used instead. SigmaPlot 14.0 (Systat, San Jose, CA, USA) was used for statistical analysis and statistical significance was defined by $\alpha = 0.05$. P -values near the threshold ($p < 0.1$) were reported in order to minimize type II error of not detecting a real difference in behavioural changes. The rationale was that behavioural experiments may have high individual variation (Shamchuk & Tierney, 2012). All values are presented as mean ± SEM.

Results

Finding an attraction-associated amino acid

Overall, L-alanine evoked attraction and avoidance responses ($\chi_{3,40} = 38$; $p < 0.001$) (Figure 3.2). Specifically, L-alanine at 0.05 mM caused zebrafish to swim 74 ± 6.8% of the time period with the amino acid once it was introduced (Figure 3.2A). This preference towards L-alanine was higher than that of any other concentrations used ($p < 0.001$ between 0.05 mM and 0.01, 0.1 and 0.5 mM). The response was also time-dependent ($\chi_{10,40} = 3.1$, $p < 0.001$) and lasted for approximately 7 min, with L-alanine attraction beginning within 1 min and peaking at the 5th and 6th min intervals ($p = 0.031$, 0.071, 0.032, 0.068, 0.009, 0.013, and 0.068 from 1–7 min, respectively; Figure 3.2B). This was associated with a decrease in swim speed of 1 cm/s or more, until the last 2 min of exposure ($p < 0.001$ for 1–6, 8 min and $p = 0.003$ for 7 min; Figure 3.2C). When L-alanine concentration was at 0.1 mM, fish demonstrated a delayed avoidance response that peaked at the 8th min interval of the exposure period, in which 8 out of 10 fish sampled had at least a 20% difference in time spent with L-alanine ($p = 0.046$, 0.043, 0.005 for 5th, 6th and 8th min interval, respectively; Figure 3.2B). Soon after 0.1 mM L-alanine was introduced, swim speed increased by 1 cm/s for 2 min ($p = 0.023$ and 0.043 for 2nd and 3rd min of exposure; Figure 3.2C). Between 0.05 and 0.1 mM, the amount of time fish spent in the zone of L-alanine inflow diverged within the third and 8th min interval of the exposure period ($\chi_{30,40}$

= 2.0, p = 0.002). Interestingly, zebrafish demonstrated indifference towards L-alanine when its concentration further increased to 0.5 mM, which may have overstimulated their olfactory system (Figure 3.2A, B). As a result, fish may not have been able to properly process the stimulus.

Finding an avoidance-associated amino acid

Zebrafish did not respond to L-serine and avoided L-cysteine (Figure 3.3, 3.4). In regard to L-serine, concentration did not influence zebrafish behaviour ($\chi_{2,3}$ = 0.28, p = 0.76; Figure 3.3A). That said, there was a gradual decline of fish spending time with the amino acid at 0.5 mM, but this trend did not come close to reaching statistical significance at any time intervals once L-serine was introduced ($\chi_{10,30}$ = 1.1, p = 0.39; Figure 3.3B). In contrast, while concentration of L-cysteine did not appear to influence zebrafish behaviour ($\chi_{2,3}$ = 2.1, p = 0.13; Figure 3.4A), the fish avoided 0.1 mM of the amino acid for nearly half of exposure period, with the biggest reductions being more than half of the basal period ($\chi_{10,30}$ = 2.4, p = 0.009; p-values of 0.037 and 0.014 for the 4th and 8th min interval, respectively; Figure 3.4B). Zebrafish swim speed was not affected by 0.1 mM L-cysteine (Figure 3.4C). Altogether, L-cysteine at 0.1 mM demonstrated being the more robust avoidance cue and therefore was used for further experimentation.

Zebrafish attraction-avoidance response towards L-alanine/L-cysteine mixtures

The amino acid mixture introduced to zebrafish consisted of 0.05 mM L-alanine and 0.1 mM L-cysteine as individually these concentrations caused the strongest attraction and avoidance responses, respectively (Figures 3.3 and 3.4). Overall, the concentration of either amino acid within the mixtures strongly affected zebrafish behaviour ($\chi_{4,50}$ = 4.5, p = 0.001; Figure 3.5A). More specifically, when fish were exposed to 0.05 mM L-alanine and 0.1 mM L-cysteine simultaneously, they responded in a time-dependent manner ($\chi_{10,50}$ = 3.1, p < 0.001) with avoidance during the second half of the exposure period (p values of 0.067 and 0.1 for 6 and 7th min, respectively; Figure 3.5B). This was characterized by often spending half as much time in the inflow zone as that during the basal activity period. Being the dominant cue, L-cysteine's concentration was decreased by 10-fold until it reached 0.001 mM. At this point, zebrafish demonstrated an attraction response and in turn the percent time they spent with the mixture

deviated from when the concentration of L-cysteine was at 0.1 or 0.01 mM (p-values of 0.019 and <0.001 between L-alanine/L-cysteine concentrations of 0.05/0.001 mM and 0.05/0.1 or 0.05/0.01 mM, respectively; Figure 3.5A, B). With the mixtures, swim speed did not change (Figure 3.5C). When increasing L-alanine concentration while holding L-cysteine concentration at 0.1 mM, the avoidance response initially seen by zebrafish did not become an attraction response (Figure 3.5A, B). At most, zebrafish were indifferent to the mixture at a 5 and 10-fold increase of L-alanine. Its concentration was not raised further due to the apparatus's excessive amount required to reach a 100-fold increase and potentially oversaturating the fish's olfactory system.

Olfactory involvement in response towards L-alanine and L-cysteine

Olfactory epithelium ablation surgery suggested that the observed attraction and avoidance response caused by 0.05 mM L-alanine and 0.1 mM L-cysteine, was mainly through the olfactory system (Figure 3.6). More specifically, anosmic zebrafish subjected to either stimulus did not change the amount of time they spent in the inflow zone. The surgery did not have an influence since unilateral cauterized zebrafish showed a gradual increase in time spent with L-alanine within the first two minutes of its introduction ($p = 0.066$ at the 2nd min interval, Figure 3.6A), and had a delayed avoidance response towards 0.1 mM L-cysteine, peaking at the 8th min interval of the exposure period ($p = 0.027$ and 0.028 for the 7th and 8th min interval, respectively, Figure 3.6B). The unilateral cauterized fish responses towards both amino acids were weaker than those not undergoing surgery, suggesting the important role for both nares. Ablation of both nasal cavities led to no response to either L-alanine or L-cysteine.

Discussion

Animals encounter a multitude of odourants simultaneously, including blends of attractive and aversive odourants. However, there has been a lack of examination on how animals respond to odourant mixtures that contain competing signals. The present study using a model aquatic vertebrate demonstrated that aversion-evoking odourants may outweigh attraction-evoking odourants until their concentration is greatly reduced.

L-alanine as an attraction stimulus

On its own, L-alanine was attractive to zebrafish only when its concentration was 0.05 mM. In general, L-alanine tends to elicit foraging behaviours in aquatic animals as it is a feeding cue found in many prey species (Steele et al., 1990; Carre et al., 1996). The concentration of L-alanine often must be greater than 0.01 mM to be considered attractive (Steele et al., 1990; Zippel et al., 1993). However, there have been exceptions such as with arctic charr showing attraction to 0.01 μM L-alanine (Jones & Hara, 1985). Interestingly, we found that 0.1 mM L-alanine eventually elicited an avoidance response in zebrafish. This may have owed to the L-alanine concentration reaching a threshold where the odourant became overwhelming and therefore evoked aversion (Laing et al., 1978; Giattina et al., 1982; Tierney, 2016). Because zebrafish have previously shown attraction towards L-alanine at 0.1 mM, I recommend further examination of what concentration this amino acid may become aversive (Steele et al., 1990).

In association with the attraction response towards L-alanine at 0.05 mM, zebrafish reduced their swim speed. This was unexpected as foraging behaviours are usually associated with an increase in swim speed and distance travelled, accompanied with frequent turns (Steele et al., 1990; Hara, 2006; Kalueff et al., 2013). A possible explanation for our findings is that individuals were fixating on the inflow source, as suggested by Jones & Hara (1985), who reported reduced swim speed in whitefish exposed to food extract.

L-serine and L-cysteine as avoidance stimuli

Of the two potential avoidance cues used in the present study, L-cysteine at 0.1 mM was noticeably more aversive than L-serine at any given concentration. A similar difference between the two amino acids has previously been reported by Vitebsky et al. (2005), who demonstrated that juvenile zebrafish displayed weaker avoidance responses towards 0.03 mM L-serine than L-cysteine. These observations could be because the avoidance towards L-cysteine is innate while that of L-serine is acquired. For example, in Vitebsky's (2005) same study, larval zebrafish exposed to L-cysteine also elicited an avoidance response, which carried through to adulthood, while they did not respond to L-serine. Therefore, fish exposed to L-serine may need aversive conditioning, such as detecting the amino acid while observing a predator kill conspecifics. As a reference, salmon learn to avoid L-serine, which is found on bear skin (Idler et al., 1956;

Rehnberg & Schreck, 1987). The current study may suggest that zebrafish associate L-serine with human net handling (Croxton et al., 2006). On the other hand, L-cysteine is a sulphurous compound, which generally cause aversion in animals (Nolte et al., 1994). A physiological explanation for why L-cysteine was more aversive than L-serine is that L-cysteine is one of the most potent olfactory amino acids in fish, including catfish, goldfish, salmonids and zebrafish (Byrd & Caprio, 1982; Sorensen et al., 1987; Michel & Lubomudrov, 1995; Hara & Zhang, 1997). This conclusion mainly comes from studies that have measured amino acid sensitivity using electro-olfactograms (EOGs). For example, Michel & Lubomudrov (1995) exposed adult zebrafish to a wide array of amino acids at 100 μM and found that L-cysteine evoked a large EOG response, while L-serine evoked a moderate response. Therefore, L-cysteine may also tend to induce a greater behavioural response than L-serine.

Zebrafish responding to L-alanine/L-cysteine mixture

The simultaneous introduction of 0.05 mM L-alanine and 0.1 mM L-cysteine evoked an avoidance response. This odourant mixture could have been perceived as its individual components or as a new odour entirely (Tabor et al., 2004; Valentincic et al., 2011; Thomas-Danguin et al., 2014). Usually, animals detect individual odourants when there are fewer within a mixture, and the more stimulatory one drives the response (Valentincic et al., 2011). Since a binary mixture was used for the current study, L-cysteine being more stimulatory than L-alanine likely drove the behavioural response of zebrafish (Michel & Lubomudrov, 1995). Only when the concentration of L-cysteine decreased from 0.1 mM to 0.001 mM, did L-alanine become more stimulatory and zebrafish again displayed attraction towards the mixture. L-cysteine was likely still detected in the mixture, as its decreased concentration was higher than the detection threshold of 0.01 μM (Michel & Lubomudrov, 1995). Overall, these findings suggest that the risk of a potential negative encounter outweighs the reward for an attractive one until the presence of the former diminishes greatly. For example, animals avoid foraging in areas that have high risk of predation (Holbrook & Schmitt, 1988; Dupuch et al., 2009; Suselbeek et al., 2014). Animals avoiding a foraging event are likely to encounter another opportunity in the near future. However, an encounter with a predator, may well mean the end of an animal's life. Zebrafish only demonstrated indifference towards the odourant mixture when the concentration of L-alanine increased. This implies that as long as the avoidance cue is present, attraction

towards the mixture will not occur, regardless of how attracting each component is. This supports the recent finding that the presence of one odourant can suppress the signal of another odourant in the mice olfactory epithelium (Xu et al., 2020).

Olfaction is involved in L-alanine attraction and L-cysteine avoidance

Ablation of the zebrafish nasal cavity demonstrated that their attraction towards L-alanine and avoidance of L-cysteine was primarily from the olfactory system. Besides the olfactory system, amino acids can be detected through other chemosensory systems. For example, rats can detect L-alanine with their gustatory system (Taylor-Burds et al., 2004). In the case of zebrafish, chemical cues can drive behavioural responses from olfaction, gustation and/or SCCs (Lindsay & Vogt, 2004). In fact, zebrafish facial nerves have previously responded strongly to L-alanine and moderately to L-cysteine (Oike et al., 2007). The fact that unilaterally-cauterized zebrafish showed behavioural responses towards L-alanine and L-cysteine, suggests that both nasal cavities are not required for fish to effectively respond to odourants. However, both nasal cavities may enhance the signal received from olfactory input, as the behavioural responses observed from unilateral cauterized fish were weaker than those that did not undergo any surgery. It is unlikely that this lesser olfactory response was from the surgery as the fish returned to behaving normally early on during their 7-day recovery period.

Conclusions

The present study demonstrated that if adult zebrafish were to encounter a mixture made up of an odourant that attracts (L-alanine) and an odourant that repels (L-cysteine), they would avoid the mixture until the concentration of the repelling stimulus was diminished greatly.

Acknowledgements

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made or revised the manuscript. This work was supported by Natural Sciences and Engineering Research Council (grant number 2019-04478).

Figures

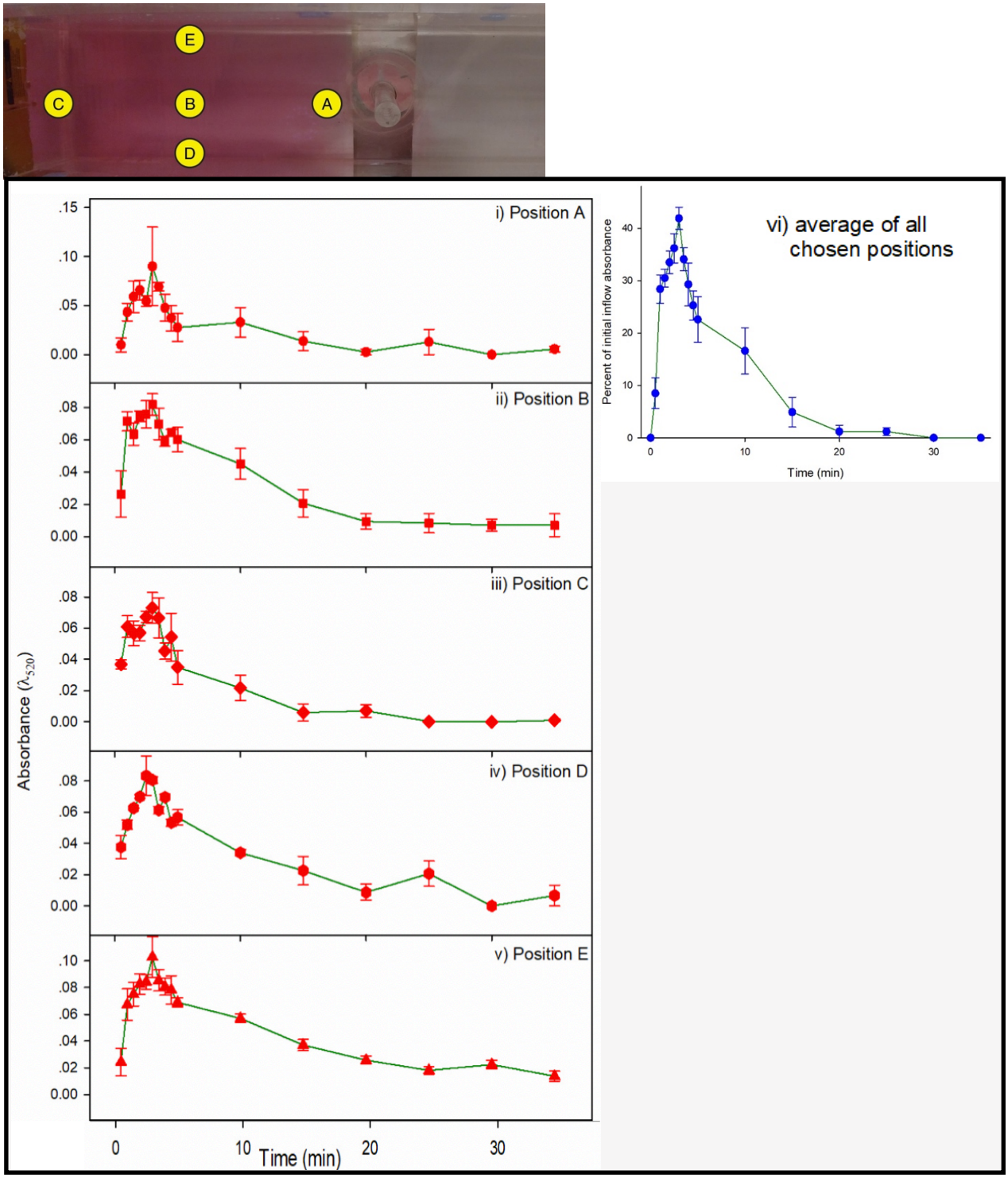
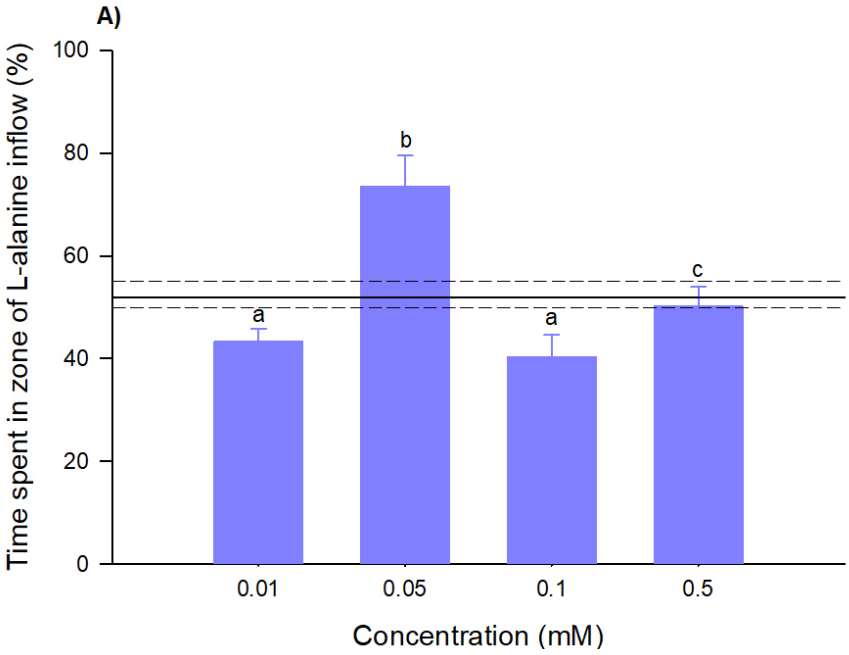
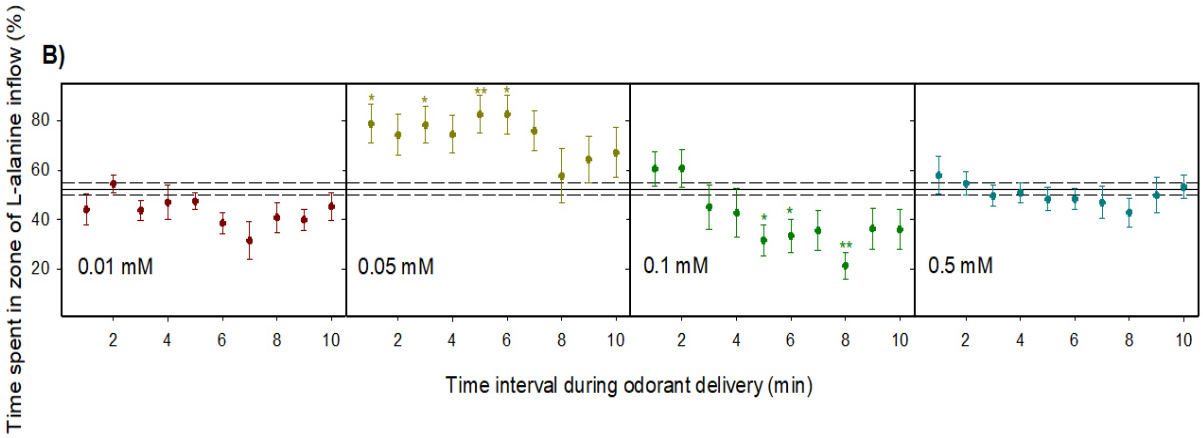


Figure 3.1. (A) 5 distinct positions (A–E) of the avoidance-attraction trough where 100-µl samples of red dye (Club House, Canada) were taken over 35 min (N = 3 for position and time); (B) i–v, mean absorbance ±SEM for each of the 5 locations over 35 min; vi, the mean percent

amount of dye ± SEM that was transferred to the trough in respect to the inflow's absorbance when the dye was initially added.


A)
Time spent in zone of L-alanine inflow (%)
100
80
60
40
20
0
a
b
a
c
0.01
0.05
0.1
0.5
Concentration (mM)


B)
Time spent in zone of L-alanine inflow (%)
80
60
40
20
0.01 mM
0.05 mM
0.1 mM
0.5 mM
2
4
6
8
10
Time interval during odorant delivery (min)

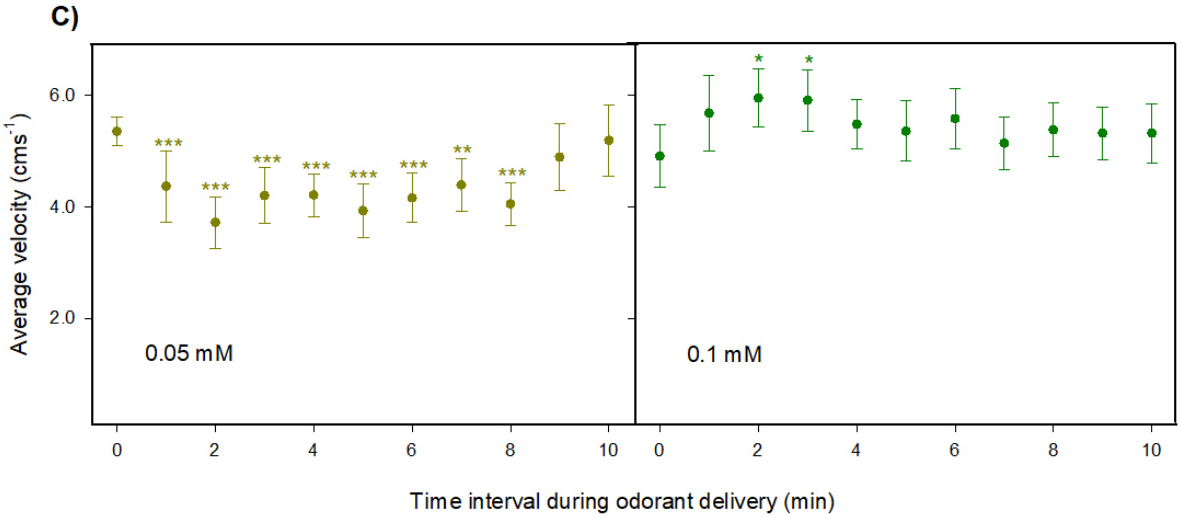

C)
Average velocity (cms-1)
6.0
4.0
2.0
0.05 mM
0.1 mM
0
2
4
6
8
10
Time interval during odorant delivery (min)

Figure 3.2. Adult zebrafish preference response towards L-alanine (0.01, 0.05, 0.1, 0.5 mM). Attraction or avoidance was determined by comparing the percentage of time fish spent in the zone of L-alanine inflow for (A) the entire exposure period, or (B) 1-min intervals of the exposure period (t = 1–10) to that of the average basal activity period pooled across the multiple concentrations used (horizontal solid line denotes the pooled average and the two horizontal dashed line denote the SEM). At the concentrations that evoked a behavioural change, (C) swim speed across the basal activity period (t = 0) was also compared to that of each minute of the exposure period (t = 1–10). All datapoints are denoted as mean ± SEM. Statistical analyses were via two-way ANOVA on ranked data ($\alpha = 0.05$). Different letters represent concentration-dependent effects, while asterisks represent statistical differences between the pooled basal activity period and 1-min intervals of the exposure period (*$p < 0.05$, **$p < 0.01$, * * *$p < 0.001$). All treatments had 10 fish.

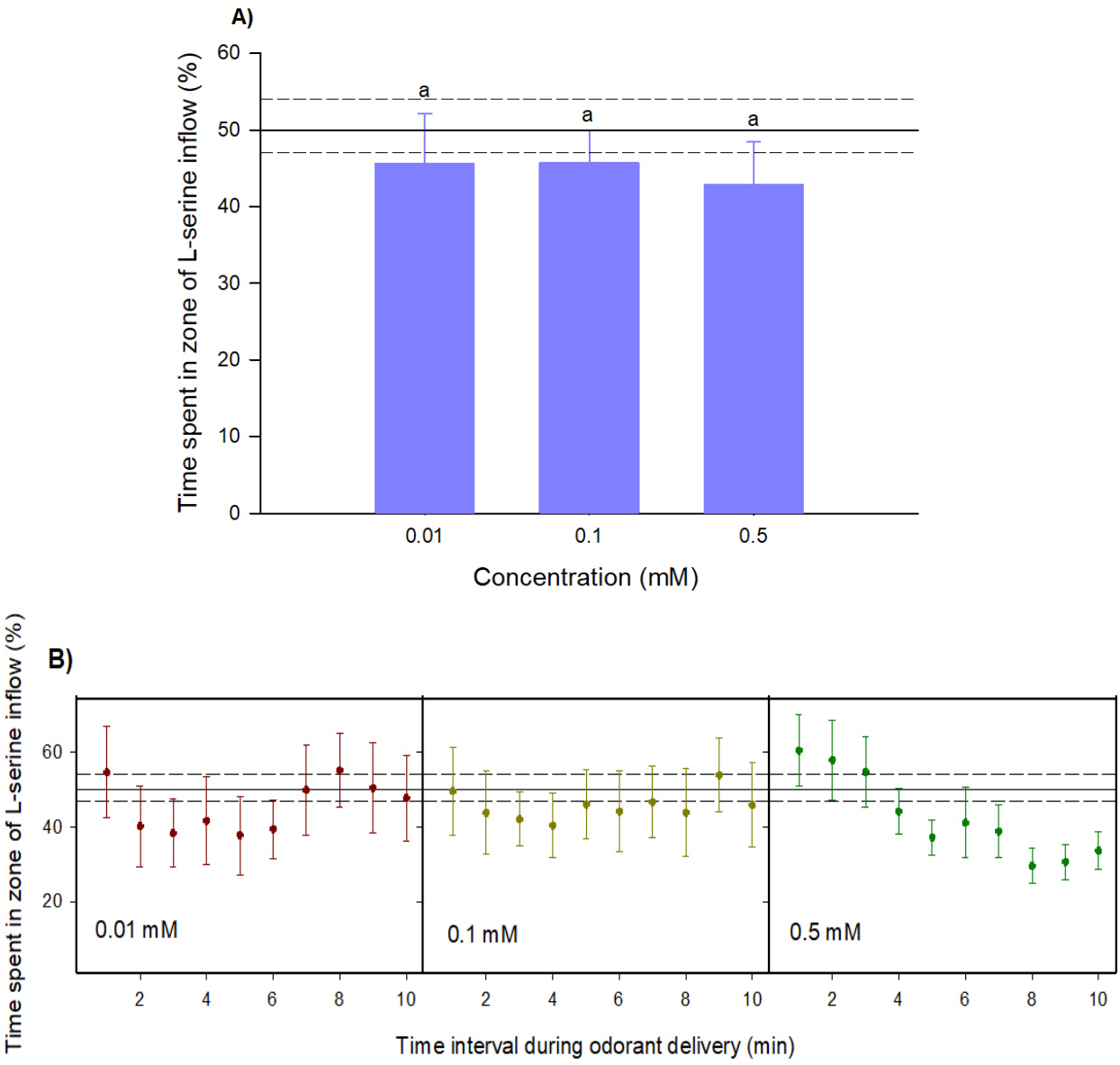
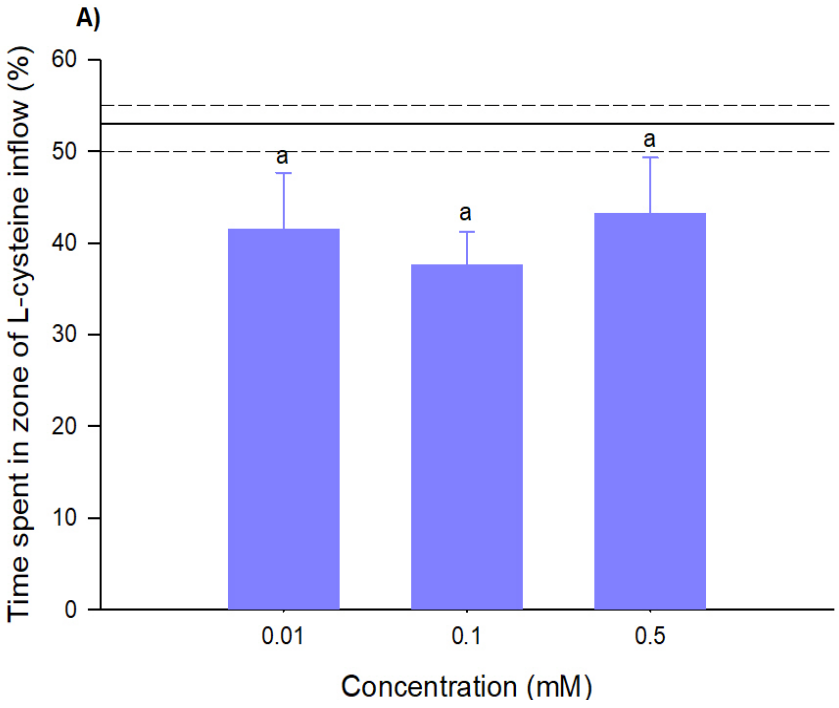
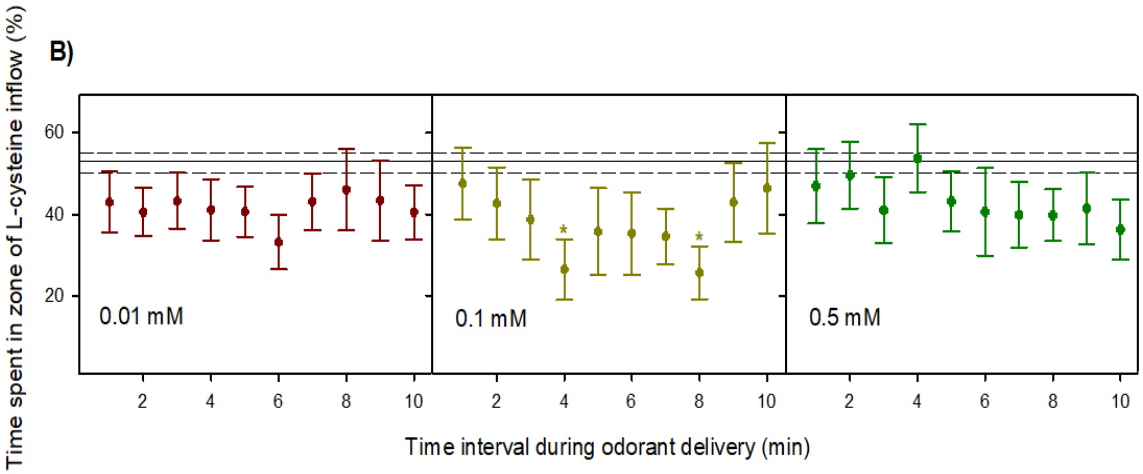


Figure 3.3. Adult zebrafish preference response towards L-serine (0.01, 0.1, 0.5 mM). Attraction or avoidance was determined by comparing the percentage of time fish spent in the zone of L-serine inflow for (A) the entire exposure period, or (B) 1-min intervals of the exposure period (t = 1–10) to that of the average basal activity period pooled across the multiple concentrations used (horizontal solid line denotes the pooled average and the two horizontal dashed line denote the SEM). All datapoints are denoted as mean ± SEM. Statistical analyses were via two-way ANOVA on ranked data ($\alpha = 0.05$). Different letters represent concentration-dependent effects,

while asterisks represent statistical differences between the pooled basal activity period and 1-min intervals of the exposure period (*$p < 0.05$, **$p < 0.01$, * * *$p < 0.001$). All treatments had 10 fish.


A)
Time spent in zone of L-cysteine inflow (%)
60
50
40
30
20
10
0
a
a
a
0.01
0.1
0.5
Concentration (mM)


B)
Time spent in zone of L-cysteine inflow (%)
60
40
20
0.01 mM
0.1 mM
0.5 mM
2
4
6
8
10
Time interval during odorant delivery (min)

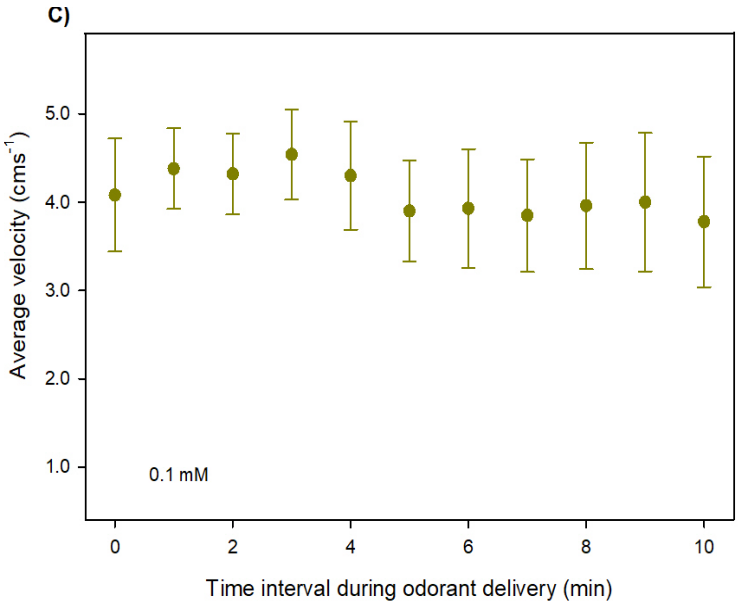

C)
Average velocity (cms-1)
5.0
4.0
3.0
2.0
1.0
0.1 mM
0
2
4
6
8
10
Time interval during odorant delivery (min)

Figure 3.4. Adult zebrafish preference response towards L-cysteine (0.01, 0.1, 0.5 mM). Attraction or avoidance was determined by comparing the percentage of time fish spent in the zone of L-cysteine inflow for (A) the entire exposure period, or (B) 1 min intervals of the exposure period (t = 1–10) to that of the average basal activity period pooled across the multiple concentrations used (horizontal solid line denotes the pooled average and the two horizontal dashed line denote the SEM). At the concentrations that evoked a behavioural change, (C) swim speed across the basal activity period (t = 0) was also compared to that of each minute of the exposure period (t = 1–10). All datapoints are denoted as mean ± S.E.M. Statistical analyses were via two-way ANOVA on ranked data ($\alpha = 0.05$). Different letters represent concentration-dependent effects, while asterisks represent statistical differences between the pooled basal activity period and 1-min intervals of the exposure period (*$p < 0.05$, **$p < 0.01$, * * *$p < 0.001$). All treatments had 10 fish.

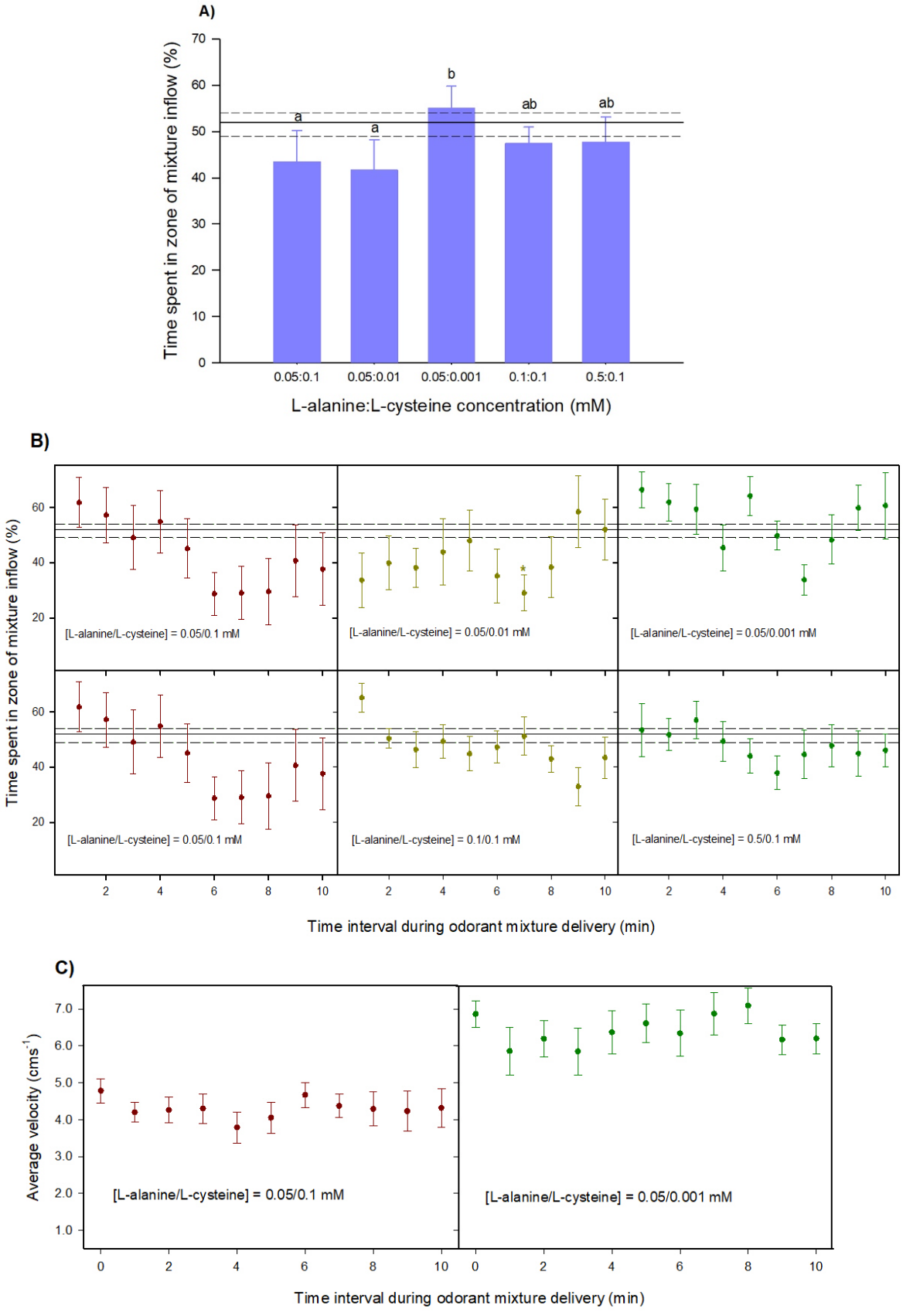

A)
Time spent in zone of mixture inflow (%)
70
60
50
40
30
20
10
0
a
a
b
ab
ab
0.05:0.1
0.05:0.01
0.05:0.001
0.1:0.1
0.5:0.1
L-alanine:L-cysteine concentration (mM)
B)
Time spent in zone of mixture inflow (%)
[L-alanine/L-cysteine] = 0.05/0.1 mM
[L-alanine/L-cysteine] = 0.05/0.01 mM
[L-alanine/L-cysteine] = 0.05/0.001 mM
[L-alanine/L-cysteine] = 0.05/0.1 mM
[L-alanine/L-cysteine] = 0.1/0.1 mM
[L-alanine/L-cysteine] = 0.5/0.1 mM
Time interval during odorant mixture delivery (min)
C)
Average velocity (cms-1)
[L-alanine/L-cysteine] = 0.05/0.1 mM
[L-alanine/L-cysteine] = 0.05/0.001 mM
Time interval during odorant mixture delivery (min)

Figure 3.5. Adult zebrafish preference response towards an attractive-aversive mixture when decreasing L-cysteine or increasing L-alanine concentration ([L-cysteine]/[L-alanine] = 0.1/0.05 mM, 0.01/0.05 mM, 0.001/0.05 mM, 0.1/0.1 mM, 0.1/0.5 mM). Attraction or avoidance was determined by comparing the percentage of time fish spent in the zone of odorant mixture inflow for (A) the entire exposure period, or (B) 1-min intervals of the exposure period (t = 1–10) to that of the average basal activity period pooled across the multiple concentrations used (horizontal solid line denotes the pooled average and the two horizontal dashed line denote the SEM). At the concentrations that evoked a behavioural change, (C) swim speed across the basal period (t = 0) was also compared to that of each minute of the exposure period (t = 1–10). All datapoints are denoted as mean ± SEM. Statistical analyses were via two-way ANOVA or two-way ANOVA on ranked data ($\alpha = 0.05$). Different letters represent concentration-dependent effects, while asterisks represent statistical differences between the pooled basal period and 1-min intervals of the exposure period (*$p < 0.05$, **$p < 0.01$, * * *$p < 0.001$). All treatments had 10 fish.

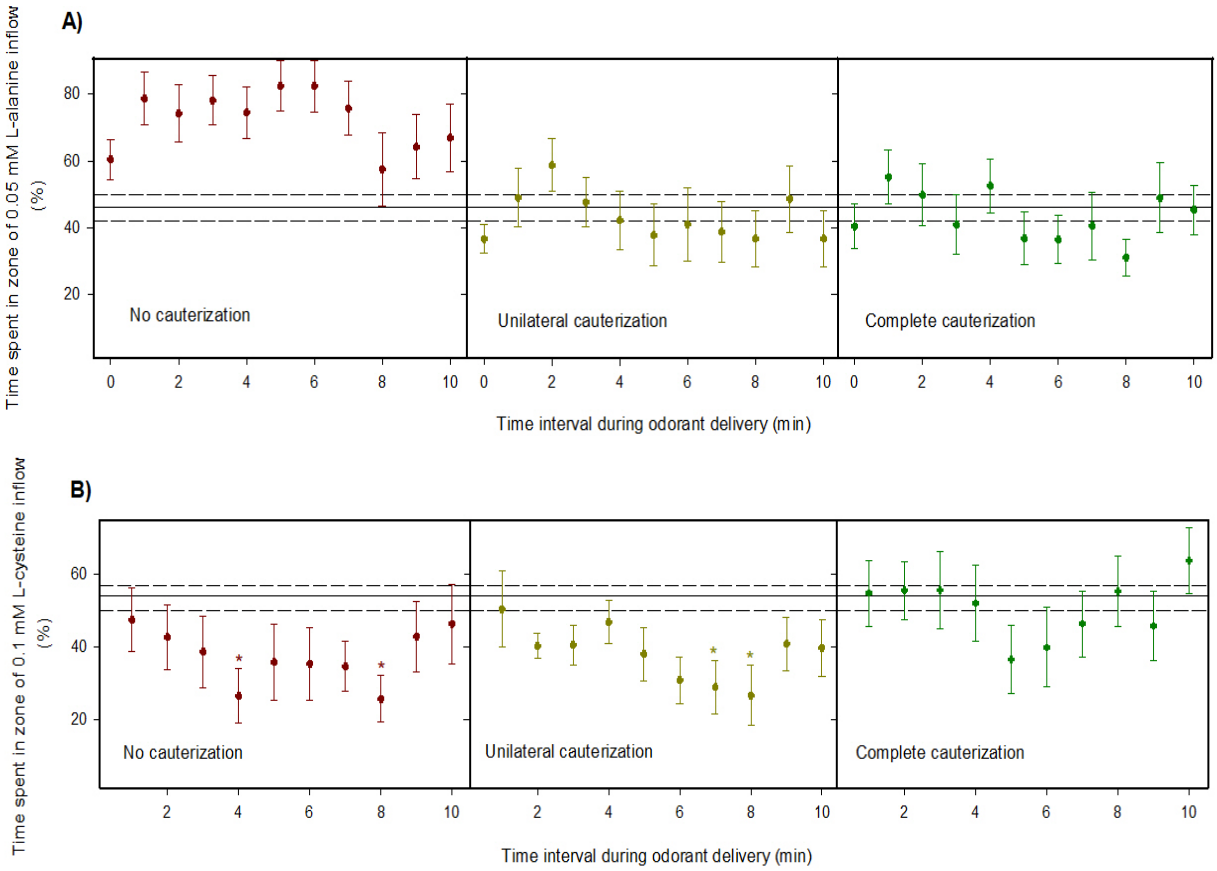


Figure 3.6. Olfactory involvement in attraction towards L-alanine and avoidance towards L-cysteine. Adult zebrafish underwent no, unilateral or complete nasal cavity cauterization and were then exposed to either (A) 0.05 mM L-alanine, or (B) 0.1 mM L-cysteine. Attraction or avoidance was determined by comparing the percentage of time fish spent with the amino acids during 1-min intervals of the exposure period (t = 1–10) to that of the basal activity period for each treatment (t = 0). Basal activity values were pooled across treatment type only for L-cysteine. The horizontal line represents the average basal preference towards the zone of oduorant inflow across all treatments and the two horizontal dashed lines represent the corresponding SEM. Data set for fish that did not undergo surgery were pulled from those that were used to determine if L-alanine and L-cysteine elicited a response. All datapoints are denoted as mean ± SEM. Statistical analyses were via a two-way ANOVA on ranked data or two-way repeated measures ANOVA on ranked data ($\alpha = 0.05$). Asterisks represent statistical differences between the basal period and 1-min intervals of the exposure period (*$p < 0.05$, **$p < 0.01$, * * *$p < 0.001$). All treatments had 10 fish.

Chapter 4: Single odourant imprinting is disrupted by a second odourant in a model vertebrate, *Danio rerio*

Abstract

Animals use olfaction to gather information about their environment. How animals respond to chemosensory information is often shaped by their rearing environment, including through olfactory imprinting. Mechanisms underlying olfactory imprinting are often studied using animals that are exposed to single odourants, but in the natural world, odourants rarely occur in isolation. The purpose of the current study was to determine how olfactory imprinting in zebrafish is affected by additional odourants in the environment during developmental imprinting and behavioural testing of imprinted odourants in adulthood. Fish were first tested for their ability to imprint to single odourants including β-phenylethyl alcohol and the amino acids L-leucine, L-lysine, and L-valine. Zebrafish were exposed to the odourants during development and then grown to adulthood, at which point their behavioural responses to odourants were compared to those of adult fish that were exposed to embryo media only during development. Fish imprinted to β-phenylethyl alcohol, L-leucine, and L-lysine, but not to L-valine. Imprinting experiments on L-leucine were then repeated, but with L-lysine also added to the environment during imprinting and/or behavioural testing in adulthood as these two amino acids individually evoked opposite responses in imprinted fish. The addition of L-lysine to the environment of early developing fish disrupted their ability to imprint to L-leucine or to L-lysine. Furthermore, fish did not imprint to a mixture of L-leucine and L-lysine. This study demonstrates the relevance of considering the complexity of the olfactory environment when examining imprinting.

Introduction

Animals sense their environment through chemical cues, which are detected in part by the gustatory and olfactory sensory systems. With the latter, the olfactory system of animals detects odourants to facilitate activities such as foraging, avoiding predators and finding mates (Laska & Freyer, 1997; Martin et al., 2010; Zhang et al., 2010). How animals respond to olfactory stimuli is shaped by their environment during early development, which can be considered the most sensitive period of sensory development (Berardi et al. 2000).

During a brief window in early development, animals imprint to olfactory stimuli that they encounter, forming long-lasting memories that shape how they respond to the odourants when encountered later in life (Fillion & Blass, 1986; Marr & Gardner, 1965). For example, during parr-smolt transformation, salmonids imprint to amino acids that help them perform future homing behaviour after seaward migration (Shoji et al. 2000; Yamamoto, et al. 2010; Yamamoto et al. 2013). Zebrafish as young as 2 or 3 days post fertilization (dpf) were shown to imprint to β-phenylethyl alcohol (PEA) through changes in preference response towards the odourant as adults (Harden et al. 2006; Calfún et al. 2016). In songbirds and guinea pigs, olfactory imprinting is important for nest and littermate recognition, respectively (Carter & Marr, 1970; Caspers et al., 2013).

Animals in their natural environment detect 'bouquets' of odourants arising from the biotic and abiotic components of an ecosystem. Studies that have examined the mechanisms underlying olfactory imprinting have often used single odourants (Harden et al. 2006; Yamamoto et al. 2010; Inoue et al. 2021; Armstrong et al. 2022). Nevertheless, odourants in a natural setting rarely, if ever, occur in isolation. During the detection of multiple odourants simultaneously, the signaling input received by the olfactory system may be modulated by synergistic or suppressive interactions between odourants (Kang and Caprio 1997; Ishii et al. 2008; Miyazawa et al. 2008; Chaput et al. 2012; McClintock et al. 2020). For example, the addition of an aversive odourant may dampen the signal generated by an attracting odourant, as aversive odours often evoke stronger responses than attracting odours (Ehrlichman et al., 1995; Boesveldt et al., 2010). As well, animals have difficulty discriminating between odourants when faced with multiple odourants simultaneously (Livermore and Laing 1998). Therefore, the different interactions between multiple odourants in the rearing environment may influence olfactory imprinting ability in animals.

Responding to imprinted odourants or mixtures later in life may be altered by the presence of other odourants. For example, odourants can mask the responses normally seen towards other odourants present, such as in the case of mice not responding to the aversive odourant dimethyl sulfide, in the presence of citrous odourants (Cain, 1975; Osada et al., 2013). As mentioned above, the ability to discriminate between odourants decreases when animals are presented with multiple odourants simultaneously (Livermore and Laing 1998). This is the

classic issue of detecting a signal from surrounding noise. Therefore, responses to imprinted odourants may be lost or obscured by the addition of other odourants to the environment, either through masking responses normally observed towards imprinted odourants or a decreased discrimination capacity.

The purpose of the current study was to determine how olfactory imprinting is affected by additional odourants, either during imprinting or during behavioural testing at adulthood. I used the common aquatic vertebrate model, the zebrafish, which have previously been shown to imprint to odourants (Harden et al., 2006; Gerlach et al., 2008; Hinz et al., 2013, Calfún et al., 2016;). In my experiments, I tested if zebrafish could imprint to single amino acids and binary amino acid mixtures with the expectation that the assay would less likely show that fish could imprint to multiple amino acids simultaneously over single amino acids.

Methods

Fish Husbandry

A breeding colony of adult tüpfel longfin (TL) strain zebrafish were housed in a self-circulating aquatic racks system (Aquaneering, San Deigo, CA, USA) containing 28 ± 0.5°C reverse-osmosis (RO)-supplied water. Fish were fed twice daily of Zeigler zebrafish diet (Gardners, PA, USA) and held under 14:10 hr light: dark cycle. All animals were treated in accordance with the University of Alberta's Animal Care and Use committee (AUP no. 052).

Developmental exposure of single and paired odourants

I first tested the imprinting paradigm by replicating the findings from previous studies that demonstrated that zebrafish imprint to β-PEA (Harden et al. 2006; Calfún et al. 2016). β-PEA is a synthetic odourant that zebrafish are not preconditioned to and so has relevance to demonstrating whether long-term associations towards the odourant can be made during early development (Nevitt et al., 1994). Fish were then tested for their ability to imprint to amino acids individually. L-leucine, L-lysine and L-valine were selected as they all generate neuronal generator signals in the zebrafish olfactory system (Michel and Lubomudrov 1995; Friedrich and Korsching 1997). All chemicals were purchased from Sigma-Aldrich (purity > 98%; St. Louis, MO, USA).

Eggs were collected immediately after fertilization and transferred to glass petri dishes, with 60 to 80 embryos in each dish. For single odourant imprinting trials, groups of 60 - 80 embryos were exposed from 0.5 to 7 dpf to 0.001 mM β-PEA (W285811), 0.1 mM L-leucine (L8000), 0.1 mM L-lysine (L5501) or 0.1 mM L-valine (A12720), which were solubilized in 40 mL embryo media (EM). Groups of 60 - 80 non-imprinted control zebrafish were exposed to EM only. 95% of exposure media was replaced daily. All embryos were housed at 28 ± 0.5°C and subjected to a photoperiod of a 12:12 hr light: dark cycle. Once fish reached 7 dpf, they were moved to an aquatics rack system and raised (Winter 2021) without being exposed to β-PEA, L-leucine, L-lysine, or L-valine by the experimenters until they reached adulthood.

After determining which amino acids zebrafish imprinted to individually, a second series of experiments were conducted using L-leucine and L-lysine. These amino acids were selected because fish imprinted to them separately. Imprinting experiments were replicated with groups of 60 to 80 embryos exposed from 0.5 to 7 dpf to EM or 0.1 mM L-leucine only, or 0.1 mM L-leucine and L-lysine simultaneously. As above, fish were reared in water free of amino acid addition (Winter 2021, Summer 2022) until they reached adulthood.

Adult zebrafish behavioural response towards odourants and odourant mixtures

Behavioural responses of adult zebrafish to all odourants or mixtures (β-PEA, L-leucine, L-lysine, L-valine, or a mixture of L-leucine and L-lysine) were determined in an avoidance-attraction trough, detailed in Shahriari et al. (2021). In short, the apparatus consisted of two inflows of 28°C RO water, situated on opposite ends of the trough. Water from the two inflows continuously drained into the trough at 0.7 l/min and subsequently drained through a central fenestrated pipe (inner diameter of 7 mm), which created two zones with minimal intermixing (5 cm in length). Therefore, when an odourant was introduced to the trough through one of the two inflows, only the corresponding half of the trough contained the odour, confirmed visually with dye trials (Figure 3.1). A water column depth of at least 5 cm was maintained in order to minimize anxiety in zebrafish (Córdova et al., 2016). Overhead dome colour IR cameras (SAV-CD120; Matcow, Stow, OH USA) connected to an Elgato Video Capture software (Elgato Systems, CA USA) were used to record fish movement. The trough was surrounded in an opaque curtain and environmental auditory input was kept to a minimum.

To determine if zebrafish exposed to β-PEA, L-leucine, L-lysine, or L-valine, had been imprinted, their preference response towards these odourants at adulthood were compared to those that were reared only in EM. For this, adult fish were fasted for 1 day prior to behavioural experiments. Behavioural trials began with a randomly selected fish being placed into one side of the avoidance-attraction trough. Fish were given a 30 min acclimation period, in which their behaviour during the last 10 min was recorded. After this 10 min period, β-PEA, L-leucine, L-lysine or L-valine (0.001, 0.01, 0.1 mM) was added into one of the two inflows, chosen at random, and the behaviour of the fish was recorded for another 10 min. Olfactory imprinting was considered to have occurred only if fish reared in β-PEA, L-leucine, L-lysine, or L-valine, had a preference response towards the odourants that differed from those reared only in EM. Each odour at a given concentration had 9 to 10 naïve fish and 10 to 11 odour-exposed fish.

To investigate if zebrafish imprint to a mixture of two odourants that evoke imprinting on their own, adult fish reared in L-leucine and L-lysine, L-leucine only, or EM only, were exposed to either 0.1 mM L-leucine, 0.1 mM L-lysine, or a mixture of 0.1 mM L-leucine and 0.1 mM L-lysine in the avoidance-attraction trough. L-leucine and L-lysine were chosen as they evoked opposite preference responses in imprinted fish. Here, zebrafish underwent the same experimental procedures as the fish that were tested for their ability to imprint to single odourants above. With this experiment, 15 – 25 fish per group were used for each treatment.

Data and Statistical Analysis

All adult zebrafish were manually scored for the total time they spent in the area of an avoidance-attraction trough that contained the odourants during a 10 min odour exposure period. The area of the trough containing the odourant was termed *odour zone*. The time fish spent in this odour zone during the 10 min exposure period was compared to the total time fish spent in the same area of the trough during a 10 min period prior to odour addition. The time fish spent in the central trough where intermixing between the two inflows occurred was not considered.

Statistical analysis on determining zebrafish behavioural response to β-PEA, L-leucine, L-lysine, L-valine, and a mixture of L-leucine and L-lysine, was based on a two-way (treatment type × time period) repeated measures analysis of variance (ANOVA). Holm-Sidak post hoc tests were used to perform pairwise comparisons between basal and exposure behaviour within

treatment groups for each concentration of the odourants administered. For all analyses, when normality or equal variance failed, log transformations were done, but if this failed to resolve the assumptions, two-way repeated measures ANOVA on ranked data were used. SigmaPlot 14.0 (Systat, San Jose, CA, USA) was used for all statistical analyses with statistical significance being defined by $\alpha = 0.05$. Group averages are represented as mean ± SEM.

Results

Zebrafish imprint to β-PEA

Exposing zebrafish to β-PEA during early development affected their behavioural response to the odourant 3 months later at adulthood (Figure 4.1). When 0.001 mM β-PEA was added to the avoidance-attraction trough, the change in time imprinted fish spent in the odour zone differed from that of non-imprinted control fish ($F_{1,17} = 9.95$, $p = 0.006$). While control fish decreased their time spent in the odour zone by 21 ± 11 s ($p = 0.095$), imprinted fish increased their time spent by 34 ± 12 s ($p = 0.015$) (Figure 4.1A). Introducing concentrations of β-PEA that were higher than those that fish imprinted to (i.e., 0.01 and 0.1 mM) did not evoke a response (Figure 4.1B, C).

Zebrafish imprint to L-leucine and L-lysine, but not to L-valine

Zebrafish imprinted to L-leucine with changes in behaviour seen in a concentration-specific manner (Figure 4.2). With the introduction of 0.001 mM L-leucine to the avoidance-attraction trough, fish decreased the amount of time spent in the odour zone ($\chi_{1,18} = 19.5$, $p < 0.001$; Figure 4.2A). Specifically, non-imprinted control fish decreased the amount of time spent in the odour zone by 14 ± 17 s ($p = 0.035$), while imprinted fish decreased the amount of time spent in the odour zone by 33 ± 12 s ($p < 0.001$). When L-leucine concentration increased to 0.01 mM, control and imprinted fish showed no preference response towards the odourant during the exposure period (Figure 4.2B). When 0.1 mM L-leucine was added to the trough, imprinted fish displayed a preference response that differed from control fish ($F_{1,18} = 18.0$, $p < 0.001$; Figure 4.2C). While control fish decreased the amount of time spent in the odour zone by 64 ± 20 s ($p = 0.005$), imprinted fish increased the amount of time spent in the odour zone by 56 ± 20 s ($p = 0.012$).

Zebrafish imprinted to L-lysine (Figure 4.3). With the addition of 0.001, 0.01 or 0.1 mM L-lysine to the avoidance-attraction trough, fish changed the amount of time spent in the odour zone ($F_{1,18}$ = 10.4, p = 0.005 for 0.001 mM; $F_{1,18}$ = 5.28, p = 0.034 for 0.01 mM; $F_{1,18}$ = 9.56, p = 0.006 for 0.1 mM). However, this change in behaviour was only seen in imprinted fish. Specifically, non-imprinted fish spent a similar amount of time in the odour zone after L-lysine introduction (p = 0.283, 0.300, 0.179 for 0.001, 0.01, 0.1 mM, respectively), while imprinted fish decreased the amount of time spent in the odour zone after L-lysine introduction, regardless of what concentration was administered (p = 0.003, 0.043, 0.008, for 0.001, 0.01, 0.1 mM, respectively).

Fish did not imprint to L-valine (Figure 4.4). Specifically, with the introduction of 0.001 and 0.01 mM L-valine to the avoidance-attraction trough, changes in behaviour indicative of imprinting were not found (Figure 4.4A, B). There was, however, a tendency to respond to L-valine at 0.1 mM, which was evident in control fish ($F_{1,18}$ = 5.95, p = 0.025; Figure 4.4C). However, post-hoc analysis revealed no differences in time EM and L-valine reared fish spent in the odour zone between the odour exposure period and the 10 min interval prior to L-valine addition to the trough (p = 0.094 and p = 0.11 for EM and L-valine reared fish, respectively).

Since L-leucine and L-lysine induced contrasting imprinting-based responses, I then investigated how the presence of both amino acids during development affected olfactory imprinting. Specifically, I examined if fish exposed to a mixture of L-leucine and L-lysine during early development would still be attracted to 0.1 mM L-leucine and would still avoid 0.1 mM L-lysine. I also determined if they would imprint to the mixture of L-leucine and L-lysine.

Zebrafish reared in L-leucine and L-lysine together did not imprint to L-leucine, L-lysine, or a mixture of L-leucine and L-lysine

Overall, zebrafish did not imprint to L-leucine or L-lysine when fish were exposed to the two amino acids together during early development (Figure 4.5). In response to L-leucine being added to an avoidance-attraction trough, changes in the amount of time spent in the odour zone was different between adult fish that were exposed to EM only, L-leucine only, or a mixture of L-leucine and lysine during early development ($F_{2,67}$ = 4.06, p = 0.022; Figure 4.5A). Adult fish exposed to L-leucine and L-lysine simultaneously during early development did not change the

amount of time they spent in the odour zone with the introduction of L-leucine to the trough ($p = 0.28$). Repeating the single odourant experiments for L-leucine, naïve fish showed no behavioural change in response to the addition of L-leucine to the trough ($p = 0.083$). Only fish exposed to L-leucine alone during early development continued to increase the time spent in the odour zone of the trough with the introduction of L-leucine, replicating findings from the first study ($p = 0.043$). In regard to L-lysine exposures, adult fish that were exposed to EM only or to a mixture of L-leucine or L-lysine during early development did not change how much time spent in the odour zone after L-lysine was introduced to the trough.

Even though zebrafish imprinted to L-leucine and L-lysine separately, fish did not imprint to a mixture of the two amino acids (Figure 4.5C). Specifically, with the simultaneous introduction of L-leucine and L-lysine to the trough during testing, adult fish exposed to EM only, L-leucine only, or a mixture of L-leucine and L-lysine during early development had no behavioural change.

Discussion

Imprinting is known to guide some of the most sensitive and important behaviours that we know. A question is, just how sensitive is the imprinting response? The current study demonstrates that the ability to imprint to single amino acids is not seen with the addition of a second odourant to the rearing environment of zebrafish.

Zebrafish imprint to β-PEA

Zebrafish imprinted to 0.001 mM β-PEA, as adult fish exposed to the odourant during early development were attracted to 0.001 mM β-PEA, whereas adult fish that were reared only in EM showed no such response. This finding replicates previous work demonstrating that zebrafish imprinted to 0.001 mM β-PEA are attracted to the odourant at 0.001 mM (Harden et al., 2006) and validates my imprinting paradigm. Interestingly, when adult fish from my study were exposed to higher concentrations of the odourant, differences in behavioural response by imprinted and control fish were absent. This identification of imprinting was also concentration specific in L-leucine imprinted-fish, which showed an altered behavioural response to the amino

acid only at 0.1 mM. Therefore, behavioural assays for observing olfactory imprinting in animals are likely sensitive to the concentration of odourants administered.

Zebrafish imprint to L-leucine and L-lysine, but not to L-valine

Overall, zebrafish imprinted to L-leucine and L-lysine, but not to L-valine. Like zebrafish, salmonids have also imprinted to amino acids including l-proline and l-glutamic acid, exhibiting an attraction response in adulthood (Yamamoto et al. 2010, 2013). While salmonids use imprinted amino acids for homing behaviour, zebrafish have been suggested to imprint to amino acids for the purposes of kin odour recognition (Shoji et al. 2000; Hinz et al. 2013; Yamamoto et al. 2013). While zebrafish imprinted to L-leucine were attracted to the amino acid at 0.1 mM in adulthood, fish that imprinted to L-lysine avoided 0.001, 0.01 and 0.1 mM L-lysine in adulthood. Olfactory imprinting that leads to animals avoiding odourants in adulthood have been reported in an ecological context, such as fish, birds and mammals avoiding kin odour to prevent inbreeding (Isles et al., 2002; Gerlach & Lysiak, 2006; Gerlach et al., 2008; Bonadonna & Sanz-Aguilar, 2012; Caspers et al., 2013;). The three amino acids that zebrafish were exposed to during early development have previously been shown to elicit an electrical signal in the olfactory system, but to varying degrees of magnitude (Michel and Lubomudrov 1995; Friedrich and Korsching 1997). Among the three amino acids, the signal generated from L-valine detection had the weakest input. This reduced olfactory signal generated by L-valine may have posed a greater difficulty in its detection, which may have contributed to no evidence of imprinting to the odourant. Even though fish exposed to L-leucine or L-lysine from 0.5 to 7 dpf was sufficient for olfactory imprinting to occur, a longer duration may have been needed for fish to imprint to L-valine. As a comparison, sockeye salmon imprinted to l-proline and l-glutamic acid only after they were exposed to the amino acids for two weeks during parr-smolt transformation (Yamamoto et al. 2010).

Zebrafish reared in L-leucine and L-lysine together did not imprint to L-leucine, L-lysine, or a mixture of L-leucine and L-lysine

Exposing zebrafish to a mixture of L-leucine and L-lysine during early development abolished the attraction response towards 0.1 mM leucine seen in fish imprinted to L-leucine alone. The avoidance response towards 0.1 mM L-lysine seen in fish imprinted to L-lysine alone

was also abolished. Therefore, fish did not imprint to L-leucine or L-lysine when detecting the two odourants together. In fish detecting two amino acids simultaneously, the signals generated from each odourant may have suppressed one another, lessening the olfactory signal of each odourant compared to each detected alone (Kang & Caprio, 1997; Oka et al., 2004; Chaput et al., 2012; McClintock et al., 2020). Specifically, the addition of L-lysine may have suppressed the signal generated by L-leucine detection, and vice versa, rendering zebrafish unable to imprint to L-leucine or L-lysine. For example, the activation of neurons in mice by isoamyl acetate was suppressed in the presence of whiskey lactone (Chaput et al., 2012). As L-leucine imprinted-fish were attracted to L-leucine and L-lysine imprinted-fish avoided L-lysine, fish may have still imprinted to the two odourants when simultaneously added to the rearing environment. The conflicting signals perceived by the fish during behavioural testing during adulthood may have masked one another, causing the fish to neither approach or avoid the binary mixture (Ehrlichman et al., 1995; Boesveldt et al., 2010). However, the idea that fish imprinted to both odourants successfully, but such a result was masked during binary mixture testing is not supported by the current study as adult fish exposed to L-leucine and L-lysine during early development were not attracted to L-leucine on its own as adults.

Even though zebrafish imprinted to L-leucine and L-lysine separately, fish did not imprint to a mixture of the two amino acids. Not only did they face a challenge in imprinting to the mixture due to potential suppressive interactions in the signal generated from the amino acids, but also from difficulty in discriminating between the odourants. The capacity of animals to discriminate between odourants decreases as they detect more odourants simultaneously (Livermore and Laing 1998). As fish exposed to L-leucine and L-lysine during early development did not respond to L-leucine in adulthood, the mixture was likely not perceived as its individual components during the imprinting window. However, I propose that future studies examine how processing of odourant mixtures affect olfactory imprinting capacity (Valentincic et al. 2011).

Mechanisms underlying olfactory imprinting are often studied using animals that are exposed to single odourants. Because odourants rarely occur in isolation, I tested if adding a second odourant to the environment of early developing zebrafish affects olfactory imprinting. I demonstrated that the inclusion of a second odourant can disrupt imprinting to single odourants,

suggesting future studies should consider the complexity of the olfactory environment when examining olfactory imprinting. Overall, this study provides an intermediary link between studies that demonstrate mechanisms of olfactory imprinting by exposing animals to single odourants and the natural world where animals sense a ‘bouquet’ of odourants.

Figures

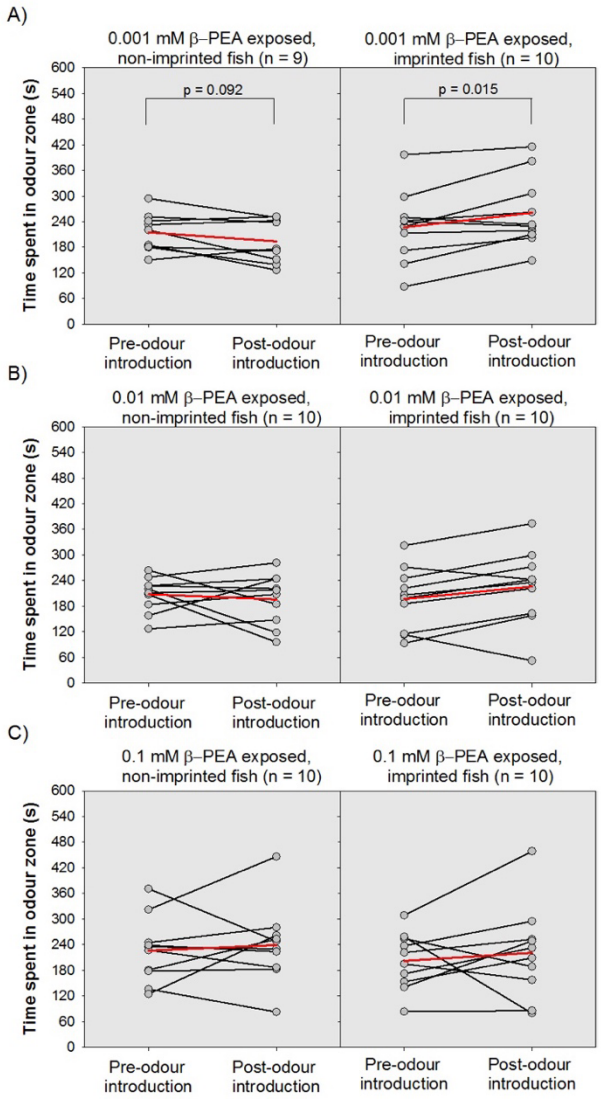


Figure 4.1. Zebrafish imprint to β-phenylethyl alcohol (β-PEA). Fish from 0.5 to 7 dpf were reared with embryo media (non-imprinted fish) or 0.001 mM β-PEA (imprinted fish), and then as adults observed for their behavioural response to the odourant at A) 0.001 mM, B) 0.01 mM, or C) 0.1 mM. The amount of time individual fish spent in the odour zone of an avoidance-attraction trough pre- and post-odour introduction are represented by grey circles, with the difference traced for each fish by a solid black line. The difference in means for how much time fish spent with the odourant after its introduction is shown by the solid red line. A two-way repeated measures ANOVA ($\alpha = 0.05$) was used to determine the effects on treatment type (non-imprinted and imprinted fish) and 10 min time period (pre- and post- odour introduction). All treatments had 9 – 10 fish.

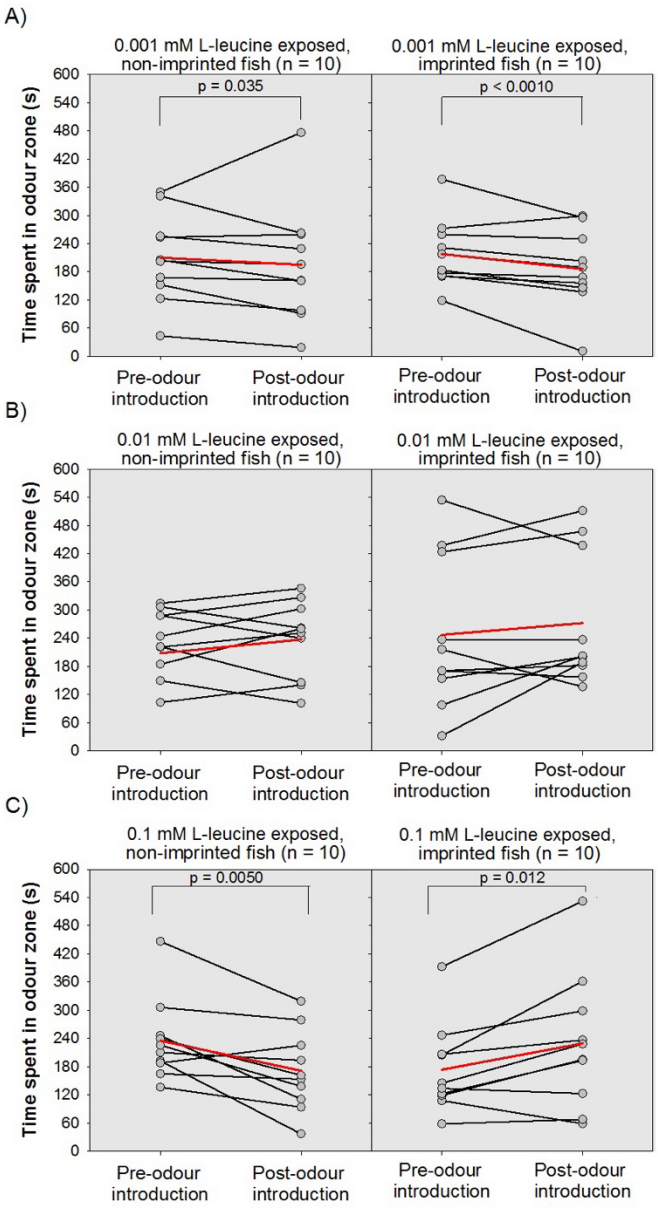


Figure 4.2. Zebrafish imprint to L-leucine. Fish from 0.5 to 7 dpf were reared with embryo media (non-imprinted fish) or 0.001 mM L-leucine (imprinted fish), and then as adults observed for their behavioural response to the odourant at A) 0.001 mM, B) 0.01 mM, or C) 0.1 mM. The amount of time individual fish spent in the odour zone of an avoidance-attraction trough pre- and post-odour introduction are represented by grey circles, with the difference traced for each fish by a solid black line. The difference in means for how much time fish spent with the odourant after its introduction is shown by the solid red line. A two-way repeated measures ANOVA (α = 0.05) was used to determine the effects on treatment type (non-imprinted and imprinted fish) and 10 min time period (pre- and post- odour introduction). All treatments had 10 fish.

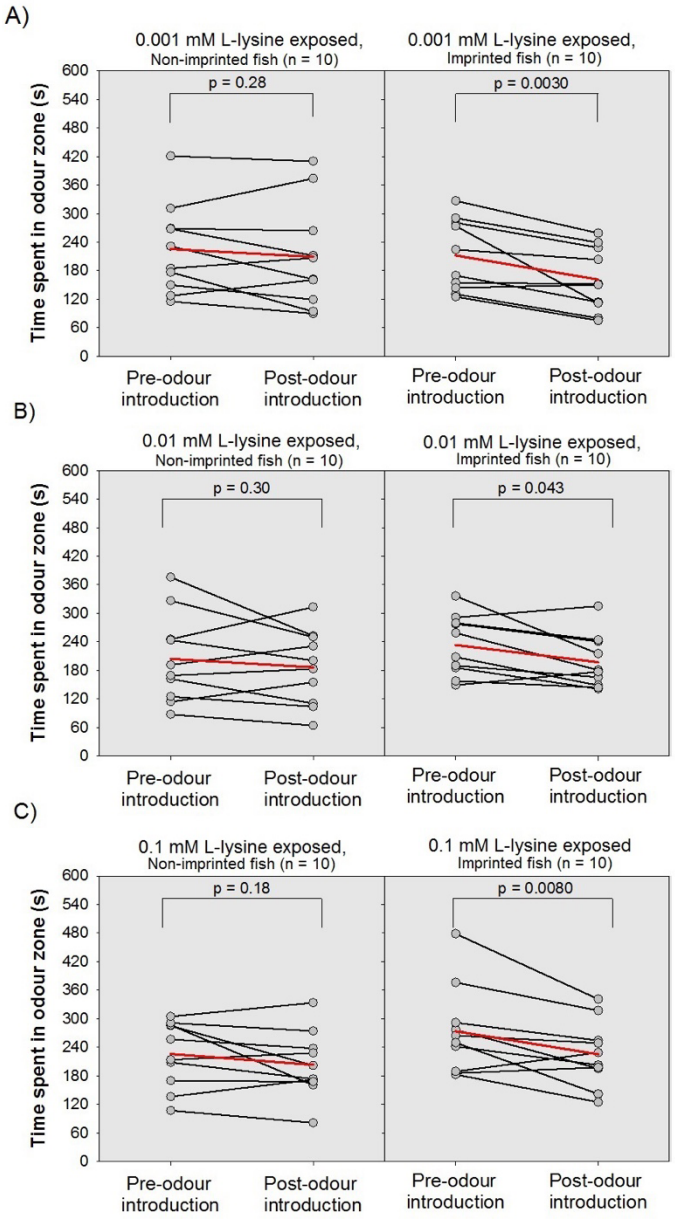


Figure 4.3. Zebrafish imprint to L-lysine. Fish from 0.5 to 7 dpf were reared with embryo media (non-imprinted fish) or 0.001 mM L-lysine (imprinted fish), and then as adults observed for their behavioural response to the odourant at A) 0.001 mM, B) 0.01 mM, or C) 0.1 mM. The amount of time individual fish spent in the odour zone of an avoidance-attraction trough pre- and post-odour introduction are represented by grey circles, with the difference traced for each fish by a solid black line. The difference in means for how much time fish spent with the odourant after its introduction is shown by the solid red line. A two-way repeated measures ANOVA (α = 0.05) was used to determine the effects on treatment type (non-imprinted and imprinted fish) and 10 min time period (pre and post- odour introduction). All treatments had 10 fish.

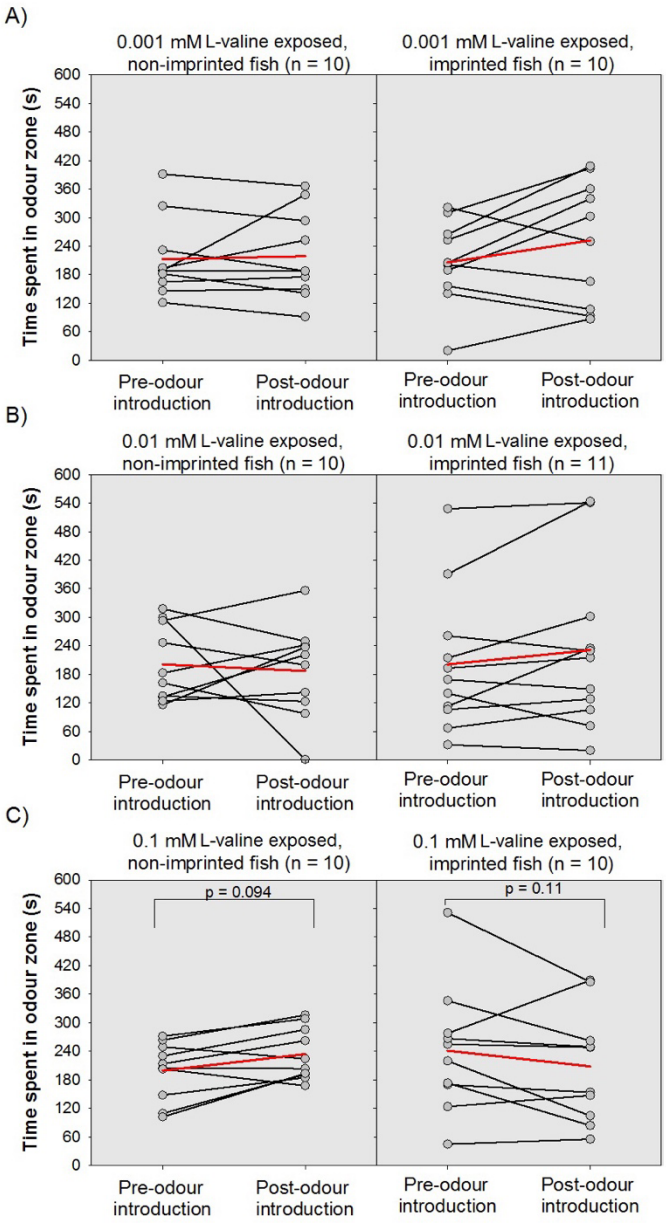


Figure 4.4. Zebrafish imprint to L-valine. Fish from 0.5 to 7 dpf were reared with embryo media (non-imprinted fish) or 0.001 mM L-valine (imprinted fish), and then as adults observed for their behavioural response to the odourant at A) 0.001 mM, B) 0.01 mM, or C) 0.1 mM. The amount of time individual fish spent in the odour zone of an avoidance-attraction trough pre- and post-odour introduction are represented by grey circles, with the difference traced for each fish by a solid black line. The difference in means for how much time fish spent with the odourant after its introduction is shown by the solid red line. A two-way repeated measures ANOVA (α = 0.05) was used to determine the effects on treatment type (non-imprinted and imprinted fish) and 10 min time period (pre- and post- odour introduction). All treatments had 10 - 11 fish.

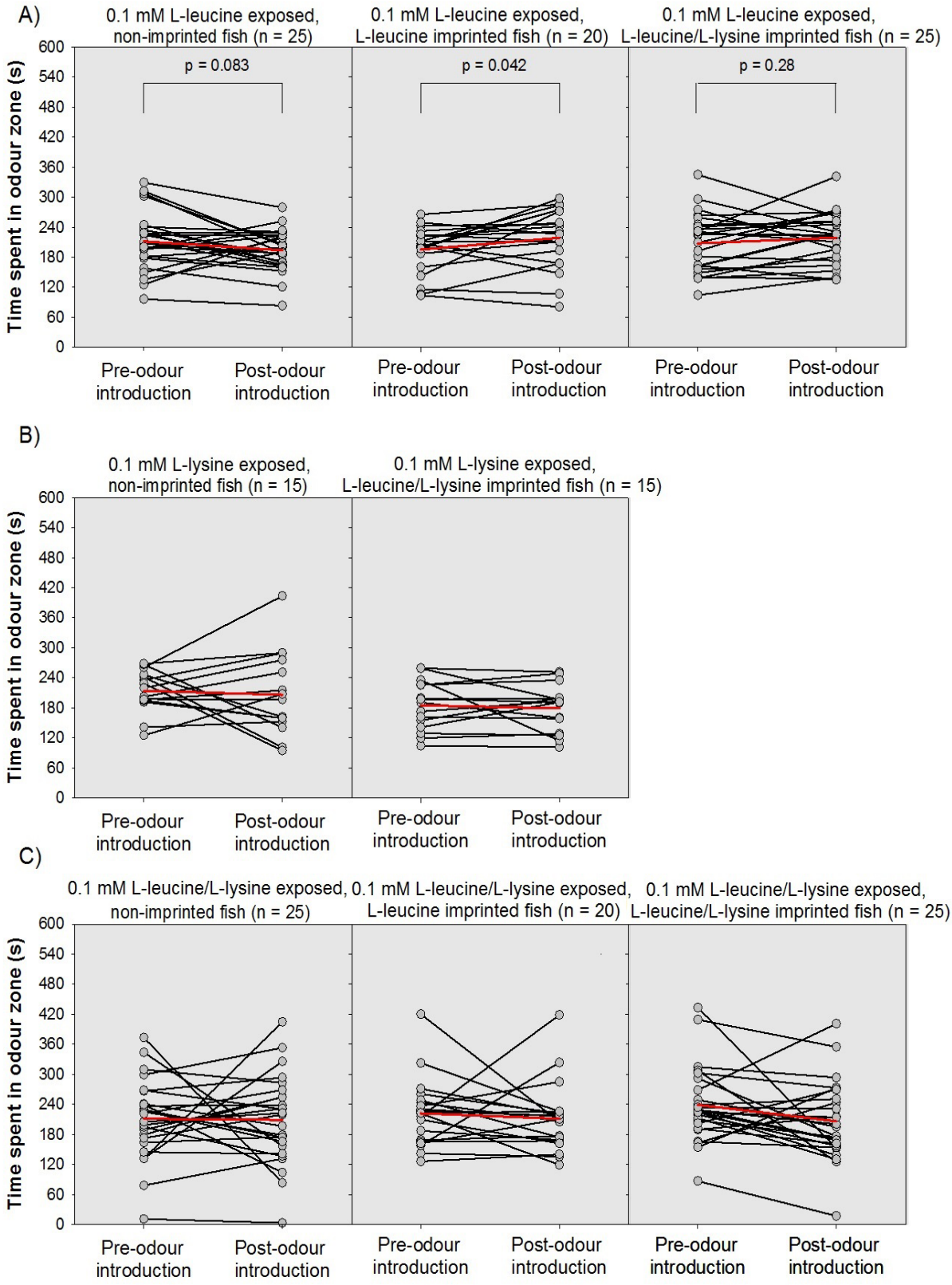

A)
0.1 mM L-leucine exposed, non-imprinted fish (n = 25)
0.1 mM L-leucine exposed, L-leucine imprinted fish (n = 20)
0.1 mM L-leucine exposed, L-leucine/L-lysine imprinted fish (n = 25)
p = 0.083
p = 0.042
p = 0.28
Time spent in odour zone (s)
600
540
480
420
360
300
240
180
120
60
0
Pre-odour introduction
Post-odour introduction
B)
0.1 mM L-lysine exposed, non-imprinted fish (n = 15)
0.1 mM L-lysine exposed, L-leucine/L-lysine imprinted fish (n = 15)
C)
0.1 mM L-leucine/L-lysine exposed, non-imprinted fish (n = 25)
0.1 mM L-leucine/L-lysine exposed, L-leucine imprinted fish (n = 20)
0.1 mM L-leucine/L-lysine exposed, L-leucine/L-lysine imprinted fish (n = 25)

Figure 4.5. Zebrafish imprint to a mixture of L-leucine and L-lysine. Fish from 0.5 to 7 dpf were reared with embryo media only (non-imprinted fish), 0.1 mM L-leucine only (L-leucine imprinted fish) or a mixture of 0.1 mM L-leucine and L-lysine (L-leucine/L-lysine imprinted fish) and then as adults observed for their behavioural responses to A) 0.1 mM L-leucine B), 0.1 mM L-lysine, or to C) a mixture of 0.1 mM L-leucine and L-lysine. The amount of time individual fish spent in the odour zone of an avoidance-attraction trough pre- and post-odour introduction are represented by grey circles, with the difference traced for each fish by a solid black line. The difference in means for how much time fish spent with the odourant after its introduction is shown by the solid red line. A two-way repeated measures ANOVA ($\alpha = 0.05$) was used to determine the effects on treatment type (non-imprinted, L-leucine imprinted, and L-leucine/L-lysine imprinted fish) and 10 min time period (pre- and post- odour introduction). All treatments had 15 - 25 fish.

Chapter 5: Age-associated changes to the zebrafish (*Danio rerio*) olfactory epithelium anatomy, olfactory sensory neuron activation, and olfactory-mediated behaviours

Abstract

Aging is associated with hyposmia, which is a weakening of the sense of smell. Hyposmia is believed to at least partially result from a thinning of the olfactory epithelium and a decline in the number of olfactory sensory neurons (OSNs), of which there are multiple classes. However, aging studies have not explored whether all OSN classes decline in the same manner. Here, using a zebrafish model, I explored whether the decline in OSNs that happens over a lifetime occurred to all OSN classes or to specific OSN classes. I expected it to be class-specific because the OSN classes are differentially sensitive to stressors. I organized zebrafish into three age groups: young-aged (3 mo – 1 yrs old), middle-aged (1 – 2 years old) and old-aged (> 2 yrs old). Age did not affect olfactory epithelium thickness, but it did consistently decrease microvillus but not ciliated OSN density. This change was not associated with any apparent differences in OSN class-specific genes including gnal2, adcy3b and plcb3. There were, however, some differences in OSN activation and downstream behavioural responses to odourants. As microvillus OSNs detect amino acids, I expected that there might have been a diminished activation of these neurons by L-cysteine. Surprisingly, the expression of pERK, a neuronal activation marker, was increased at middle age, not young or old, and was not different between OSN classes. Furthermore, a behavioural response to L-cysteine was also apparent, but only at middle age. The data confirm that age brings changes in the cellular structure of the olfactory epithelium, and that these changes can be OSN class-specific. The data also indicate that the relationship between olfactory tissue anatomy and function are not straightforward and can in fact oppose each other at times in life. It is possible that there may be compensatory mechanisms that maintain or even enhance olfactory responsiveness at middle age that no longer apply at old age, but this remains for study.

Introduction

As animals age they lose their ability to sense their environment. For example, aging weakens olfaction, and this decreased sense of smell is referred to as hyposmia (Patel & Larson, 2009; Rawson et al., 2012; Rey et al., 2012; Suzuki et al., 2021). Aging-associated hyposmia is often associated with structural changes to the olfactory system including at the peripheral olfactory organ. This organ contains an olfactory epithelium, which consists of stem cells, supporting cells, and OSNs that detect odourants (Morrison & Costanzo, 1990). Structural changes include the thinning of the olfactory epithelium and decreased OSN density and a turnover rate that correlates with reduced progenitor cell proliferation (Child et al., 2018; Jia & Hegg, 2015; Kondo et al., 2010; Ueha et al., 2018; Zhang et al., 2018). As OSNs generate signals that are mediated by signal-transduction pathways, decreases in OSN density may reduce signalling amplitude at the olfactory epithelium in response to odourants (Buiakova et al., 1996; Thesen & Murphy, 2001; Zhang et al., 2017). Therefore, aging may affect olfactory-mediated behaviours as signals are relayed from OSNs to higher-order processing centers (Xu et al., 2005). However, recent studies suggest that signalling sensitivity to certain odourants can be maintained despite decreased OSN density, and therefore, aging may not affect OSN signalling sensitivity or behaviour (Lee et al., 2009; Kass et al., 2018; Sabiniewicz et al., 2023).

Multiple OSN classes exist within the olfactory epithelium and each class has different sensitivity towards specific subsets of odourants. Two classes that are consistently found across vertebrates are ciliated and microvillus OSNs (Kratzing, 1975; Zuri et al., 1998; Lipschitz, 2002; Saito et al., 2010; Benzekri & Reiss, 2012). In fish, ciliated OSNs respond most strongly to bile acids while microvillus OSNs respond most strongly to amino acids (Lipschitz and Michel, 2002; Sato et al., 2005, Koide et al., 2009). Detection of bile acids and amino acids activates class-specific transduction pathways, in which ciliated OSNs have an adenylyl cyclase (AC) pathway and microvillus OSNs have a phospholipase-C (PLC) pathway (Wong et al., 2000; Hansen et al., 2003). While no studies have yet focused on age-associated effects on multiple OSN classes, toxicological studies using fish have demonstrated that different OSN classes have differential regenerative capacity owing to differences in precursor proliferation rates (Lazzari et al., 2017; Heffern et al., 2018; Ma et al., 2018). Furthermore, toxicants may differentially affect responses to different odourants, including bile acids and amino acids (Tierney et al., 2007;

Kolmakov et al., 2009; Dew et al., 2014). It is therefore possible that with aging, changes in the number of OSNs are class-specific due to differences in precursor proliferation rates. Such changes could be apparent in changing physiological or behavioural responses to OSN class-specific odourants.

The objective of the current study was to use molecular, anatomical, physiological, and behavioural endpoints to determine how aging-associated anatomical changes to the olfactory epithelium correspond to the development of hyposmia (Figure 5.1). The focus on age-associated effects between OSN classes and their impact on olfactory functionality will demonstrate if olfaction changes with certain odours only. I used the popular vertebrate model, the zebrafish, which have five OSN classes including ciliated and microvillus OSNs (Ahuja et al., 2014; Hansen & Eckart, 1998; Wakisaka et al., 2017). The findings from this study have implications beyond zebrafish as the olfactory system is highly conserved across vertebrates.

Methods

Fish Husbandry

A colony of tüpfel longfin zebrafish were housed in a self-circulating aquatic racks system (Aquaneering, San Deigo, CA, USA) that contained 28 $\pm$ 0.5°C reverse-osmosis (RO) water. Fish were fed twice daily consisting of artemia and Zeigler zebrafish diet (Gardners, PA, USA), and were subjected to a photoperiod of 14:10 hr light: dark cycle. Prior to any experiments, zebrafish were organized into one of three age groups: young-aged fish (3 mo – 1 yr old), middle-aged fish (1 – 2 yrs old), or old-aged fish (> 2 yrs old). Sex was balanced or near balanced between each age group. All animals were treated in accordance with the University of Alberta's Animal Care and Use committee (AUP no. 052)

Olfactory epithelium anatomy

I first examined whether the olfactory epithelium would change with age. In fish, the olfactory epithelium is located within finger-like projections called lamellae (Hansen & Eckart, 1998). Hematoxylin and eosin (H&E) staining was used to determine olfactory epithelium thickness and overall cell density across young-, middle-, and old-aged zebrafish. Fish were first euthanized from an overdose of tricaine mesylate (TMS; Syndel, Nanaimo, BC, Canada) that

was buffered with bicarbonate (stock concentration = 2.5 g/L, pH = 7.2). Fish were decapitated with the heads immediately transferred into 10% neutral-buffered formalin for fixation for 1 day at 4°C. After fixation, the samples were decalcified using Cal-Ex^{TM} (Fisher Scientific, Waltham, Massachusetts, USA) for 4 to 7 hrs. The heads were dehydrated by ascending ethanol washes and then embedded in paraffin. Five micrometer horizontal sections were cut using a microtome (Lecia, RM2125 RTS), mounted on Superior Quality Microscope Slides (Bio Nuclear Diagnostics, Toronto, Ontario, Canada), and incubated overnight at 37°C. Sections were deparaffinized in toluene, rehydrated in descending ethanol washes and then H&E stained. Observers were blinded prior to imaging slides. Sections were imaged under the same light conditions on a Zeiss Axio Scope A1 Microscope at 20x and 40x magnification to measure olfactory epithelium thickness and cell density, respectively. Images were analyzed on FIJI (ImageJ). Olfactory epithelium thickness was measured across 4 sections per fish. For each section, three lamellae were randomly selected to measure the olfactory epithelium thickness from the basal membrane to the apical surface that excluded cilia. Three measurements were taken along the sensory region of a lamella. Cell density was counted for three chosen lamellae that appeared across three sections per fish. Cell density was determined on three 30 μm in length regions from the basal membrane to the apical surface of a lamella.

After measuring the lamellae, separate sections were taken for immunohistochemistry (IHC). IHC was used to quantify ciliated and microvillus OSN abundance across young-, middle-, and old-aged fish. Fish were overdosed with TMS and then decapitated. Heads were fixed in 4% paraformaldehyde for 20 $\pm$ 4 hrs at 4°C and then decalcified with 0.5 M ethylenediaminetetraacetic acid (EDTA) for 7 days at 4°C. Following decalcification, heads were dehydrated in ascending ethanol washes and embedded in paraffin. Five micrometer-thick horizontal sections were cut, mounted on Superfrost Plus Microscope Slides (Fisherbrand, Ottawa, Ontario, Canada) and incubated overnight at 37°C.

IHC staining of the olfactory epithelium was done by using a Vectastain Elite ABC: Universal Kit, HRP (Horse Anti-mouse/rabbit IgG) kit (BioLynx, Brockville, Ontario, Canada). Slides were kept at room temperature unless specified. Sections were first deparaffinized with xylenes (Sigma-Aldrich, St. Louis, MO, USA) and dehydrated with ascending ethanol washes. Sections were rinsed with three 30 min washes in phosphate-buffered saline (PBS). Sections

were immersed in 1x sodium citrate buffer solution + 0.05% tween (pH = 6; Millipore, Burlington, Massachusetts, USA) for 10 min at 95°C in an oven. Samples were rinsed again in three 30 min PBS washes. Samples were quenched for endogenous peroxidase activity using BLOXALL for 10 min. Samples were rinsed with three 30 min washes in PBS-tween (PBS-T). Slides were blocked for 2 hrs with 2.5% normal horse serum (NHS) + 1% bovine serum albumin. The olfactory epithelium was incubated in primary antibodies with blocking solution overnight at 4°C. Ciliated OSNs were stained with monoclonal-mouse $G_{\alpha/olf}$ antibody (1:200, sc-55545; Santa Cruz Biotechnology, Dhallas, Texas, USA) and microvillus OSNs were stained with polyclonal-rabbit TRPC2 antibody (1:200, LS-C95010; LSBio Lynnwood, Washington, USA). Slides were returned to room temperature the following day and rinsed in three 30 min PBS-T washes. Sections were exposed to biotinylated anti-mouse/anti-rabbit secondary antibodies for an hour and then rinsed in two 30 min PBS-T washes followed by two 30 min PBS washes. Sections were incubated for 30 min in an ABC reagent and then rinsed in two 30 min PBS washes. Staining of ciliated and microvillus OSNs was visualized with 3,3'-diaminobenzedine (DAB) that was optimized for a one min exposure period.

The olfactory epithelium stained for ciliated or microvillus OSNs were imaged on a Zeiss Axio Scope A1 Microscope at 40x magnification under the same light conditions. Observers were blinded prior to measuring ciliated and microvillus OSN abundance, which was indirectly determined by taking optical density (OD) measurements on ImageJ; OD = log (background grey value/region of interest grey value) as reported in Bettini et al. (2016). Optical density values were averaged from three sections, in which three lamellae were randomly chosen per section. Optical density values were determined across three 30 μm in length regions on each side of a given lamella from the basement membrane to the apical side excluding cilia. Optical density was not determined for 10 μm intervals that interconnected between the 30 μm in length measured regions.

Olfactory signal-transduction pathway gene expression

Real-time PCR (qPCR) was used to quantify genes involved in the olfactory AC and PLC signal-transduction pathway of ciliated and microvillus OSNs, respectively. Six young-, middle-, or old-aged male or female fish were placed in a 5.7 L tank containing 5 L RO water where they

acclimated for 24 hrs. Three out of the six fish were transferred into a second 5.7 L tank and following another 24 hrs, they were used for RNA extraction. These fish were euthanized from a TMS overdose, and then decapitated anteriorly from the eye as the tissue contained the olfactory epithelium. The tissues were mechanically homogenized and pooled into TRIzol™ (Ambion, Carlsbad, CA, USA) in order to initiate RNA extractions, which was based on the manufacturer's guidelines. RNA samples were stored in RNase-free water at -80°C until analysis. This was replicated three to four times per age group.

RNA purity was measured on a NanoDrop ND-100, in which all samples had acceptable 260:230 nm and 260:280 nm ratios of being above 1.5. RNA integrity was assessed by an RNA Nano 6000 Assay Kit for the Agilent 2100 Bioanalyzers, in which all samples had an RNA Integrity Number above 7. RNA concentration was determined using a Qubit fluorometer (Invitrogen™, Carlsbad, CA, USA). Following quality control, cDNA was synthesized out of total RNA using a SuperScript™ III First Strand Synthesis System (Invitrogen™) as described by the manufacturer on Mastercycler Pro S (Eppendorf, Hamburg, Germany).

Primer efficiencies were determined before running qPCR experiments, with acceptable values falling in the range of between 90 and 110%. Experiments were run on 96 well plates on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each reaction contained 2.5 μL forward/reverse gene specific primers, 2.5 μL cDNA template, and 5.0 μL custom SYBER Green master mix. Every cDNA amplification sequence was run in triplicates. Each qPCR reaction was denatured at 95°C for 2 min and then cycled 40x through a 15 s denature step at 95°C followed by a 1 min annealing step at 60°C. The generation of a single product was confirmed from dissociation curves. All genes were normalized to the endogenous control, tubulin-α1 (TUB-A1). The threshold cycle (Ct value) was used to determine target cDNA amplification levels. Relative changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method.

Targeted genes for the ciliated OSN signal transduction pathway included adenylyl cyclase 3b (adcy3b) and $G_{\alpha olf2}$ (gnal2). Phospholipase-C β3 (PLC-b3) was used to target the microvillus OSN signal transduction pathway. Specific primer sequences are listed in Table 5.1.

Behaviour assay

Young-, middle-, and old-aged fish were recorded for their responses towards an amino acid (L-cysteine; Sigma-Aldrich) and a bile acid (taurocholic acid, TCA; Millipore), which are detected by microvillus and ciliated OSNs, respectively (Lipschitz and Michel, 2002; Sato et al., 2005; Koide et al., 2009). Fish response towards L-cysteine and TCA were compared to their response towards RO water, which served as a negative control. Fish were placed in a 20 × 9 × 8 cm (l × w × h) tank containing 800 mL of 28°C RO water and their behaviour was recorded using an overhead dome colour IR camera (SAV-CD120; Matcow, Stow, Ohio, USA). EthoVisionXT10 (Noldus, Wageningen, Netherlands) was used for live tracking fish movement to measure differences in average velocity (cm/s) and number of sharp turns (>90°) in response to the three odour stimuli. The tank was situated in a dark chamber devoid of any visual stimuli. Once the fish were placed in the tank, they were given a 50 min acclimation period, followed by the recording of a 5 min activity period without any odours injected into the tank. After this 5 min period was over, water, 0.01 mM TCA or 0.1 mM L-cysteine was injected into the tank and fish behaviour was recorded for another 5 minutes. Tanks were rinsed three times with RO water between experiments.

Neuronal activity

A subset of zebrafish that underwent behaviour experiments were immediately euthanized with an overdose of TMS to determine if water, TCA, or L-cysteine increased neuronal activity at the olfactory epithelium. Following euthanasia, fish were decapitated, fixed in 4% PFA overnight at 4°C and then decalcified in 0.5 M EDTA for 7 days at 4°C. Heads were dehydrated in ascending ethanol washes and embedded in paraffin. Five micrometer thick sections were mounted on SuperFrost Plus Microscope Slides and incubated overnight at 37°C. The olfactory epithelium was stained for the neuronal activation marker, pERK (1:100, p-44/42; New England Biolabs, Ipswich, Massachusetts, USA), using the same IHC protocol as described above in *Olfactory epithelium morphology and anatomy*, except for the fact that nickel-intensified DAB (BioLynx) was used for 1 min to enhance visualization of pERK immunoreactivity. Images of olfactory epithelial tissue stained for pERK were captured on a Zeiss Axio Scope A1 Microscope at 40x magnification under the same light conditions.

Observers were blinded before using ImageJ to count cells that had increased pERK immunoreactivity. Three random lamellae were chosen per section with 3 sections analyzed per fish. A standardized staining intensity threshold across all images was used to discriminate cells that had increased pERK activity from background noise. On each side of a lamella, cells that passed the threshold were manually counted within three 30 μm in length regions from the basement membrane to the apical side excluding cilia. Ten micrometer intervals that interconnected between the 30 μm in length measured regions were excluded from data collection.

Statistical analysis

All statistical analyses were run on SigmaPlot 13.0 (Systat, San Jose, CA, USA). Analysis on olfactory epithelium thickness and cell density, ciliated and microvillus OSN optical density, and ciliated and microvillus OSN signal-transduction pathway gene expression were based on one-way analysis of variance (ANOVA). A non-linear regression model was also used to examine changes in microvillus and ciliated OSN optical density values across age to ensure that binning data into three age groups did not misrepresent conclusions drawn from the dataset. Analysis on zebrafish behaviour and pERK immunoreactivity in response to water, L-cysteine, and TCA were based on two-way (Age group × Treatment type) ANOVAs. Tukey and holm-sidak post hoc tests were used for one-way ANOVA and two-way ANOVA analyses, respectively. All analyses were tested for the assumptions of normality and equal variance. Log transformations were performed on data that failed either assumption. If log transformations failed, analyses were used on ranked data instead. Outliers were removed if they passed Grubb's Method on GraphPad v9.5. Statistical significance was defined by $\alpha = 0.05$. All data are represented as mean $\pm$ SEM.

Results

Changes in olfactory epithelium anatomy over age

Olfactory epithelium thickness was similar between young-, middle-, and old-aged zebrafish ($F_{2,28} = 0.907$, $p = 0.417$; Figure 5.2A). However, cell density varied by age ($F_{2,25} =$

6.03, p = 0.008) as young-aged fish had a greater number of cells compared to middle- (p = 0.046) and old-aged fish (p = 0.010; Figure 5.2B).

Ciliated OSN optical density for $G_{\alpha/olf}$ immunoreactivity was similar between young-, middle-, and old-aged fish ($F_{2,27} = 1.63$, p = 0.217; Figure 5.3A), potentially owing to moderate biological variation across age ($R^2 = 0.16$, p = 0.11, Figure 5.4A) Microvillus OSN optical density for TRPC2 immunoreactivity varied by age ($F_{2,26} = 5.15$, p = 0.014; Figure 5.3B) with an apparent decrease between middle- and old-aged fish (p = 0.011). Regression analysis also revealed that TRPC2 immunoreactivity varied by age despite the moderate biological variation between samples ($R^2 = 0.23$, p = 0.045, Figure 5.4B).

Age-related changes in expression of ciliated and microvillus OSN signal-transduction markers

The genetic markers selected for revealing age-related changes to the AC signal-transduction pathway of ciliated OSNs did not reveal any differences between young-, middle- and old-aged fish ($F_{2,3} = 0.427$, p = 0.67; $F_{2,3} = 0.359$, p = 0.71 for adcy3b and gnal, respectively; Figure 5.5). The genetic marker selected for revealing age-related changed to the PLC signal-transduction pathway of microvillus OSNs also did not reveal any differences ($F_{2,3} = 0.273$, p = 0.77 for PLC-b3).

Age-related changes in behavioural response to water, L-cysteine, and TCA

Zebrafish did not alter swim speed in response to water, L-cysteine, or TCA, regardless of their age ($F_{2,3} = 0.00803$, p = 0.99; Figure 5.6A). However, there was a difference in the number of turns made in response to L-cysteine ($F_{4,122} = 2.51$, p = 0.046). After L-cysteine introduction, the number of turns made by middle-aged fish was higher than in young-aged fish (p = 0.004; Figure 5.6B). This suggested that the assay did work, at least for the one odourant.

Age-related changes in neuronal activity during water, L-cysteine, and TCA exposures

An immediate neuronal response was apparent following the introduction of L-cysteine and TCA ($\chi_{2,3} = 5.09$, p = 0.010; Figure 5.7). Responses in young- and old-aged fish were not significant to either odourant whereas they were in middle-aged fish (p = 0.022 and 0.040 for L-

cysteine and TCA-exposed fish, respectively). This suggests that olfaction was most functional at middle age. Having said that, neuronal activity did tend to be apparent in some of the young-aged fish, but differences were likely obscured by responses to control.

Discussion

As with other vertebrates, a change in the anatomy of the olfactory epithelium was noted with zebrafish. While a general thinning was not observed, as was expected, a decrease in density of OSNs was determined, but of interest, for one OSN class only. Specifically, only microvillus OSN density decreased with aging. The difference in olfactory epithelium anatomy was associated with a change in their behaviour that was only evident in middle-aged fish exposed to L-cysteine. Neuronal activation during odourant exposure was also only seen in middle-aged fish. This suggests that age-associated anatomical changes to the olfactory epithelium do not dictate changes to neuronal activation or olfactory-mediated behaviours. Furthermore, olfaction may be most functional at middle age.

Overall, age did not affect zebrafish olfactory epithelium anatomy. Olfactory epithelium thickness was similar between young-, middle-, and old-aged zebrafish, despite a decline in cell density as fish aged. The decreased cell density may have derived from a decreased quantity or proliferation of stem cells that give rise to other cell types in the olfactory epithelium (Jia & Hegg, 2015; Medrano et al., 2009; Ueha et al., 2018). It is also possible that the decreased cell density led to larger sized cells and larger gaps between the cells, thereby maintaining olfactory epithelium thickness across the three age groups (Greenberg et al., 1977). Furthermore, zebrafish were raised in an aquatic racks system that minimized the introduction of contaminants, thereby likely demonstrating the effects of physiological aging processes on olfactory epithelium anatomy. Nevertheless, this finding contradicts other studies that found that olfactory epithelium thickness decreases with aging (Jia & Hegg, 2015; Ueha et al., 2018; Zhang et al., 2018).

Changes to OSN density were class specific. Specifically, microvillus OSN density decreased in old-aged zebrafish while ciliated OSN density was similar across age. This differential effect between OSN classes may be explained by proliferation differences in progenitor cells, with microvillus and ciliated OSNs having decreased and unchanged turnover rates during aging, respectively (Kondo et al., 2010; Lazzari et al., 2017; Ma et al., 2018). It is

worthwhile to examine how age affects OSN classes besides ciliated and microvillus OSNs. In the zebrafish olfactory epithelium, there are also crypt cells, kappe neurons, and pear-shaped neurons, which detect odourants that are different from those that are detected by ciliated and microvillus OSNs, but may be equally important to the fish (Ahuja et al., 2014; Hansen & Eckart, 1998; Wakisaka et al., 2017). As ciliated OSNs are most sensitive to bile acids and microvillus OSNs are most sensitive to amino acids, the differential age effect on OSN classes is a major new finding, in part because animals may be able to respond to bile acids regardless of age while responses to amino acids may be absent in the aging population (Lipschitz, 2002; Sato et al., 2005).

There was an expectation that activation of OSNs would be apparent to varying degrees between young-, middle-, and old-aged zebrafish that were exposed to L-cysteine or TCA, which are detected by microvillus and ciliated OSNs, respectively (Lipschitz and Michel, 2002; Sato et al., 2005; Koide et al., 2009). This was not the case as the neuronal activation marker, pERK, was elevated only in middle-aged fish that were exposed to either odourants. Furthermore, only middle-aged fish responded to L-cysteine by increasing the number of sharp turns elicited, and fish across the three age groups did not respond to TCA. In regard to swimming activity during odourant exposures, one study also demonstrated that L-cysteine increases the number of sharp turns zebrafish elicit (Kermen et al., 2020). However, this study also demonstrated that bile acids increase fish movement. Nevertheless, my findings suggest that olfaction was most functional in middle-aged fish. In contrast, exposing old-aged fish to the two odourants did not activate OSNs or evoke behavioural responses. In comparison to middle-aged fish, this finding may imply aging-associated hyposmia that was non-specific to L-cysteine and TCA even though microvillus OSN and not ciliated OSN density decreased in old-aged fish. However, neuronal activation was also absent in young-aged fish that were exposed to the two odourants. This is surprising as cell density within the olfactory epithelium was highest in this age group. Furthermore, microvillus OSN density was similar between young- and middle-aged fish, yet only middle-aged fish responded to L-cysteine. Therefore, age-associated changes to the olfactory epithelium anatomy may not dictate changes to neuronal acuity or behavioural responses to odourants. Instead, age-associated changes in olfactory sensitivity to TCA and L-cysteine may depend on changes in AC and PLC signal-transduction pathways of ciliated and microvillus OSNs, respectively (Carlson et al., 2008; Gerschütz et al., 2014). The current study found that expression of genetic markers

involved in either transduction pathways were not different between young-, middle- and old-aged fish. It is important to note that gene expression does not necessarily dictate activity.

Hyposmia is often defined as a decreased sense of smell. A recent discussion has been to improve the clarity of olfactory nomenclature, including the definition of hyposmia (Hernandez et al., 2023). This study's findings demonstrate the importance of defining hyposmia clearly. Aging induced hyposmia in terms of decreasing microvillus OSN density and OSN activation during L-cysteine and TCA exposures. However, aging did not affect olfactory epithelium thickness or ciliated OSN density.

In conclusion, age-associated structural changes to the olfactory epithelium do not dictate changes in OSN activation or behaviour during odourant detection. Specifically, this study's findings suggest that age may have OSN class-specific effects as ciliated OSN density remained the same across age while microvillus OSN density decreased with aging. However, increased neuronal activation and changes in behaviour towards an amino acid and a bile acid were seen only in middle-aged fish. Throughout an animal's lifespan, olfaction may be most functional at middle age.

Tables

Table 5.1. Primer sequences and accession numbers for target genes.

Target gene name	Forward sequence (5' – 3')	Reverse sequence (5' – 3')	Accession number
TUB-A1	GCC TGG GCT CGT CTG GAT	AAC TCG CCC TCC TCC ATA CC	AF029250.1
gnal2	GTC ACT ACT GTT ACC CCC ATT TCA C	CTG CCG CAG GTG CAT TC	NM_001366715.1
adcy3b	ATC CTG AAG GAG TAC GGT TTT CG	CAA GAT TTG CCT TGT ATC TGA TGT G	XR_659225.3
PLC-b3	GAG GCA GAG CTA AAG CGT TTG	AGA GTT TGG GCC GTC ATT G	NM_001122773.1

Figures

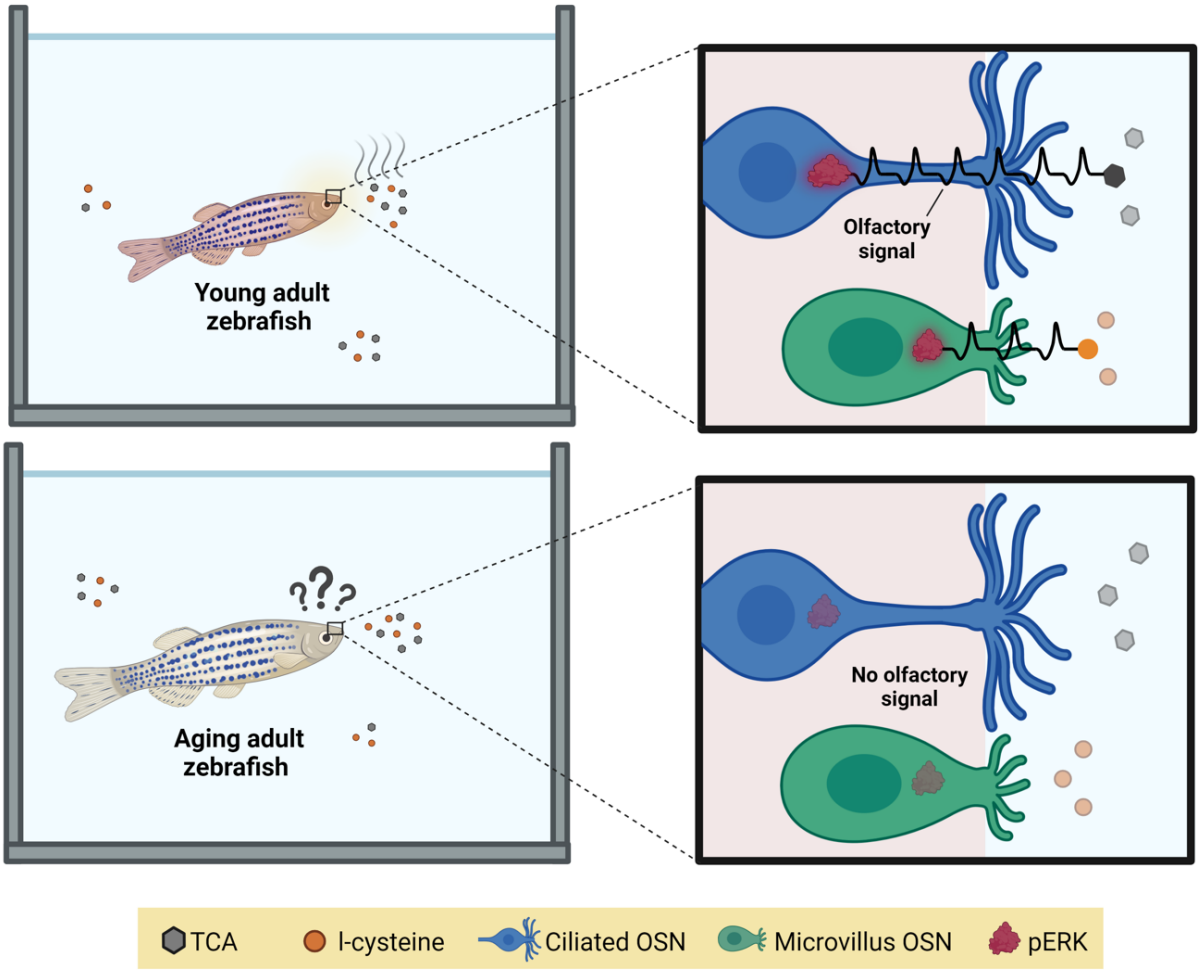


Figure 5.1. A schematic on how age may affect behaviour and activation of ciliated and microvillus OSNs in response to TCA or L-cysteine. This image was made using BioRender.com

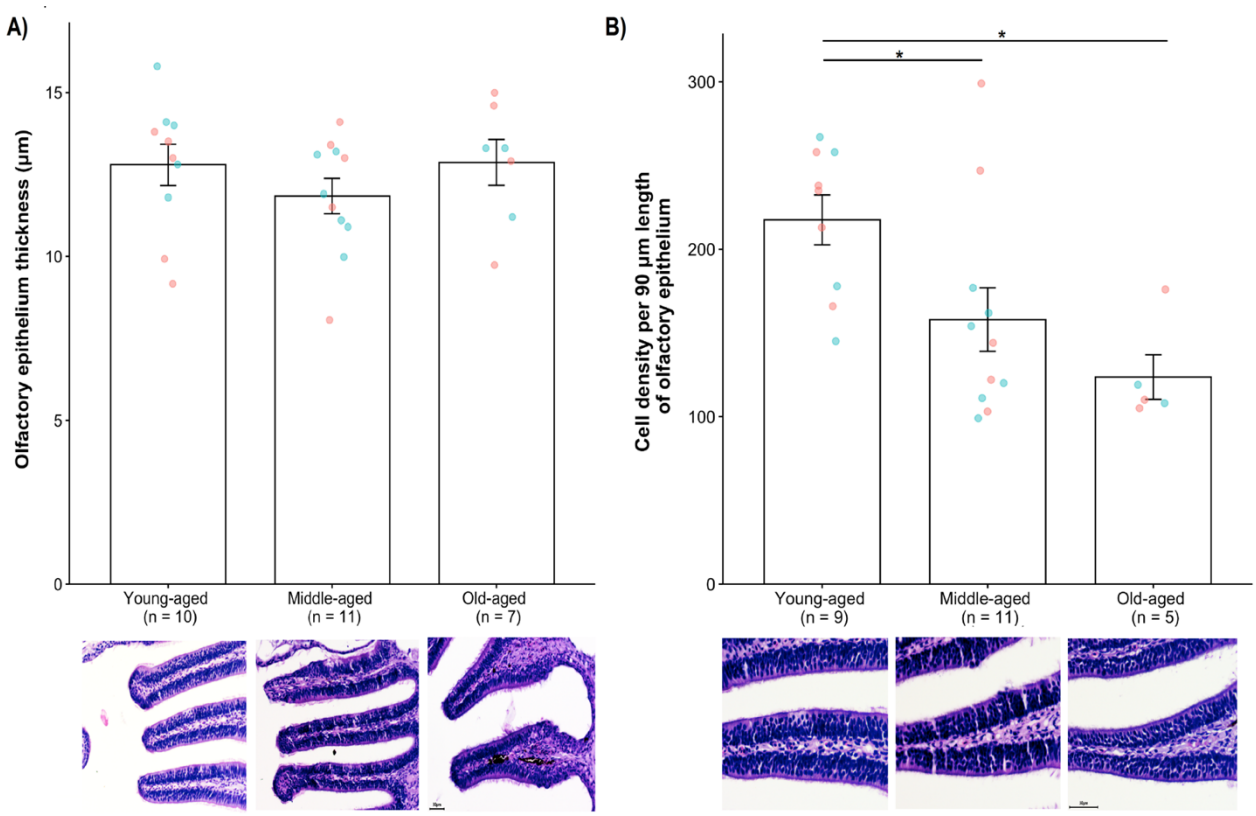


Figure 5.2. Changes in olfactory epithelium morphology across young-, middle-, and old-aged fish. Measurements were A) olfactory epithelium thickness at 20x magnification, and B) olfactory epithelium cell density at 40x magnification. H&E was used to stain the olfactory epithelium. Bar graphs represent mean ± SEM. Data points from individual female and male fish are shown in red and blue dots, respectively. Statistical analysis was based on a one-way ANOVA ($\alpha = 0.05$) with asterisks indicating significant differences.

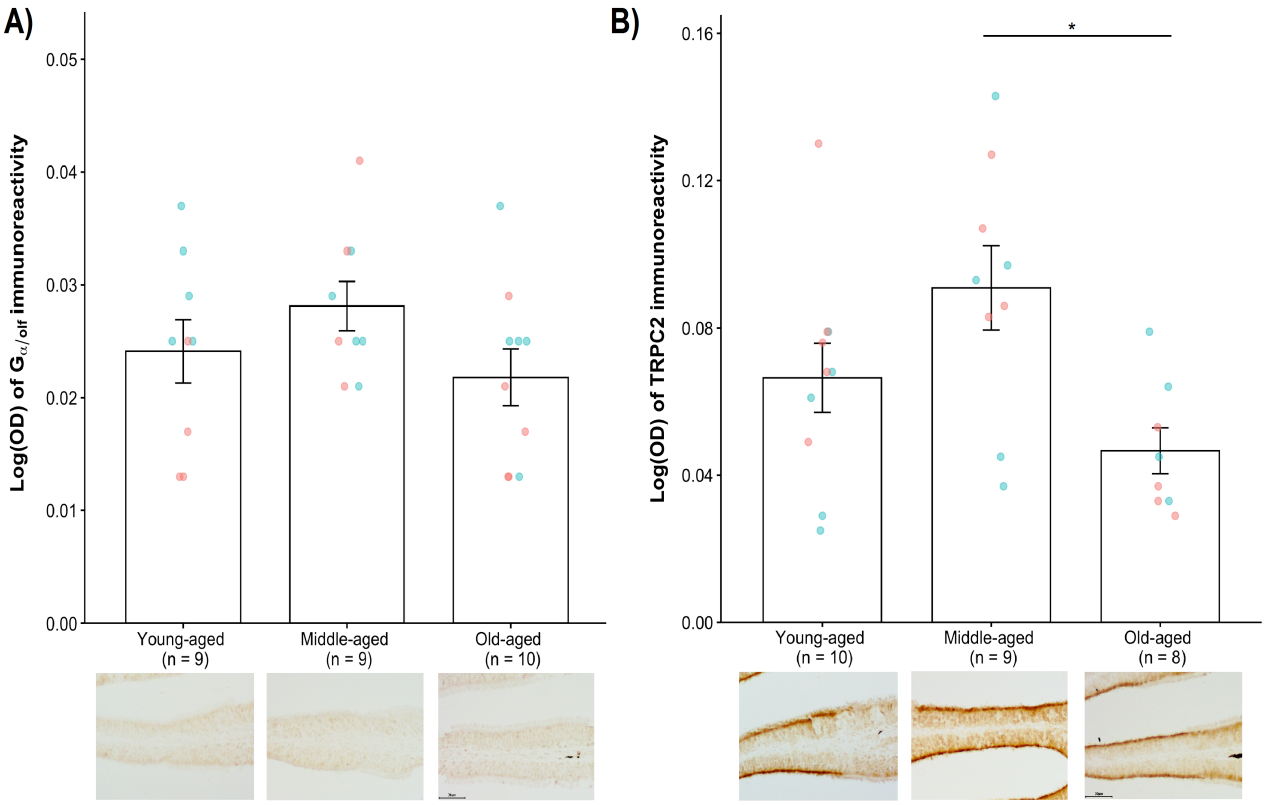


Figure 5.3. Changes in ciliated and microvillus OSN density across young-, middle- and old-aged fish, Measurements were A) $G_{\alpha/olf}$ immunoreactivity for ciliated OSN density, and B) TRPC2 immunoreactivity for microvillus OSN density. IHC was used to stain the olfactory epithelium. Bar graphs represent mean ± SEM. Data points from individual female and male fish are shown in red and blue dots, respectively. Statistical analysis was based on a one-way ANOVA ($\alpha = 0.05$) with asterisks indicating significant differences.

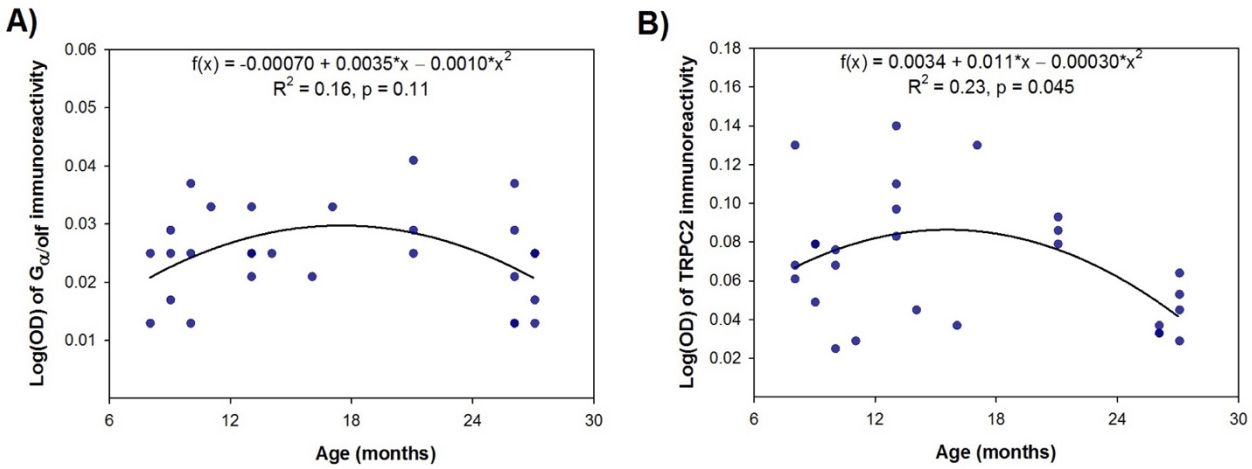


Figure 5.4. Changes in ciliated and microvillus OSN density across age (months). Measurements were A) $G_{\alpha/olf}$ immunoreactivity for ciliated OSN density, and B) TRPC2 immunoreactivity for microvillus OSN density. IHC was used to stain the olfactory epithelium. Data points from individual fish are shown in dark blue dot. Statistical analysis was based on a non-linear regression ($\alpha = 0.05$).

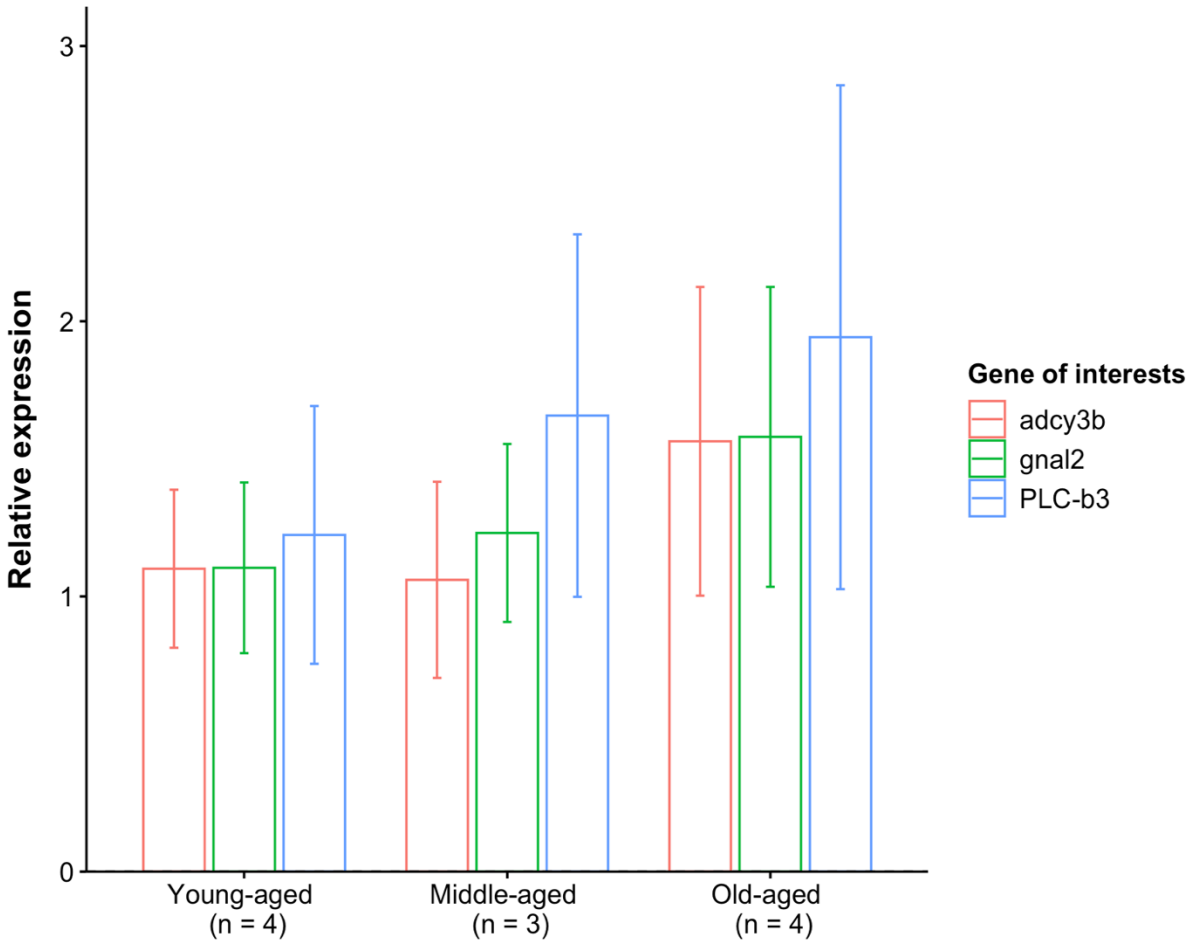
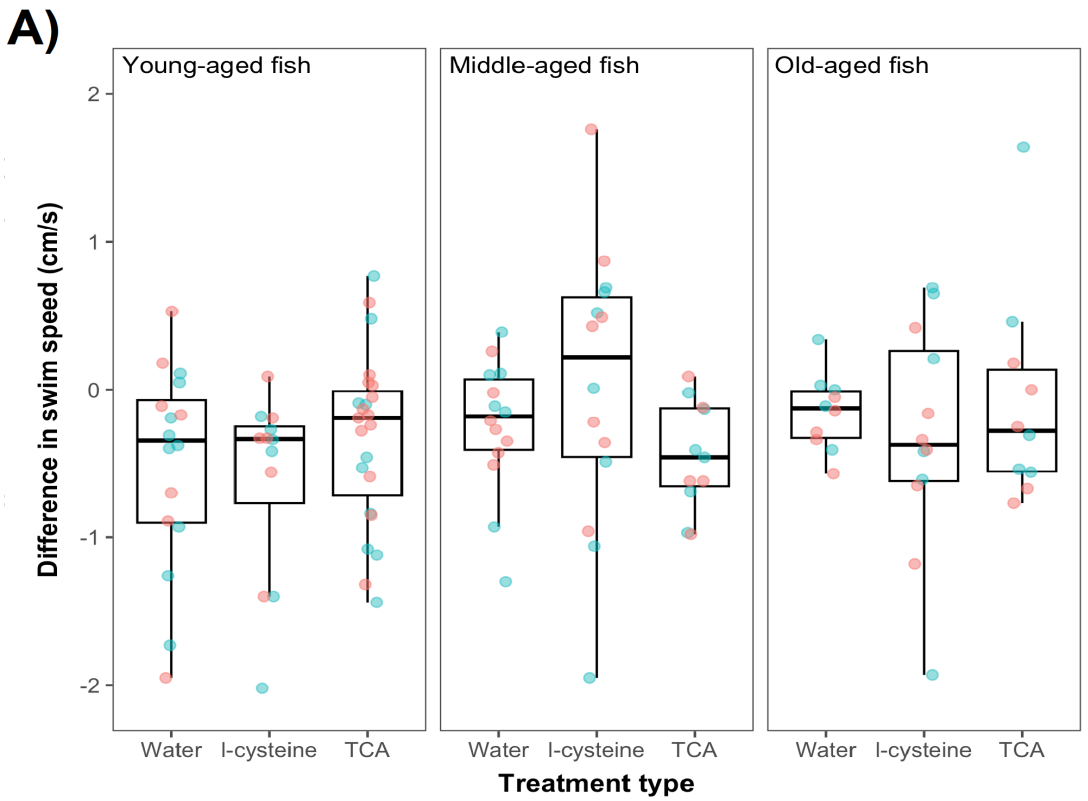


Figure 5.5. Relative expression of genes involved in the olfactory AC pathway (adcy3b and gnal2) and PLC pathway (PLC-b3), based on qPCR experiments. Genes were normalized to TUB-A1. Relative changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method. Bar graphs represent mean $\pm$ SEM. Statistical analysis was based on a one-way ANOVA (α = 0.05).


A)
Young-aged fish
Middle-aged fish
Old-aged fish
Difference in swim speed (cm/s)
2
1
0
-1
-2
Water
l-cysteine
TCA
Treatment type

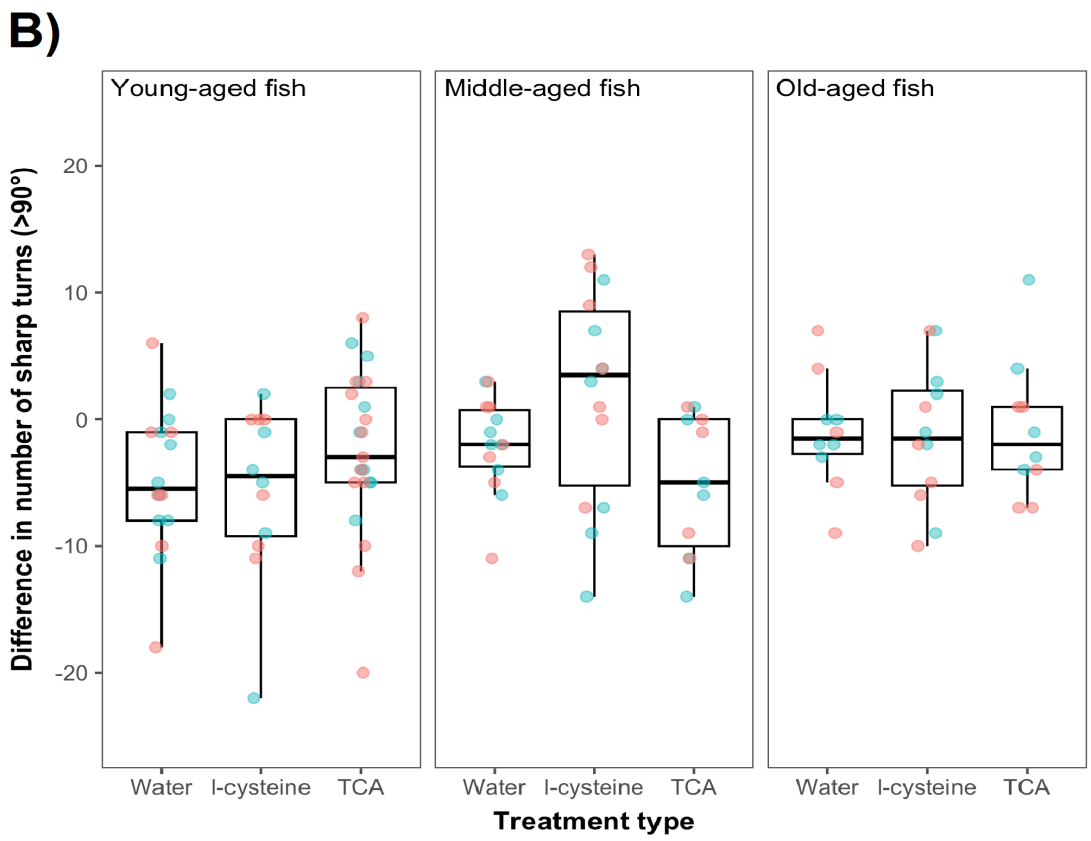

B)
Young-aged fish
Middle-aged fish
Old-aged fish
Difference in number of sharp turns (>90°)
20
10
0
-10
-20
Water
l-cysteine
TCA
Treatment type

Figure 5.6. Young-, middle- and old-aged zebrafish responses to water, 0.1 mM L-cysteine and 0.01 mM taurocholic acid (TCA). Behavioural parameters were changes in A) swim speed (cm/s), and B) number of sharp turned (>90°) elicited (n = 10 – 23 for all treatments per age group). Fish were exposed to one of the three stimuli for 5 minutes after a 5-minute period where no odourants were introduced. Box and whisker plots show the spread of data with the box representing 50% of the data, the whiskers representing the bottom and top 25% of data, and the horizontal line representing the median. Data points from individual female and male fish are shown in red and blue dots, respectively. Statistical analyses were based on two-way ANOVAs on ranked data ($\alpha = 0.05$).

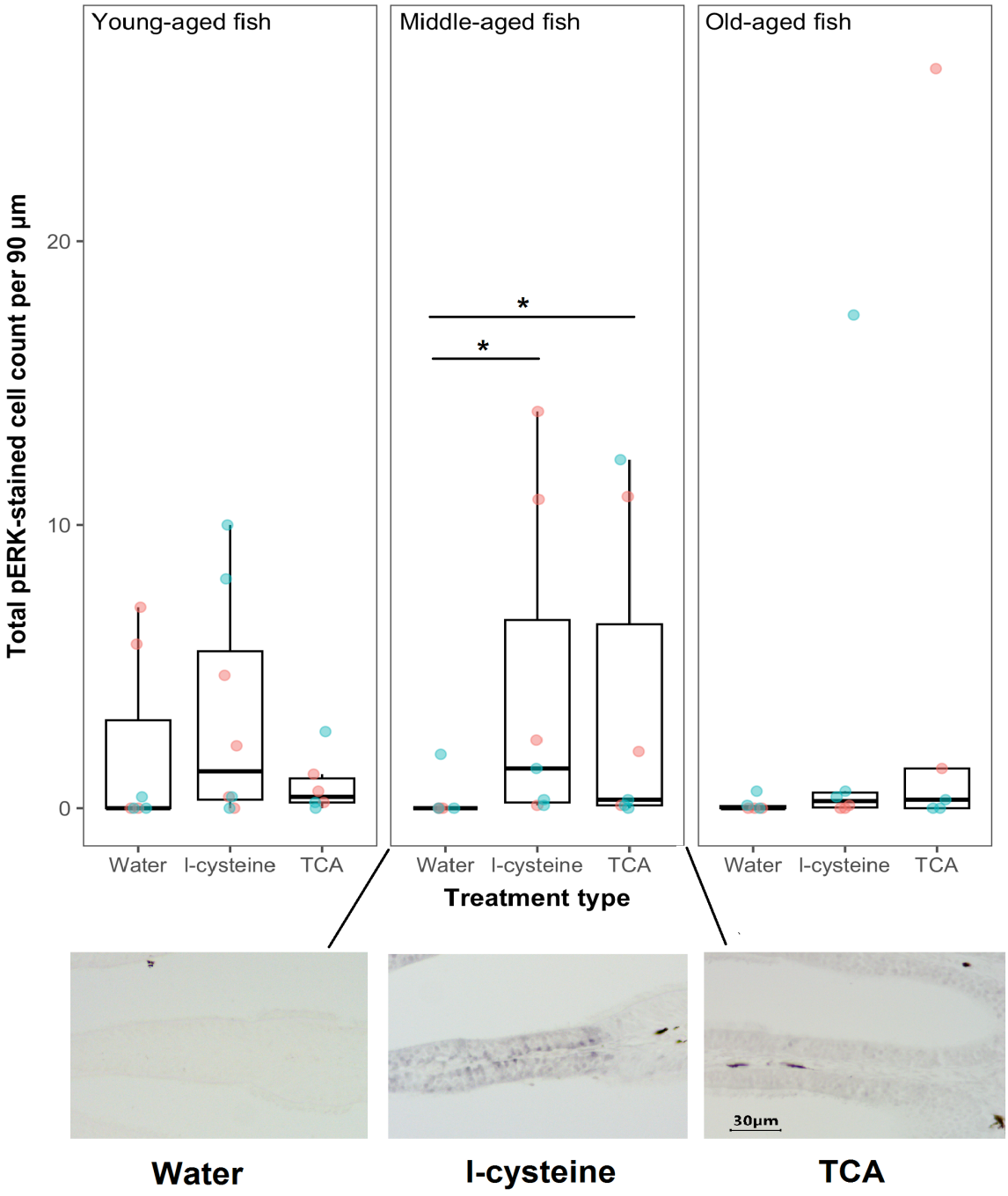


Figure 5.7. Activation of OSNs in young-, middle- and old-aged fish exposed to water, 0.1 mM L-cysteine or 0.01 mM taurocholic acid (TCA) (n = 5 – 8 for all treatments per age group). Fish were exposed to one of the three stimuli for 5 minutes after a 5-minute period where no odourants were introduced. Measurements for neuronal activation was based on IHC staining for pERK activity with images captured at 40x magnification. Sample images were taken from

middle-aged fish exposed to water, L-cysteine, or TCA. Box and whisker plots show the spread of data with the box representing 50% of the data, the whiskers representing the bottom and top 25% of data, and the horizontal line representing the median. Data points from individual female and male fish are shown in red and blue dots, respectively. Statistical analyses were based on two-way ANOVAs on ranked data ($\alpha = 0.05$) with asterisks indicating significant differences.

Chapter 6: Age affects olfactory toxicity from copper sulfate exposures in zebrafish (*Danio rerio*)

Abstract

The olfactory system is continuously vulnerable to damage from toxicants. For example, copper induces apoptosis at the olfactory epithelium and reduces the number of olfactory sensory neurons (OSNs), of which there are multiple classes including bile acid-detecting ciliated OSNs and amino acid detecting-microvillus OSNs. In regard to these two OSN classes, copper exposures may affect their numbers differently. Such damage is reversible, at least for environmentally realistic copper concentrations. However, regenerative capacity of the olfactory epithelium may weaken with aging as proliferation of progenitor cells decreases. Using zebrafish, I demonstrated that age affects olfactory toxicity from copper exposures with a focus on differential toxic effects between microvillus and ciliated OSNs. I specifically examined changes to olfactory epithelium thickness, density of ciliated and microvillus OSNs, expression of genes specific to ciliated and microvillus OSN signal-transduction pathways, and preference responses to an amino acid (L-cysteine) and a bile acid (taurocholic acid). Copper exposures decreased olfactory epithelium thickness and did not affect ciliated or microvillus OSN density, regardless of age. Differential toxicity was seen in aging fish as copper downregulated expression of genes specific to ciliated OSNs including gnal2 and adcy3b. Age influenced how copper affected olfactory-mediated responses. While copper did not affect a behavioural response to taurocholic acid, it did affect those of middle-aged fish to L-cysteine. As old-aged fish did not respond to L-cysteine, copper exposures were not associated with a response. Throughout an animal's lifespan, olfaction may be most functional at middle age, but during this time olfactory functionality may also be most vulnerable to disruption by toxicants such as from copper exposures. As animals age, effects from toxicants on olfaction become less relevant as aging-associated hyposmia is induced.

Introduction

Olfaction facilitates invaluable interactions between animals and their chemical environment. Chemical cues may be referred to as odourants, are detected by the olfactory

system, which begins with the peripheral olfactory organ. This organ contains an olfactory epithelium that consists of odourant-detecting olfactory sensory neurons (OSNs), supporting cells, and stem cells that give rise to all cell types within the epithelium (Morrison & Costanzo, 1990, 1992). Regarding OSNs, there are multiple classes, and each class has a different sensitivity to specific subsets of odourants. Ciliated and microvillus OSNs are two classes found across vertebrates, and in fish, they are most sensitive to bile acids and amino acids, respectively (Lipschitz, 2002; Sato et al., 2005). During odourant detection, signals are generated within OSNs by transduction pathways that are class-specific (Pace et al., 1985; Sklar et al., 1986; Berghard & Buck, 1996). Ciliated OSNs have an adenylyl cyclase (AC) pathway while microvillus OSNs have a phospholipase-C (PLC) pathway (Hansen et al., 2003; Wong et al., 2000). Olfactory signals propagate along OSNs to higher-order processing centers that influence behaviour (Xu et al., 2005). Olfactory-mediated behaviours include foraging, avoiding predators and finding kin or mates (Petranka et al., 1987; Nevitt, 1999; Barata et al., 2007; Roberts et al., 2018).

Throughout an animal's lifespan, the olfactory system will continuously receive chemical pressure from the environment. In fact, the olfactory epithelium is in direct contact with the environment and is therefore vulnerable to damage by toxicants. For example, copper enters the hydrosphere through urban runoffs and causes apoptosis at the olfactory epithelium, which correspondingly decreases its thickness (Julliard et al., 1996; Wang et al., 2013; Lazzari et al., 2017). Interestingly, there is data to suggest that not all OSNs are equally affected by copper exposures. For example, two studies using zebrafish demonstrated that ciliated OSNs were more sensitive to copper toxicity than microvillus OSNs (Lazzari et al., 2017; Ma et al., 2018). This differential toxicity can also be seen in various toxicants that disrupt either the AC or PLC signal-transduction pathways of ciliated and microvillus OSNs, respectively (Shahriari et al., 2023). Other toxicants can also cause differential OSN toxicity (Tierney et al., 2010; Shahriari et al., 2023). As toxicants may have class-specific effects on OSNs and their associated signal-transduction pathways, toxicants may also affect responses to certain odourants only. For fish species exposed to copper, olfactory signals from bile acids may be disrupted to a greater extent than those from amino acids (Kolmakov et al., 2009; Dew et al., 2014; Razmara et al., 2019). However, this differential impact on olfaction has not always been observed as one study demonstrated that copper affected the generation of olfactory signals in salmon exposed to amino

acids and bile acids (Baldwin et al., 2003). This may have owed to differences in species sensitivities or copper concentrations.

The olfactory epithelium has remarkable resistance to toxic agents. There is considerable activity of biotransformation enzymes, which inactivate toxicants (Ben-Arie et al., 1993; Monod et al., 1994; Matsuo et al., 2008). As well, metal exposures will increase the expression of metallothioneins, which chelate metals to limit adverse effects (Williams & Gallagher, 2013; Williams et al., 2016). Furthermore, damage to the olfactory epithelium after toxicant exposures is reversible as progenitor cells proliferate to replace damaged cells (Lazzari et al., 2017; Ma et al., 2018; Lazzari et al., 2019). However, the capacity of OSN regeneration after toxicant exposures may be class specific.

Resistance to olfactory toxicants and regenerative capacity may reduce as animals age. This may owe to several factors. For example, the expression of biotransformation enzyme and metallothioneins decreases past middle age (Malavolta et al., 2008; Xu et al., 2019) Aging also decreases progenitor cell proliferation (Jia & Hegg, 2015; Ueha et al., 2018; Zhang et al., 2018). Changes such as these may affect all OSN classes together (Lazzari et al., 2017; Ma et al., 2018; Lazzari et al., 2019). Furthermore, aging is associated with hyposmia, which refers to a weakened olfactory sense (Patel & Larson, 2009; Rawson et al., 2012; Suzuki et al., 2021). The addition of toxicant exposures may exacerbate hyposmia.

The objective of the current study was to determine how age affects olfactory toxicity with a focus on examining differential toxicity to ciliated and microvillus OSNs. I used the popular model, the zebrafish, in which I exposed them to copper sulfate. Copper was chosen as it was previously found to differentially affect ciliated and microvillus OSNs in the context of their specific responses to associated odourants (Kolmakov et al., 2009; Dew et al., 2014; Lazzari et al., 2017; Ma et al., 2018; Razmara et al., 2019)

Methods

Fish Husbandry

A colony of tüpfel longfin zebrafish were housed in a self-circulating aquatic racks system (Aquaneering, San Deigo, CA, USA) that contained 28 $\pm$ 0.5°C reverse-osmosis (RO)

water. Fish were fed twice daily consisting of artemia and Zeigler zebrafish diet (Gardners, PA, USA), and were subjected to a photoperiod of 14:10 hr light: dark cycle. Prior to any experiments, zebrafish were categorized as middle-aged fish (1 – 2 yrs old) or old-aged fish (> 2 yrs old). Sex was balanced or near balanced between the two age groups. All animals were treated in accordance with the University of Alberta's Animal Care and Use committee (AUP no. 052).

Treatment exposures

Copper (II) sulfate (Sigma-Aldrich, St. Louis, MO, USA) was used to make 5 g/L of copper stock solutions, which were treated with 1% HNO_3 (FisherBrand, Ottawa, Ontario, Canada) to maximize ion dissociation. Stock solutions were stored at 4ºC.

Prior to exposures, six middle- or old-aged fish were acclimated for 24 $\pm$ 3 hrs in a 5.7 L glass tank that contained 5 L RO water that was heated to 28 $\pm$ 0.5°C. Following acclimation, half of the fish were placed in a new 5.7 L tank containing 5 L RO water only. This treatment acted as a control. The other half were placed in a third tank containing 5 L of 25 μg/L copper solution, which is a concentration high enough to affect olfaction without impairing movement (Tilton et al., 2011; Lazzari et al., 2017; Ma et al., 2018). Copper exposure tanks were pretreated with 25 μg/L copper solution for one day to reach a stable state and copper concentration would not fluctuate during exposures. Fish were exposed to water only or to copper for 24 $\pm$ 3 hrs prior to any experimentation. Copper exposure tanks were rinsed with 5% HNO_3 followed by a rinse with water for one day prior to any reuse.

Olfactory epithelium morphology and anatomy

Zebrafish were euthanized from an overdose of tricaine mesylate (TMS; Syndel, Nanaimo, BC, Canada) that was buffered with bicarbonate (stock concentration = 2.5 g/L, pH = 7.2). Fish were decapitated with the heads immediately transferred into 4% paraformaldehyde for fixation for 20 $\pm$ 4 hrs at 4°C. After fixation, samples were decalcified using 0.5 M ethylenediaminetetraacetic acid (EDTA) for seven days at 4°C. Following decalcification, the heads were dehydrated by ascending ethanol washes and then embedded in paraffin. A microtome (Lecia, RM2125 RTS) was used to cut samples into five micrometer thick sections.

Sections were mounted on Superior Quality Microscope Slides (Bio Nuclear Diagnostics, Toronto, Ontario, Canada) for hematoxylin and eosin (H&E) staining or mounted on Superfrost Plus Microscope Slides (Fisherbrand, Ottawa, Ontario, Canada) for immunohistochemistry (IHC). Slides were incubated overnight at 37°C prior to tissue staining.

Olfactory epithelium thickness was quantified on sections that were H&E stained. Sections were first deparaffinized in toluene, rehydrated in descending ethanol washes and then H&E stained. Sections were imaged under the same light conditions on a Zeiss Axio Scope A1 Microscope at 20x magnification. Observers were blinded prior to analyzing images on FIJI (ImageJ). Olfactory epithelium thickness was measured across three sections per fish. For each section, three lamellae were randomly selected to measure the olfactory epithelium thickness from the basal membrane to the apical surface that excluded cilia. Three measurements were taken along the sensory region of a lamella.

Ciliated and microvillus OSN density were quantified using IHC. A Vectastain Elite ABC: Universal Kit, HRP (Horse Anti-mouse/rabbit IgG) kit (BioLynx, Brockville, Ontario, Canada) was used. Slides were kept at room temperature unless specified. Sections were deparaffinized with xylenes (Sigma-Aldrich, St. Louis, MO, USA) and dehydrated with ascending ethanol washes. Sections were rinsed with three 30 min washes in phosphate-buffered saline (PBS) and then immersed in 1x sodium citrate buffer solution + 0.05% tween (pH = 6; Millipore, Burlington, Massachusetts, USA) at 95°C in an oven for 10 min. Samples were rinsed again in three 30 min PBS washes. Samples were quenched for endogenous peroxidase activity using BLOXALL for 10 min. Samples were rinsed with three 30 min washes in PBS-tween (PBS-T). Slides were blocked for 2 hrs with 2.5% normal horse serum (NHS) + 1% bovine serum albumin. Sections were incubated in primary antibodies with blocking solution overnight at 4°C. Ciliated OSNs were stained with monoclonal-mouse $G_{\alpha/olf}$ antibody (1:200, sc-55545; Santa Cruz Biotechnology, Dhallas, Texas, USA) and microvillus OSNs were stained with polyclonal-rabbit TRPC2 antibody (1:200, LS-C95010; LSBio Lynnwood, Washington, USA). After primary antibody incubation, slides were returned to room temperature and rinsed in three 30 min PBS-T washes. Sections were incubated in biotinylated anti-mouse/anti-rabbit secondary antibodies for an hour and then rinsed in two 30 min PBS-T washes followed by two 30 min PBS washes. Sections were incubated for 30 min in an ABC reagent and then rinsed in two 30 min

PBS washes. Staining of ciliated and microvillus OSNs was visualized from 1 min 3,3'-diaminobenzedine (DAB).

Images of the olfactory epithelium that were stained for ciliated or microvillus OSNs were captured on a Zeiss Axio Scope A1 Microscope at 40x magnification under the same light conditions. Observers were blinded prior to measuring ciliated and microvillus OSN density. OSN density was indirectly determined by taking optical density (OD) measurements on ImageJ; OD = log (background grey value/region of interest grey value) as reported in Bettini et al. (2016). Optical density values were averaged across three sections, in which three lamellae were randomly chosen per section. On each side of a lamellae, optical density values were determined across three 30 μm in length regions from the basement membrane to the apical side excluding cilia. Ten micrometer intervals that interconnected between the 30 μm in length measured regions were excluded from data collection.

Olfactory signal-transduction pathway gene expression

Real-time PCR (qPCR) was used to quantify expression of genes involved in the AC and PLC signal-transduction pathway of ciliated and microvillus OSNs, respectively. Three middle- or old-aged fish that underwent water or copper exposures were euthanized from a TMS overdose. Fish were then decapitated anteriorly from the eye as the tissue contained the olfactory epithelium. The tissues were mechanically homogenized and pooled into TRIzol™ (Ambion, Carlsbad, CA, USA) in order to initiate RNA extractions, which was based on the manufacturer's guidelines. RNA samples were stored in RNase-free water at -80°C until analysis. This was replicated three to five times per age group.

RNA purity was measured on a NanoDrop ND-100, in which all samples had acceptable 260:230 nm and 260:280 nm ratios of being above 1.5. RNA integrity was assessed by an RNA Nano 6000 Assay Kit for the Agilent 2100 Bioanalyzers, in which all but two samples had an RNA Integrity Number (RIN) above 7. A middle- and old-aged fish that were exposed to copper had a RIN number of 5.4 and 5.3, respectively. A Qubit fluorometer (Invitrogen™, Carlsbad, CA, USA) was used to determine RNA concentrations. After quality control, total RNA was used to synthesize cDNA via a SuperScript™ III First Strand Synthesis System (Invitrogen™) as described by the manufacturer on Mastercycler Pro S (Eppendorf, Hamburg, Germany).

Primer efficiencies were determined prior to running qPCR experiments. Acceptable values were considered between 90 and 110%. Experiments were run on 96 well plates on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each qPCR reaction contained 2.5 μL forward/reverse gene specific primers, 2.5 μL cDNA template, and 5.0 μL custom SYBER Green master mix. Every cDNA amplification sequence was run in triplicates. Each qPCR reaction was denatured at 95°C for two minutes and then cycled 40x through a fifteen second denature step at 95°C followed by a one-minute annealing step at 60°C. Dissociation curves were used to confirm the generation of a single product. All genes were normalized to the endogenous control, tubulin-α1 (TUB-A1). The threshold cycle (Ct value) was used to determine target cDNA amplification levels. Relative changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method.

Targeted genes specific to the ciliated OSN signal-transduction pathway included adenylyl cyclase 3b (adcy3b) and $G_{\alpha olf2}$ (gnal2), while the targeted gene that was specific to the microvillus OSN signal-transduction pathway was phospholipase-C β3 (PLC-b3). Primer sequences are listed in Table 5.1.

Behaviour assay

Zebrafish were observed for their response to L-cysteine and taurocholic acid (TCA), which are detected by microvillus and ciliated OSNs, respectively (Lipschitz, 2002; Sato et al., 2005). Fish preference responses to the two odourants were observed in an avoidance-attraction trough, detailed in Shahriari et al., 2021. In short, two inflows that were situated on opposite ends of the trough drained water into the trough at a flowrate of 0.7 l/min. The opposing flows subsequently drained through a central fenestrated pipe (inner diameter of 7 mm), which created two contiguous bodies of water that had minimal intermixing (an area of roughly 5 cm contained mixed water). This permitted the addition of an odourant through one of the two inflows to be restricted to the corresponding half of the trough, as visualized with dye trials (Figure 3.1). A water column depth of at least 5 cm was maintained. Fish movement was recorded using overhead dome colour IR cameras (SAV-CD120; Matcow, Stow, OH USA) that were connected to an Elgato Video Capture software (Elgato Systems, CA USA). An opaque curtain surrounded the apparatus and environmental auditory input was kept to a minimum.

A single zebrafish was placed in an avoidance-attraction trough and allowed to acclimate for 30 min. Fish movement was recorded for the last 10 min of this acclimation period, after which 0.001 mM TCA or 0.1 mM L-cysteine was added into one of the two inflows, chosen at random. These concentrations were selected as they evoke similar generator potential responses at the olfactory epithelium (Michel & Lubomudrov, 1995). Fish response was recorded for another 10 minutes. The amount of time fish spent in the body of water that contained the odourant where there was no intermixing was manually scored for this 10 min period and compared to how much time the fish spent in the same area for the 10 min period prior to odour introduction. This area of the trough containing the odourants was termed as *odour zone*.

Data analysis

SigmaPlot 13.0 (Systat, San Jose, CA, USA) was used to run all statistical analyses. Statistical analyses for olfactory epithelium thickness, ciliated and microvillus OSN density, ciliated and microvillus OSN signal-transduction pathway gene expression, and preference responses, were based on two-way (Age group × Treatment type) analysis of variance (ANOVA). Holm-sidak tests were performed for post hoc analyses. Assumptions of normality and equal variance were tested for all analyses. Log transformations were performed on data that failed either assumption. If log transformations failed for two-way ANOVAs, analyses were used on ranked data instead. Outliers were removed if they passed Grubb's Method on GraphPad v9.5. Statistical significance was defined by $\alpha = 0.05$. All data are represented as mean $\pm$ SEM.

Results

Anatomical changes to the olfactory epithelium in middle- and old-aged fish after copper exposures

Copper reduced olfactory epithelium thickness in middle- and old-aged fish ($F_{1,2}$ = 40.3, $p < 0.001$; Figure 6.1). Specifically, olfactory epithelium thickness of middle- and old-aged fish were 17 ± 0.76 μm and 15 ± 0.76 μm, respectively. Copper exposures decreased olfactory epithelium thickness of middle- and old-aged fish to 12 ± 0.83 μm and 9.8 ± 0.76 μm, respectively ($p < 0.001$ for both middle- and old-aged fish; Figure 6.1). Copper, however, did not

affect ciliated or microvillus OSN density regardless of age ($F_{1,2}$ = 0.164, p = 0.690 for ciliated OSN density, and $F_{1,2}$ = 3.49, p = 0.074 for microvillus OSN optical density; Figure 6.2A, B).

Changes in expression of genes specific to the AC and PLC signal-transduction pathways in middle- and old-aged fish after copper exposures

Copper reduced expression of the two target genes specific to the AC signal-transduction pathway of ciliated OSNs ($F_{1,2}$ = 5.19, p = 0.042 for gnal2, and $F_{1,2}$ = 9.48, p = 0.010 for adcy3b; Figure 6.4A, B) and did not affect the target gene specific to the PLC signal-transduction pathway of microvillus OSNs ($F_{1,2}$ = 3.78, p = 0.076; Figure 6.4C). In regard to the AC signal-transduction pathway, copper affected gene expression only in old-aged fish (p = 0.024 and 0.020 for gnal2 and adcy3b, respectively).

Changes in response to an amino acid and a bile acid in middle- and old-aged fish after copper exposures

Age affected zebrafish behavioural response to L-cysteine ($\chi_{1,2}$ = 5.87, p = 0.021; Figure 6.5A). This age effect was seen only in control fish (p = 0.027). While middle-aged fish decreased the amount of time spent in the odour zone by 83 $\pm$ 24 s after L-cysteine was introduced into the trough, old-aged fish increased the amount of time spent in the odour zone by 37 $\pm$ 57 s after the amino acid was introduced into the trough. Copper affected responses to L-cysteine with near statistical significance ($\chi_{1,2}$ = 3.84, p = 0.059). The response seen in middle-aged control fish was absent in copper-exposed fish from the same age group (p = 0.056) as copper-exposed fish decreased the amount of time spent in the odour zone by only 2.9 $\pm$ 30 s after L-cysteine was introduced to the trough. Copper exposures did not affect responses to the amino acid in old-aged fish.

Responses to taurocholic acid were biphasic, with age driving whether an avoidance or attraction response was noted ($\chi_{1,2}$ = 8.09, p = 0.007). Middle-aged fish decreased the amount of time spent in the odour zone by 37 $\pm$ 25 s while old-aged fish increased the amount of time spent by 27 $\pm$ 15 s after TCA was introduced to the trough (p = 0.020; Figure 6.5B). Copper did not affect responses to TCA, regardless of age ($\chi_{1,2}$ = 1.45, p = 0.24).

Discussion

Copper is a well-known neurotoxicant that has been shown to reduce the ability of ciliated and microvillus OSNs to respond to bile acids and amino acids, respectively. What has not been explored, however, is whether such effects change over a lifetime. My study indicates that the toxicity of copper does change over age, and in an OSN-specific manner. Even though age did not affect copper toxicity to the zebrafish olfactory epithelium, it was only with middle-aged fish that copper disrupted behavioural responses to L-cysteine and not TCA. As aging-associated hyposmia was seen in old-aged fish responding to L-cysteine, copper toxicity to the olfactory epithelium and olfactory signal-transduction pathway were negligible.

Copper exposures decreased olfactory epithelium thickness in zebrafish regardless of age. This finding further supports the notion that copper induces apoptosis at the olfactory epithelium (Julliard et al., 1996; Wang et al., 2013; Lazzari et al., 2017). Even though damage to the olfactory epithelium progresses over multiple days, recovery is possible after pressure from acute copper exposures is removed, as progenitors cells proliferate to replace damaged cells (Julliard et al., 1996; Lazzari et al., 2017; Ma et al., 2018; Lazzari et al., 2019). Age may affect this regenerative capacity of the olfactory epithelium as proliferation of progenitor cells decreases with aging (Jia & Hegg, 2015; Ueha et al., 2018; Zhang et al., 2018). Therefore, copper-induced decreases in olfactory epithelium thickness may be reversible in middle-aged fish while irreversible in old-aged fish.

Even though copper exposures decreased olfactory epithelium thickness, it did not affect the density of ciliated or microvillus OSNs regardless of age. This contradicts previous findings of differential toxicity to ciliated and microvillus OSN density, of which ciliated OSNs are more sensitive to copper exposures than microvillus OSNs are (Lazzari et al., 2017; Ma et al., 2018). It is possible that zebrafish from our study had high metallothionein activity, which is expressed in OSNs and binds to heavy metals to minimize olfactory toxicity (Skabo et al., 1997; Williams & Gallagher, 2013; Williams et al., 2016). Besides metallothioneins, biotransformation enzymes including glutathione-S transferase are expressed in OSNs (Starcevic & Zielinski, 1995). It is also possible that indirect quantification using optical density values may not capture changes in ciliated and microvillus OSN numbers when olfactory epithelium thickness decreases.

Age influenced subcellular olfactory toxicity. Specifically, copper exposures downregulated expression of genes specific to the AC signal-transduction pathway of ciliated OSNs in old-aged zebrafish only. This aligns with previous studies that also demonstrated that copper affected gene expression in the AC signal-transduction pathway (Tilton et al. 2008; Tilton et al. 2011; Pfister et al. 2020). As bile acids activate the AC signal-transduction pathway, copper may disrupt olfactory signalling during bile acid stimulation even though copper did not affect ciliated OSN density (Lipschitz, 2002; Sato et al., 2005). However, it is important to note that changes in gene expression of the AC transduction pathway may not dictate changes in transduction. Since copper exposures did not affect expression of plcb3, which is specific to the PLC signal-transduction pathway of microvillus OSNs, differential toxicity was seen in the aging population.

There was an expectation that copper exposures would affect zebrafish response to L-cysteine or TCA to some degree, as copper exposures affected olfactory epithelium thickness regardless of age and affected gene expression of the AC-signal transduction pathway in old-aged fish. Middle-aged fish avoided L-cysteine and TCA. While this avoidance to L-cysteine was expected, fish avoiding TCA contradicts previous findings of attraction towards the bile acid (Vitebsky et al., 2005; Koide et al., 2009). However, TCA is a social cue and therefore, may elicit different responses between fish. Copper exposures affected middle-aged fish response to L-cysteine and not to TCA. While this is the first study to demonstrate that copper disrupts responses to L-cysteine, previous work on cadmium toxicity, which affects ciliated and microvillus OSNs, showed that avoidance responses to the amino acid disappeared after cadmium exposure (Williams & Gallagher, 2013; Williams et al., 2016). Furthermore, there was a poor translation in how copper exposures affected olfactory epithelium anatomy and how the contaminant affected olfactory functionality. Even though olfactory epithelium thickness decreased while ciliated and microvillus OSN density remained unchanged, only responses to L-cysteine were affected. Besides copper, aging disrupted fish avoidance to L-cysteine and to TCA as this preference response seen in middle-aged fish disappeared in old-aged fish. This demonstrates aging-associated hyposmia. Therefore, when old-aged zebrafish were exposed to copper, no effects on olfactory functionality were observed. While copper may not have affected responses to TCA regardless of age, it only affected responses to L-cysteine within middle-aged fish. Altogether, copper decreased olfactory epithelium thickness and signal-transduction gene

expression, but these anatomical and molecular changes had a negligible impact on olfactory functionality due to aging already inducing hyposmia. Differential toxicity typically seen from copper exposures may also be minimized in the aging population.

In conclusion, age influenced how copper affected olfactory toxicity in zebrafish. This influence may come in one of two ways. First, aging may exacerbate olfactory toxicity such as what was observed in the downregulation of genes specific to the AC-signal transduction pathway in old-aged fish only. However, aging may also minimize the significance of olfactory toxicity from copper exposures as hyposmia is already induced. Throughout an animal's lifespan, olfaction may be most functional at middle age, but it is also during this time when olfaction may be most sensitive to disruption from toxicants including copper exposures.

Figures

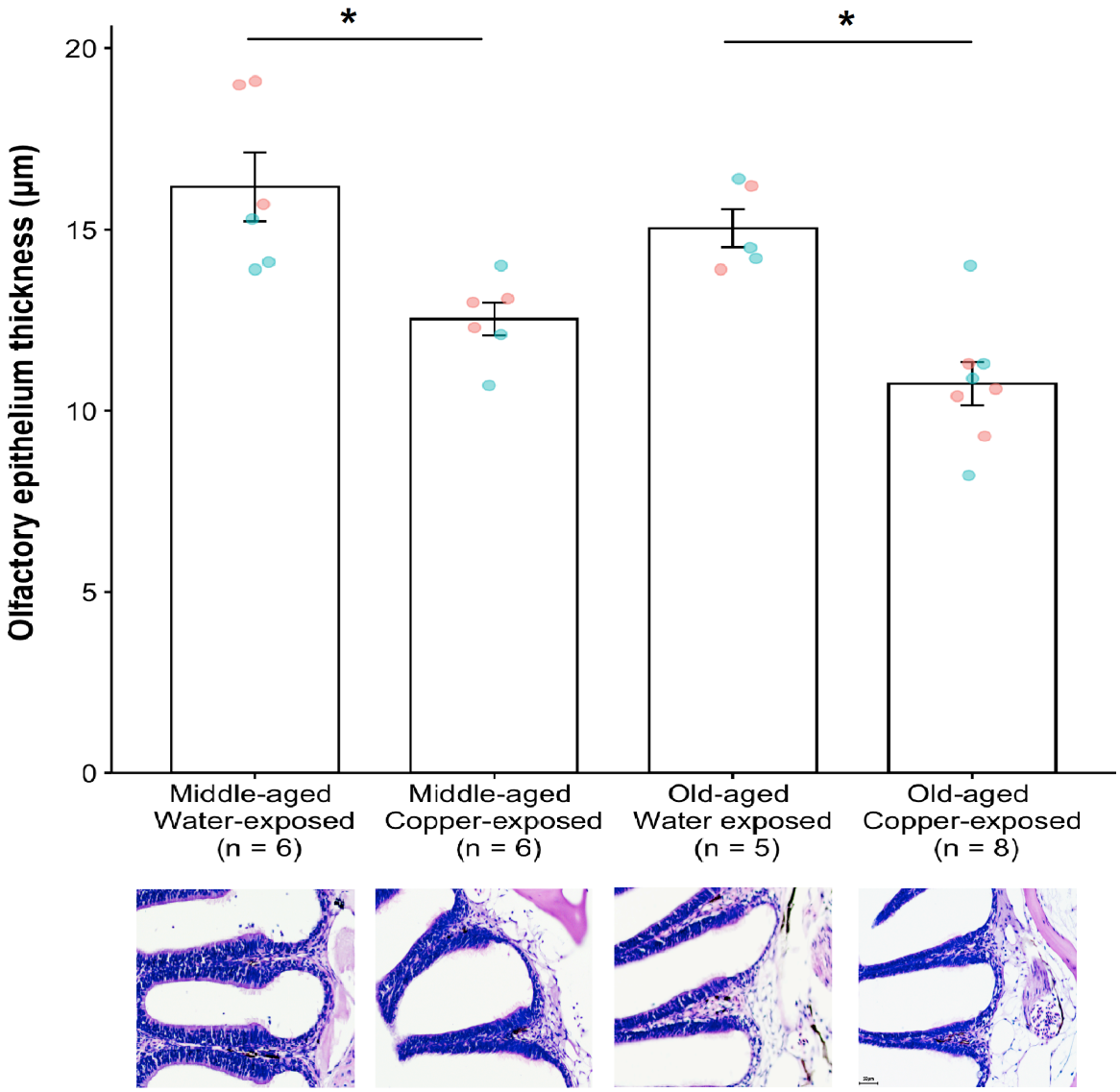


Figure 6.1. Olfactory epithelium thickness in middle- and old-aged zebrafish that underwent water or copper exposures. H&E was used to stain the olfactory epithelium with images captured at 20x magnification. Bar graphs represent mean ± SEM. Data points from individual female and male fish are shown in red and blue dots, respectively. Statistical analysis was based on a two-way ANOVA ($\alpha = 0.05$) with asterisks indicating significant differences.

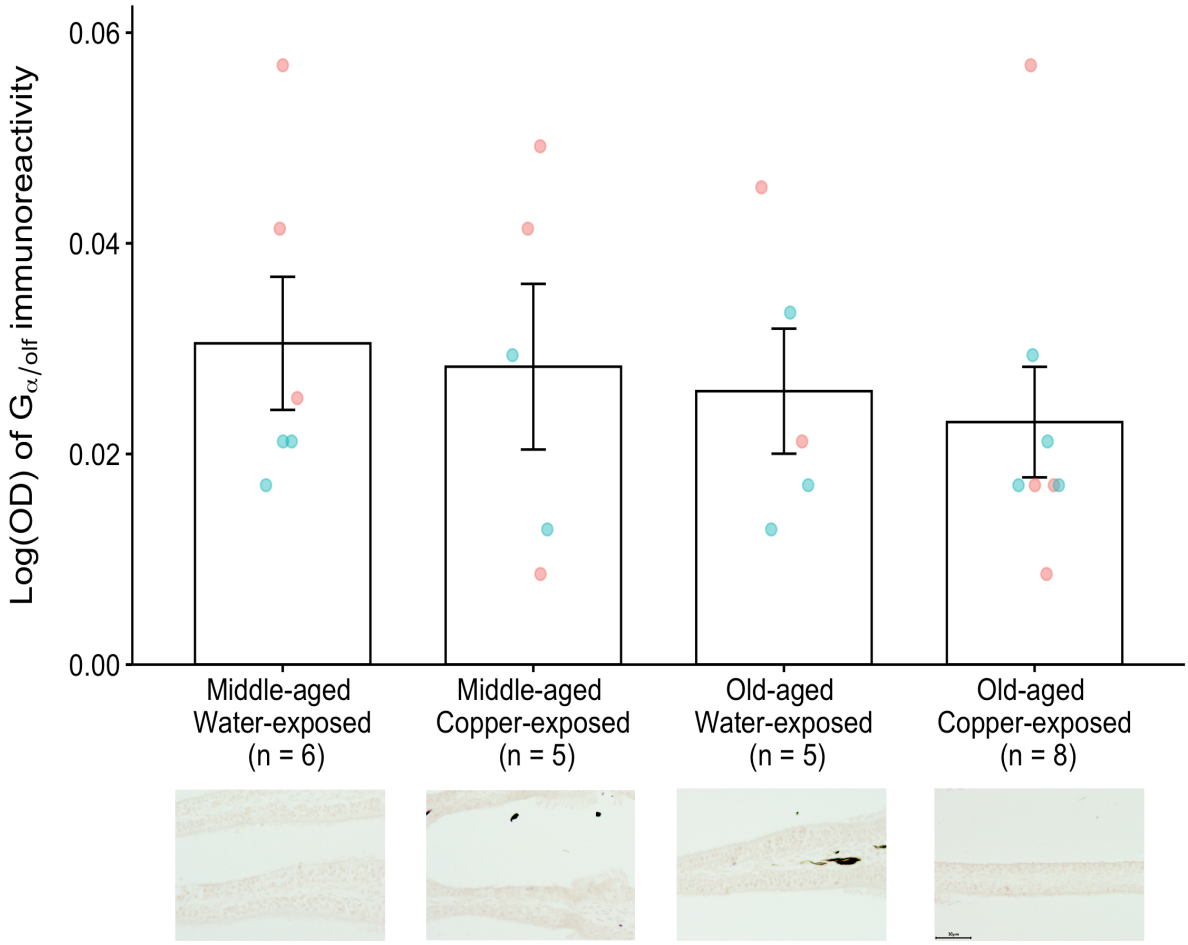


Figure 6.2. Ciliated OSN density in middle- and old-aged zebrafish that underwent water or copper exposures. Density was indirectly measured through optical density values of $G_{\alpha/olf}$ immunoreactivity from IHC staining of the olfactory epithelium. Bar graphs represent mean ± SEM. Data points from individual female and male fish are shown in red and blue dots, respectively. Statistical analysis was based on a two-way ANOVA ($\alpha = 0.05$).

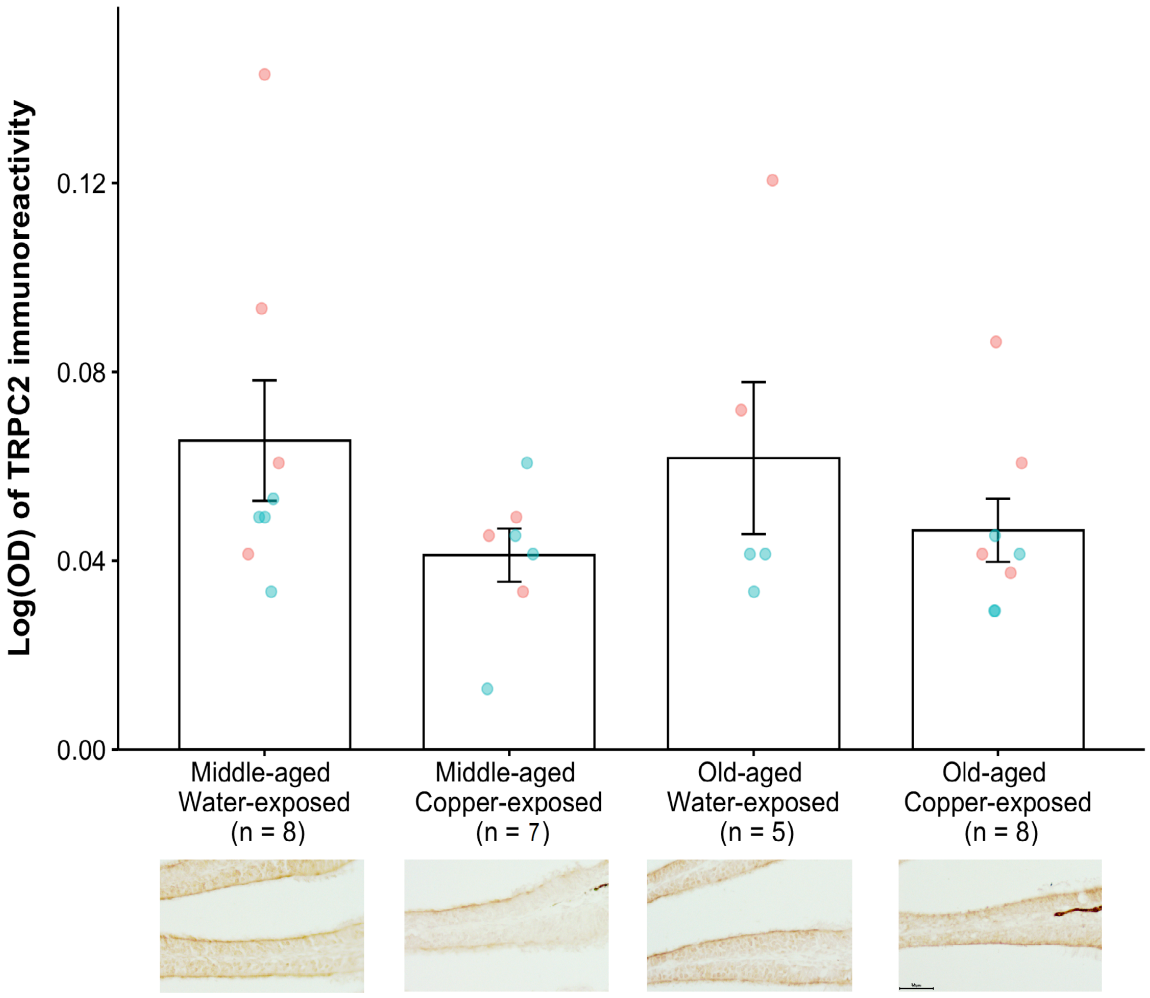
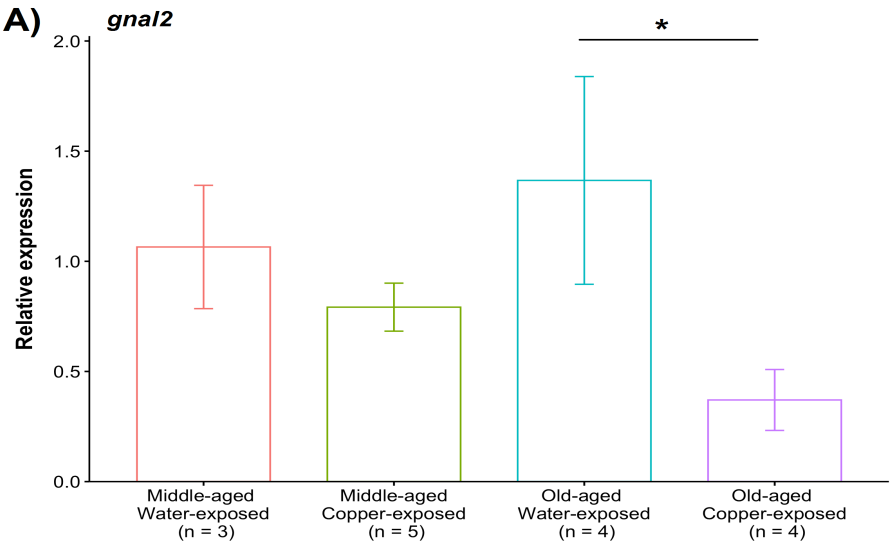
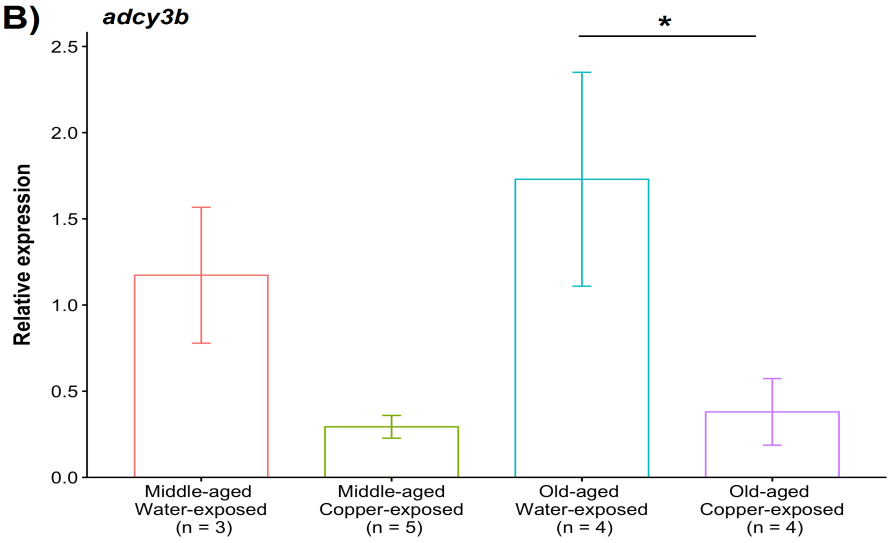


Figure 6.3. Microvillus OSN density in middle- and old-aged zebrafish that underwent water or copper exposures. Density was indirectly measured through optical density values of TRPC2 immunoreactivity from IHC staining of the olfactory epithelium. Bar graphs represent mean ± SEM. Data points from individual female and male fish are shown in red and blue dots, respectively. Statistical analysis was based on a two-way ANOVA (α = 0.05).


A)
gnal2
*
Relative expression
2.0
1.5
1.0
0.5
0.0
Middle-aged Water-exposed (n = 3)
Middle-aged Copper-exposed (n = 5)
Old-aged Water-exposed (n = 4)
Old-aged Copper-exposed (n = 4)


B)
adcy3b
*
Relative expression
2.5
2.0
1.5
1.0
0.5
0.0
Middle-aged Water-exposed (n = 3)
Middle-aged Copper-exposed (n = 5)
Old-aged Water-exposed (n = 4)
Old-aged Copper-exposed (n = 4)

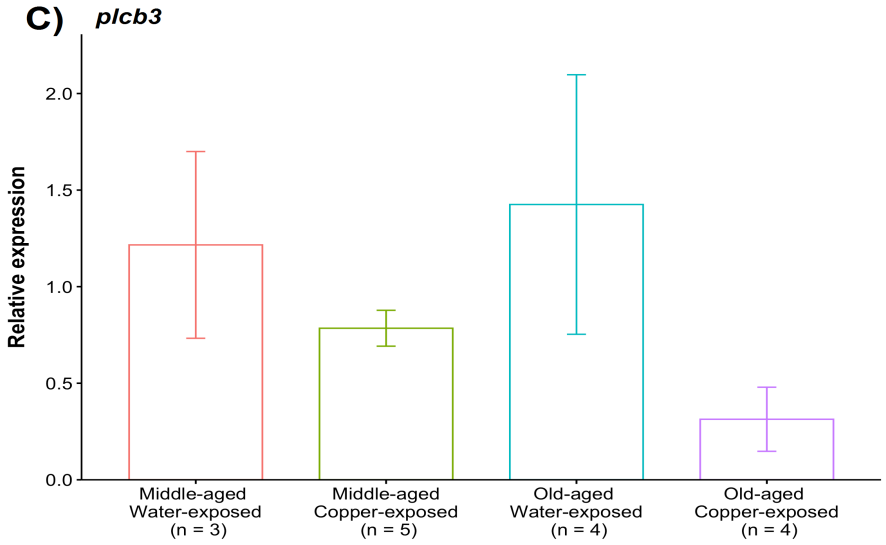
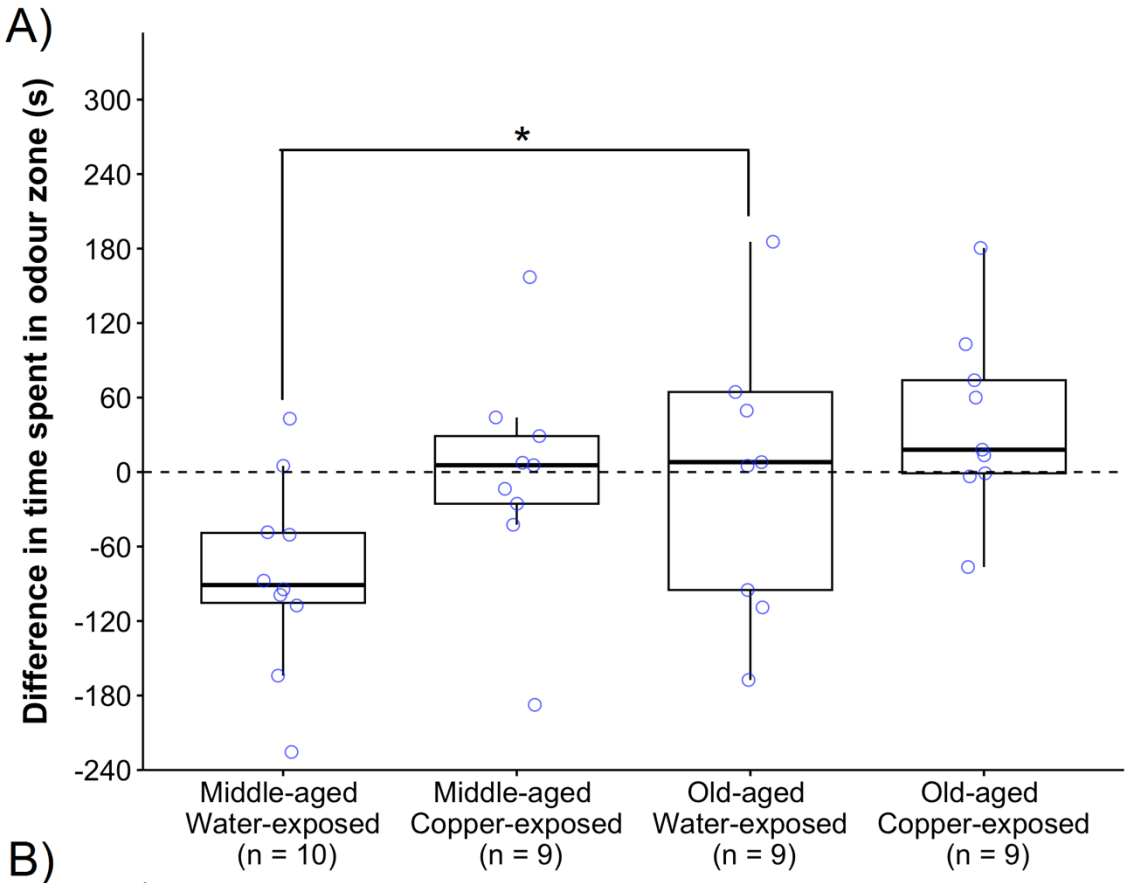

C)
plcb3
Relative expression
2.0
1.5
1.0
0.5
0.0
Middle-aged Water-exposed (n = 3)
Middle-aged Copper-exposed (n = 5)
Old-aged Water-exposed (n = 4)
Old-aged Copper-exposed (n = 4)

Figure 6.4. Relative expression of A) gnal2 and B) adcy3b, which are involved in the AC pathway, and C) plcb3, which is involved in the PLC pathway, based on qPCR experiments. Genes were normalized to TUB-A1. Relative changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method. Bar graphs represent mean $\pm$ SEM. Statistical analysis was based on a two-way ANOVA (α = 0.05) with asterisks representing statistical significance.


A)
Difference in time spent in odour zone (s)
300
240
180
120
60
0
-60
-120
-180
-240
*
Middle-aged Water-exposed (n = 10)
Middle-aged Copper-exposed (n = 9)
Old-aged Water-exposed (n = 9)
Old-aged Copper-exposed (n = 9)

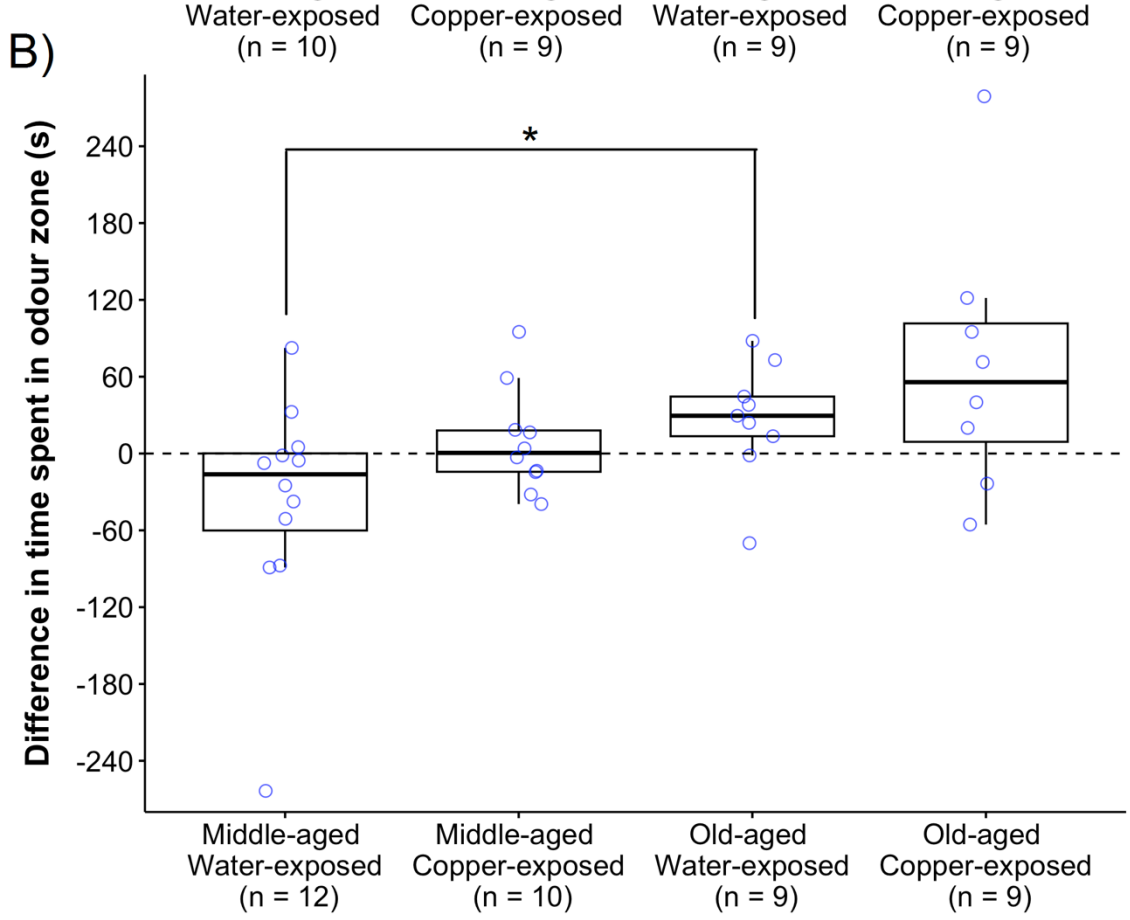

B)
Difference in time spent in odour zone (s)
240
180
120
60
0
-60
-120
-180
-240
*
Middle-aged Water-exposed (n = 12)
Middle-aged Copper-exposed (n = 10)
Old-aged Water-exposed (n = 9)
Old-aged Copper-exposed (n = 9)

Figure 6.5. Behaviour of middle- and old-aged zebrafish that underwent water or copper exposures. Fish preference responses to A) 0.1 mM L-cysteine and B) 0.001 mM taurocholic acid, were observed in an avoidance-attraction trough. Preference responses were determined by measuring the difference in how much time fish spent in the odour zone between the 10 min period after odourant introduction and the 10 min period prior to odourant introduction. Box and whisker plots show the spread of data with the box representing 50% of the data, the whiskers representing the bottom and top 25% of data, and the horizontal line representing the median. Data points from individual fish are shown in blue unfilled dots. Statistical analysis was based on two-way ANOVAs on ranked data ($\alpha = 0.05$) with asterisks representing statistical significance.

Chapter 7: Summary and general conclusions

Olfaction allows animals to receive invaluable chemical information about their environment, such as the location of food, predators, and kin or mates (Petranka et al., 1987; Nevitt, 1999; Barata et al., 2007; Roberts et al., 2018). Odourants are detected by OSNs, which are located at the olfactory epithelium (Morrison & Costanzo, 1990, 1992). Generation of signals during odourant detection are mediated by signal-transduction pathways of OSNs (Pace et al., 1985; Sklar et al., 1986; Berghard & Buck, 1996). These signals propagate from the OSNs to higher-order processing centers that mediate physiology and behaviour (Pace et al., 1985; Levy et al., 1991; Xu et al., 2005). In chapter 2 of my thesis, I reviewed the olfactory signal-transduction pathways of OSNs with an emphasis on how their impairment may change olfactory functionality (Shahriari et al., 2023).

Even though the olfactory system develops early, olfaction changes throughout an animal's lifespan. This is largely influenced by the environment. For example, the chemical environment during early development shapes future olfactory-mediated responses (Schaal et al., 2000; Gerlach & Lysiak, 2006). Moreover, toxicants may damage the olfactory epithelium and disrupt responses normally seen towards odours (Tierney et al., 2010). Besides the environment, physiological processes also affect olfaction. While olfaction continues to develop through to adulthood, there is a certain point at which olfaction deteriorates from physiological aging-related processes (Patel & Larson, 2009; Rawson et al., 2012; Suzuki et al., 2021). This reduced sense of smell is referred to as hyposmia. These environmental and physiological factors are hardly if ever isolated, and instead interact to mediate olfaction. For example, age may affect olfactory toxicity from toxicant exposures. For my thesis, I examined how the nature of olfaction changes throughout life in the popular vertebrate model, the zebrafish. My thesis objective was split into two research questions: how does the chemical environment of early developing zebrafish affect future olfactory-mediated behaviours and how does olfaction change with age?

Responses to a binary mixture of attracting and repelling odourants speculated across age

In a natural setting, animals often detect multiple odourants simultaneously. For example, animals may encounter predator- and prey-relevant odourants in mixture. In this situation, a

decision may need to be made on whether to avoid or move towards this mixture of repelling and attracting odourants. In chapter 3 of my thesis, I examined how animals may respond to a binary mixture of odourants that on their own elicit opposite responses. Specifically, I observed adult zebrafish response to a binary mixture that contained an attracting amino acid and a repelling amino acid (Shahriari et al., 2021). I built and validated an avoidance-attraction trough to observe fish preference responses to odourants. Zebrafish elicited an attraction response to L-alanine and an avoidance response to L-cysteine. These behaviours were olfactory-mediated, which was confirmed by nasal tissue ablation. When fish were exposed to L-alanine and L-cysteine together, an avoidance response was observed. When the repelling odourant's concentration decreased, the avoidance response toward the mixture became attraction. In comparison to another study, finches displayed a combination of avoidance or delayed attraction to food odours in the presence of predator odours (Roth et al., 2008). In an ecological context, animals avoid foraging in areas that have a high risk of predation (Holbrook & Schmitt, 1988; Brinkerhoff et al., 2005; Dupuch et al., 2009; Suselbeek et al., 2014). Altogether, this means that repelling odourants outweigh attracting odourants.

My findings on how animals respond to a binary odourant mixture of an attracting amino acid and a repelling amino acid were based on behaviours elicited in zebrafish from a particular age group. Avoidance responses towards this binary odourant mixture of L-alanine and L-cysteine may change with age, especially in aging fish that have hyposmia. Specifically, the avoidance response to the mixture may be absent in the aging population, especially since amino acid-detecting microvillus OSNs decrease in number with age (Lipschitz, 2002; Sato et al., 2005). Furthermore, responses to odourant mixtures may depend on what subset of odourants are being detected. Zebrafish exposed to a binary mixture of bile acids that attract and repel may demonstrate a response that is different from the amino acid mixture of L-alanine and L-cysteine. While the avoidance response to the amino acid mixture may be absent in aging zebrafish, responses to a mixture of attracting and repelling bile acids may be observed across all ages as the number of bile acid-detecting ciliated OSNs stays the same across age. Furthermore, animals tend to have higher sensitivity to repelling odourants than to attracting odourants (Michel & Lubomudrov, 1995; Boesveldt et al., 2010; Valentincic et al., 2011). Therefore, responses to repelling odourants or to a binary mixture that contained a repelling and an attracting odourant may persist throughout life.

Olfactory imprinting

Animals imprint to odourants during early development (Carter & Marr, 1970; Shoji et al., 2000; Atema et al., 2002; Gerlach & Lysiak, 2006). Olfactory imprinting is associated with the formation of long-term memories that lead to changes in behavioural responses to the imprinted odourants (Marr & Gardner, 1965; Fillion & Blass, 1986). As animals detect multiple odourants simultaneously, interactions between odourants during their detection may affect olfactory imprinting (Kang & Caprio, 1997; Ishii et al., 2008; McClintock et al., 2020). Furthermore, the addition of an odourant may mask responses normally seen towards imprinted odourants (Cain, 1975; Osada et al., 2013). For the fourth chapter of my thesis, I tested if zebrafish could imprint to single amino acids and to a binary amino acid mixture with the expectation that the assay would less likely show that fish could imprint to multiple amino acids simultaneously over single amino acids. The rationale for this was that interactions between odourants in mixture would disrupt olfactory imprinting or mask responses to imprinted odourants. To test imprinting, I used the avoidance-attraction trough from the third chapter since fish responded to single odourants and to a binary odourant mixture. The imprinting assay was validated by demonstrating that zebrafish imprint to β-phenylethyl alcohol, which acted as a positive control (Harden et al., 2006; Calfún et al., 2016). Fish imprinted to L-leucine and L-lysine, but not to L-valine. However, when early developing zebrafish were exposed to a binary mixture of L-leucine and L-lysine, fish did not imprint to either amino acid separately or as a mixture. As a result, olfactory imprinting was easily disrupted by the addition of an odourant to the environment of early developing zebrafish. Future studies should consider the complexity of the olfactory environment when examining imprinting.

Olfactory imprinting often leads to the formation of positive associations with odourants, such as the odourants used for homing behaviour, kin recognition, and forming maternal attachments (Carter & Marr, 1970; Russell, 1976; Shoji et al., 2000; Harden et al., 2006; Hinz et al., 2013). I also demonstrated this with zebrafish imprinting to L-leucine. While naïve fish avoided L-leucine, imprinted fish displayed an attraction response to the amino acid. This change from an avoidance response to an attraction response in L-leucine-imprinted fish suggests that a similar finding may be observed with other amino acids that evoke avoidance responses. For example, zebrafish as early as 3 dpf avoid L-cysteine and this avoidance persists through to

adulthood (Vitebsky et al., 2005; Shahriari et al., 2021). However, early developing fish that are continuously exposed to L-cysteine may associate this amino acid with their rearing environment and therefore display attraction responses when re-exposed as adults. While positive associations are often formed during olfactory imprinting, I demonstrated an example of imprinting that may have led to a negative association as fish that imprinted to L-lysine avoided the amino acid when as adults. This avoidance response to imprinted odours is also seen with kin odour to prevent inbreeding (Roberts & Gosling, 2003; Gerlach & Lysiak, 2006; Gerlach et al., 2008; Bonadonna & Sanz-Aguilar, 2012). Regardless of whether positive or negative associations are formed to individual odourants during imprinting, the addition of a second odourant to the environment of early developing animals may disrupt imprinting altogether.

The mechanism underlying olfactory imprinting may be mediated by hormones. For example, thyroid hormones are elevated in salmon during imprinting and correspondingly, increase proliferation of neuronal precursors at the olfactory epithelium (Lema & Nevitt, 2004). This increased number of OSNs may explain why imprinting of amino acids potentiates the generator potential in the salmon olfactory epithelium during odourant detection (Yamamoto et al., 2010). Furthermore, long-term potentiation may be involved in the imprinting process. Prior to seaward migration when salmon imprint to odours from their natal stream, increased thyroid hormone levels lead to an upregulation of N-methyl-D-aspartate receptors (NMDARs) at the olfactory bulb (Ueda et al., 2016). Later in life when salmon return to their natal stream for spawning, NMDARs are upregulated by gonadotropin releasing hormone, demonstrating memory retrieval. As imprinting is associated with increased generator potential and long-term upregulation of NMDARs, responses to imprinted odours may persist during olfactory degradation from aging processes.

Olfaction across age

The olfactory system forms very early in development and continues to develop as animals reach adulthood. For example, the olfactory epithelium of zebrafish increases in size as the fish grow, all the way to old age (Hansen & Zeiske, 1998). Moreover, this epithelium is organized into finger-like projections known as lamellae, and the number of lamellae increases with age (Hansen & Zeiske, 1998). However, towards the end of an animal's lifespan,

physiological aging processes may cause a decrease in olfactory epithelium thickness and a reduction in OSN density (Jia & Hegg, 2015; Ueha et al., 2018; Zhang et al., 2018). The natural decline in OSNs derives from increased apoptosis of these cells that exceeds an age-associated reduction in precursor proliferation.

Vertebrates often have multiple OSN classes, which have different sensitivities to subsets of odourants. Two OSN classes found across vertebrates including in zebrafish are ciliated and microvillus OSNs, which in fish detect bile acids and amino acids, respectively (Lipschitz, 2002; Sato et al., 2005). It has been shown that OSNs have class-specific regenerative capacity due to differences in precursor proliferation, as demonstrated using toxicant exposures (Lazzari et al., 2017; Ma et al., 2018). Therefore, it is possible that age also has class-specific effects on OSN density with impacts on responses to associated odourants. In chapter five of my thesis, I demonstrated that olfactory epithelium thickness remained the same as zebrafish aged, but overall cell density decreased. Class-specific changes to OSN density were observed, as ciliated OSN density was similar as fish aged, but microvillus OSN density decreased with aging. Since age affected microvillus OSNs only, there was an expectation that age would affect neuronal activation and behaviour to amino acids and not to bile acids. However, age-associated changes to the olfactory epithelium did not translate to changes in neuronal activation or olfactory-mediated behaviour as seen in chapters five and six. Specifically, neuronal activation within the olfactory epithelium increased in middle-aged fish and not young- or old-aged fish that were exposed to an amino acid (L-cysteine) or to a bile acid (TCA). Also, only middle-aged fish elicited an avoidance response to both odourants and had an increased number of sharp turns in response to L-cysteine. Therefore, olfaction was most functional in zebrafish at middle age. In comparison to a study that examined age-associated changes to human olfaction, frequency of participants accurately detecting an odour was highest in a middle-aged cohort of between 40 and 60 years old (Barber, 1997). Performance dramatically decreased with people over 60 years old. At middle age, zebrafish body size is at its highest. It is possible that the development of olfaction correlates with body size in addition to age. While this notion has not been tested in regard to the development of the olfactory system, sexual maturity has been suggested to depend more on size than age (Spence et al., 2008). Furthermore, as body size is influenced by thyroid hormones and growth hormones, changes in olfaction across age is likely to be mediated by hormones (Bartke et al., 1998; Wen & Shi, 2015).

Throughout an animal's lifespan, there is constant chemical pressure to the olfactory system from the environment such as through toxicant exposures. The olfactory epithelium is especially vulnerable to damage by toxicants as it is in direct contact with the environment. For example, copper enters the hydrosphere and induces apoptosis at the olfactory epithelium (Julliard et al., 1996; Wang et al., 2013; Lazzari et al., 2017). Copper also imposes class-specific toxicity on OSNs as ciliated OSNs are more sensitive to copper toxicity than microvillus OSNs (Lazzari et al., 2017; Ma et al., 2018). Even though the olfactory epithelium has high resistance to toxicants due to high biotransformation activity and OSN class-specific regenerative capacity, this resistance to olfactory toxicity decreases with age (Ben-Arie et al., 1993; Monod et al., 1994; Malavolta et al., 2008; Williams & Gallagher, 2013; Xu et al., 2019). For chapter six of my thesis, I examined how age affects olfactory toxicity from copper exposures with a focus on differential effects between ciliated and microvillus OSNs. Age did not affect copper toxicity on the zebrafish olfactory epithelium anatomy. Specifically, olfactory epithelium thickness decreased while ciliated and microvillus OSN density were unaffected after copper exposures regardless of age. Middle-aged fish and not old-aged fish responded with avoidance to L-cysteine and to TCA. Therefore, when middle- or old-aged fish were exposed to copper, the neurotoxicant only affected middle-aged fish response to the amino acid. Copper did not affect fish response to the bile acid, demonstrating that olfactory toxicity may depend on the subset of odourants that is being detected. This specificity in olfactory toxicity has been seen in other studies that exposed fish to copper and demonstrated differential effects on responses towards amino acids and bile acids (Kolmakov et al., 2009; Dew et al., 2014; Ma et al., 2018; Razmara et al., 2019). Overall, olfaction may be most sensitive to disruption from toxicants including copper when it is most functional.

While olfaction may be most functional at middle age and therefore have the most meaningful disruption from toxicants, it does not mean that consequences to olfaction will be minimal if animals were exposed to toxicants at other ages. For example, toxicant exposures during early development may have lasting adverse effects on olfactory-mediated behaviours as imprinting to odourants may be disrupted. In fact, resistance to toxicants may be comparable between early developing and aging animals as biotransformation activity is low in both age groups (Xu et al., 2019). Due to low resistance towards toxicants, toxicant exposures during imprinting may induce a rate of apoptosis of OSNs that exceeds enhanced proliferation of

neuronal precursors from elevated thyroid hormone levels (Lema & Nevitt, 2004). As a result, enhanced generator potentials to imprinted odourants may be absent (Yamamoto et al., 2010). Olfactory toxicity during imprinting may adversely affect behaviours relevant to increased fitness including homing, kin and maternal recognition, and inbreeding avoidance (Carter & Marr, 1970; Russell, 1976; Shoji et al., 2000; Gerlach & Lysiak, 2006; Hinz, Namekawa, et al., 2013).

A focus of my thesis was to examine age and toxicant effects to the olfactory epithelium anatomy. In chapters five and six, I demonstrated that toxicity to the olfactory epithelium may be limiting as it translates poorly to changes in olfactory functionality. Instead, age and toxicant-associated changes to olfactory functionality may depend more heavily on how higher-order processing centers are affected. For example, copper inhibited the electrical current at the olfactory bulb of rats exposed to an amino acid (Trombley & Shepherd, 1996). This inhibition was through copper targeting NMDARs. Therefore, toxicants that disrupt olfactory processing at the olfactory bulb such as through targeting NMDARs may affect fundamental olfactory processes including imprinting, even if signals generated at the olfactory epithelium are not affected (Ueda et al., 2016).

Future Direction

For my thesis, I examined changes to the nature of olfaction throughout the life of zebrafish by investigating how the olfactory environment during early development changes future olfactory-mediated behaviours and how olfaction changes throughout the adult zebrafish life span. A future direction would be to link the two investigations by examining how the olfactory environment of early developing animals affects olfaction throughout an animal's lifespan. For example, olfactory imprinting studies expose animals to odourant(s) early on in life and observe response to the odourants at one time point later in life (Carter & Marr, 1970; Shoji et al., 2000; Atema et al., 2002; Gerlach & Lysiak, 2006; Hinz, Kobbenbring, et al., 2013). However, olfaction changes as animals age. Therefore, future studies should examine how age affects changes to behaviour from imprinting processes.

How age affects olfaction are often studied in regard to changes in olfactory epithelium morphology, OSN density and olfactory-mediated behaviours. However, studies have not

focused on subcellular mechanisms underlying age-associated changes to olfaction. As animals age, the prevalence of free radicals and reactive oxygen species within cells increases (Liochev, 2013; Harman, 2002). These toxic agents damage organelles. In chapter 5 of my thesis, I demonstrated that age differentially affects ciliated and microvillus OSNs. Therefore, it is possible that free radicals and reactive oxygen species affect organelles within ciliated and microvillus OSNs differently. Furthermore, there may be class-specific resistance towards their toxicity with differences in activity of biotransformation enzymes such as glutathione-s-transferase, which protect the cell from oxidative stress (Ketterer, 1998). I suggest that studies explore mechanisms underlying OSN class-specific changes across age.

Age affects olfactory-mediated behaviours and this has been associated with a change in olfactory epithelium anatomy. However, there was a poor translation between the two endpoints. This may derive from the possibility that olfactory-mediated behaviours depend more heavily on the processing of odours rather than the sensitivity of their detection. Therefore, future studies should focus on examining how age affects odour processing that may lead to changes in behaviour.

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