8-3-2017

Description of olfactory sensory neurons, olfactory bulb glomeruli and effect of sensory experience on glomerular development in early life history of Chinook salmon, Oncorhynchus tshawytscha

Alexandra Zygowska
University of Windsor

DESCRIPTION OF OLFACTORY SENSORY NEURONS, OLFACTORY BULB GLOMERULI AND EFFECT OF SENSORY EXPERIENCE ON GLOMERULAR DEVELOPMENT IN EARLY LIFE HISTORY OF CHINOOK SALMON, *Oncorhynchus tshawytscha*

By

Alexandra Zygowska

A Thesis
Submitted to the Faculty of Graduate Studies
through the Department of Biological Sciences
in Partial Fulfillment of the Requirements for
the Degree of Master of Science
at the University of Windsor

Windsor, Ontario, Canada

2017

Description of olfactory sensory neurons, olfactory bulb glomeruli and effect of sensory experience on glomerular development in early life history of Chinook salmon, *Oncorhynchus tshawytscha*

By

Alexandra Zygowska

APPROVED BY:

_____

S. Ananvoranich,
Department of Chemistry and Biochemistry

_____

T. Pitcher,
Department of Biological Sciences

_____

B. Zielinski, Advisor
Department of Biological Sciences

June 9th 2017

DECLARATION OF ORIGINALITY

I hereby certify that I am the sole author of this thesis and that no part of this thesis has been published or submitted for publication.

I certify that, to the best of my knowledge, my thesis does not infringe upon anyone's copyright nor violate any proprietary rights and that any ideas, techniques, quotations, or any other material from the work of other people included in my thesis, published or otherwise, are fully acknowledged in accordance with the standard referencing practices. Furthermore, to the extent that I have included copyrighted material that surpasses the bounds of fair dealing within the meaning of the Canada Copyright Act, I certify that I have obtained a written permission from the copyright owner(s) to include such material(s) in my thesis and have included copies of such copyright clearances to my appendix.

I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis committee and the Graduate Studies office, and that this thesis has not been submitted for a higher degree to any other University or Institution.

ABSTRACT

In teleosts, G_{olf} positive ciliated, calretinin positive microvillous and S100 positive crypt morphotypes of olfactory sensory neurons (OSNs) respond to different odorants (bile acids, amino acids and sex steroids respectively) and project axons to glomeruli in specific stereotyped areas of the olfactory bulb. In this thesis, the diversity of OSNs and their olfactory bulb glomeruli was described using calretinin, G_{olf} and S100 immunocytochemistry in Chinook salmon embryos, larvae and fingerlings. Olfactory learning, such as imprinting to natal waters, occurs during these early stages. Calretinin immunoreactivity was present in lateral glomeruli lG_1, $lG_{3/4}$, lG_6 and dorsal lateral glomerular chain (dlG) from embryonic stages. At late larval and fingerling stages calretinin also labeled some medial anterior glomeruli (maG), ventromedial glomeruli (vmG) and a ventroposterior glomerulus (vpG_1). G_{olf} immunoreactivity was seen in medial and ventral olfactory bulb regions, specifically in ventral medial glomeruli vmG_x, vmG_7 and the dlG in embryos and additional dorsal glomeruli (dG), maG, smaller vmGs and vpG_2 starting from late yolk-sac larval stage. S100 immunoreactivity was present in lateral regions of the olfactory bulb at the fingerling stage. Amino acid (40-450 nM), a potential imprinting odour, or phenylethyl alcohol (10^{-7}M), an odour used in salmon imprinting studies and not found endogenously in municipal water, exposure over larval development resulted in decreased glomerular volumes of $lG_{3/4}$ or vmG_7 at early yolk-sac larvae and late yolk-sac larvae stages, respectively. No difference was observed at other larval periods or for other lGs and vmGs. Different timing of development of various glomeruli may be important for understanding their role in salmon behaviour and the learning of olfactory cues.

ACKNOWLEDGMENTS

I would like to thank the following people for their help and support during my Masters research. My family, Arkadiusz, Krystyna, Dominika and Christopher, thank you for your encouragement, love and always being there for me.

I am very thankful for the guidance I received from my Masters supervisor Dr. Barbara Zielinski. Thank you for sharing your knowledge with me and allowing me the opportunity to do research. You opened up the world of olfactory neurobiology to me and challenged me to keep building upon my knowledge and improving my research. Thanks to you I grew as a researcher.

I am grateful for the help I received from my committee members Dr. Trevor Pitcher and Dr. Sirinart Ananvoranich. Your questions and suggestions helped focus my research and made me realise how much more there still is to learn.

Thank you Linda Sterling and Theresa Warner for your help with raising and taking care of the Chinook salmon. I appreciate all the technical support I received from Bob Hodge. Additionally, I would like to thank Cory Ochs for introducing me to olfactory research with the Chinook salmon and teaching me experimental techniques. Thank you Tina Suntres for your help with optimizing experimental procedures, and Yucca Albano and Kristyn Quenneville for your assistance with the analysis. Thank you all for your help and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

OSN – olfactory sensory neuron

CR – calretinin

dlG – dorsal lateral glomerular chain

dG – dorsal glomeruli

lG – lateral glomeruli

maG – medial anterior glomeruli

vmG – ventromedial glomeruli

vpG – ventroposterior glomeruli

PEA – phenyl ethyl alcohol

PBS – phosphate buffered saline

CHAPTER 1: OLFACTORY SENSORY NEURONS AND THEIR OLFACTORY BULB PROJECTIONS IN TELEOSTS AND THEIR IMPORTANCE TO OLFACTORY IMPRINTING IN HOMING MIGRATION

1.1 General Overview

Olfaction in fish is important in mediating various behaviours and interactions with their environment. Fish can respond differentially to specific signals and cues they encounter in their aquatic environments. Some of the behaviours mediated by the olfactory system include finding mates, avoiding predators, feeding activity, social interactions and guiding fish back to their natal streams for spawning (Sorensen and Caprio 1998). Of particular interest in this thesis is the role of olfaction in mediating the long distance homing migration of salmonids for spawning. Behavioural studies of salmonids after olfactory occlusion have indicated that fish rely on their sense of smell to guide their homing migration and without this sense they are unable to reliably differentiate their natal stream (silver salmon, *O. kitsutch*: Wisby and Hasler 1954, Chinook salmon, *O. tshawytscha*: Groves et al. 1968). Since olfaction plays a critical role in the fidelity of salmon migration the olfactory imprinting hypothesis was formed, suggesting that salmon imprint to the unique chemical composition of their natal streams during a sensitive developmental period after hatch, and use these odour memories as adults to return to their waters of origin for spawning (Hasler et al. 1978). Imprinting refers to the rapid learning of a stimulus and the long-term ability to recognize the cue that was learned during a critical period during development (Bateson 1990). Experiments tracking movements of adult salmon exposed to synthetic chemicals morpholine or phenylethyl alcohol (PEA) at early life history stages confirmed the

occurrence of odour imprinting and use of the olfactory memory in guiding the migrating fish to streams scented with the artificial odour (Coho salmon, *O. kisutch*: Cooper et al. 1976, Scholz et al. 1976; sockeye salmon, *O. nerca*, Tilson et al. 1994).

Even though the role of olfaction for homing has been studied for a long time the neural mechanism of olfactory imprinting has yet to be fully understood. Dissolved odorants in the water are detected through their binding to olfactory receptor proteins on olfactory sensory neurons (OSNs), which relay the information first to the olfactory bulb and then onto higher brain structures (Laberge and Hara 2001). Ciliated, mircovillous and crypt morphotypes of OSNs respond to different types of odorants (rainbow trout: Sato and Suzuki 2001, Bazaes and Schmachtenberge 2012) and each send their axons into specific and stereotypical regions of the olfactory bulb where they terminate onto units of odour discrimination called glomeruli (rainbow trout, *O. mykiss*: Riddle and Oakley 1992, zebrafish, *Danio rerio*: Sato et al. 2005, Braubach et al. 2013, 2013, Ahuja et al. 2013). How fish encode olfactory memories within the olfactory circuitry has yet to be explained. However, the fish brain shows life-long plasticity and early development is particularly affected by early experiences with the fish's environment (review: Ebbesson and Braithwaite 2012).

This thesis aims to describe three OSN morphotypes and their axonal projections into the olfactory bulb in wild-caught Chinook salmon (a species that undergoes a long-distance upstream spawning migration) during early developmental stages when imprinting is likely to occur (Dittman et al. 2015). An understanding of the development of different OSNs in wild populations of salmon, such as the Chinook salmon, is necessary as a basis for further study into how imprinting affects the olfactory system in

salmon. Chapter 2 describes the ciliated, microvillous and crypt OSN morphotypes found in the olfactory epithelium and where their axons terminate within the olfactory bulb of Chinook salmon during early developmental stages. The developmental of the OSNs and their bulbar axons was examined in two embryonic stages (early and late eyed embryo), three larval stages (early yolk-sac, late yolk-sac and fry) and the fingerling stage. Chapter 3 aims to provide some insight into the effect of olfactory experience during the larval stage in Chinook salmon on the development of glomeruli in the olfactory bulb. This chapter examines the changes in glomerular development after exposure of larval Chinook salmon to olfactory enrichment with either an amino acid mixture or phenyl ethyl alcohol (PEA). Overall, the goal of this thesis is to add to the knowledge of olfactory biology in a non-model species and investigate anatomical evidence of imprinting after odour exposure in larval Chinook salmon. Particularly, greater insight in the neurological mechanism of imprinting may have applications in restocking efforts and restorative biology.

1.2 Olfactory sensory neurons and the olfactory epithelium

Fish live in aquatic environments where they use their olfactory system to detect and process information regarding a wide variety of dissolved odorants that they use to guide behaviours and physiological responses necessary for their survival. These olfactory cues are composed of combinations of dissolved chemicals including amino acids, bile acids, nucleotides, steroids and even alcohols. Fish utilize the olfactory system to respond to feeding cues, reproductive pheromones, alarm cues and kin odours (Sorensen and Caprio 1998). However, since these odorants are dissolved together in the water, the olfactory system, from the OSNs in the olfactory epithelium to the olfactory

bulb and telencephalon, is designed to recognize certain molecules and to organize this sensory information to provide an appropriate behavioural response (Laberge and Hara 2001).

The peripheral component of the olfactory system in fish is the pseudostratified olfactory epithelium which opens into the external environment through the nares (Hansen and Zeiske, 1998). The olfactory epithelium has folds called lamellae with both non-sensory and sensory portions. The non-sensory epithelium contains ciliated non-sensory cells. The sensory epithelium contains basal cells and support cells and also includes the OSNs that bind and respond to different odorants (Yanagi et al. 2004, Hansen and Zielinski 2005). The axons of OSNs extend from the olfactory epithelium into the olfactory bulb and form synaptic contacts onto second order sensory neurons called mitral cells that carry olfactory information to higher brain structures including the telencephalon (Laberge and Hara 2001).

In teleosts there are four types of OSNs that differ functionally and morphologically within the sensory region of the olfactory epithelium. These are ciliated OSNs, microvillous OSNs, crypt cells and Kappe cells (recently reported in zebrafish) (review: Kermen et al. 2013, Ahuja et al. 2014). Each of these OSN morphotypes expresses different classes of olfactory receptors that bind specific odorant molecules and uses different proteins in signal transduction. Unlike the mammalian olfactory system where ciliated OSNs are present in the main olfactory epithelium and microvillous OSNs are found in the vomeronasal organ (Eisthen 1992, 1997), fish only have one main olfactory epithelium where all OSN morphotypes are randomly distributed throughout (Hansen et al. 2003).

The cell bodies of ciliated OSNs are located deep in the olfactory epithelium and these neurons have long dendrites and cilia. These express OR-type olfactory receptors and trace amino receptors (TAARS) that are associated with $G_{\alpha olf}$ (zebrafish: Sato et al. 2005, Oka and Korsching 2011, goldfish: Hansen et al. 2004, Hussain et al. 2009) and have shown responsiveness to bile acids (catfish: Nikonov and Caprio 2001; Hansen et al. 2003; rainbow trout: Sato and Suzuki 2001, Schmachtenberg and Bacigalupo 2004). The nuclei of microvillus OSNs are located at an intermediate depth in the olfactory epithelium with microvilli protruding from the dendritic knob. These cells express V1R-type and V2R-type olfactory receptors (zebrafish *D. rerio*: Sato et al. 2005, goldfish: Hansen et al. 2005, *D. rerio, Gasterosteus aculeatus, Oryzias latipes, Tetraodom nigroviridis, Takifugu rubripes*: Saraiva and Korsching 2007). Microvillous OSNs primarily respond to amino acids (catfish: Hansen et al 2003, rainbow trout: Sato and Suzuki 2001). Both ciliated and microvillar OSNs have been found in embryonic rainbow trout (Zielinski and Hara 1988), embryonic zebrafish and non-teleost fish such as sturgeon (Zeiske et al. 2009).

The crypt and kappe cells have only been described in fish. Pear or ovoid shaped crypt cells are located apically in the olfactory epithelium and are both microvillar and ciliated (Hansen and Zeiske 1998). The crypt cells express ora4 of the V1R-types olfactory receptors (zebrafish: Oka et al. 2012). Crypt cells have shown responsiveness to amino acids (mackerel: Vielma et al. 2008), some bile acids during early life stages and also sex steroids during later life stages (rainbow trout: Bazaes and Schmachtenberge 2012). They have also been linked to kin recognition in zebrafish (Biechl et al. 2016, Biechl et al. 2017). Crypt cells were first seen in salmon the olfactory epithelium of chum

salmon (*O.keta*) (Sandahl et al. 2006). Lastly, Kappe cells have been recently identified as a separate class of olfactory sensory neuron in zebrafish similar to the crypt cells with not much yet known about its function (zebrafis*h D. rerio*: Ahuja et al. 2014). These cells were labeled by an antibody to $G_{\alpha o}$ and found to also be located apically in the olfactory epithelium with a round shape and tuft of cilia that gives the cells the appearance of a toque (zebrafish *D. rerio* Ahuja et al. 2014). As of yet little is known about what these cells respond to or which olfactory receptors they express.

The OSN morphotypes can be studied using their characteristic protein expression. Ciliated OSNs express the G protein $G_{\alpha olf}$ which has been used to label ciliated OSNs in the olfactory epithelium and their axonal projections (goldfish: Hansen et al. 2004, zebrafish *D. rerio*: Koide et al. 2009, Gayoso et al. 2011, Braubach et al. 2012, 2013). However, $G_{\alpha olf}$ labeling of ciliated cells has not been described in salmon. The calcium binding protein calretinin has been used to visualize microvillous OSNs in zebrafish (embryo: Duggan et al. 2008, larvae: Kress et al. 2015, Koide et al. 2009, adult: Braubach et al. 2012, Kress et al. 2015) and larval Chinook salmon (Ochs et al. 2017). However, calretinin has also been shown to label ciliated cells in zebrafish (Germana et al. 2007, Koide et al. 2009, Braubach et al. 2012, Kress et al. 2015). In salmonids calretinin labeling has only been shown in larvae and has not been analyzed for labeling of ciliated cells. Crypt cells have been labeled by immunohistochemistry to the calcium binding protein S100 in zebrafish (Germana et al. 2004, 2007, Sato et al. 2005, Gayoso et al. 2012, Oka et al. 2012, Braubach et al, 2012, Kress et al. 2015, Ahuja et al. 2013) but some studies also indicated that S100 labeling was also seen in a subpopulation of microvillous OSNs (Gayoso et al. 2012, Kress et al. 2015). S100 labeling of crypt cells or

microvillous OSNs has yet to be reported in salmon. Lastly, Kappe cells were reported through $G_{\alpha o}$ straining in zebrafish (Ahuja et al. 2014) but no studies describe these cells in salmon.

1.3 Olfactory bulb

Odorants are first detected in the most peripheral portion of the olfactory system, the olfactory epithelium, where OSN receptors bind specific dissolved molecules (Hansen and Zielinski 2005; Sato et al. 2007; Hamdani and Døving 2007). Once odorants bind to olfactory receptors of OSNs, the cells relay the information electrically through its axon as they extend into the olfactory nerve to the olfactory bulb. In addition to expressing different classes of olfactory receptors, different morphotypes of olfactory sensory neurons are further distinguished by the organization of where their axonal projections terminate within the olfactory bulb, the primary location for odour processing. Depolarization in the neuron conducts a signal through the axons along the olfactory nerve into the olfactory bulb terminating onto regions of high synaptic activity called glomeruli, where the terminals of the OSNs synapse onto dendrites of mitral cells that relay the signal to higher brain regions (Mori et al. 1999; Kerman et al. 2013). Glomeruli are the functional units of odour discrimination and demonstrate a highly stereotyped organization where different olfactory sensory neuron morphotypes project their axons to different glomeruli (Zielinski and Hara 2007) which are stimulated by particular odorants (Hansen and Zielinski 2005; Sato et al. 2007; Hamdani and Døving 2007). The clustering of the glomeruli create localized areas for odour detection through formation of glomerular chains (Friedrich and Korsching 1997; Zielinski and Hara 2007).

The segregation of odorants binding particular OSN morphotypes also translates into a spatial segregation in the teleost olfactory bulb. In particular, amino acids and nucleotides stimulate the lateral regions of the olfactory bulb (zebrafish *D. rerio*: Friedrich and Korsching 1998, Doving et al. 1980, Hara and Zhang 1998, Koide et al. 2009) which are innervated by axons of microvillous OSNs (zebrafish *D. rerio*: Sato et al. 2005), while bile acids stimulate the medial regions of the olfactory bulb (zebrafish *D. rerio*: Friedrich and Korching 1997/1998, chars *Salmo alpinus L.* and graylings *Thymallus thymallus L.:* Doving et al. 1980) innervated by ciliated OSN axons (zebrafish *D. rerio*: Sato et al. 2005). Axons of crypt cells and kappe cells project their axons to a different dorsomedial glomerulus in zebrafish (zebrafish *D. rerio*: Braubach et al. 2012, 2013, Gayoso et al. 2012, Ahuja et al. 2013, Ahuja et al. 2014, Kress et al. 2015), while crypt cells project their OSNs to ventral regions of the olfactory bulb in catfish (*Ictalurus punctatus*: Hansen et al. 2003) and crucian carp (*Carassius carassius*: Hamdani and Doving 2007). However, axonal projections of crypt cells have not been described in the salmon olfactory bulb.

The glomerular regions that OSN axons innervate form repeatable territories (rainbow trout *O. mykiss*: Riddle and Oakley 1992) and show a spatial segregation by stimulus type (zebrafish *D. rerio*: Friedrich and Korsching 1997, 1998). Individual glomeruli in these glomerular territorial regions have been mapped and characterized in zebrafish (Braubach et al. 2012, 2013). Calretinin labeling of the olfactory epithelium and olfactory bulb of brown trout (*Salmo trutta*) from embryonic to adult stages showed positive labeling in microvillous and ciliated cells and calretinin positive glomeruli in lateral glomeruli but most dorsomedial glomeruli being calretinin negative (Castro et al.

2008). Ochs et al. (2017) have mapped the glomerular territories and individual lateral glomeruli in larval Chinook salmon. However, the complete organization of glomeruli within all the glomerular territories has not fully been elucidated in wild non-laboratory fish such as the Chinook salmon. Particularly, a description of glomeruli during the embryonic and fingerling stages has not been reported. In addition, only lateral glomerular development labeled with calretinin antibody has been characterized in detail in Chinook salmon while course glomerular territory organization was described in the non-lateral (dorsal, medial, ventral) regions (Ochs et al, 2017). The glomeruli of medial and ventral portions of the olfactory bulb are of particular interest as these regions undergo extensive growth during the larval period than the lateral and dorsal regions (Ochs et al. 2017). The mapping of these dorsal, medial and ventral glomeruli and chronicling when they develop in these fish may provide better insight into their function based on odorants present in the water at the time and may also indicate the relevance of the OSN morphotypes in wild fish populations. The properties of different OSN morphotypes are summarized in Table 1.1.

1.4 Olfactory imprinting for homing migration

Of particular interest in this thesis is how fish imprint to olfactory cues and create odour memories that they use later in life. Imprinting refers to the rapid learning that occurs during a sensitive learning period or critical period and is associated with the ability to recognize the cue the individual was exposed to at a later time (Bateson 1990). With regards to the olfactory system, imprinting has been suggested to play a role is such behaviours as kin recognition (Gerlach et al. 2008, Biechl et al. 2016, 2017) and natal homing in salmonids (Hasler et al. 1978). Hasler and Wisby first proposed in 1951 that

olfactory imprinting plays an important role in natal homing of salmonids. Various behavioural studies in fish exhibiting homing abilities showed early evidence that the olfactory system plays an important role in fish being able to differentiate their natal streams during homing migration. In a study by Wisby and Hasler (1954) silver salmon (*O. kitsutch*) that had their olfactory pits occluded were less likely than control fish to retrace their original migratory path when displaced a mile before a junction of two streams. In studies tracking homeward migration of black rockfish *(Sebastes inermis)* and Chinook salmon (*O. tshawytscha*), fish either had their visual or olfactory sense ablated (Mitamura et al., 2005; Groves et al., 1968). Both studies found that fish deprived of their visual senses were still able to reach their specific waters of origin, but when deprived of their olfactory sense there was a significant reduction in fish reaching their natal streams. Additionally, in a study tracking the movement of Lacustrine sockeye salmon (*O. nerka*) deprived of either there visual or magnetic senses, the fish were still able to navigate to their natal streams (Ueda et al., 1998). Therefore, evidence from behavioural experiments in migrating fish indicated that olfaction is important for guiding fish back to their particular stream of origin.

Studies tracking migrating behaviour of salmonids imprinted to artificial odours phenylethyl alcohol (PEA) and morpholine have also supported the role of olfaction for homing migration and the process of olfactory imprinting (Scholz et al. 1976, Dittman et al. 1996). PEA is a compound found in nature but not usually in municipal water (Erbas and Baydar 2016, Medjahed et al. 2016), that salmon can learn to recognize (Nevitt et al. 1994, Dittman et al. 1996) and has been shown to elicit olfactory responses (Nevitt et al. 1994, Harden et al. 2006). Coho salmon (*O. kisutch*) that were exposed to morpholine or

PEA during early life history stages were shown to preferentially migrate towards streams scented with the artificial odour (Cooper et al. 1976, Scholz et al. 1976). In studies by Tilson et al. (1994) and reiterated by Ditman et al. (2015) Lacustrine sockeye salmon (*O. nerca*) were exposed to an artificial imprinting odour (either PEA or morpholine) at various points in their life cycle from fertilization to the par stage. Fish that were exposed to the imprinting odour during the hatch and larval stages showed the greatest percentage of fish that were attracted to the imprinting odours after they reached maturity, identifying a potential window for olfactory imprinting. In addition to this, the egg and larval stages are the only life stage when fish remain in the redds and are guaranteed exposure to the natal stream waters. Hence, the larval stage appears to be a critical developmental period that may be significant as a time when imprinting can occur.

1.5 Imprinting odour

The olfactory imprinting hypothesis suggests that salmon imprint to unique chemical cues within their natal streams that are consistent year to year and are distinct between different streams (Hasler and Scholz, 1983; Dittman et al., 2015). As juvenile salmon may imprint to the specific chemical signature of the streams in which they were hatched and use the odour memory as adults to return for spawning in their natal streams after years away in open waters (Hasler et al. 1978). Dissolved free amino acids in water have been put forth as the potential odours to which salmonids imprint to in the wild. Salmonids show electrophysiological and behavioural responsiveness to the naturally occurring composition of dissolved free amino acids of stream water (review: Ueda 2012).Electro-physiological experiments on Masu salmon (*O. masou*) showed that

application of artificial stream water with a composition of dissolved free amino acids reflective of their natural waters resulted in electro-physiological responses in the olfactory epithelium similar to that from applications of their natural water (Shoji et al. 2000). The same study also showed that the fish had larger responses to the artificial amino acid water than responses to artificial river water composed of a similar bile acid composition as the natural river water or the amino acid composition from a non-home stream (Shoji et al., 2000). Hence, this study showed that fish respond preferentially to water with an amino acid composition similar to their home stream water. Furthermore, behavioural studies looking at the responsiveness of chum salmon (*O. keta*) to different amino acids showed that fish preferred artificial stream water that had a dissolved free amino acid composition similar to that of their natural natal stream water and were not as attracted to artificial stream water with a dissolved free amino acid composition not typical of their natal streams (Shoji et al., 2003; Yamamoto and Ueda, 2009). These studies suggest that salmon are able to distinguish different streams by their amino acid composition. Hence, amino acids have been implicated as a key component of the signature odour of the water of a fish's natal stream.

1.6 Neural mechanism of imprinting

The focus of this paper is to investigate anatomical evidence of imprinting relating to homing in wild Chinook salmon. Sensory experience and olfactory imprinting can have effects on various levels of the olfactory system. In 1965 Hara et al. reported that spawning adult Chinook (*O. tshawytscha*) and Coho salmon (*O. kisutch*) showed high amplitude electroencephalographic responses in the olfactory bulb to application of home pond water and little or no change to application of water from other pond sources.

This study showed the importance of the olfactory bulb in discriminating home water during spawning migration.

Biechl et al. (2016 and 2017) showed that zebrafish can be imprinted to kin odour. Crypt cells were activated when exposed to a kin odour but not a non-conspecific odour or food odour. After exposure of fish to the kin odour, increased activation of crypt cells in the olfactory epithelium and olfactory bulb cells located at the dorsomedial glomerulus that crypt cell axons project to was seen in imprinted zebrafish compared to control non-imprinted fish. These studies helped link crypt cells to imprinting and recognition of kin odour (Biechl et al., 2016). Studies looking into the effect of imprinting on the peripheral olfactory organ showed an increased response to PEA in electrophysiological recordings of the olfactory epithelium of Coho salmon (*O. kisutch*) that were imprinted to the odour a year earlier year later (Nevitt et al. 1994). Additionally, zebrafish showed a downregulation of several OR olfactory receptor genes when imprinted to PEA (Calfun et al. 2016). Calfun et al. (2016) suggest that the downregulation may occur due to an enhancement of the signal-to-noise ratio as some ORs are repressed and others are activated. Studies in insects and mammals have indicated that sensory experience can affect the size of and number of glomeruli in the olfactory bulb, and that these changes can be translated to higher brain regions leading to alterations in behavioural responses (Devaud et al., 2001, Todrank et al., 2011). In insects, an increase in glomerular volume was observed in *Drosophila* and *Apis* after olfactory exposure during a critical developmental stage (Sachse et al., 2007; Sigg et al., 1997; Arenas et al., 2012). In mammals, mouse pups showed a preference for an odour they were exposed to *in utero* and while nursing, with an increase in the volume of the

glomeruli corresponding to the odour (Todrank et al., 2011). Additionally, olfactory experience to an amino acid mixture in larval zebrafish resulted in more numerous small glomeruli with decreased cross-sectional area (Braubach et al 2012). However, zebrafish do not undergo a homing migration so it would be more beneficial to work with a wild population of fish that do home back to their natal streams. Ochs et al. 2015 did not see changes in volumes of several lateral glomeruli in pairs of amino acid imprinted and non-imprinted Chinook salmon larvae. However, this study did not look into a difference in the average glomerular volume between all amino acid exposed and control fish and did not take into account endogenous amino acids in the water.

1.7 Chinook salmon as a model species undergoing homing migration

Of particular interest is the imprinting of Chinook salmon to amino acids during the larval stage. Chinook salmon (*O. tshawytscha*) is an anadromous fish species that undergoes homing migration as adults from open waters back to their natal streams. They were introduced to the Great Lakes in the 1970s in response to declining native trout populations and increasing populations of non-native alewife fish prey in Lake Ontario (Wurster et al. 2005; Jones et al. 1993). The stocking of Chinook salmon allowed alewife population control and growth of recreational fishing of the Chinook salmon with economic benefits for local coastal communities (Wurster et al. 2005; Talhelm 1988). Chinook salmon spawn in the fall, depositing eggs in the gravels of their natal streams. These eggs hatch during the winter and the larval fish remain within the redds or nests, not feeding during this period as they grow by absorbing nutrients from their yolk sacs (Scott and Crossman 1998). Once they have absorbed their yolk sac juvenile Chinook salmon, first called fry or fingerlings, spend time in different estuaries before they

become smolts and migrate to open waters at the beginning of summer (Scott and Crossman 1998). As they mature, the salmon swim downstream to open waters where adults feed for a few years before returning through natal homing migration to their natal streams for one spawning event before dying and completing their life cycle (Quinn, 2005). Accurate homing is important for Chinook salmon homeward migration to natal streams and with recent descriptions of its glomerular organization during the larval stage (Ochs et al., 2017), it is a candidate for experiments in how imprinting affects the olfactory system.

1.8 Thesis objective

The aim of this thesis is to gain a better understanding of the development and function of OSN morphotypes in a wild population of Chinook salmon, and the anatomical evidence of imprinting in olfactory bulbar projections of the OSN axons. There is a lack of understanding of the ontogeny of different OSN morphotypes during early developmental stages in salmonids, a period when olfactory imprinting can potentially occur (Tilson et al. 1994) and feeding behaviour in beginning (Mearns 1986). The ability of fish to recognize these cues should presumably require a developed olfactory system. Chapter 2 describes the development of ciliated, microvillous and crypt OSNs in the Chinook salmon olfactory epithelium and their axonal projections into the olfactory bulb from embryonic to larval and fingerling stages. In Chapter 3 the objective is to analyze the effect of olfactory experience to an amino acid mixture or PEA odour during larval stages in Chinook salmon on the development of glomeruli in the olfactory bulb. Since amino acids, which have been proposed as imprinting odours, are found endogenously in the water (Shoji et al. 2000), this may dampen the effects of amino acids

on glomerular development. Hence, the experiments described in Chapter 3 look into the composition of amino acids in the water of the natal streams and investigate an alternative imprinting odour (PEA) to investigate the glomerular effects. Hence, this thesis aims to better understand the effects of olfactory experience at the level of the OSNs innervating specific glomeruli in wild Chinook salmon.

1.9 References

Arenas, A., Giurfa, M., Sandoz, J.C., Hourcade, B., Devaud, J.M., Farina, W.M. (2012). Early olfactory experience induces structural changes in the primary olfactory center of an insect brain. *European Journal of Neuroscience*. 35:682-690.

Ahuja, G., Ivandic, I., Salturk, M., Oka, Y., Nadler, W., Korsching, S. (2013). Zebrafish crypt neurons project to a single, identified mediodorsal glomerulus. *Scientific Reports*. 3:2063.

Ahuja, G., Bozorg Nia, S., Zapilka, V., Shiriagin, V., Kowatschew, D., Oka, Y., Korsching, S.I. (2014). Kappe neurons, a novel population of olfactory sensory neurons. *Scientific Reports*. 4:4037.

Bateson, P. (1990). Is imprinting such a special case? *Philosophical Transactions of the Royal Scoiety of B: Biological Sciences.* 1990;329:125-131.

Bazaes, A., Schmachtenberg, O. (2012). Odorant tuning of olfactory crypt cells from juvenile and adult rainbow trout. *Journal of Experimental Biology*. 215:1740-1748.

Biechl, D., Tietje, K., Gerlach, G., Wullimann, M.F. (2016). Crypt cells are involved in kin recognition in larval zebrafish. *Scientific Reports.* 6: article 24590.

Biechl, D., Tietje, K., Ryu, S., Grothe, B., Gerlach, G., Wulliman, M.F. (2017). Identification of accessory olfactory system and medial amygdala in the zebrafish. *Scientific Reports*.

Braubach, O., Fine, A., Croll, P. (2012). Distribution and functional organization of glomeruli in the olfactory bulbs of zebrafish (Danio rerio). *Journal of comparative neurology.* 520(11): 2317-39.

Braubach, O., Miyasaka, N., Koide, T., Yoshihara, Y., Croll, R.P., Fine, A. (2013). Experience-dependent versus experience-independent postembryonic development of distinct groups of zebrafish olfactory glomeruli. *The Journal of Neuroscience.* 33(16): 6905-16.

Castro, A., Becerra, M., Anadon, R., Manso, M.J. (2008). Distribution of calretinin during development of olfactory system in the brown trout, *Salmo trutta fario:* comparison with other immunohistochemical markers. *Journal of Chemical Neuroanatomy.* 35(4):306-316.

Calfun, C., Dominguez, C., Perez-Acle, T., Whitlock, K.E. (2016). Changes in olfactory receptor expression are correlated with odor exposure during early development in the zebrafish (*Danio rerio). Chemical Senses.* 41(4):301-312.

Devaud, J.M., Acebes, A., Ferrus, A. (2001). Odor exposure causes central adaptation and morphological changes in selected olfactory glomeruli in Drosophila. *The Journal of Neuroscience*. 21: 274-272.

Dittman, A.H., Quinn, T.P., Nevitt, G.A. (1996). Timing of imprinting to natural and artificial odors by coho salmon (Oncorhynchus kisutch). *Canadian Journal of Fisheries and Aquatic Sciences*. 53: 434-442.

Dittman, A.H., Quinn, T.P., Nevitt, G.A., Hacker, B., Storm, D.R. (1996). Sensitization of olfactory guanylyl cyclase to a specific imprinted odorant in coho salmon. *Neuron*. 19: 381-389.

Dittman, A.H., Pearsons, T.N., May, D., Couture, R.B., Noakes, D.L.G. (2015). Imprinting of hatchery-reared salmon to targeted spawning locations: a new embryonic imprinting paradigm for hatchery programs. *Fisheries.* 40(3): 114-123.

Duggan, C.D., DeMaria, S., Baudhuin, A., Stafford, D., Ngai, J. (2008). Foxg1 is required for development of the vertebrate olfactory system. *Journal of Neuroscience.* 28:5229-5239.

Ebbesson, L.O.E., Braithwaite, V.A. (2012). Environmental effects on fish neural plasticity and cognition. *Journal of Fish Biology*. 81(7): 2151-2174.

Eisthen, H.L. (1992). Phylogeny of the vomeronasal system and of receptor cell types in the olfactory and vomeronasal epithelia of vertebrates. *Microscopy Research and Technique.* 23:1-21.

Eisthen, H.L. (1997). Evolution of vertebrate olfactory systems. *Brain, behaviour and evolution.* 50: 222-233.

Erbas, S., Baydar, H. (2016). Variation in scient compounds of oil-bearing rose (*Rosa damascene* Mill.). Produced by headspace solid phase microextraction, hydrodistillation and solvent extraction. *Records of Natural Products*. 10: 555-565.

Friedrich, R.W., Korsching, S. (1997). Combinatorial and chemotopic odorant coding in the zebrafish olfactory bulb visualized by optical imaging. *Neuron.* 18:737-752.

Friedrich, R.W., Korsching, S. (1998). Chemotopic, combinatorial, and noncombinatorial odorant representations in olfactory bulb revealed using a voltage-sensitive axon tracer. *Journal of Neuroscience.* 18(23):9977-9988.

Gayoso, J., Castro, A., Anadón, R., Manso, M.J. (2012). Crypt cells of the zebrafish Danio rerio mainly project to the dorsomedial glomerular field of the olfactory bulb. *Chemical Senses*. 37:357–369.

Gerlach, G., Hodgins-Davis, A., Avolio, C. Schunter, C. (2008). Kin recognition in zebrafish: a 24-hour window for olfactory imprinting. *Proceedings. Biological sciences/The Royal Society*. 275: 2165-2170.

Germana, A., Paruta, Simona., Germana, G.P., Ochoa-Erena, J., Montalbano, G., Cobo, Juan., Vega, J.A. (2007). Differential distribution of S100 protein and calretinin in mechanosensory and chemosensory cells of adult zebrafish (*Danio rerio). Brain Research.* 1163:48-55.

Groves, A.B., Collins, G.B., Trefethen, P.S. (1968). Roles of olfaction and vision in choice of spawning site by homing adult Chinook salmon (*Oncorhynchus tshawytscha*). *Journal of the Fisheries Research Board of Canada*. 25(5): 867-876.

Hamdani, E.H., Doving, K.B. (2007). The functional organization of the fish olfactory system. *Progress in Neurobiology*. 82(2):80-86.

Hansen, A., Anderson, K.T., Finger, T.E. (2004). Differential distribution of olfactory receptor neurons in goldfish: structural and molecular correlates. *Journal of Comparative Neurology*. 477: 347-359.

Hansen, A., Rolen, S.H., Anderson, K., Morita, Y., Caprio, J., Finger, T.E. (2003). Correlation between olfactory receptor cell type and function in the channel catfish. *Journal of Neuroscience.* . 23: 9328-9339.

Hansen, A., Zeiske, E. (1998). The peripheral olfactory organ of zebrafish, *Danio rerio*: an ultrastructure study. *Chemical Senses*. 23(1): 39-48.

Hansen, A., Zielinski, B. (2005). Diversity in the olfactory epithelium of bony fishes: development, lamellar arrangement, sensory neuron cell types and transduction components. *Journal of Neurocytology*. 34(3): 183-208.

Hasler, D., Scholz, A.T., Horrall, R.M. (1978). Olfactory imprinting and homing in salmon: recent experiments in which salmon have been artificially imprinted to a synthetic chemical verify the olfactory hypothesis for salmon homing. *American Scientist*. 66: 347-355.

Hara, T.J., Ueda, K., Gorbman, A. (1965). Electroencephalographic studies of homing salmon. *Science*. 149(3686): 884-885.

Harden, M.V, Newton, L.A., Lloyd, R.C., Whitlock, K.E. (2006). Olfactory imprinting is correlated with changes in gene expression in the olfactory epithelia of the zebrafish. *Journal of Neurobiology*. 66: 1452-1466.

Hussain, A., Saraiva, L.R., Korsching, S.I. (2009). Positive Darwinian selection and the birth of an olfactory receptor clade in teleosts. *Proceedings fo the National Academyu of Sciences.*. 106(11): 4313-4318.

Kerman, F., Franco, L.M., Wyatt, C., Yaksi, E. (2013). Neural circuits mediating olfactory-driven behaviour in fish. *Front Neural Circuit 7*.

Koide, T., Miyasaka, N., Morimoto, K., Asakawa, K., Urasaki, A., Kawakami, K., Yoshihara, Y. (2009). *Proceedings of trhe National Academy of Sciences.* 106:9884-9889.

Kress, S., Biechl, D., Wullimann, M.F. (2015). *Brain Structure and Function.* 220: 1951-1970.

Laberge, F., Hara, T.J. (2001). Neurobiology of fish olfaction: a review. *Brain Research Reviews.* 36(1): 46-59.

Mearns, K.J. (1986). Sensitivity of brown tout (*Salmo trutta L.)* and Atlantic salmon (*Salmo salar L.)* fry to amino acids at the start of exogenous feeding. *Aquaculture.* 55(3):191-200.

Medjahed, F., Merouane, A., Saadi, A., Bader, A., Cioni, P.L., Flamini, G. (2016). Chemical profile and antifungal potential of essential oils from leaves and flowers of *Salvia algeriensis* (Desf.): A comparative study. *Chilean Journal of Agricultural Research*. 76: 195-200.

Mitamura, H., Arai, N., Sakamoto, W., Mitsunaga, Y., Tanaka, H., Mukai, Y., Nakamura, K., Sasaki, M., Yoneda, Y. (2005). Role of olfaction and vision in homing

behaviour of black rockfish *Sebastes inermis*. *Journal of Experimental Marine Biology and Ecology*. 322(2): 123-134.

Mori, K., Nagao, H., Yoshihara, Y. (1999). The olfactory bulb: coding and processing of odor molecule information. *Science*. 286(22): 711-715.

Nevitt, G.A., Dittman, A.H., Quinn, T.P., Moody, W.J. (1994). Evidence for a peripheral olfactory memory in imprinted salmon. *Proceedings of the national Academy of Sciences.* USA. 91: 4288-4292.

Nikonov, A.A., Caprio, J. (2001). Electrophysiological evidence for a chemotopy of biologically relevant odors in the olfactory bulb of the channel catfish. *Journal of Neurophysiology.* 86:1869-1976.

Ochs, C.L. (2015). Ontogeny of glomerular territory patterning in the olfactory bulb of juvenile Chinook salmon (*Oncoryhynchus tshawytscha*) and exploring for potential effects of sensory experience on glomerular development. Electronic Theses and Dissertations. 5319.

Ochs, C.L., Suntres, T., Zygowska, A., Pitcher, T., Zielinski, B.S. (2017). Organization of glomerular territories in the olfactory bulb of post-embryonic wild Chinook salmon *Oncoryhynchus tshawytscha*. *Journal of Morphology.* 278(4): 464-474.

Oka, Y., Korsching, S.I. (2011). Shared and unique G alpha proteins in the zebrafish versus mammalian senses of taste and smell. *Chemical Senses*. 36(4): 357-365.

Oka Y., Saraiva, L.R., Korsching, S.I. (2012). Crypt Neurons express a single V1R-related ora gene. *Chemical senses*. 37: 219-227.

Quinn., T. P. (2005). The behavior and ecology of Pacific salmon and trout. *University of Washington Press*, Seattle, WA.

Quinlan, E.M., Philpot, B.D., Huganir, R.L., Bear, M.F. (1999). Rapid, experience-dependent expression of synaptic NMDA receptors in visual cortex in vivo. *Nature Neuroscience*. 2: 352-357.

Riddle, D.R.,Oakley, B. (1992). Immunocytochemical identification of primary olfactory afferents in rainbow trout. *Journal of Comparative Neurology.* 324:575-589.

Sachse, S., Ruechert, E., Keller, A., Okada, R., Tanaka, N.K., Ito, K., Vosshall, L.B. (2007). Activity-dependent plasticity in an olfactory circuit. *Neuron.* 56: 838-850.

Sandahl, J.F., Muyasaka, G., Koide, N., Ueda, H. (2006). Olfactory inhibition and recovery in chum salmon. *Canadian Journal of Fisheries and Aquatic Sciences.* 63:1840-1847.

Saraiva, L.R, Korsching, S.I. (2007), A novel olfactory receptor gene family in teleost fish. *Genome Research.* 14: 1448-1457.

Sato, Y., Miyasaka, N., Yoshihara, Y. (2005). Mutually exclusive glomerular innervation by two distinct types of olfactory sensory neurons revealed in transgenic zebrafish. *Journal of Neuroscience.* 25: 4889-4897.

Sato, Y., Miyasaka, N., Yoshihara, Y. (2007). Hierarchical regulation of odorant receptor gene choice and subsequent axial projection of olfactory sensory neurons in zebrafish. *Journal of Neuroscience.* 27:1606-1615.

Sato, K., Suzuki, N. (2001). Whole-cell response characteritics of ciliated and microvillous olfactory receptor neurons to amino acids, pheromone candidates and urine in rainbow trout. *Chemical Senses*. 26: 1145-1156.

Schmachtenberg, O. (2006). Histological and electrophysiological properties of crypt cells from the olfactory epithelium of the marine teleost *Trachurus symmetricus.* *Journal of Comparative Neurology*. 495, 113-121.

Schmachtenberg, O., Bacigalupo, J. (2004). Olfactory transduction in ciliated receptor neurons of the Cabinza grunt, *Isacia conceptionis* (teleostei: haemulidae). *European Journal of Neuroscience.* 20: 3378-3386.

Scholz, A.T., Horrall, R.M., Cooper, J.C., Hasler, A.D. (1976). Imprinting to chemical cues: the basis for home stream selection in salmon. *Science*. 192: 1247-1248.

Scholz, A.T., Goy, R.W., Hasler, A.D. (1983). Harmonal regulation of smolt transformzation and olfactory imprinting in salmon. *Olfactory imprinting and homin in salmon: investigations into the mechanism of the imprinting process, AD Hasler, AT Scholz.*

Scott, W.B., Crossman, E.J. (1973). Freshwater fishes of Canada. *Fisheries Research Board of Canada Bulletin,* 184.

Shoji, T., Ueda, H., Ohgami, T., Sakamoto, T., Katsuragi, Y, Yamauchi, K., Kurihara, K. (2000). Amino acids dissolved in steam water as possible home stream odorants for masu salmon. *Chemical Senses*. 25: 533-540.

Shoji, T., Yamamoto, Y., Nishikawa, D., Kurihara, K., Ueda, H. (2003). Amino acids in stream water are essential for salmon homing migration. *Fish Physiology and Biochemistry*. 28:249-2003.

Sigg, D., Thompson, C.M., Mercer, A.R. (1997). Activity-dependent changes to the brain and behaviour of the honey bee, *Apis mellifera (L.). The Journal of Neuroscience*. 17: 7148-7156.

Sorensen, P.W., Caprio, J. (1998). Chemoreception. In: Evans DH, editor. *The Physiology of fishes*. Boca Raton (FL): CRC Press. p. 375-405.

Talhelm, D.R. (1988). The international great lakes sport fishery of 1980. *Great Lakes Fishery Commission.*

Tilson, M.B., Scholz, A.T., White, R.J., Galloway, H. (1994). Thyroid-induced chemical imprinting in early life stages and assessment of smoltification in kokanee salmon hatcheries. 1993 Annual report. Prepared for Bonneville Power Administration, Portland, Oregon.

Todrank, J., Heth, G., Restrepo, D. (2011). Effects of in utero odorant exposure on neuroanatomical development of the olfactory bulb and odour preferences. *Proceedings of the Royal Society B: Biological Sciences*. 278(1714):1949–1955.

Ueda, H. (2012). Physiological mechanisms of imprinting and homing migration in Pacific salmon *Oncorhynchus* spp. *Journal of Fish Biology*. 81: 543-558.

Ueda, H., Nakamura, S., Nakamura, T., Inada, K., Okubo, T., Furukawa, N., Murakami, R., Tsuchida, S., Zohar, Y., Konno, K., Watanabe, M. (2016). Involvement of hormones in olfactory imprinting and homing in chum salmon. *Scientific Reports*. 6: article 21102.

Ueda, H., Kaeriyama, M., Mukasa, K., Urano, A., Kudo, H., Shoji, T., Tokumitsu, Y., Yamauchi, K., Kurihara, K. (1998). Lacustrine sockeye salmon return to their

natal area from open water using both visual and olfactory cues. *Chemical Senses*. 23: 207-212.

Vielma, A., Alvaro, A., Delgado, Luz., Schmachtenberg, O. (2008). The elusive crypt olfactory receptor neuron: evidence for its stimulation by amino acids and cAMP pathway agonists. *Journal of Experimental Biology.* 211:2417-2422.

Wisby, W.J., Hasler, A.D. (1954). Effect of olfactory occlusion on migrating silver salmon (*O. kitsutch). Journal of the Fisheries Research Board of Canada*. 11(4): 472-478.

Wurster, C.M., Patterson, W.P., Stewart, D.J., Bowlby, J.N., Stewart, T.J. (2005). Thermal histories, stress, and metabolic rates of chinook salmon (*Oncoryhynchus tshawystscha*) in Lake Ontario: evidence from intra-otolith stable isotope analyses. *Canadian Journal of Fisheries and Aquatic Sciences.* 62: 700-713.

Yamamoto, Y., Ueda, H. (2009). Behavioural responses by migratory chum salmon to amino acids in natal stream water. *Zooligical Science*. 26(11): 778-782.

Yamamoto, Y., Hino, H., Ueda, H. (2010). Olfactory imprinting of amino acids in lacustrine sockeye salmon. *PLoS ONE*. 5(1): e8633. Doi:10.1371journal.pone.0008633

Yanagi, S., Kudo, H., Doi, Y., Yamauchi, K., Ueda, H. (2004). Immunohistochemical demonstration of salmon olfactory gluthione S-transferase class pi (N24) in the olfactory system of lacustrine sockeye salmon during ontogenesis and cell proliferation. *Anatomy and Embryology*. 208(3): 231-238.

Zeiske, E. Bartsch, P. Hansen, A. (2009). Early ontogeny of the olfactory organ in a basal actinopterygian fish: *Polypterus. Brain, Behaviour and Evolution.* 73(4):259-272.

Zielinski, B., Hara, T. (1988). Morphological and physiological development of olfactory receptor cells in rainbow trout (*Salmo gairdneri)* embryos. *Journal of Comparative Neurology*. 271(2):300-311.

Zielinski, B., Hara, T. Olfaction. (2007). Sensory Systems Neuroscience. *Fish Physiology.* Volume 25. San Diego: Academic Press. 1-33.

1.10 Tables and Figures

Table 1.1: Summary of OSN morphotype properties in zebrafish and responsiveness to odorants in rainbow trout. The asterisk (*) denotes the antibody each morphotype is mainly labeled with.

		Ciliated	Microvillous	Crypt	Kappe
OSN description	Cell body in olfactory epithelium	-Basally located	-Located at intermediate depths	-Pear shaped -In apical portion	-Ovoid shaped -Apically located
	Dendrite	-Long -Cilia extending from apical surface	-Short dendrite with microvilli	-No dendrite -Cilia and microvilli	-No dendrite -Tuft of cilia
Olfactory receptor expression		OR-type TAARs	V1R-type V2R-type	Ora4 of the V1R-type	Unknown
Responsiveness to odorants	Amino acids	Response	Main response	Response	Not seen
	Bile acids	Main response	Not seen	Response	Not seen
	Steroids	Not seen	Not seen	Main response	Not seen
Olfactory bulb glomerular location of axon terminals		-Anterior dorsal and ventromedial glomeruli	-Dorsolateral and lateral glomeruli	-Dorsomedial glomerulus	-Dorsomedial glomerulus
Immunolabeled with		$G_{\alpha olf}$* Calretinin	Calretinin* S100	S100	$G_{\alpha o}$

CHAPTER 2: OLFACTORY SENSORY NEURON MORPHOTYPES AND THEIR OLFACTORY BULB PROJECTIONS IN DEVELOPING CHINOOK SALMON *(Oncorhynchus tshawytscha)*

2.1 Introduction

Stochastically dispersed throughout the teleost olfactory epithelium are ciliated, microvillous, crypt and kappe morphotypes of olfactory sensory neurons (OSNs), that bind diverse dissolved odorants (Friedrich and Korsching 1997, 1998, Braubach et al. 2013, Kerman et al. 2013) to mediate different behaviours such as prey avoidance, foraging and finding mates (Sorensen and Caprio 1998). These OSN morphotypes display distinctive morphological, molecular and physiological properties (Gayoso et al. 2011), with their nuclei situated at different depths of the pseudostratified olfactory epithelium. The cell bodies of ciliated OSNs are found in the basal layer of the olfactory epithelium (Hansen et al. 2004, Sato et al. 2005), microvillous OSN cell bodies at intermediate depths (Hansen et al. 2004, Sato et al. 2005), and crypt and Kappe cells are situated in the superficial layers (Morita and Finger 1996, Hansen and Zeiske 1998, Ahuja et al. 2014). Axons from different OSN morphotypes project to specific and consistent regions of the olfactory bulb (Sato et al. 2005), where they terminate on discrete units of odour discrimination called glomeruli, creating a highly stereotyped organization of glomerular regions in the olfactory bulb (Baier and Korsching 1994, Riddle and Oakley 1992, Braubach et al. 2013).

Past studies in teleosts have employed immunohistochemical techniques with various calcium binding proteins and G-protein subunits to describe OSN morphtoypes in the olfactory epithelium and their axonal projections in the olfactory bulb. However, most

extensive studies have been conducted in laboratory raised zebrafish (Braubach et al. 2012, 2013), while studies in wild-caught fish are less common. Ciliated OSNs, which are responsive to bile acids (rainbow trout: Sato and Suzuki 2001, Schmachtenberg and Bacigalupo 2004) and have been linked to alarm responses in fish (carp: Hamdani and Doving 2007), express the G protein $G_{\alpha olf}$ (goldfish: Hansen et al. 2004). G_{olf} has been used to label ciliated OSNs and their axons within the dorsoanterior, medial anterior, ventroanterior and ventromedial olfactory bulb in embryonic, larval and adult zebrafish (Koide et al. 2009, Gayoso et al. 2011, Braubach et al. 2012, 2013). However, G_{olf} labeling has not been described in the olfactory system at any stage of development in wild salmon. The calcium binding protein calretinin has been used in the past to label microvillous OSNs projecting to distinct lateral dorsal lateral glomeruli in the olfactory bulb of zebrafish (Braubach et al. 2012, 2013), embryonic to fry stages of brown trout (Castro et al. 2006) and larval Chinook salmon (Ochs et al. 2017). However, some studies indicate that calretinin also labels ciliated OSNs (Braubach et al. 2012, Gayoso et al. 2011, Castro et al. 2006). This has not yet been determined in Chinook salmon where only larval stages have been analyzed for calretinin immunoreactivity.

Several studies in zebrafish indicate that crypt cells exhibit S100 immunoreactivity (Germana et al. 2004, Gayoso et al. 2011) and innervate a dorsomedial glomerulus (Braubach et al. 2012, 2013, Gayoso et al. 2012, Ahuja et al. 2013). Crypt cells have been found in salmon (chum salmon: Sandahl et al. 2006) but S100 labeling of crypt cells and which glomeruli the crypt cells project their axons to within the olfactory bulb have not been established in salmon. Kappe neurons were labeled with the G-protein

$G_{\alpha o}$ in zebrafish (Ahuja et al. 2014) and have yet to be described in salmon. However, this OSN morphotype was not a focus of this paper.

In this paper G_{olf}, calretinin and S100 antibody markers of OSNs are used to label the ciliated, microvillous and crypt OSNs and their axons in wild Chinook salmon, a commercially important fish in the Great Lakes. Examining these OSN morphotypes and their glomerular projections in a wild caught fish may provide insights on the relevance of these morphotypes in wild fish populations. Early developmental periods are of particular interest as the olfactory organ in fish species undergoes dramatic maturation during this period (sturgeon and zebrafish: Zeiske et al. 2003). It is also a period when imprinting of salmon to their natal waters is proposed to occur (Tilson et al. 1994, Dittman et al. 2015) and when active feeding behaviour commences (Mearns 1986). Hence, the development of OSN morphotypes was examined in eyed embryo, larvae and fingerling stages of development in Chinook salmon. Ciliated and microvillous OSNs were expected to appear early in embryonic development as seen in rainbow trout (Zielinski and Hara 1988) and brown trout (Castro et al. 2006). Crypt cells develop at different times in various fish, being present in early zebrafish embryos (Ferrando et al. 2007) but not appearing until the end of larval development in the turbot (*Psetta maxima*) (Doldan et al. 2011). Hence, crypt cells are expected to develop in Chinook salmon between embryo and fingerling stages. Additionally, G_{olf}, calretinin and S100 labeling was expected to innervate similar glomeruli as described in zebrafish (Braubach et al. 2012, 2013) based on similarities of calretinin immunoreactivity in the olfactory bulb of larval Chinook salmon (Ochs et al. 2017) and zebrafish (Braubach et al. 2012).

2.2 Materials and Methods

Fish rearing

Animal care and handling were approved by the University of Windsor Animal Care Committee complying to guidelines set by the Canada Council of Animal Care. Spawning Chinook salmon from the Credit River (Mississauga, Ontario) were electro-shocked (described in: Pitcher and Neff 2006, Butts et al. 2012) for collection of eggs from females and milt from males. Eggs and milt were transported to the University of Windsor Animal Care Facilities where eggs from each female were then fertilized with milt from a unique male activated in river water from the Credit River. The eggs were subsequently raised in Heath trays sourced by a continuous recirculating flow of dechlorinated municipal water. A chiller was used to control the water temperature which was decreased roughly 1 degree per week starting from 12^{o}C until the temperature reached approximately 7^{o}C to mimic a decrease in water temperature like seen in the wild during the winter. Collection of fish began once all embryos were eyed and continued weekly through the embryonic, larval and early fry stages. Since salmon development is influenced by the water temperature they are raised at, fish age was determined based on degrees days measured as degree days (dd) calculated by summing up the average daily temperatures of the water from fertilization to each collection date (Crisp 1981). Table 2.1 summarizes the developmental stages assessed in this paper and is based on naming of early developmental stages in Chinook salmon (Ochs et al. 2017). Embryonic (266-470dd), larval (505-920dd), and fingerling (1257-1537dd) stages were analyzed with the embryo and larval stages further subdivided to facilitate a more in depth investigation of OSN development during each period (Table 2.1).

Sample Preparation

Chinook salmon alevin were euthanized by anaesthetic overdose (1g/L MS-222) and dropped-fixed in 4% paraformaldehyde in 0.1M PB overnight, while eggs shells were first removed from embryonic specimens before drop-fixation. Fixed tissue was rinsed in PBS overnight before the head was isolated with a cut half-way through the optic tectum and removal of the mandible. The salmon heads were cryoprotected in a sucrose gradient in 0.1M PBS buffer (20% sucrose solution overnight then 30% sucrose solution overnight). Fish heads were sectioned in horizontal sections at 18µm from the olfactory bulb to olfactory epithelium on a Leica CM 3050A cryostat onto Fisherbrand Superfrost Plus microscope slides. Sections were dried at room temperature before storage at -20^{o}C. Slides were rehydrated with three rinses over 3 hours of PBS-T before being placed in HistoVTOne (06380-05, lot# L6R0020, Nacalai tesque, Kyoto) for 20 minutes at 70^{o}C for antigen retrieval. The slides were then rinsed three times and placed in a blocking solution with 10% goat serum (Sigma-Aldrich, Oakville ON, G9023) in 0.1M PBS with 0.1% Triton-X for an hour. Slides were then labelled with primary antibody 1:500 monoclonal calretinin antibody raised in mouse (Swant, Switzerland, 6B3) and 1:250 polyclonal $G_{\alpha s/olf}$ raised in rabbit (Santa Cruz Biotechnology, Texas USA, sc-383, lot# D2011) or 1:250 polyclonal rabbit anti-S100 (Dako Cytomation, Denmark, Z0311, lot# 00020766) for 3 days at 4^{o}C under gentle agitation. The slides were then rinsed with PBS over 5 hours before being placed in secondary antibody of 1:500 Alexafluor 488 goat anti-rabbit IgG (Sigma-Aldrich Oakville ON, A11008 lot #1829924) and 1:500 goat anti-mouse Alexafluor 568 IgG (Sigma-Aldrich Oakville ON, A11004 lot# 1698376) for two days at 4^{o}C with gentle agitation. The slides were then rinsed a second time with PBS

over 5 hours and coverslipped with VectaShield (H-1200, Vector Laboratories, Burlingame, CA) mounting medium for fluorescence with DAPI counterstain.

Image analysis

Immunolabeled slides were viewed and photographed under a Nikon Eclipse E600 microscope under a 10x, 20x, 40x and 60x objective lens. The glomeruli were named according previous studies in larval and adult zebrafish (Baier and Korsching 1994, Braubach et al. 2012, 2013) and adult rainbow trout (Riddle and Oakley 1992). $G_{\alpha olf}$ labeling was compared to labeling in zebrafish (Braubach et al. 2012, 2013). Calretinin labeling was compared to calretinin labeling in embryo to adult zebrafish (Gayoso et al. 2011, Braubach et al. 2012, 2013) and brown trout (Castro et al. 2008), as well as labeling in larval Chinook salmon (Ochs et al. 2017). S100 labeling was compared to labeling in zebrafish (Gayoso et al. 2011, Braubach et al. 2012, 2013, Ahuja et al. 2013). Patterning and distinguishing of individual OSNs and glomeruli was performed with the analysis of immunohistochemical labelling of the olfactory epithelium and olfactory bulb of the Chinook salmon through early developmental stages with calretinin, G_{olf} and S100 antibodies. Counterstaining with DAPI was used to assist in recognizing the cell bodies of the OSNs and determining the borders of the individual glomeruli within the olfactory bulb.

2.3 Results

Immunolabeling of OSNs in the embryonic, larval and fingerling olfactory epithelium

During the embryonic stage the olfactory epithelium of Chinook salmon appeared as two pits at either side of the rostral end of the head (Figure 2.1 A-B). The olfactory

bulb region was approximately parallel to the epithelium and found in the same horizontal plane at the early eyed embryonic stage (Figure 2.4 A) but appeared to become more caudal in the head as the fish developed. At the early yolk sac larval stage the olfactory epithelium appeared similar in shape with no prominent lamellae formed (Figure 2.1 C). A greater number of lamellae appeared within the olfactory epithelium during the late yolk-sac larval and fry periods (Figure 2.1 D-E), while individual lamellae became more prominent in the fingerling stage (Figure 2.1 F-H). Micrographs of horizontal sections taken at the same depth (216µm) of DAPI counterstained olfactory epithelium showed how the olfactory epithelium started as a simple pit in the embryonic stage, developed 2-3 lamellae in the larval stage and 4-5 in the fingerling stage (Figure 2.1 A-F). At greater depths (486µm) of the fingerling olfactory epithelium even more lamellae were visible (Figure 2.1 G) before the reaching the ventral portion (720µm) of the olfactory epithelium where few lamellae were present (Figure 2.1 H). It appeared the greatest increase in number of invaginations occurred in the dorsal portion of the olfactory epithelium. Additionally, no secondary foldings were visible at any of these early developmental stages.

G_{olf}, calretinin (CR) and S100 labeling was present in the olfactory epithelium at the early eyed embryo stage (Figure 2.2 A-F). However, cells appeared to be poorly differentiated, though G_{olf} labeled cells appeared deep in the olfactory epithelium (Figure 2.2 C), calretinin cells were mostly in intermediate depths (Figure 2.2 A, B) and S100 cells were small and situated close to the apical surface (Figure 2.2 D). The beginning of the olfactory nerve was visible extending from the OSNs towards the olfactory bulb of early eyed embryo (Figure 2.2 A, C, D, Figure 2.4 A). Since CR immunoreactivity was

much more prominent than G_{olf} immunoreactivity it was difficult to distinguish if there was co-localization of CR and G_{olf}-positive cells during this stage.

In larval and fingerling stages CR, G_{olf} and S100 positive cells became widespread in the olfactory epithelium (fingerling olfactory epithelium: Figure 2.3 A-C) and began to take on a bipolar appearance more typical of OSNs (Figure 2.3 D-F). CR positive cells in the olfactory epithelium took on a bipolar shape with cell bodies of most cells present at intermediate epithelial depths (Figure 2.3 B, E). G_{olf} labeling in the olfactory epithelium was weaker than labeling seen with CR antibody. G_{olf} positive cell, which became more prominently visible at later stages of development, were bipolar in shape with somata present in the deeper layers of the olfactory epithelium and cilia protruding from dendritic knobs at the lamellar surface, (Figure 2.3 A, D). The S100 immunoreactive cell bodies were predominantly found in the most apical layer of the olfactory epithelium, while some S100 positive cells were situated at intermediate depths with a visible dendrite (Figure 2.3 C, F). Labeling with each OSN marker was present in the olfactory epithelium from embryonic stages and cells labeled with different antibodies appeared to exhibit specific characteristics.

Immunolabeling of OSN axons in the olfactory bulb

Embryonic olfactory bulb

During the early eyed Chinook salmon embryo, the olfactory bulb region was not developed in the forebrain and the axons of the G_{olf} and CR positive OSNs did not extend into the olfactory bulb until late eyed embryo stage (Figure 2.4). At the late eyed embryo stage CR-ir and G_{olf} –ir fibers extended into the olfactory bulb and onto characteristic

glomeruli. CR labeled lateral glomeruli lG_1, $lG_{3/4}$ and lG_6 (Figure 2.4 C-F) along with small nerve terminals in the dorsal lateral (dlG) territory (Figure 2.4 B). These glomeruli were shown to be CR-ir in larval Chinook salmon (Ochs et al. 2017) and this study shows they are present during the late eyed embryo stage. dlG was present in the dorsal region of the olfactory bulb but did not appear fully developed as only a few small glomeruli made up the cluster (Figure 2.4 B). lG_1 was a small oval shaped glomerulus present posterior and ventral to the dlG (Figure 2.4 C). $lG_{3/4}$ glomerulus was larger than lG_1 with a spherical shape and situated ventral and somewhat anterior to lG_1 (Figure 2.4 D). Calretinin also labeled lG_6, located in the lateral region of the olfactory bulb sometimes appearing at the same depth as $lG_{3/4}$ but situated close to the posterior edge of the bulb and extending further ventrally in the bulb (Figure 2.4 D-F). These known CR positive glomeruli were used as landmarks to describe glomeruli labeled with G_{olf} or S100 and any new glomeruli CR labeled. G_{olf} labeling was visible in ventromedial glomeruli vmG_x and vmG_7 (Figure 2.4 E, F) situated at the depths similar or ventral to $lG_{3/4}$ and found directly medial to $lG_{3/4}$. At this stage vmG_x and vmG_7 were difficult to tell apart due to their close proximity to each other. Double labeling in the dlG was seen with G_{olf} and CR (Figure 2.4 B). No S100 labeling was seen in the olfactory bulb of Chinook salmon embryos.

Larval olfactory bulb

Similar to calretinin labeling in larval Chinook salmon described by Ochs et al. (2017), CR labeling of lG_1, $lG_{3/4}$ and lG_6 and dlG was present in stereotypical locations and shapes throughout the larval stage. The arrangement of these glomeruli remained consistent to what was described earlier in this study in late yolk sac larvae. Through the

larval stages lG_1 (Figure 2.5 A-C), $lG_{3/4}$ (Figure 2.5 D-F) and lG_6 (Figure 2.5 E-I) remained immunoreactive only to CR antibody.

G_{olf} labeling of fibers was present in glomeruli of the ventromedial region of the olfactory bulb from early yolk-sac larvae. The positions of these vmG_x and vmG_7 (Figure 2.5 E-F) glomeruli in the olfactory bulb were consistent to what was described in late eyed embryos and remained consistently identifiable throughout the late yolk-sac and fry stages. The vmG_x was irregularly shaped and sometimes appeared to be two oblong glomeruli (Figure 2.5 F). The vmG_7 was larger than vmG_x and showed an oval shape (Figure 2.5 I). In some specimens the vmG_x and vmG_7 were difficult to distinguish from one another due to their close proximity. Visualization of the fibers entering each glomerulus with confocal microscopy may help distinguish the two glomeruli. The glomeruli vmG_x and vmG_7 also appeared to considerably increase in size from early yolk-sac larvae to fry stage (visually assessed: Figure 2.5 E-F) consistent with reports of development of ventral regions of the olfactory bulb in larval Chinook salmon (Ochs et al. 2017).

During the late yolk-sac stage fibers of the dorsal glomeruli (dG), medial anterior glomeruli (maG) and additional smaller vmGs appeared within the olfactory bulb predominantly immunoreactive to G_{olf}. G_{olf}-ir dG appeared in the dorsal region of the olfactory bulb medial to dlG (Figure 2.5 B). maG, also G_{olf} positive, was composed of many small circular and oval shaped glomeruli situated anterior and medial to $lG_{3/4}$ and vmGx, along the midline of the olfactory bulb (Figure 2.5 E). The small vmGs (not vmG_x or vmG_7) were found in the ventral region of the olfactory bulb and appeared to be innervated by G_{olf} and CR fibers (Figure 2.5 K). Even though the dG, maG and small

vmGs were present in late yolk-sac larvae these glomeruli were underdeveloped with labeling appearing diffuse in these regions. However, the G_{olf} and CR immunoreactive dlG appeared to have developed so that individual glomeruli of the chain could be seen (Figure 2.5 B).

Calretinin and G_{olf} glomeruli were prominent during the fry stage (Figure 2.5 C, F, I, L). The dlG was clearly immunoreactive to both G_{olf} and calretinin (shown double labeled in orange Figure 2.5 C). The dG (Figure 2.5 C), maG (Figure 2.5 F, I), small vmGs (Figure 2.5 L) were prominent in fry. dG and maG were G_{olf} positive while the small vmGs were both G_{olf} and CR positive. In the ventroposterior (vpG) region of the olfactory bulb two vpG glomeruli become visible during the fry stage (Figure 2.5 I, L). Both glomeruli were located in the ventral and posterior region of the olfactory bulb. vpG_2 was a G_{olf} immunoreactive circular shaped glomerulus situated posterior to lG_6 (Figure 2.5 I). vpG_1 was an oval shaped calretinin immunoreactive glomerulus found ventral and medial to vpG_2 at the posterior part of the olfactory bulb (Figure 2.5 L). The labeling of the vpGs with either G_{olf} or CR antibodies was not described in zebrafish (Braubach et al. 2012, 2013) and was not described in previously described CR labeling in the Chinook salmon olfactory bulb (Ochs et al. 2017).

During the larval stage no immunostaining of the glomerular region of the olfactory bulb with the S100 antibody was observed.

Fingerling olfactory bulb

During the fingerling stage the most dorsal glomeruli were the dorsal glomeruli (dG) and dorsal lateral glomerular chain (dlG) (Figure 2.6 A-C). dlG was a large oval

shaped glomerular chain composed of many smaller glomeruli exhibiting double labeling with both CR and G_{olf} antibodies (Figure 2.6 A-C). However, some of the most posterior glomeruli of dlG appeared weakly labeled by CR, consistent to descriptions of dlG in zebrafish (Braubach et al. 2012) and larval Chinook salmon (Ochs et al. 2017). The G_{olf} immunoreactive dG was found anterior and medial in the bulb and medial to dlG. In the dorsal region of the olfactory bulb some lateral glomeruli are also visible. The region of the dorsal most lateral glomerulus, lG_2, was diffusely labeled by G_{olf} in the fingerling olfactory bulb (Figure 2.6 A-C) similar to how lG_2 is labeled by G_{olf} in zebrafish (Braubach et al. 2012). Ventral and anterior to lG_2 was the CR immunoreactive lG_1 glomerulus (Figure 2.6 B, C).

The calretinin immunoreactive $lG_{3/4}$ was present ventral and anterior to lG_1 (Figure 2.7 A). This glomerulus is larger than lG_1 and spherical in shape. The posteriorly situated lateral glomerulus lG_6 was also only calretinin positive (Figure 2.7 B-D) and often showed a different pattern of axonal projections from one specimen to the next. The medial portion of the olfactory bulb showed prominent glomeruli in the maG region (Figure 2.7 B-D). maG was composed of small circular or oval shaped glomeruli situated in the medial and anterior portion of the olfactory bulb, ventral to dG and medial to $lG_{3/4}$. These glomeruli were predominantly G_{olf} immunoreactive but some calretinin positive fibers also appeared enter the region. Several ventral medial glomeruli were also present. vmGx was a G_{olf} immunoreactive glomeruli usually appearing as two oval pieces found very close together and connected by fibers (Figure 2.7 B-D). It was situated medial to $lG_{3/4}$. In more ventral sections G_{olf} positive vmG_7 appeared ventral and posterior to vmGx (Figure 2.7 D, Figure 2.8 A-C). vmG_7 was a large glomerulus with an elongated

appearance, extending to ventral regions of the olfactory bulb. Additionally, in the ventral and posterior portion of the olfactory bulb the G_{olf} immunoreactive vpG_2 was situated directly posterior to lG_6 (Figure 2.7 C, D). In the posterior regions of the olfactory bulb a single calretinin positive glomerulus named vpG_1 was located medial and ventral to vpG_2 (Figure 2.8 A-C). vpG_2 was circular in shape, while vpG_1 had a crescent shape. In the ventral and medial region of the olfactory bulb small vmGs were both G_{olf} and calretinin immunoreactive (Figure 2.8 A-C). S100 fibers appeared in lateral regions throughout the olfactory bulb in fingerlings (Figure 2.9 A-C) with some fibers also labeling calretinin labeled lateral glomeruli lG_1, $lG_{3/4}$, lG_6 and non-calretinin positive lG_2 regions. However, S100 labelling was not present in the dlG region that calretinin labeled (Figure 2.9 A).

In this study we showed that CR labeling of lG_1, $lG_{3/4}$ and lG_6 was present before hatching in the late eyed embryo stage and remain easily identifiable throughout development (Figure 2.4, Figure 2.5). During late yolk-sac larvae and fry stages CR immunolabeled additional non-lateral glomeruli. In particular, calretinin labeled some medial anterior (maG), ventromedial glomeruli (vmG) (Figure 2.5 K, L) and one ventroposterior glomerulus (vpG1) (Figure 2.5 L, Figure 2.6). G_{olf} labeling was first observed in two ventromedial glomeruli from late eyed embryo (vmG_x and vmG_7, Figure 2.4) which remained consistently identifiable in shape and location through larval and fingerling stages (Figure 2.5, 2.7). During late yolk-sac larvae and fry stages G_{olf} labeling was also present in dorsal glomeruli dG, maG, small vmGs, vpG_2 and diffusely labeled the lG_2 glomerular region (Figure 2.5, 2.7, 2.8, 2.9). S100 labeling of the olfactory was

only visible in the fingerling stage where S100 fibers innervated lateral regions of the olfactory bulb.

2.4 Discussion

This study examined OSNs and their axons in the olfactory bulb of developing Chinook salmon from embryo to fingerling using calretinin, G_{olf} and S100 antibodies to label microvillous, ciliated and crypt OSNs respectively, expanding upon a previous study of calretinin and KLH (a general glomerular marker) labeling in larval Chinook salmon (Ochs et al. 2017). Based on labeling of OSNs with these antibodies in zebrafish (Gayoso et al. 2011, Braubach et al. 2012, 2013, Kress et al. 2015), and calretinin labeling in brown trout (Castro et al. 2008) and larval Chinook salmon (Ochs et al. 2017), it was hypothesized that G_{olf} would label dorsal anterior, medial anterior and ventromedial regions of the olfactory bulb, calretinin would label lateral regions and S100 would label a mediodorsal glomerulus. Additionally, we predicted that G_{olf} positive ciliated OSNs and calretinin positive microvillous OSNs would be present in Chinook salmon embryos as seen in rainbow trout (Zielinski and Hara 1988). In different fish species crypt cells appear at different times during early development (zebrafish: Ferrando et al. 2007, turbot: Doldan et al. 2011), so crypt cells were expected to appear sometime during early development between embryonic and larval stages in Chinook salmon. Our data suggests that the Chinook salmon shows many similarities in glomerular patterning to zebrafish but the timing of when glomeruli appear during early development was not always comparable.

As previously described by Ochs et al. (2017), calretinin labeling in the olfactory bulb of larval Chinook salmon was most prominent in glomeruli of the lateral region.

However, this study is the first to show that calretinin positive lateral glomeruli (lG_1, $lG_{3/4}$ and lG_6) were visible and clearly identifiable even before the Chinook salmon hatched, during the late eyed embryo stage. The development of calretinin positive lateral glomeruli into distinct units in the late embryo stage of Chinook salmon is similar to calretinin labeling reported in brown trout embryo, where only a short nerve was present in early embryos that reached the olfactory bulb to branch into characteristic glomeruli by late embryo stages (Castro et al. 2008). Consequently, this may indicate a common ontogeny of calretinin immunoreactive cells and glomeruli in salmonids and imply early functional significance of these glomeruli in salmonid behaviours, perhaps for imprinting to amino acids in their natal streams (Shoji et al. 2003).

The immunolabeling of calretinin was also described in the fingerling stage when labeling was also present in glomeruli outside the lateral region of the olfactory bulb. Calretinin labeled medial anterior glomeruli (maG) and small ventromedial glomeruli (vmGs). Calretinin positive fibers present in medial and ventral regions of the olfactory bulb, regions that are innervated by ciliated OSNs in zebrafish (Sato et al. 2005), and the lateral region, innervated by microvillous OSNs (Sato et al. 2005), may indicate that calretinin labeled subsets of both ciliated and microvillous OSNs. This finding is supported by observation of calretinin labeling in medial and ventral regions of the olfactory bulb in zebrafish (Braubach et al. 2012, Kress et al. 2015), and calretinin labeling of OSNs in the zebrafish olfactory epithelium with properties typical of ciliated cells (Gayoso et al. 2011, Braubach et al. 2012, Kress et al. 2015), an observation also noted in brown trout (Castro et al. 2008). Additionally, Chinook salmon were unique in that calretinin fibers appeared to reach the ventroposterior region of the olfactory bulb

terminating onto vpG_1 in fry and fingerling stages. This was not described in a study of calretinin labeling in larval Chinook salmon (Ochs et al. 2017) where vpG_1 was only reported to be immunoreactive to KLH, similar to zebrafish (Braubach et al. 2012). This paper shows calretinin positive fibers in the vpG_1 in which calretinin fibers only reached this glomerulus at fry and fingerling stages.

To our knowledge this is the first study in which G_{olf} and S100 labeling has been reported at any life history stage in Chinook salmon. In this study G_{olf} antibody was used as a label specific to ciliated OSNs, which respond to bile acids (rainbow trout *O. mykiss*: Sato and Suzuki 2001, Schmachtenberg and Bacigalupo 2004) and project their axons to medial anterior and ventral medial regions of the olfactory bulb in zebrafish (Braubach et al. 2012, 2013). In Chinook salmon, G_{olf} was observed to chiefly immunolabel cells with their somata situated deep in the olfactory epithelium, and dorsal anterior, medial anterior and ventromedial territories of the olfactory bulb. This is consistent with G_{olf} labeling of these territories in zebrafish (Braubach et al. 2012, 2013) and reports that ciliated cells project axons to medial regions of the zebrafish olfactory bulb (Sato et al. 2005). Similar to zebrafish (Braubach et al. 2013), in late eyed embryo G_{olf} labeled two ventromedial glomeruli, vmG_x and vmG_7. Consistent to labeling in zebrafish (Gayoso et al. 2011, Braubach et al. 2012), in fry and fingerling stages G_{olf} labeled the dorsal glomeruli (dG), the dorsal lateral glomerular chain (dlG), lateral glomerulus (lG_2) , maG and additional smaller vmGs. maG and small vmGs were also immunoreactive to calretinin at fry stage. These glomeruli were consistently identified at late yolk-sac larvae, fry and fingerling stages in Chinook salmon based on comparable position and characteristic shape within the olfactory bulb to G_{olf} labeled glomeruli in zebrafish (Braubach et al. 2012, 2013).

However, some clear differences were determined between G_{olf} labeling in the zebrafish and Chinook salmon olfactory bulbs. In zebrafish several smaller vmG glomeruli are described along with vmG_x and vmG_7 in ventral medial regions of the olfactory bulb named $vmG_{1\text{-}6}$, vmG_y and another vmG_x, were differentially labeled by either G_{olf}, calretinin or both antibodies (Braubach et al. 2012). However, in the Chinook salmon olfactory bulb numerous small vmG glomeruli are present in the same region but these glomeruli were immunolabeled by both G_{olf} and calretinin. Since this study focussed on early developmental periods, smaller vmG glomeruli solely immunoreactive to either G_{olf} or calretinin may not have developed in the olfactory bulb at this point. Confocal microscopy could be useful in this situation for visualizing individual vmG fibers to confirm if they were labeled by both G_{olf} and calretinin.

Another clear difference between zebrafish and Chinook salmon was the innervation of a ventroposterior glomerulus (vpG_2) with G_{olf} in the Chinook. In zebrafish this glomerulus was only reported to by immunoreactive to KLH antibody (Braubach et al. 2012). Hence, this may suggest that the vpGs, vpG_1 being calretinin immunoreactive and vpG_2 being G_{olf} immunoreactive in Chinook salmon, may have different functional importance compared to zebrafish. Calretinin positive vpG_1 and G_{olf} positive vpG_2 may be innervated by microvillous and ciliated OSNs respectively. Retrograde tracing from these glomerular regions in the olfactory bulb to the olfactory epithelium could be used to identify from which populations of OSNs the vpGs receive inputs.

In a previous study describing the glomerular territories in Chinook salmon larvae from the early yolk-sac larval period to swim up, most glomerular territories described in zebrafish (Braubach et al. 2012) were also labeled from hatch in Chinook salmon with

KLH antibody, a general OSN label that labeled all the glomerular territories (Ochs et al. 2017). Medial anterior and other ventral medial territories corresponding to ciliated OSNs were labeled with KLH in Chinook salmon early yolk-sac larvae (Ochs et al. 2017) but G_{olf} labeling in individual glomeruli did not begin to develop in these regions until the late yolk-sac larvae or fry stages. This could indicate that even though the territories may already have the glomerular architecture in place, the glomeruli may not be functional if factors such as the G-protein used for signal transduction have not yet developed.

Unlike zebrafish which show G_{olf} labeling of glomeruli in embryo comparable to that of adults (Braubach et al, 2012, 2013), labeling with G_{olf} was only present in vmG_x and vmG_7 of the ventromedial olfactory bulb from late eyed embryo to late yolk-sac larvae. Chinook salmon appeared to undergo rapid development of G_{olf} positive regions during late yolk-sac to fry stages when dG and vpG_2 developed, along with maG and vmG glomeruli which also exhibited calretinin labeling. This is consistent with reports that the Chinook salmon undergoes asynchronous growth of the ventral portion of the olfactory bulb during larval development (Ochs et al. 2017). Additionally, a similar pattern of asynchrony in development was seen with calretinin antibody. At late yolk-sac larvae stage characteristic labeling of lateral glomeruli lG_1, $lG_{3/4}$ and lG_6 was present in the olfactory bulb (similar to G_{olf} vmG_x and vmG_7 being present at this stage) but labeling of non-lateral regions with calretinin did not occur until fry and fingerling stages. Calretinin labeled several of the same G_{olf} immunoreactive glomeruli that develop in this period, particularly maG and small vmGs. As medial and ventral regions are typically innervated G_{olf} fibers (Sato et al. 2005), this indicated that calretinin could also be labeling ciliated OSNs.

The apparent asynchrony in the development of different glomerular regions could be a result of experience to certain relevant odours that are in the water that stimulate glomerular growth once they have been detected (Miyasaka et al. 2013, Todrank et al. 2011). For instance, lateral regions of the olfactory bulb are amino acid responsive (Friedrich and Korsching 1998, Sato and Suzuki 2001) and appear to develop distinct glomeruli as early as in late eyed embryo (Figure 2.4) (Castro et al. 2008). This may be because amino acids are a potential component of stream odour that larval salmon imprint to (Shoji et al. 2003) and are also feeding cues that fish to learn to respond to for foraging once their yolk sac is depleted (Hara et al. 2006). Salmon have been shown to respond to behaviourally at the end of larval development to feeding-specific cues, as at this point salmon have absorbed their yolk sac (Mearns 1986). Hence, the various potential functions amino acids play in early fish development may itself be a factor that contributes to development of lateral glomeruli.

Additionally, two G_{olf} positive ventromedial glomeruli are also present in the late eyed embryo. G_{olf} labels ciliated OSNs, which are widely responsive to bile acids (Sato and Suzuki 2001, Schmachtenberg and Bacigalupo 2004), and have been shown to be related to alarm response reaction (Hamdani and Doving 2007). Since salmon exhibit anti-predator behaviour to olfactory cues released by predators even in the larval stages, these G_{olf} positive vmG_x and vmG_7 glomeruli may have a possible function in mediating alarm responses in salmonids (Louhi et al. 2011). Additionally, bile acids, that stimulate ciliated OSNs, are also relevant to other types of olfactory mediated functions including fish having general awareness of surrounding individuals (Huertas et al. 2010) and

foraging (Sorensen 1986), opening different possibilities for the function of G_{olf} positive glomeruli in early development.

Additional G_{olf} immunoreactive ventromedial and medial anterior glomeruli do not develop until fry and fingerling stages. In particular the ventral region of the olfactory bulb has been shown to be responsive to pheromone components (zebrafish: Friedrich and Korsching 1997, 1998, Lastein et al. 2006), which are important for reproductive behaviours in adult fish (Stacey et al. 1986). Additionally, prostaglandin PGF2α, which is a reproductive hormone in female fish (goldfish: Kobayashi et al. 2002) that functions as a sex pheromone in various fish species such as zebrafish (Friedrich and Korsching 1998), carp (Irvine and Sorensen 1988) and salmon (Moore et al. 1996), has recently been shown to activate two ventromedial glomeruli in the olfactory bulb of zebrafish (Yabuki et al. 2016). As these odours are related to reproductive behaviours in fish they may not be functionally relevant in juvenile fish stages, which may help explain why in this study many G_{olf} positive ventromedial glomeruli and ventroposterior glomeruli do not develop until later fry or fingerling stage in Chinook salmon.

S100 labeling, used to visualize crypt cells, was present in the olfactory epithelium from embryonic stages and through larval development in cells situated apically in the olfactory epithelium, sometimes exhibiting a short dendrite. This cell description is consistent with either crypt or microvillous cells. S100 fibers were only apparent in the olfactory bulb during the fingerling stage. However, the fibers did not terminate onto the dorsalmedial glomerulus where crypt cells project their axons to in zebrafish (Ahuja et al. 2013), nor did they terminate in ventral glomeruli of the olfactory bulb like in catfish (Hansen et al. 2003). In Chinook salmon fingerlings S100 fibers were

present in lateral regions of the olfactory bulb. However, the lateral olfactory bulb typically receives axonal innervation from microvillous OSNs (Sato et al. 2005). S100 marker has been shown in some studies to also label a subset of microvillous OSNs (Gayoso et al. 2011, Kress et al. 2015, Biechl et al. 2016) which may explain S100 labeling in lateral glomeruli. Braubach et al. 2012 mentioned that S100 labeling was occasionally present in the lateral glomeruli and lateral plexus of zebrafish but did not attribute this labeling to crypt cells, noting them as inconsistencies in the labeling due to the immunohistochemical procedure. Due to conflicting reports of S100 labeling using a more reliable marker for crypt cells or tract tracing experiments could help in understanding if these fibers truly come from microvillous or crypt cells or if they are artifacts of the labeling procedure. In particular, anti-Trk-A antibody has been used to mark crypt cells in zebrafish and their axonal projections into the olfactory bulb (Ahuja et al. 2013). This may be a potential marker to label cryt cells and their axons to confirm if there is a specific glomerulus crypt cells project their axons to in salmon.

Overall, this study gives a description of olfactory system development in early life stages of Chinook salmon and opens questions into the significance of specific groups of glomeruli developing at different times. The ontogeny of different OSN morphotypes may be reflective of biological relevance of different classes of odours at different points of development of salmon. Additionally, our knowledge of glomerular patterning in the developing olfactory bulb of a wild fish species was expanded with similarities observed to patterning in the model species zebrafish, particularly the general location and shape of glomeruli. However, some noticeable differences were also seen, such as in the timing of when certain glomeruli emerge in the olfactory bulb.

Understanding the glomerular patterning in the olfactory bulb of wild caught salmon species is a useful tool for determining the relevance of glomerular maps in model fish species to wild fish populations. This study builds on previous research in calretinin immunoreactivity in larval Chinook salmon (Ochs et al. 2017) to create a more complete view of the glomerular patterning in the olfactory bulb, which is a basis for neuroanatomical studies for olfaction.

2.5 References

Ahuja, G., Ivandic, I., Salturk, M., Oka, Y., Nadler, W., Korsching, S. (2013). Zebrafish crypt neurons project to a single, identified mediodorsal glomerulus. *Scientific Reports*. 3:2063.

Ahuja, G., Bozorg Nia, S., Zapilka, V., Shiriagin, V., Kowatschew, D., Oka, Y., Korsching, S.I.(2014). Kappe neurons, a novel population of olfactory sensory neurons. *Scientific Reports*. 4:4037.

Baier, H., Korsching, S. (1994). Olfactory glomeruli in the zebrafish form an invariant pattern and are identifiable across animal. *Journal of Neuroscience*. 14: 219-230.

Bazaes, A., Schmachtenberg, O. (2012). Odorant tuning of olfactory crypt cells from juvenile and adult rainbow trout. *The Journal of Experimental Biology*. 215:1740 1748.

Biechl, D., Tietje, K., Gerlach, G., Wullimann, M.F. (2016). Crypt cells are involved in kin recognition in larval zebrafish. *Scientific Reports.* 6: article 24590.

Braubach, O., Fine, A., Croll, P. (2012). Distribution and functional organization of glomeruli in the olfactory bulbs of zebrafish (*Danio rerio*). *Journal of Comparative Neurology*.520(11): 2317-39.

Braubach, O., Miyasaka, N., Koide, T., Yoshihara, Y., Croll, R.P., Fine, A. (2013). Experience dependent versus experience-independent postembryonic development of distinct groups of zebrafish olfactory glomeruli. *The Journal of Neuroscience.* 33(16): 6905-16.

Butts IAE, Johnson K, Wilson CC, Pitcher TE. (2012). Ovarian fluid enhances sperm velocity based on relatedness in lake trout, *Salvelinus namaycush*. *Theriogenology*. 78:2105—2109.

Castro, A., Becerra, M., Anadon, R., Manso, M.J. (2008). Distribution of calretinin during development of the olfactory system in brown tout, *Salmo trutta fario:* comaparison with other immunohistochemical markers. *Journal of Chemical Neuroanatomy.* 35(4):206-316.

Crisp DT. (1981). A desk study of the relationship between temperature and hatching time for the eggs of 5 species of salmonid fishes. *Freshwater Biology*. 11:361—368.

Dittman, A.H., Pearsons, T.N., May, D., Couture, R.B., Noakes, D.L.G. (2015). Imprinting of hatchery-reared salmon to targeted spawning locations: a new embryonic imprinting paradigm for hatchery programs. *Fisheries.* 40(3): 114-123.

Doldan, M.J., Mantilla, L., de Miguel Villegas, E. (2011). Development of the olfactory system in turbot (*Psetta maxima L.). Journal of Chemical Neuroanatomy.* 41(3): 148-157.

Ferrando, S., Bottaro, M., Gallus, L., Girosi, L., Vacchi, M., Tagliafierro, G. (2007) First detection of olfactory marker protein (OMP) immunoreactivity in the olfactory epithelium of a cartilaginous fish. *Neuroscience Letters.* 413(2):173-176.

Friedrich, R.W., Korsching, S. (1997). Combinatorial and chemotopic odorant coding in the zebrafish olfactory bulb visualized by optical imaging. *Neuron.* 18:737-752.

Friedrich, R.W., Korsching, S. (1998). Chemotopic, combinatorial, and noncombinatorial odorant representations in olfactory bulb revealed using a voltage-sensitive axon tracer. *Journal of Neuroscience.* 18(23):9977-9988.

Gayoso, J.A., Castro, A., Anadon, R., Manso, M.J. (2011). Differential bulbar and extrabulbar projections of diverse olfactory receptor neuron population in the adult zebrafish *(Daniorerio*). *Journal of Comparative Neuroscience.* 519: 247 276.

Gayoso, J., Castro, A., Anadón, R., Manso, M.J. (2012). Crypt cells of the zebrafish Danio rerio mainly project to the dorsomedial glomerular field of the olfactory bulb. *Chemical Senses*. 37:357–369.

Germana, G., Montalbano, R., Ciriaco, E., del Valle, M.E., Vega, J.A. (2004). S100 protein-like immunoreactivity in the crypt olfactory neurons of the adult zebrafish. *Neuroscience Letters.* 371(2-3): 196-198.

Hamdani, E.H., Doving, K.B. (2007). The functional organization of the fish olfactory system. *Progress in Neurobiology*. 82(2):80-86.

Hansen, A., Rolen, S.H., Anderson, K., Morita, Y., Caprio, J., Finger, T.E. (2003). Correlation between olfactory receptor cell type and function in channel catfish. *Journal of Neuroscience.* 23(28):9328-9339.

Hansen, A., Anderson, K.T., Finger, T.E. (2004). Differential distribution of olfactory receptor neurons in goldfish: structural and molecular correlates. *Journal of Comparative Neurology*. 477: 347-359.

Hansen, A., Zeiske, E. (1998). The peripheral olfactory organ of zebrafish, *Danio rerio*: an ultrastructure study. *Chemical Senses*. 23(1): 39-48.

Hara, T.J. (2006). Feeding behaviour in some teleosts is triggered by single amino acids primarily through olfaction. *Journal of Fish Biology*. 68(3): 810-825.

Huertas, M., Hagey, L., Hofmann, A.F., Cerga, J., Canario, A.V.M., Hubbard, P.C. (2010). Olfactory sensitivity to bile fluid and bile salts in the European eel (*Anguilla anguilla),* goldfish (*Carassius auratus)* and Mozambique tilapia (*Oreochromis mossambicus)* suggests a 'broad range' sensitivity not confined to those produced by conspecifics alone. *Journal of Experimental Biology.* 213:308 317.

Irvine, I.A.S., Sorensen, P.W. (1993). Acute olfactory sensitivity of wild common carp, *Cyprinus carpio,* to goldfish hormonal sex pheromones is influenced by gonadal maturity. *Canadian Journal of Zoology.* 71:2199-2210.

Kerman, F., Franco, L.M., Wyatt, C., Yaksi, E. (2013). Neural circuits mediating olfactory-driven behaviour in fish. *Front Neural Circuit 7.*

Kobayashi, M., Sorensen, P.W., Stacey, N.E. (2002). Hormonal and pheromonal control of spawning behavior in the goldfish. *Fish Physiology and Biochemistry.* 26:71 84.

Koide, T., Miyasaka, N., Morimoto, K., Asakawa, K., Urasaki, A., Kawakami, K., Yoshihara, Y. (2009). *Proceedings of the National Academy of Sciences.* 106:9884-9889.

Kress, S., Biechl, D., Wullimann, M.F. (2015). Combinatorial analysis of calcium-binding proteins in larval and adult zebrafish primary olfactory system identifies differential olfactory bulb glomerular projection fields. *Brain Structure and Function.* 220: 1951-1970.

Lastein, S., Hamadi, E.H., Doving, K.B. (2006). Gender distinction in neural discrimination of sex pheromones in the olfactory bulb of crucian carp, *Carassius carassius. Chemical Senses.* 31(1):69-77.

Louhi, P., Ovasaka, M., Maki-Petays, A., Erkinaro, J., Muotka T. (2011). Does fine sediment constrain salmonid alevin development and surivival? *Canadian Journal of Fisheries and Aquatic Sciences.* 68:1819-1826.

Mearns, K.J. (1986). Sensitivity of brown tout (*Salmo trutta L.)* and Atlantic salmon (*Salmo salar L.)* fry to amino acids at the start of exogenous feeding. *Aquaculture.* 55(3):191-200.

Moore, A., Waring, C.P. (1996) Electrophysiological and endocrinological evidence that F series prostaglandins function as priming pheromones in mature male Atlantic salmon (*Salmo salar parr). Journal of Experimental Biology.* 199:2307-2316.

Miyasaka, N., Wanner, A.A., Li, Jun., Mack-Bucher, J., Genoud, C., Yoshishara, Y., Friedrich, R.W. (2013). Functional development of the olfactory system in zebrafish. *Mechanisms of Development.* 130(6-8):336-346.

Ochs, C.L., Suntres, T., Zygowska, A., Pitcher, T., Zielinski, B.S. (2017). Organization of glomerular territories in the olfactory bulb of post-embryonic wild Chinook salmon *Oncoryhynchus tshawytscha*. *Journal of Morphology*. 278(4): 464-474.

Pitcher TE, Neff BD. (2006). MHC class IIB alleles contribute to both additive and nonadditive genetic effects on survival in chinook salmon. *Molecular Ecology*. 15:2357—2365.

Riddle, D.R., Oakley, B. (1992). Immunocytochemical identification of primary olfactory afferents in rainbow trout. *Journal of Comparative Neurology*. 324 (4): 575-589.

Sandahl, J.F., Muyasaka, G., Koide, N., Ueda, H. (2006). Olfactory inhibition and recovery in chum salmon. *Canadian Journal of Fisheries and Aquatic Sciences.* 63:1840-1847.

Sato,Y., Suzuki, N. (2001). Whole-cell response characteristics of ciliated and microvillous olfactory receptor neurons to amino acids, pheromone candidates and urine in rainbow trout. *Chemical Senses.* 26(9):1145-1156.

Sato, Y., Miyasaka, N., Yoshihara, Y. (2005) Mutually exclusive glomerular innervation by two distinct types of olfactory sensory neurons revealed in transgenic zebrafish. *The Journal of Neuroscience*. 25(20): 4889-4897.

Schmachtenberg, O., Bacigalupo, J. (2004). Olfactory transduction in ciliated receptor neurons of the Cabinza grunt, *Isacia conceptionis* (teleostei: haemulidae). *European Journal of Neuroscience.* 20: 3378-3386.

Shoji, T., Yamamoto, Y., Nishikawa, D., Kurihara, K., Ueda, H. (2003). Amino acids in stream water are essential for salmon homing migration. *Fish Physiology and Biochemistry. 28:249.*

Sorensen, P.W., Bianchinni, M.L., Winn, H.E. (1986). Diel foraging activity of American eels, *Anguilla-rostrata (L),* in a Rhode-island estuary. *Fishery Bulletin.* 84(3):746-747.

Sorensen, P.W., Caprio, J. (1998). Chemoreception. In: Evans DH, editor. *The Physiology of fishes*. Boca Raton (FL): CRC Press. p. 375-405.

Stacey, N.E., Kyle A.L., Liley, N.R. (1986). Fish reproductive pheromones. *Chemical Signals in Vertebrates*. (4).

Tilson, M.B., Scholz, A.T., White, R.J., Galloway, H. (1994). Thyroid-induced chemical imprinting in early life stages and assessment of smoltification in kokanee salmon hatcheries. *1993 Annual report*. Prepared for Bonneville Power Administration, Portland, Oregon.

Todrank, J., Heth, G., Restrepo, D. (2011). Effects of in utero odorant exposure on neuroanatomical development of the olfactory bulb and odour preferences. *Proceedings of the Royal Society B: Biological Sciences*. 278(1714):1949–1955.

Yabuki, Y., Koide, T., Miyasaka, N., Wakisaka, N., Masuda, M., Ohkura, M., Nakai, J., Tsuge, K., Tsuchiya, S., Sugimoto, Y., Yoshihara, Y. (2016). Olfactory receptor for prostaglandin F-2alpha mediates courtship behavior of male zebrafish. *Chemical Senses.* 41(9):143.

Zielinski, B., Hara, T. (1988). Morphological and physiological development of olfactory receptor cells in rainbow trout (Salmo gairdneri) embryos. *The Journal of Comparative Neurology*. 271(2): 300-311.

2.6 Tables and Figures

Table 2.1: Chinook salmon early developmental stages from embryo to fingerling. Temperature units (degree days) were calculated by summing average daily temperatures (Crisp 1981) and stage naming was based on Ochs et al. 2017 and visual observations.

Stage		Description	Degree days (dd)
Embryonic	Early eyed embryo	All embryos have eyes visible through the egg shell	266
	Late eyed embryo	Eye and fish body very pronounced under egg shell.	410-470
Larval	Early yolk sac larvae	Fish have hatched. Large yolk sac weighs fish down within the water.	505
	Late yolk-sac larvae	Fish have absorbed approximately half their yolk sacs. Have started swimming in Heath trays.	714
	Fry	Fish have almost completely reabsorbed their yolk sacs. Fish are very active in Heath trays.	920
Fingerling		Fish transferred to hatchery tanks and are now actively feeding	1257-1537

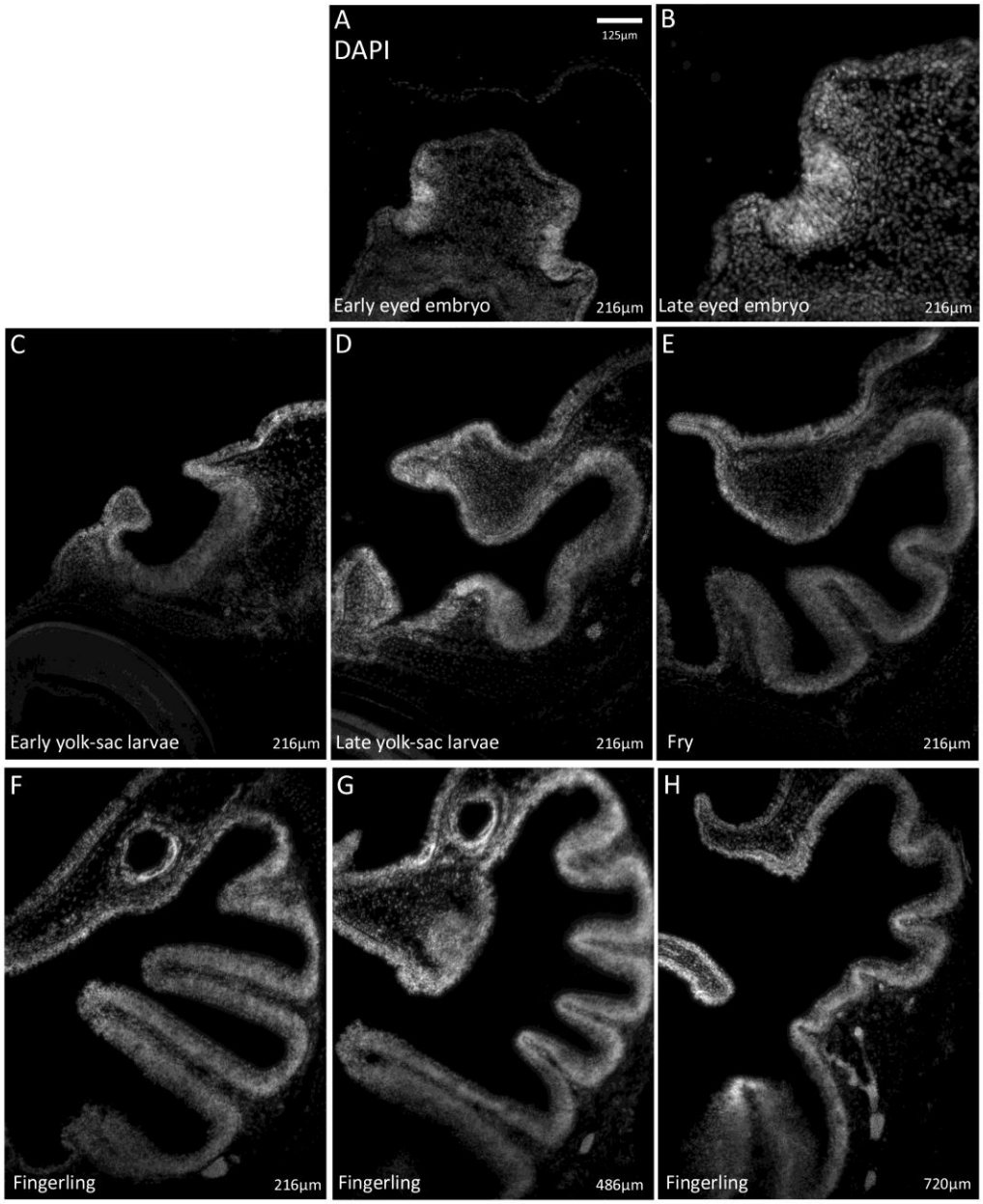

Figure 2.1: Increase in number of lamellae in Chinook salmon olfactory epithelium embryonic, larval and fingerling stages. DAPI stained nuclei of cells in olfactory epithelium showing outline of lamellae. A-F show the olfactory epithelium at the same depth (216µm) across early developmental stages and increase in epithelial folds from 0 folds in embryo (A-B), to 2 small invaginations in late yolk-sac larvae (D) and then 3 to 5 distinguishable lamellae in fry and fingerlings (E-G). G and H show olfactory epithelium at greater depths in the fingerling stage where even more lamellae are found (G: 486µm depth) and at the caudal edge of the peripheral olfactory organ (H: 720µm). Scale bar in A of 125µm applies to A-H. Depth in the olfactory epithelium is on the lower right corner of each micrograph.

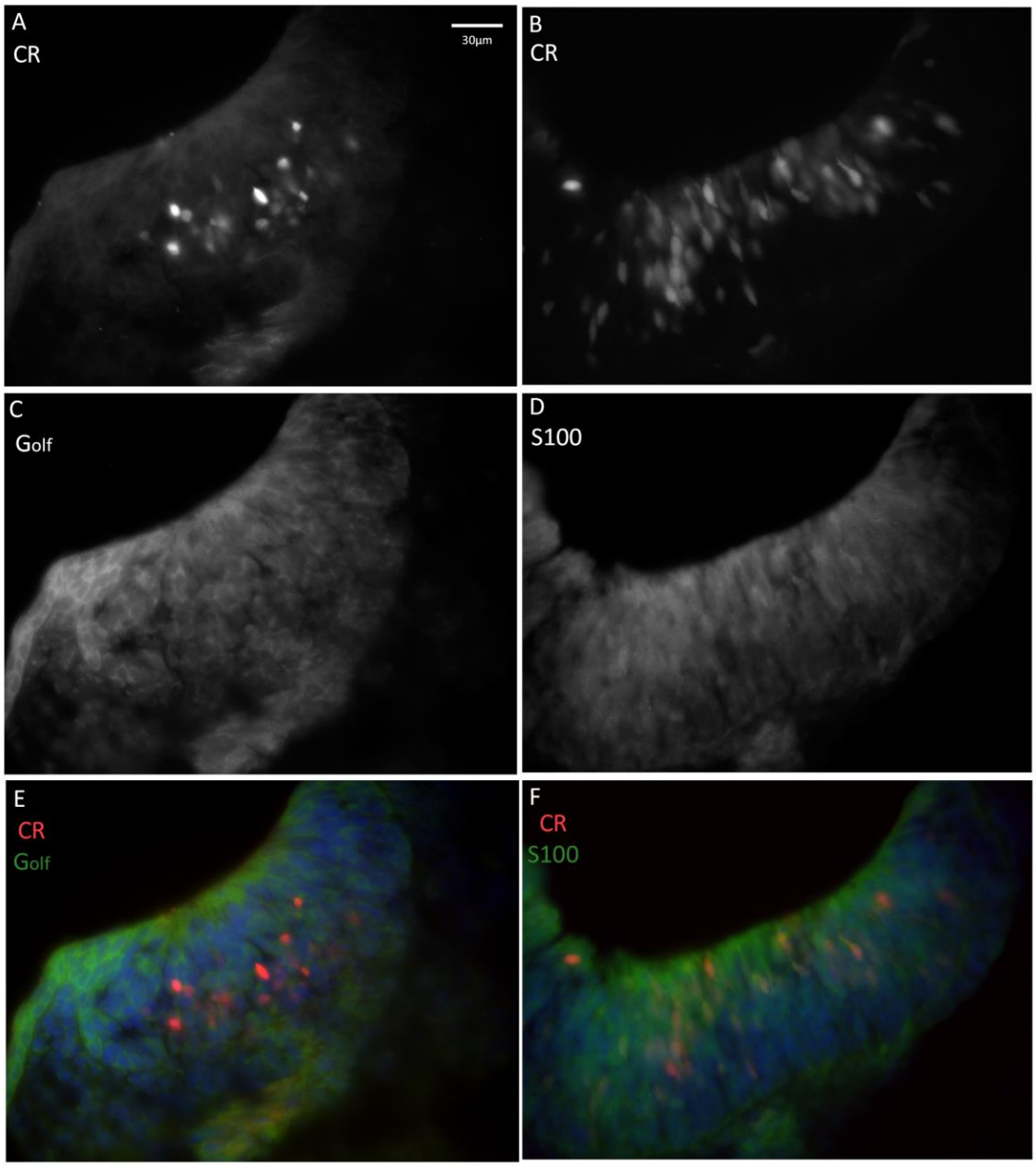

Figure 2.2: Olfactory epithelium of early eyed embryo exhibits calretinin, G_{olf} and S100 labeling. A, B: Calretinin immunolabeled cells were visible in the olfactory epithelium with some fibers extending from a primitive nerve at an early eyed embryonic stage (A). C: G_{olf} labeling was present dispersed throughout the olfactory epithelium. D: S100 labeling is present at apical portions of the olfactory epithelium. E and F: merged image of calretinin (red) and Golf (green) (E) and calretinin (red) and S100 (green) immunoreactivity in eyed embryo olfactory epithelium with DAPI counterstain. Scale bar in A of 30µm applies to A-F.

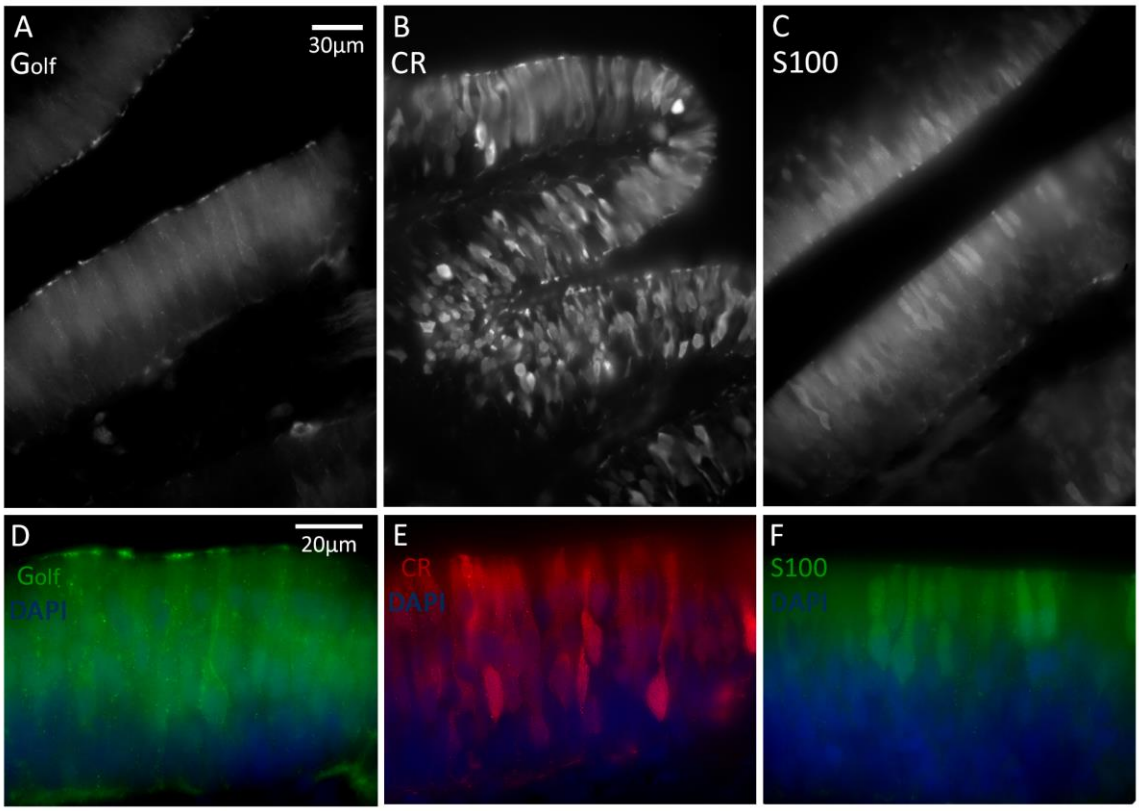

Figure 2.3: Calretinin, G_{olf} and S100 positive OSNs in fingerling olfactory epithelium. A-C: G_{olf}, CR and S100 cells were widespread throughout the epithelium. D: G_{olf} positive cells appeared to have their cell bodies deep in the epithelium with a long dendrite. E: CR positive cells were present at various levels of the epithelium but most were in intermediate layers. F: S100 positive cells were situated close to the apical layer of the epithelium and some cells also had small dendrites. Scale bar in A corresponds to micrographs A-C, while the scale bar in D corresponds to D-F.

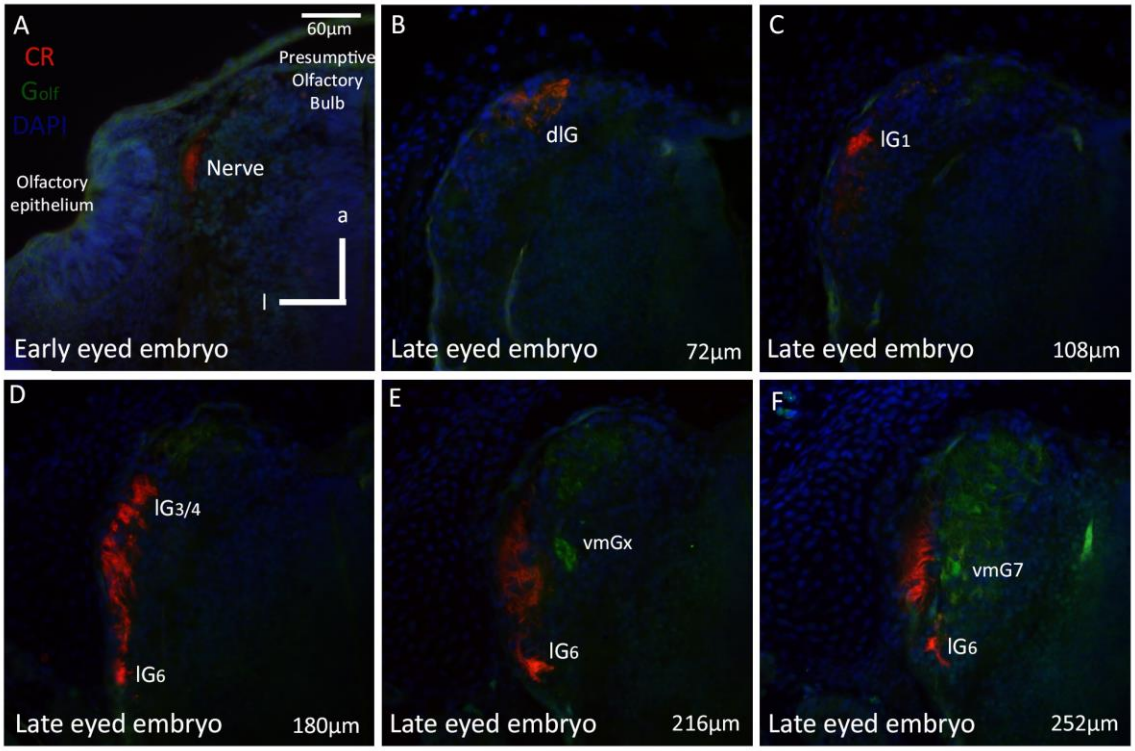

Figure 2.4: Late eyed embryo olfactory bulb exhibited calretinin positive lateral glomeruli and two G_{olf} positive ventromedial glomeruli. A: calretinin labeling is present in nerve outside the presumptive olfactory bulb. B-F: calretinin labeled the dlG and lateral region (lG) of the late eyed stage olfactory bulb, particularly lG_1 (C), $lG_{3/4}$ (D) and lG_6 (D-F). E-F: G_{olf} labeling is seen in two small ventromedial glomerulus (vmG_x and vmG_7). Bulbar depths are noted at the lower right hand corner of each micrograph. Scale bar in A of 60µm applies to A-F. Depth of the olfactory bulb section is in the lower right corner of each micrograph.

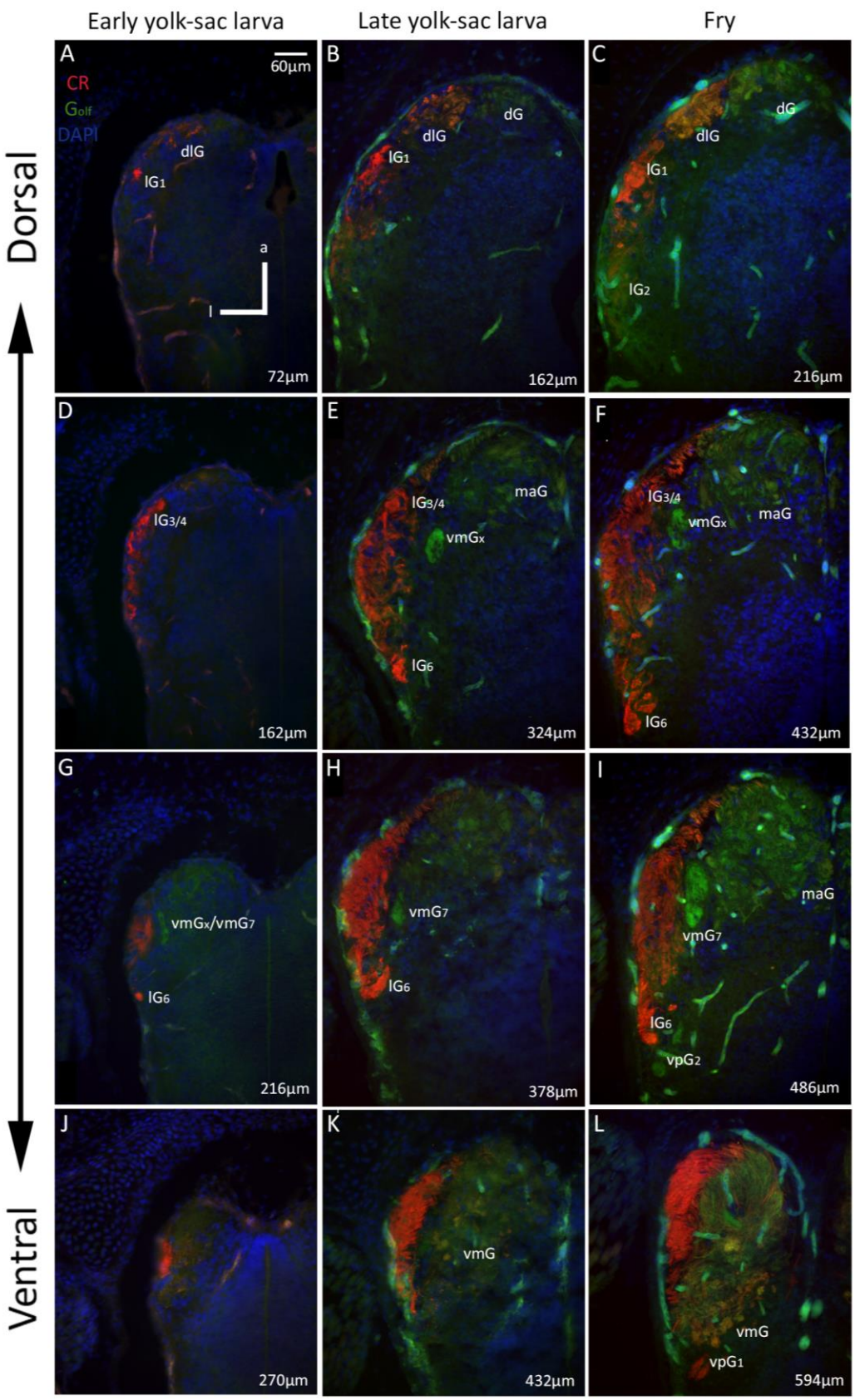
Early yolk-sac larva
Late yolk-sac larva
Fry
Dorsal
Ventral
A
60µm
CR
Golf
DAPI
dlG
lG1
a
l
72µm
B
dG
dlG
lG1
162µm
C
dG
dlG
lG1
lG2
216µm
D
lG3/4
162µm
E
maG
lG3/4
vmGx
lG6
324µm
F
lG3/4
vmGx
maG
lG6
432µm
G
vmGx/vmG7
lG6
216µm
H
vmG7
lG6
378µm
I
maG
vmG7
lG6
vpG2
486µm
J
270µm
K
vmG
432µm
L
vmG
vpG1
594µm

Figure 2.5: Calretinin and G_{olf} immunoreactive glomeruli during larval stages. DAPI counterstaining marks cell nuclei outlining of the olfactory bulb in the micrographs. Calretinin (red) positive glomeruli are present primarily in the lateral region of the olfactory bulb. A-C: In dorsal regions of the olfactory bulb CR immunoreactivity is present in dlG and lG_1 throughout larval stages. G_{olf} (green) also labeled dlG (double labeled in orange) and dG at late yolk-sac larvae and fry stages. D-I: midway through the olfactory bulb calretinin labels anteriorly situated $lG_{3/4}$ and lG_6 in the posterior bulb. G_{olf} fibers innervate vmG_x and vmG_7 medial to $lG_{3/4}$ and medial anterior glomeruli in late yolk-sac larvae and fry (E,F, H,I). Golf also labels vpG_2 in the ventral posterior olfactory bulb of fry. J-L: calretinin and G_{olf} positive nerve layer is visible in ventral regions of olfactory bulb with some double labeled vmGs present in late yolk-sac and fry stages. Calretinin labels posteriorly located vpG_1 (L). 60µm scale bar in A applies to A-L. Depth of the olfactory bulb section is in the lower right corner of each micrograph.

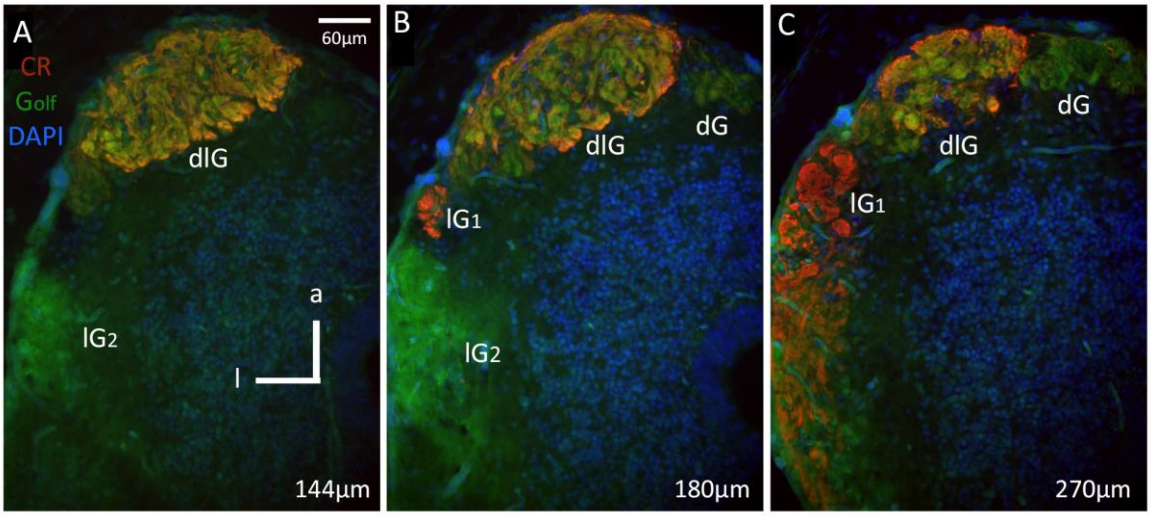

Figure 2.6: Dorsally located calretinin and G_{olf} positive glomeruli of the fingerling Chinook salmon olfactory bulb. A-C: Calretinin (red) and G_{olf} (green) both labeled dlG in the dorsal olfactory bulb (double labeled area coloured orange), but only Golf diffusely labeled the lG_2 region (A,B). B-C: Calretinin labeling of lG_1 and G_{olf} labeling of dG appear at 180µm bulbar depth. Scale bar of 60µm corresponds to A-C. Depth of the olfactory bulb section is in the lower right corner of each micrograph.

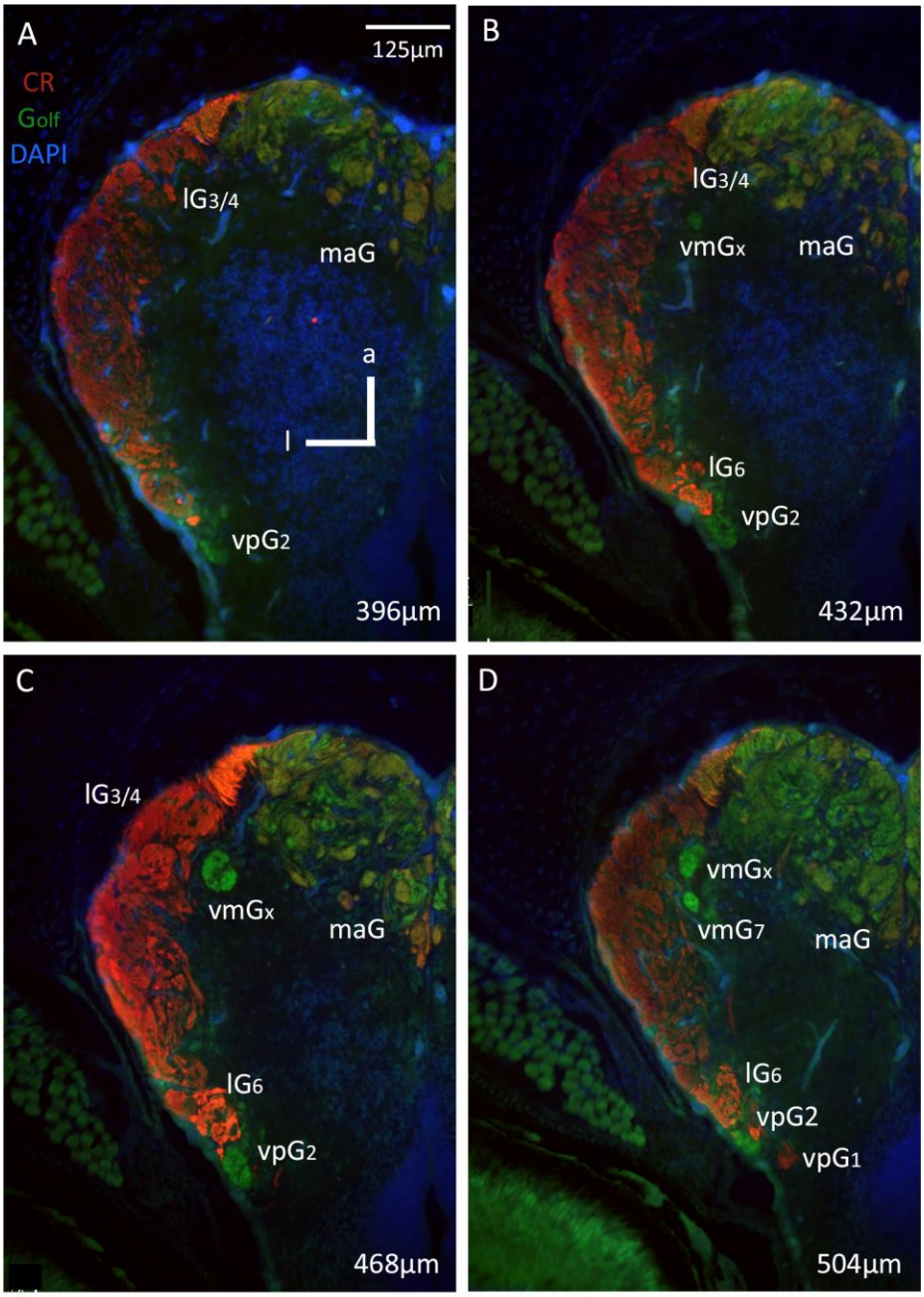

Figure 2.7: Calretinin and G_{olf} positive glomeruli in the medial and ventral regions of the fingerling olfactory bulb. Calretinin (red) labeling is present in lateral and anteriorly situated $lG_{3/4}$ (A-C) and posteriorly situated lG_6 (B-D). G_{olf} (green) immunoreactivity is present in vmGx (B-D) and ventral to this glomerulus vmG_7 (D). G_{olf} labeled vpG_2 situated posterior to lG_6 (A-D). Additionally, G_{olf} labels the maG region with calretinin fibers innervating some maG glomeruli (A-D: orange double labeled). A-D: scale bar of 125µm. Depth of the olfactory bulb section is in the lower right corner of each micrograph.

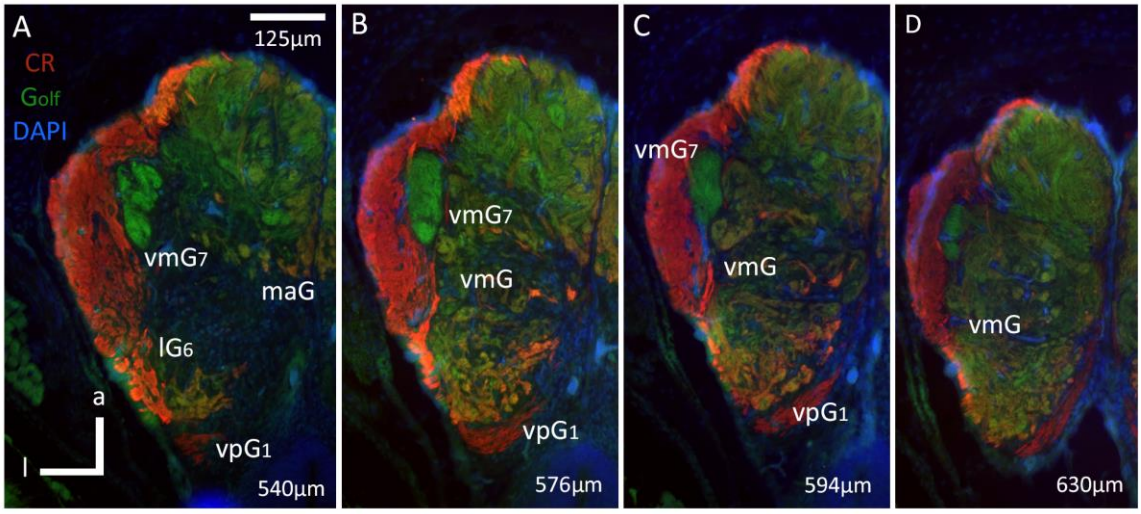

Figure 2.8: Calretinin and G_{olf} positive glomeruli in the ventral olfactory bulb and nerve layer of the Chinook salmon fingerling. G_{olf} (green) is present in vmG_7 (A-C) in ventral portions of the olfactory bulb and numerous smaller vmG glomeruli (B-D) which also show innervation with calretinin fibers (orange double labeled). Calretinin (red) labeling was present in ventrally and posteriorly situated vpG_1 (A-C). Scale bar of 125µm in A applies to micrographs A-D. Depth of the olfactory bulb section is in the lower right corner of each micrograph.

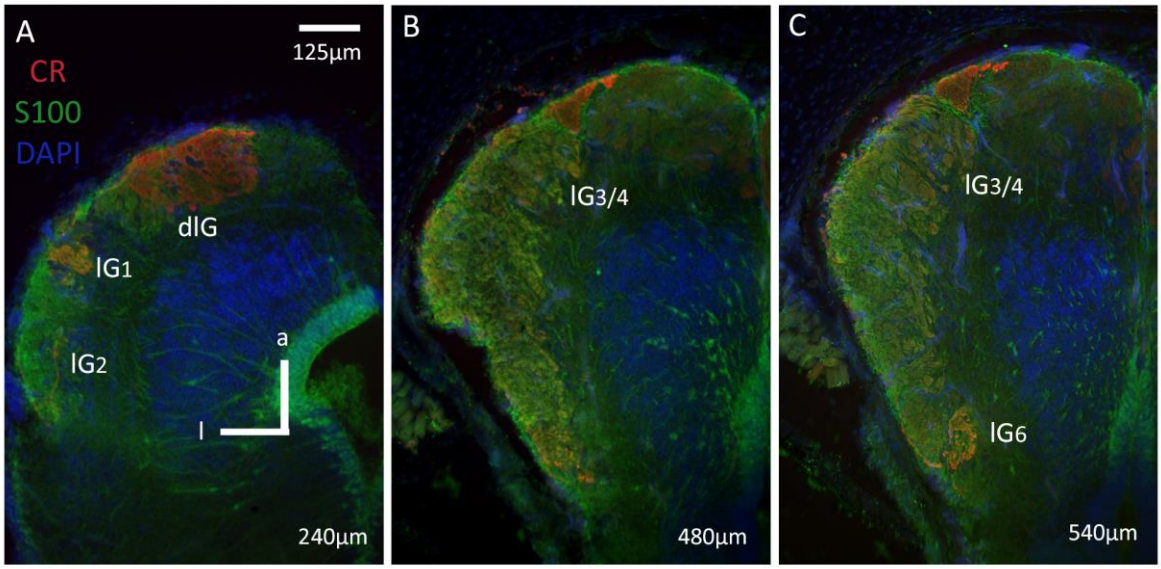

Figure 2.9: S100 and calretinin labeling in lateral regions of fingerling olfactory bulb. A-C: S100 (green) showed labeling in lateral regions of the olfactory bulb. A: S100 fibers did not innervate calretinin (red) positive regions of dlG but double labeling is seen in lG_1 (orange). B-C: S100 and calretinin label $lG_{3/4}$. C: posterior and ventral lG_6 regions shows labeling with S100 and CR. The scale bar represents 125µm in A-C. Depth of the olfactory bulb section is in the lower right corner of each micrograph.

Table 2.2: Summary of calretinin, G_{olf} and S100 labeling of olfactory bulb glomeruli over embryonic, larval and fingerling stages in Chinook salmon. The plus sign (+) denotes that the glomerulus was immunoreactive to antibody while the minus sign (-) means the glomerulus was not immunoreactive to the antibody at the particular stage. Glomeruli were described as "absent" if they were not present in the olfactory bulb at the life stage.

	Embryo			**Larval**			**Fingerling**		
	CR	G_{olf}	S100	CR	G_{olf}	S100	CR	G_{olf}	S100
dlG	+	+	-	+	+	-	+	+	-
dG	Absent			-	+	-	+	+	-
lG_1	+	-	-	+	-	-	+	-	+
lG_2	Absent			-	+	-	-	+	+
$lG_{3/4}$	+	-	-	+	-	-	+	-	+
lG_6	+	-	-	+	-	-	+	-	+
maG	Absent			+	+	-	+	+	-
vmG_x	-	+	-	-	+	-	-	+	-
vmG_7	-	+	-	-	+	-	-	+	-
vpG_1	Absent			-	+	-	-	+	-
vpG_2	Absent			+	-	-	+	-	-

CHAPTER 3: EFFECT OF OLFACTORY EXPERIENCE ON GLOMERULAR DEVELOPMENT DURING THE LARVAL PERIOD IN CHINOOK SALMON (*Oncorhynchus tshawytscha*)

3.1 Introduction

Imprinting is the rapid learning during a sensitive temporal window called a critical period forming a memory that can be recognized without reinforcement (Bateson 1990). Imprinting has been linked to various types of learning in different species, such as song learning of juvenile male birds through mimicry of adult male's songs (Doupe and Kuhl 1999) and fish imprinting to kin odour for kin recognition (Biechl et al. 2016). The olfactory imprinting hypothesis suggests that during early development salmon imprint to the unique odour composition of the stream where they were hatched and use the odour memory as adults to return to the same stream for spawning (Hasler et al. 1978). Studies exposing salmonids to artificial odourarnts phenylethyl alcohol (PEA) or morpholine at different developmental stages indicated that a sensitive window for imprinting may occur during the larval stage of development (Tilson et al. 1994, Dittman et al. 2015). During the larval stage, when rapid maturation of the olfactory organ is seen in several fish species (Zeiske et al. 2003, Ochs et al. 2017, see Chapter 2 of this thesis), chinook salmon remain in the redds where they are directly exposed to their natal stream water. This makes the larval stage a period that could potentially be very responsive to olfactory stimulation for imprinting.

The distinctive amino acid composition of streams, which show a stable composition year to year (Yamamoto et al. 2013), is a component of a stream's odour signature that salmon can potentially imprint to during early development and use to

guide homing migration later in life (Shoji et al. 2003). Recognition of home stream water by its signature amino acid composition was shown in chum salmon (*O. Keta*) that preferred artificial stream water with a dissolved free amino acid composition similar to that of their natural natal stream water over artificial stream water with a dissolved free amino acid composition not typical of their natal streams (Shoji et al., 2003; Yamamoto and Ueda, 2009). However, how the brain translates olfactory experience during a critical stage of development into a lasting odour memory is not well understood.

The olfactory system in fish is composed of the peripheral olfactory epithelium and central brain structures such as the olfactory bulb and telencephalon (Laberge and Hara 2001). Olfactory sensory neurons (OSNs) in the olfactory epithelium bind odorant molecules and conduct the odour information electrically along the OSN axon to the olfactory bulb (Ngai et al. 1993). Axon terminals of OSNs binding specific odorants aggregate together within the olfactory bulb giving rise to discreet glomeruli, which are functional units of odour discrimination with a highly stereotyped organization (Friedrich and Korsching 1997, Baier and Korsching 1994, Gayoso et al. 2011, Braubach et al. 2012). Glomeruli, with their predictable organization and ability to facilitate odorant identification, may be units on which sensory stimulation could leave long lasting impacts.

Studies in insects (*Drosophila* and *Apis)* and mammals (mice) have indicated that olfactory imprinting results in an increase in the volume of the corresponding glomerulus (Sachse et al. 2007, Sigg et al. 1997, Arenas et al. 2012, Todrank et al. 2011). In teleosts, zebrafish imprinted to a kin odour early in development showed increased activation of specific OSNs and olfactory bulb cells surrounding the corresponding glomerulus

compared to non-imprinted fish when exposed to the kin odour again as adults (Biechl et al. 2016, 2017). Coho salmon (*O. kisutch*) imprinted to PEA showed increased sensitivity to the PEA odour compared to naïve fish in individual patch clamp recordings of OSNs a year after odour imprinting took place (Nevitt et al. 1994). Alternatively, zebrafish imprinted as embryos to PEA showed downregulation of several OR olfactory receptor genes as juveniles and adults (Calfun et al. 2016). A study of amino acid enrichment in zebrafish indicated that fish exposed to the 1μM amino acid mixture during embryonic and larval development had more numerous small lateral glomeruli (lG_x) but with decreased cross sectional areas compared to control fish (Braubach et al. 2013). Exposure of Chinook salmon larvae to an amino acids mixture showed no difference in volume of large lateral glomeruli between paired control and amino acid exposed fish (Ochs 2015). Hence, measures such as glomerular volume have been used to investigate the effects of olfactory experience in the brain of many organisms but results have not been clear.

The aim of this paper is to investigate olfactory sensory neuroanatomical evidence of olfactory stimulation in wild Chinook salmon during larval development, which is a sensiritve period for imprinting for homing migration in salmon (Tilson et al. 1994). To compare river and laboratory water composition, we measured the amino acid concentrations between river water and water used to raise the fish in salmon trays over a four month period (October to January) when Chinook salmon would be found at embryonic and larval stages in the wild. This was also used to see which amino acids could potentially be used by salmon for imprinting. In this study amino acids were used for olfactory enrichment because of their potential as imprinting odours for homing. Odour exposure began 3 days before the fish hatched and continued through the larval

period, a potential critical period for imprinting. However, compared to the 2015 study by Ochs stock concentrations of added amino acids were increased by approximately three times to take into account endogenous amino acids in municipal water. Since amino acids stimulate the lateral region of the olfactory bulb (Friedrich and Korsching 1997) a calretinin antibody was used to label lateral glomeruli lG_1, $lG_{3/4}$ and lG_6 for volume analysis, as seen in larval Chinook salmon (Ochs et al. 2017).

Chinook salmon were also exposed to PEA, a compound found in plants (Erbas and Baydar 2016, Medjahed et al. 2016) and commonly used in salmon imprinting experiments, but not usually found in municipal water (Bett et al. 2016). Odorant exposure began once the unhatched embryos were eyed and continued throughout larval development as these stages are a critical period for PEA imprinting in salmon (Tilson et al. 1994, Dittman et al. 2015). Calfun et al. (2016) found that PEA imprinting downregulated some OR olfactory receptor genes in zebrafish and suggested that PEA also causes upregulation of other olfactory receptors not yet identified. However, since PEA seemed to be affect the regulation of OR olfactory receptors that correspond to ciliated cells that are marked by G_{olf} antibody (Sato et al. 2005, Oka and Korsching 2011**,** Hansen et al. 2004**,** Hussain et al. 2009), this study used a G_{olf} antibody (which labeled vmG_x and vmG_7 in Chinook salmon larvae - see Chapter 2) to label potential glomerular targets of PEA in the Chinook salmon olfactory bulb. In the Chinook salmon olfactory bulb the only other glomeruli that were present consistently from embryonic to larval stages besides amino acid responsive lateral glomeruli were two ventromedial glomeruli vmG_x and vmG_7 labeled by G_{olf} (see Chapter 2 of this thesis). Hence, since these glomeruli are present from the eyed embryonic stage they may be important for early

olfactory learning such as for imprinting and therefore were identified as possible targets of olfactory experience to the PEA odour to be measured at early yolk-sac larvae, late yolk-sac larvae and fry stages. Based on previous olfactory enrichment studies in zebrafish (Braubach et al. 2013) we expected to see a decrease in glomerular volume with olfactory experience due to a refinement of glomerular OSN terminals.

3.2 Material and Methods

Water sample collection

Water from the Credit River (43^{o}N, 79^{o}W) and the University of Windsor was collected simultaneously every month from October to January. Chinook salmon were spawning in October with eggs likely hatching in December. Hence, water samples were analyzed for the period when the Chinook salmon would be undergoing embryonic and early larval development. Water was collected from the same location at the Credit River, a spawning location for Chinook salmon, each month located approximately 1km downstream of the Streetsville Dam. Water from the University of Windsor was taken from salmon trays sourced by municipal water housing Chinook salmon eggs. 80mL of water was collected for each sample into two 50mL falcon tubes, filtered with a Millipore Stericup with 0.22µm GP Express PLUS Membrane for volumes less than 150mL (cat #: SCGPU01RE) and frozen at -20^{o}C. The water samples were sent to the SPARC Biocenter attached to the Sickkids Hospital in Toronto for amino acid analysis. There the water samples were lyophilized and then reconstituted with HCl before mass spectrometry using a Waters ACQUITY UPLC system including UV-VIS and fluorescence detectors to determine the amino acid composition of the water samples.

Fish rearing and water collection

Animal care and handling were approved by the University of Windsor Animal Care Committee in compliance to guidelines set by the Canada Council of Animal Care. Eggs and milt was collected from spawning Chinook salmon captured from the Credit River (Mississauga, Ontario) by electro-shock techniques in October of both 2015 and 2016 (described in: Pitcher and Neff 2006, Butts et al. 2012). The eggs and milt were then transported to the University of Windsor where eggs from each female were fertilized by milt from a unique male. The fertilized eggs were kept in Heath trays continuously sourced with dechlorinated municipal water.

2015 imprinting experiment – amino acids

Eggs from a unique male female pair were divided so half the eggs were one of two separate Heath trays. Both Heath trays connected to a hose controlled by a peristaltic pump delivering either an amino acid mixture or dechlorinated municipal water to each tray respectively. One Heath tray received a pulse of 4mL of 90mM stock amino acid mixture (90mM each of histidine, alanine, methionine, proline, glutamic acid and serine, Sigma) every hour, with concentration for each added amino acid reaching a peak between 0.4 and 4μM. These amino acids have been effective in eliciting responses in salmon (Hara et al. 1973) and Ala, His and Met were used in a previous study looking specifically at lateral glomeruli lG_3, lG_4 and lG_x (Braubach et al. 2013). The other Heath tray received a pulse of 4mL of dechlorinated municipal tap water every hour. Administration of the amino acid mixture and dechlorinated tap water was started 3 days before the hatching of the fish. HOBO water temperature loggers were used to monitor

temperature of the water for determination of age of the fish. Collection of fish began the day of hatch and continued weekly for both Heath trays. Chinook salmon larvae were euthanized using MS-222 (1g/L), weighed using an analytical scale to monitor development of fish and dropped-fixed in 4% paraformaldehyde in 0.1M PB.

2016 imprinting experiment – PEA

Eggs from a unique male/female pair were divided into two separate Heath trays. In 2016 the water sourcing system recirculated the water through the Heath trays. PEA (Sigma-Aldrich, W285803, lot# MKBZ9717V) was added to one salmon tray system starting when all embryos were eyed, as imprinting of salmon to PEA during eyed embryonic stages showed some success (Tilsen et al. 1994). To one Heath salmon tray a volume of PEA was added to reach a dilution of 10^{-7}M. The water was changed daily so an appropriate volume of PEA was added periodically to keep the concentration of PEA at 10^{-7}M in one Heath tray throughout embryonic and larval development. This was usually approximately 1µL of PEA every 3 days. The other Heath tray received an addition of dechlorinated municipal water instead of PEA at the same time. Temperature of salmon tray water was controlled by a chiller where water temperature was dropped approximately 1^{o}C per week from 14^{o}C to 7^{o}C to mimic the decrease in temperature seen in the wild. Water temperature readings were taken daily to keep track of salmon age. Collection of fish began once all embryos were eyed. The first addition of the odours occurred the day after the first collection, when fish were just eyed and did not yet have a developed olfactory bulb with distinguishable glomeruli to measure (see – Chapter 2). The fish were collected weekly from the time they were eyed until they had hatched and absorbed most of their yolk sac. The Chinook salmon embryos and larvae were

euthanized by addition of MS-222 to the water (1g/L), weighed with an analytical scale and drop-fixed overnight in 4% PFA.

Sectioned Tissue Immunohistochemistry for glomerular volume analysis

Fish were collected for volume measurements from three time points during the larval stage for analysis for both 2015 and 2016 experiments: the early yolk-sac, late yolk-sac and fry periods. Due to fish development being influenced by the water temperature they are raised at, fish age was determined based on degrees days measured through summation of average daily temperatures of the water from fertilization to each collection date (Crisp 1981). These stages, based on naming of early developmental stages in Chinook salmon (Ochs et al. 2017), are described in Table 3.1. Fixed tissue was rinsed in PBS before the head was cut right after the eye and the mandible removed. The heads were then placed in 20% sucrose and then 30% sucrose (in 0.1M PBS) for cryoprotection. A Leica CM 3050A cryostat was used to section the fish heads into 18µm horizontal sections from the beginning of the olfactory bulb down to the end of the olfactory epithelium and placed on Fisherbrand Superfrost Plus microscope slides. The slides dried at room temperature before storage at -20^{o}C. For immunohistochemistry, slides were rehydrated with three rinses over 3 hours of PBS-T before an antigen retrieval step using HistoVTone (06380-05, lot# L6R0020 Nacalai, Japan) and subsequently placed for an hour in a 10% goat serum in 0.1M PBS-T blocking solution (Sigma-Aldrich, Oakville ON, G9023). Slides were then placed in the primary antibody for 3 days at 4^{o}C under gentle agitation. The fish from 2015 amino acid exposure experiment were labeled with 1:500 calretinin (Swant, Switzerland, 6B3) and 1:250 KLH (Sigma) (a general label of glomeruli in the olfactory bulb) while the olfactory bulb of the fish from

the 2016 PEA olfactory enrichment experiment were placed in a primary antibody solution of 1:250 G_{olf} (Santa Cruz Biotechnology, Texas USA, sc-383, lot# D2011). The slides were then rinsed with PBS over 5 hours before being placed in secondary antibody: either 1:500 Alexafluor 488 goat anti-mouse IgG (Sigma-Aldrich Oakville ON, A11001) and 1:500 goat anti-rabbit Alexafluor 568 IgG (Sigma-Aldrich Oakville ON, A11011) or 1:250 Alexafluor 488 IgG goat anti-rabbit (Sigma-Aldrich, Oakville ON, A1108) and 1:250 Alexafluor 568 IgG goat anti-mouse (Sigma-Aldrich, Oakville ON, A1104) for two days at 4^{o}C. The slides were then rinsed a second time with PBS over 5 hours and then coverslipped with VectaShield mounting medium with DAPI counterstain (H-1200, Vector Laboratories, Burlingame, CA).

Analysis

The volumes of three clearly distinguishable lateral glomeruli (lG_1, $lG_{3/4}$, lG_6) labeled with calretinin antibody were measured for control and amino acid treated Chinook salmon larvae 2015 at three points of larval development: early yolk-sac larvae, late yolk-sac larvae and fry (Table 3.1 describes these stages in more detail). Glomerular volume of G_{olf} positive vmG_x and vmG_7 was measured for the PEA enrichment experiment in 2016 as these were the only consistently identifiable G_{olf} positive glomeruli from early yolk-sac larvae stage to fry stage. Labelled slides of the olfactory bulb were photographed using a fluorescence Axio Scan Z1 (Zeiss) microscope at 20x magnification for a final z-stack image of 30µm depth and a 3µm z-step. Glomerular areas on each slide were measured using the Zen software used to run the Zeiss microscope. Glomerular areas were measured at a zoom so that axonal fibers were visible and DAPI counterstain was used to help identify the outlines of individual glomeruli. The

volumes of the glomeruli were calculated by adding the areas in serial sections of a specific glomerulus and multiplying this by the section thickness (18µm). In total N=5 volume measurements were taken for each glomerulus at the early and late yolk-sac larvae stage, while an N of 4 was used for measurements of each glomerulus at the fry stage.

Repeated measures ANOVAs were performed to assess change in glomerular volume over time while independent t-tests were conducted to determine if there was a statistical difference in glomerular volume between control and treatment groups in a particular glomerulus and stage. Statistical analysis of glomerular volumes was performed using SPSS software.

3.3 Results

Amino acid composition of water samples from the Credit River and the University of Windsor

Amino acid concentrations (nM) were measured from water samples from the Credit River (a Chinook salmon spawning location) and salmon trays sources by municipal water used to house Chinook salmon eggs at the University of Windsor. The amino acid concentrations of 20 amino acids were determined for four months (October 2016 to January of 2017) (Table 3.2), when Chinook salmon would be undergoing embryonic and larval development in the wild. There was more variation in the monthly amino acid concentrations in water samples from the Credit River compared to water samples from the University of Windsor (Figure 3.1, Figure 3.2). However, some amino acids showed relatively stable concentrations over the four month period in both water

sources. In particular, phenylalanine, tryptophan, histidine and aspartic acid all had consistent amino acid concentrations over time (Figure 3.1, 3.2). For example, the range of concentrations measured for tryptophan was 1.9nM - 2.2nM in Credit River water and 1.2nM - 1.3nM in water from the University of Windsor (Table 3.2). However, some amino acids showed large fluctuations or differences between Credit River and salmon tray samples. Cysteine particularly showed a large increase in concentration, peaking in November, in Credit River water, but not in salmon tray water (Table 3.2, Figure 3.1). Additionally, proline concentration spiked in January in the Credit River samples, but remained relatively constant in salmon tray samples (Table 3.2, Figure 3.1). Conversely, isoleucine and leucine were consistently present at higher concentrations in salmon tray water compared to Credit River water.

However, there were similarities in which amino acids were found at relatively higher and lower concentrations in both river water and salmon tray water. Levels of glycine, valine, cysteine and phenylalanine amino acids were high in both Credit River and University of Windsor salmon tray water (3.5-18.8nM), compared to asparagine, glutamine, arginine, methionine, histidine and tyrosine, which were present in low concentrations (0.3-2.5nM and sometimes too low to be detected) in both locations (Table 3.2). Altogether, some amino acids showed more stable concentrations over time than others and there were similar group of amino acids that were present at relatively higher and lower concentrations in water from both the Credit River and the University of Windsor.

Amino acid enrichment experiment

Odour exposure of Chinook salmon to amino acids began 3 days before fish hatched and continued through the larval stage. Water samples were taken from control (CT – receiving dechlorinated water) and treated salmon trays (AA – receiving the amino acid odour mixture) to confirm an observable difference in amino acid concentration between water from each source. Measurements were conducted in duplicate and indicated that concentrations of amino acids that were added to the amino acid (AA) tray (Glu, Ser, His, Ala, Pro and Met) had nanomolar (nM) concentrations approximately 10 times greater than the concentration of these amino acids in the CT tray (Figure 3.3). This confirmed that AA tray water had a measurably increased concentration of the amino acids used for the odour enrichment above endogenous amino acid levels. Peak concentrations of each of the amino acids added to the AA tray were between 0.4 and 4μM.

Chinook salmon steadily increased in mass from hatch to the end of the larval period, showing a plateau when the yolk-sac has been absorbed at the fry stage (Figure 3.4). A repeated measures ANOVA performed on masses indicated that the Chinook salmon significantly increased in mass over larval development $P(6,276) = 351.472$, $p<0.001$ but that masses were also significantly different between control and amino acid treated groups over time ($P(6,276)=11.890$, $p<0.001$). Hence, to take into account the difference in larvae size, glomerular volumes in control and amino acid specimens at each stage (early yolk-sac larvae, late yolk-sac larvae and fry) were divided by the average mass of the control or amino acid treated larvae at that particular stage.

Lateral glomeruli show responsiveness to amino acids (Friedrich and Korsching 1998) and therefore are potential targets for olfactory imprinting to amino acids. Hence, amino acid responsive lateral glomeruli were labeled with calretinin antibody, a marker of microvillous OSNs that innervate lateral glomeruli (Braubach et al. 2013, Ochs et al. 2017). The volumes of three lateral glomeruli, lG_1, $lG_{3/4}$ and lG_6, were measured from early yolk-sac larvae to fry stages. These glomeruli are present at hatch and distinguishable throughout larval development of Chinook salmon (Ochs et al. 2017), allowing for measurement of the glomeruli at three time points during the larval stage. Regardless of the treatment group, the glomerular volume of each measured lateral glomerulus showed a significant difference over time (lG_1: $F(2,12)=51.460$, $p<0.001$; $lG_{3/4}$: $F(2,12)=107.077$, $p<0.001$; lG_6: $F(2,12)=31.324$, $p<0.001$). Graphs of the glomerular volumes compared between control (CTL) and amino acid treated (AA) Chinook salmon at early yolk-sac, late yolk-sac and fry stages show a general increase in glomerular volume over time (Figure 3.5, 3.6, 3.7).

Independent t-tests indicated that olfactory enrichment to an amino acid odour mixture had no effect on glomerular volume of lG_1 at any developmental stage (early yolk-sac larvae: $t(8)=1.236$, $p=0.251$; late yolk-sac larvae: $t(8)=0.690$, $p=0.51$; fry: $t(6)=1.228$, $p=0.265$). There was no statistically significant difference in average glomerular volume of lG_1 due to treatment (Figure 3.5).

There was a statistically significant difference in volume of $lG_{3/4}$ at the early yolk-sac larval stage $t(8)=2.653$, $p=0.029$ between control and amino acid treated fish, after three days of odour exposure . However, glomerular volume of $lG_{3/4}$ was not different between control and amino acid treated groups in the late yolk-sac larvae stage

(t(8)=0.376, p=0.716) or fry stage (t(6)=1.607, p=0.159) when the fish had been exposed to the amino acid mixture for approximately four and six weeks, respectively. The average glomerular volumes of $lG_{3/4}$ over larval development are graphed in Figure 3.6. Independent t-tests showed no statistical difference in glomerular volume of lG_6 between control and amino acid treated fish in early yolk-sac larvae t(8)=1.325, p=0.222, late yolk-sac larvae t(8)=0.580, p=0.578 or fry t(6)=0.879, p=0.413 (Figure 3.7). Overall volume of glomeruli appeared lower in amino acid treated fish than control fish (Figure 3.5-3.7) but only $lG_{3/4}$ showed a significant decrease in glomerular volume at the early yolk-sac larval stage following amino acid treatment for 3 days (Figure 3.6).

PEA enrichment experiment

Consistent growth of control and 10^{-7}M PEA treated Chinook salmon was seen in mass measurements of the fish from embryo to fry stage. There was a significant increase in mass of the larvae over development as indicated by a repeated measures ANOVA ($P(12, 456)=724.495$, $p<0.001$). Independent t-tests comparing masses between control (CTL) and PEA treated (PEA) fish at the early yolk-sac stage (505dd: t(38)=0.751, p=0.457), late yolk-sac stage (714dd: t(38)=0.604, p=0.550) and fry stage (920dd: t(38)=1.276, p=0.211) showed no statistically significant difference in mass between the two groups at any of the stages used for analyzing potential changes due to odour experience . Therefore, absolute glomerular volumes measured were not transformed for analysis.

The glomerular volumes of vmG_x and vmG_7 were measured from early yolk-sac larvae to fry stage in control and PEA treated fish. Because PEA may stimulate OR type

receptors (Calfun et al. 2016) which correspond to ciliated OSNs that express G_{olf} (Sato et al. 2005), PEA was assumed to interact with ciliated OSNs. In the Chinook salmon larval olfactory bulb G_{olf} only labeled two glomeruli consistently from hatch to fry stage – vmG_x and vmG_7 (see Chapter 2 of this thesis). Hence, these glomeruli were identified as possible targets of olfactory experience to the PEA odour to be measured at early yolk-sac larvae, late yolk-sac larvae and fry stages. However, in the early yolk-sac stage vmG_x and vmG_7 were sometimes overlapping making them difficult to distinguish, so the two glomeruli were also measured together at this stage (vmG_{x+7}).

The volumes of ventromedial glomeruli vmG_x and vmG_7 labeled by the G_{olf} antibody were measured in tissue slices of the olfactory bulb of control and PEA treated fish. The glomerular volume of vmG_x and vmG_7 showed a statistical significant increase in volume over time from late yolk-sac and fry stage (vmG_x: $F(1,6)=56.623$, $p<0.001$; vmG_7: $F(1,6)=103.805$, $p<0.001$) (Figure 3.10) regardless of odour treatment.

Glomerular volume of the both vmG_x and vmG_7 together showed no significant difference between control and PEA groups at early yolk-sac larvae: $t(8)=1.951$, $p=0.109$; and fry stage: $t(6)=0.911$, $p=0.398$, after approximately four and 13 weeks of odour exposure respectively. However, there was a significant difference in glomerular volume of vmG_{x+7} between control and PEA treated larvae at the late yolk-sac larval stage (independent t-test: $t(8)=23.682$, $p=0.028$), after approximately 8 weeks of exposure to PEA.

Independent t-tests indicated that olfactory PEA enrichment had no effect on glomerular volume of vmG_x individually between control and PEA treated fish at late

yolk-sac larvae stage t(8)=1.350, p=0.212 or fry stage t(6)=0.748, p=0.483. Similarly, no statistical significant difference in average glomerular volume in vmG_7 at the fry stage was observed t(6)=2.280, p=0.063. However, there was a significant difference in average glomerular volume of vmG_7 due to treatment during the late yolk sac larvae stage t(8)=2.948, p=0.018. As shown in Figure 3.10, vmG_7 was significantly smaller in PEA treated fish at the late-yolk sac larvae stage. Hence, only vmG_7 and vmG_{x+7} showed a significant decrease in glomerular volume after 8 weeks of exposure to PEA at the late yolk-sac larvae stage in development.

Overall, glomerular volumes measured from lateral and ventromedial glomeruli in groups that received olfactory enrichment (either amino acid mixture or PEA) was lower than control groups (receiving dechlorinated municipal water). However, a significant decrease in average volume for odour exposed fish compared to control fish was only seen for the $lG_{3/4}$ glomerulus after 3 days of amino acid odour exposure in the early yolk-sac stage, and vmG_7 after approximately 8 weeks of PEA exposure during the late yolk-sac larvae stage.

3.4 Discussion

This paper used glomerular patterns described in embryonic and larval Chinook salmon (Ochs et al. 2017, see Chapter 2 of this thesis) to investigate how sensory input during early life stages affects the development of glomerular units of odour discrimination within the olfactory bulb from early yolk-sac larval stage to fry stage in Chinook salmon. There was a general decrease in volume of lateral glomerulus $lG_{3/4}$ in amino acid exposed fish versus control fish and a decrease in ventromedial glomerulus vmG_7 volume in PEA exposed fish versus non-treated fish, similar to decreased

glomerular volume after odour treatment seen in previous studies in *Drosophila* (Devaud et al. 2003) and zebrafish (Braubach et al. 2013). The amino acids composition of water samples from the Credit River (a spawning location for Chinook salmon) and water from the salmon trays, used to raise wild-caught Chinook salmon from eggs to larvae at the University of Windsor, were also measured over a four month period (October 2016 - January 2017) to identify specific amino acids salmon could potentially be imprinting to. Chinook salmon were spawning in October in the Credit River and eggs likely hatched around December (Scott and Crossman 1998). Hence, the water samples were taken during period when Chinook salmon would be going through embryonic and early larval development. The eyed embryo, with fish egg membrane semipermeability potentially allowing exposure to the imprinting stream odour (Hagedorn et al. 1997), and larval stages are important periods for olfactory imprinting to the specific odour of the natal stream water (Tilson et al. 1994).

Credit River water concentrations of individual amino acids appeared to fluctuate more over the four month period than amino acid concentrations from University of Windsor water. Large fluctuations over time were prominent in cysteine, proline and alanine, suggesting that these amino acids may not be important components for olfactory imprinting during larval development. Additionally, zebrafish have shown aversive behavioural responses to cysteine (Vitebsky et al. 2005) which may also suggest that this amino acid likely would not be a significant component of the stream imprinting odour. A few amino acids exhibited highly consistent concentrations over the four month period (His, Trp, Phe and Asp) in both the Credit River and University of Windsor water. Hence, during the larval stage, a potential critical period for olfactory imprinting in

salmonids (Tilson et al. 1994), fish would be exposed to these consistent concentrations of amino acids which they could imprint to and recognize as adults. This may suggest that these amino acids could show a stable composition year to year, as indicated in a previous study reporting river water having a stable year to year amino acid composition of certain amino acids (Yamamoto et al. 2013), which salmon can potentially recognize years later when they return during spawning migration.

There were some general similarities in amino acid profiles and concentrations over monthly samples from river water, and between river water and salmon tray water. Amino acids that were detected at higher concentrations in river water were found at comparable concentrations in salmon tray water (Gly, Val, Cys, Phe). Furthermore, river water and municipal water showed relatively low measured concentrations of Asn, Gln, His, Arg, Tyr and Met. The concentrations of these amino acids also remained at relatively high or low concentrations, respectively, over monthly water samples. The apparent similarities in stable amino acids in river water and water from a laboratory could be because water from the University of Windsor, which comes from the Detroit River, and Credit River water are both sourced by the Great Lakes water system which may result is some commonality in amino acid composition.

Early life history stages are critical periods in which an organism's environment can have a propound effect on the development of the organism. Kihslinger and Nevitt (2006) showed that there was a significant difference in growth of the cerebella in larval rainbow trout (*O. mykiss*) that were raised in a laboratory rearing environment with an addition of gravel substrate or in a natural river environment, indicating the importance of early rearing environment on behaviour and neural development in juvenile salmon. In

particular, the olfactory system is highly receptive to olfactory experience potentially affecting rates of axon recruitment leading to changes within the olfactory bulb of the animal (Todrank et al. 2011, Braubach et al. 2013). Since amino acids are important olfactory cues early in development that may be part of the odour signature of natal stream water salmon imprint to as larvae (Shoji et al. 2003, Ueda 2012) and are also part of feeding cues that salmon learn to behaviourally respond to before fully absorbing their yolk sac (Hara et al. 2006**,** Mearns et al. 1986), the identification of several amino acids with stable concentrations over a four month period from river water may indicate that these amino acids are potential amino acids salmon could imprint to and recognize later as adults.

This paper looked into potential neuroanatomical effects of olfactory enrichment during larval development in Chinook salmon, with treatment groups generally having lower volume of glomeruli compared to control animals. In this study we exposed Chinook salmon during early development to either an amino acid odour (peak of 0.4 - 4μM of each added amino acid in 2015) or 10^{-7}M PEA (in 2016) to look at anatomical effects of olfactory experience in olfactory bulb glomeruli. Amino acids, potential imprinting odours for homing migration, with different functional groups were put together for the amino acid enrichment experiment to allow for targeting of various lateral glomeruli (Friedrich and Korsching 1997). The amino acids added, His, Ala, Met, Glu, and Ser, except Pro were some of the most effective amino acids in eliciting responses in salmon (Hara 1973). Additionally, Ala, His and Met were amino acids previously used in an amino acid mixture zebrafish were exposed to in a study looking into changes in specific lateral glomeruli lG_3, lG_4 and lG_x (Braubach et al. 2013). Salmon

have also been previously imprinted to Pro or Glu during early life stages and one year old fish shown behavioural preference to the amino acid (Yamamoto et al. 2010), indicating that salmon can be imprinted to these amino acids. Measurement of amino acid composition between control salmon tray water and amino acid enriched salmon tray water confirmed a measureable increase in concentration of added amino acids in the amino acid enriched water compared to endogenous levels in the control water, and that concentrations of these amino acids were above the 10^{-7} to 10^{-8}M detection threshold of amino acids in salmon (Hara et al. 1973).

Glomerular volume increased over the larval period calretinin labeled amino acid responsive glomeruli (lG_1, $lG_{3/4}$, lG_6) and G_{olf} positive ventromedial glomeruli (vmG_x, vmG_7), matching previous descriptions of increases in volume of some comparable zebrafish glomeruli after hatch (Braubach et al. 2013). However, this observation is different from previous work with Chinook salmon larvae that reported no overall increase in volume of the lG_1 glomerulus over larval development (Ochs 2015). Difference in results may be a reflection of different measurement techniques or low resolution due to small sample size. In this study the glomerular volume measured in treatment animals (exposed to either the amino acid mixture or PEA) was generally lower than volumes measured in control animals for calretinin positive lG_1, $lG_{3/4}$ (amino acid treatment experiment) and G_{olf} positive vmG_7 (PEA treated experiment) over larval development. There were some exceptions where average volume from treated animals was greater than control animals in early and late yolk-sac stages for lG_6 (measured for the amino acid enrichment study) and the late yolk-sac stage of vmG_x (measured for PEA odour enrichment study). This may indicate that lG_6 was not a target of the amino

acid mixture used for olfactory enrichment and that vmG_x may not be a glomerular target of PEA, or that they were not responsive to the olfactory stimulation until later stages of larval development.

However, of all the glomeruli measured and the different time points examined there was only a significant decrease in glomerular volume of fish receiving odour enrichment compared to control fish of $lG_{3/4}$ during the early yolk-sac larvae stage (in response to amino acid enrichment for 3 days) and vmG_7 during the late yolk-sac larvae stage (after PEA enrichment for 8 weeks), indicating that these could be potential targets of imprinting to each odour during larval development. Based on activity seen in the zebrafish olfactory bulb after exposure to various amino acid (Friedrich and Korsching et al. 1997, Braubach et al. 2013) alanine, histidine and methionine that stimulate anterior lateral regions of the olfactory bulb corresponding to such glomeruli as lG_3, lG_4 and lG_x could be potential amino acids affecting the changes to $lG_{3/4}$ volume seen in this study.

The observation that treatment groups generally had lower glomerular volumes (even though it was not always statistically significant) is consistent with work done in zebrafish where it was reported that small lateral glomeruli (lG_x) were more numerous but had a smaller cross-sectional area after olfactory enrichment with an amino acid mixture of 1μM Ala, His, Lys, Met, Phe, Trp and Val during early development in treatment fish compared to control fish (Braubach et al. 2013). Our study did not focus on the number of supernumerary glomeruli since they have not been reliably characterized in the Chinook salmon olfactory bulb. Additionally, due to their small size and close proximity to each other, confocal microscopy may be necessary to visualize axon fibers to distinguish individual microglomeruli. The decrease in volume of glomeruli may be

attributed to differentiation of glomeruli through refinement of glomerular axons with sensory stimulation, with pruning of axons leading to development of defined glomeruli with slightly smaller volumes (Potter et al. 2001, Takeuchi et al. 2010, Li et al. 2005). In contrast, olfactory deprivation or disruption of OSN signaling would lead to glomeruli that were less refined with alterations to glomerular targeting of axons (Zheng et al. 2000, Yu et al. 2004). Hence, the decreased volumes of glomeruli in treated fish compared to control fish to either amino acids or PEA sensory experience may be due to refinement of diffuse glomerular units into more well defined glomeruli

However, Braubach et al. (2013) did not describe any difference in larger lateral glomeruli, such as the lG_1, $lG_{3/4}$ and lG_6 that we measured in the present study. The decrease in $lG_{3/4}$ may be due to some differences in amino acids used in each study even though concentrations of amino acids were similar in both (this study: 0.4 – 4 μM peak concentration, Braubach et al. (2012): 1μM peak concentration) or sensitivity differences of these glomeruli to olfactory stimulation between different species. Additionally, even though this study used a higher concentration of added amino acids than a previous olfactory enrichment experiment in larval Chinook salmon focussing on volumes of calretinin positive lateral glomerulus lG_1 (Ochs 2015), lG_1 similarly showed no significant difference in glomerular volume between control and amino acid exposed fish in both studies, though there was an overall lower volume of lG_1 for amino acid treated larvae in this study. This may be due to differences in amino acid concentrations the treatment fish were exposed to in each study. Compared to the study by Ochs, we increased the concentration of the amino acids in the stock solution by approximately three times and doubled the volume of stock solution dispensed with each pulse. Our

final concentration of each amino acid added to the treatment salmon tray water was between 0.4 μM and 4μM, which is within the 10^{-7} to 10^{-8}M detection threshold of amino acids (Hara 1973). Since the study by Ochs used a lower stock concentration of amino acids the final amino acid concentration the fish were exposed to in that study may not have been different enough from concentrations of the endogenous amino acids in the water.

Braubach et al. (2013) suggested that small and large lateral glomeruli mature via different processes or are differentially susceptible to changes caused by olfactory stimulation which may result in differential responses to olfactory enrichment. Olfactory stimulation may direct the activity of axon guidance molecules, such as molecules aiding in cell adhesion, cell attraction molecules that direct axon growth to the appropriate glomerular destination and cell repulsion molecules that keep axons from growing in the wrong direction (Imai et al. 2006, Kaneko-Goto et al. 2008, Imai and Sakano, 2011). These factors responding to olfactory stimulation may affect the arrangement of axons into defined bundles in the olfactory bulb, or lead to pruning of unstimulated axons, resulting in glomeruli with a smaller volume. Utilization of high powered confocal microscopy could be used to visualize individual fibers of the glomeruli to determine if olfactory stimulation affects the number of OSN axons or if the axons grow closer together.

In our study G_{olf} positive vmG_7 glomerulus showed a significant decrease in glomerular volume in PEA exposure fish compared to control fish in the late yolk-sac stage, indicating that vmG_7 may be a possible target of PEA olfactory stimulation. However, in the past response to PEA, which has been used in various behavioural

experiments for olfactory imprinting in salmon (Scholz et al. 1976, Tilson et al. 1994, Dittman et al. 2015) and is not usually found in municipal water (Bett et al. 2016), has not been attributed to any specific glomeruli and PEA activation in the olfactory bulb has not been recorded (Calfun et al. 2016). Imprinting of embryonic zebrafish to PEA showed a measurable decrease in gene expression of several OR-type olfactory receptors of the olfactory epithelium (Calfun et al. 2016), which are expressed by ciliated OSNs labeled with G_{olf} antibody (Sato et al. 2005, Oka and Korsching 2011, Hansen et al. 2004, Hussain et al. 2009). Calfun et al. (2016) suggested that olfactory stimulation with PEA may lead to the repression of certain ORs (OR103-1, OR111-1, OR115-1, OR125-1) and activation of other not yet identified ORs. We found of vmG_x and vmG_7, which were present from late eyed embryonic development in Chinook salmon (see – Chapter 2), vmG_7 showed a decreased volume in PEA treated fish which manifested early in larval development. Identification of the receptor expressed by OSNs that project into vmG_7 could indicate a potential OR gene target that could be activated by PEA imprinting.

However, PEA could alternatively stimulate other glomeruli, such as other smaller G_{olf} positive vmGs not measured in this study because of their development only in the late yolk-sac and fry stages. Additionally, since PEA imprinting resulted in decreased expression of some OR genes corresponding to OR103 and OR111 subfamilies (Calfun et al. 2016) which have axons that terminate on a few medioanterior glomeruli (Sato et al. 2007), glomeruli in this areas may exhibit less refinement after PEA imprinting. However, since G_{olf} positive medioanterior glomeruli are not well developed in the olfactory bulb of Chinook salmon larvae (see Chapter 2 of this thesis) these glomeruli would have to be analyzed at later stages beyond larval development when

salmon begin feeding which would introduce new variables that could affect results. Additionally, volumes of calretinin positive lateral glomeruli could also be measured in control and PEA treated fish to determine whether the PEA treatment only affected the volume of the ventromedial glomerulus.

Potential differences between average glomerular volume of control and treatment groups at the different developmental time points may have been distorted due to large variation in volume measurements between animals. In particular, lG_6 volumes showed great variation between specimens of the same treatment. The decrease in glomerular volume of $lG_{3/4}$ and vmG_7 were only significantly decreased in odour treated fish versus control fish in the early yolk-sac larvae and late yolk-sac larval stages, respectively. Even though volumes of both $lG_{3/4}$ and vmG_7 were lower in treatment versus control animals at other larval stages, the large variation in volume measurements may have contributed to why the difference was not significant at other stages. Another point of consideration is the small sample size of the study. More replicates could have given a better representation of the experimental populations. Additionally, there was a mass difference between control and amino acid treated fish over larval development even though these fish were full siblings raised in adjacent salmon trays. While volume measurements for these samples were divided by each group's average mass to account for the difference in mass, measurements of another structure unrelated to the olfactory system in control and amino acid treated fish could have been used to standardize differences seen in the olfactory bulb. In particular, measurement of the olfactory bulb volumes of control and treatment fish could be used to determine if there are differences in olfactory bulb size between the two groups and better normalize the glomerular volumes measured.

Another consideration is that the specific glomeruli that amino acids target have not been specified and there are other potential targets of amino acid stimulation in the olfactory bulb (Braubach et al. 2013, Friedrich and Korsching 1997). Most of the amino acids we administered for olfactory enrichment were shown to target different glomeruli in a particular region of the zebrafish olfactory bulb (Braubach et al. 2013, Friedrich and Korsching 1997) but some smaller glomeruli in these regions that were not clearly distinguishable with calretinin immunohistochemistry in larval Chinook salmon (Ochs et al. 2017). Hence, it may be beneficial to look into the volumes of smaller lateral glomeruli that showed sensitivity to olfactory experience in zebrafish (Braubach et al. 2013) but these have not been reliably mapped in Chinook salmon olfactory bulb.

This study only examined one way olfactory experience could potentially affect the developing olfactory system. Changes due to sensory experience can manifest as changes in gene expression of OSNs causing variations to OSN number (Jones et al. 2008), possible increased gene expression of the corresponding olfactory receptors (Cadiou et al. 2014), increased recruitment of axons innervating glomeruli and increased number of supernumerary glomeruli (Valle-Leija et al. 2012, Braubach et al. 2013). Additionally, imprinting may potentially have postsynaptic effects such as increases in gene expression of N-methyl-D-aspartate receptor (Ueda et al. 2016), involved in memory formation (Kinoshita et al. 2004, Cox et al. 2005, Gomez et al, 2006).

This paper expanded on a previous study looking into neuro anatomical effects of olfactory experience in larval Chinook salmon to an amino acid odour mixture. In the present study we increased the amino acid concentrations (Ochs 2015) fish were exposed to and also ran another experiment with olfactory experience to PEA. Even though a

decrease in average of volume of amino acid treated fish versus control fish was seen at early yolk-sac larvae for $lG_{3/4}$ and between PEA exposed and control fish for vmG_7 at the late yolk-sac larval stage, large variation in glomerular volumes measured likely contributed to other volume changes not being supported statistically. Nevertheless, this study provided insights into growth of lateral and ventromedial glomeruli in the larval olfactory bulb and may provide a basis for future studies into effects of olfactory stimulation and experience in the brain.

3.5 References

Arenas, A., Giurfa, M., Sandoz, J.C., Hourcade, B., Devaud, J.M., Farina, W.M. (2012). Early olfactory experience induces structural changes in the primary olfactory center of an insect brain. *European Journal of Neuroscience*. 35:682-690.

Baier, H., Korsching, S. (1994). Olfactory glomeruli in the zebrafish form an invariant pattern and are identifiable across animal. *Journal of Neuroscience*. 14: 219-230.

Bateson, P. (1990). Is imprinting such a special case? *Philosophical Transactions of the Royal Society of London B: Biological Sciences*.1990;329:125-131.

Bett, N.N., Hinch, S.G., Dittman, A.H., Yun, S. (2016). Evidence of olfactory imprinting at an early life stage in pink salmon (*Oncorhynchus gorbuscha*). *Scientific Reports*. 6:36393.

Biechl, D., Tietje, K., Gerlach, G., Wullimann, M.F. (2016). Crypt cells are involved in kin recognition in larval zebrafish. *Scientific Reports.* 6: article 24590.

Biechl, D., Tietje, K., Ryu, S., Grothe, B., Gerlach, G., Wulliman, M.F. (2017). Identification of accessory olfactory system and medial amygdala in the zebrafish. *Scientific Reports*.

Braubach, O., Fine, A., Croll, P. (2012). Distribution and functional organization of glomeruli in the olfactory bulbs of zebrafish (Danio rerio). *Journal of comparative neurology.* 520(11): 2317-39.

Braubach, O., Miyasaka, N., Koide, T., Yoshihara, Y., Croll, R.P., Fine, A. (2013). Experience dependent versus experience-independent postembryonic

development of distinct groups of zebrafish olfactory glomeruli. *The Journal of Neuroscience.* 33(16): 6905-16.

Butts IAE, Johnson K, Wilson CC, Pitcher TE. (2012). Ovarian fluid enhances sperm velocity based on relatedness in lake trout, *Salvelinus namaycush*. *Theriogenology*. 78:2105—2109.

Cadiou, H., Aoudé, I., Tazir, B., Molinas, A., Fenech, C., Meunier, N., Grosmaitre, X. (2014). Postnatal odorant exposure induces peripheral olfactory plasticity at the cellular level. *Journal of Neuroscience*. 34(14):4857–4870.

Calfun, C., Dominguez, C., Perez-Acle, T., Whitlock, K.E. (2016). Changes in olfactory receptor expression are correlated with odor exposure during early development in zebrafish (*Danio rerio*). *Chemical Senses*. 41 (4): 301-312.

Cox, J. A., Kucenas, S., Voigt, M. M. (2005). Molecular characterization and embryonic expression of the family of N-methyl-D-aspartate receptor subunit genes in zebrafish. *Developmental Dynamics.* 234**,** 756–766.

Dittman, A.H., Quinn, T.P., Nevitt, G.A. (1996). Timing of imprinting to natural and artificial odors by coho salmon (Oncorhynchus kisutch). *Canadian Journal of Fisheries and Aquatic Sciences*. 53: 434-442.

Dittman, A.H., Pearsons, T.N., May, D., Couture, R.B., Noakes, D.L.G. (2015). Imprinting of hatchery-reared salmon to targeted spawning locations: a new embryonic imprinting paradigm for hatchery programs. *Fisheries*. 40(3): 114-123.

Doupe, A., Kuhl, P. (1999). Birdsong and human speech: Common themes and mechanisms. *Annual Review of Neuroscience*. 22: 567-631.

Erbas, S., Baydar, H. (2016). Variation in scient compounds of oil-bearing rose (*Rosa damascene* Mill.). Produced by headspace solid phase microextraction, hydrodistillation and solvent extraction. *Records of Natural Products*. 10: 555-565.

Friedrich, R. W., & Korsching, S. I. (1997). Combinatorial and chemotopic odorant coding in the zebrafish olfactory bulb visualized by optical imaging. *Neuron*, *18*(5): 737-752.

Gayoso, J.A., Castro, A., Anadon, R., Manso, M.J. (2011). Differential bulbar and extrabulbar projections of diverse olfactory receptor neuron population in the adult zebrafish *(Daniorerio*). *Journal of Comparative Neuroscience.* 519: 247 -276.

Gómez, Y., Vargas, J. P., Portavella, M., López, J. C. (2006). Spatial learning and goldfish telencephalon NMDA receptors. *Neurobiology of Learning and Memory.* 85: 252–262.

Groves, A.B., Collins, G.B., Trefethen, P.S. (1968). Roles of olfaction and vision in choice of spawning site by homing adult Chinook salmon (Oncorhynchus tshawytscha). *Journal of the Fisheries Research Board of Canada*. 25(5): 867-876.

Hagedorn, M., Hsu, E., Kleinhans, F.W., Wildt, D.E. (1997). New approaches for studying the permeability of fish embryos: toward successful cryopreservation. *Cryobiology*. 34(4): 335-347.

Hansen, A., Anderson, K.T., Finger, T.E. (2004). Differential distribution of olfactory receptor neurons in goldfish: structural and molecular correlates. *Journal of Comparative Neurology*. 477: 347-359.

Hara, T.J. (1973). Olfactory responses to amino acids in rainbow trout, *Salmo gairdneri*. *Comparative Biochemistry and Physiology Part A: Physiology*. 44(2): 407-416.

Hara, T.J. (2006). Feeding behaviour in some teleosts is triggered by single amino acids primarily through olfaction. *Journal of Fish Biology*. 68(3): 810-825.

Hasler, D., Scholz, A.T., Horrall, R.M. (1978). Olfactory imprinting and homing in salmon: recent experiments in which salmon have been artificially imprinted to a synthetic chemical verify the olfactory hypothesis for salmon homing. *American Scientist*. 66: 347-355.

Hussain, A., Saraiva, L.R., Korsching, S.I. (2009). Positive Darwinian selection and the birth of an olfactory receptor clade in teleosts. *Proceedings of the National Academy of Sciences*. 106(11): 4313-4318.

Imai, T., Sakano, H. (2011). Axon-axon interactions in neuronal circuit assembly: lessons from olfactory map formation. *European Journal of Neuroscience*. 24: 1647-1654.

Imai, T., Suzuki, M., Sakano, H. (2006). Odorant receptor-derived cAMP signals direct axonal targeting. *Science*. 314: 657-661.

Jones, S.V., Choi, D.C., Davis, M., Ressler, K.J. (2008). Learning-dependent structural plasticity in the adult olfactory pathway. *The Journal of Neuroscience*. 28:13106-13111.

Kanedo-Goto, T., Yoshihara, S., Miyazaki, H., Yoshihara, Y. (2008). BIG-2 mediates olfactory axon convergence to target glomeruli. *Neuron*. 57: 834-846.

Kihslinger, R.L., Nevitt, G.A. (2006). Early rearing environment impacts cerebellar growth in juvenile salmon. *The Journal of Experimental Biology*. 209: 504-509.

Kinoshita, M., Hosokawa, T., Urano, A., Ito, E. (2004). Long-term potentiation in the optic tectum of rainbow trout. *Neuroscience Letters.* 370**:**146–150.

Laberge, F., Hara, T.J. (2001). Neurobiology of fish olfaction: a review. *Brain Research Reviews.* 36(1): 46-59.

Li, J., Mack., J.A., Souren, M., Yaksi, E., Higashijima, S., Mione, M., Fetcho, J.R., Friedrich, R.W. (2005). Early development of functional spatial maps in zebrafish olfactory bulb. *The Journal of Neuroscience*. 25, 5784-5795.

Mearns, K.J. (1986). Sensitivity of brown tout (*Salmo trutta L.)* and Atlantic salmon (*Salmo salar L.)* fry to amino acids at the start of exogenous feeding. *Aquaculture.* 55(3):191-200.

Medjahed, F., Merouane, A., Saadi, A., Bader, A., Cioni, P.L., Flamini, G. (2016). Chemical profile and antifungal potential of essential oils from leaves and flowers of *Salvia algeriensis* (Desf.): A comparative study. *Chilean Journal of Agricultural Research*. 76: 195-200.

Mitamura, H., Arai, N., Sakamoto, W., Mitsunaga, Y., Tanaka, H., Mukai, Y., Nakamura, K., Sasaki, M., Yoneda, Y. (2005). Role of olfaction and vision in homing behaviour of black rockfish Sebastes inermis. *Journal of Experimental Marine Biology and Ecology*. 322(2): 123-134.

Nevitt, G.A., Dittman, A.H., Quinn, T.P., Moody, W.J. (1994) Evidence for peripheral olfactory memory in imprinted salmon. *Proceedings of National Academy of Sciences.* USA. 91: 4288- 4292.

Ngai, J., Chess, A., Dowling, M.M., Necles, N., Macagno, E.R., Axel, R. (1993). Coding of olfactory information: topography of odorant receptor expression in the catfish olfactory epithelium. *Cell*. 72:667-680.

Ochs, C.L. (2015). Ontogeny of glomerular territory patterning in the olfactory bulb of juvenile Chinook salmon (*Oncoryhynchus tshawytscha*) and exploring for potential effects of sensory experience on glomerular development. Electronic Theses and Dissertations. 5319.

Ochs, C.L., Suntres, T., Zygowska, A., Pitcher, T., Zielinski, B.S. (2017). Organization of glomerular territories in the olfactory bulb of post-embryonic wild Chinook salmon *Oncoryhynchus tshawytscha*. *Journal of Morphology*. 278(4): 464-474.

Oka, Y., Korsching, S.I. (2011). Shared and unique G alpha proteins in the zebrafish versus mammalian senses of taste and smell. *Chemical Senses*. 36(4): 357-365.

Pitcher TE, Neff BD. (2006). MHC class IIB alleles contribute to both additive and nonadditive genetic effects on survival in chinook salmon. *Molecular Ecology*. 15:2357—2365.

Potter, S., Zheng, C., Koos, D.S., Feinstein, P., Fraser, S.E., Mombaerts, P. (2001). Structure and emergence of specific olfactory glomeruli in the mouse. *The Journal of Neuroscience*. 21: 9713-9723.

Sachse, S., Ruechert, E., Keller, A., Okada, R., Tanaka, N.K., Ito, K., Vosshall, L.B. (2007). Activity-dependent plasticity in an olfactory circuit. *Neuron*. 56: 838-850.

Sato, Y., Miyasaka, N., Yoshihara, Y. (2005). Mutually exclusive glomerular innervation by two distinct types of olfactory sensory neurons revealed in transgenic zebrafish. *Journal of Neuroscience.* 25: 4889-4897.

Sato, Y., Miyasaka, N., Yoshihara, Y. (2007). Hierarchical regulation of odorant receptor gene choice and subsequent axonal projection of olfactory sensory neurons in zebrafish. *Journal of Neuroscience*. 27(7): 1606-1615.

Scholz, A.T., Horrall, R.M., Cooper, J.C., Hasler, A.D. (1976). Imprinting to chemical cues: the basis for home stream selection in salmon. *Science*. 192: 1247-1248.

Scott, W.B., Crossman, E.J. (1973). Freshwater fishes of Canada. *Fisheries Research Board of Canada Bulletin*, 184.

Shoji, T., Ueda, H., Ohgami, T., Sakamoto, T., Katsuragi, Y, Yamauchi, K., Kurihara, K. (2000). Amino acids dissolved in steam water as possible home stream odorants for masu salmon. *Chemical Senses*. 25: 533-540.

Sigg, D., Thompson, C.M., Mercer, A.R. (1997). Activity-dependent changes to the bran and behaviour of the honey bee, *Apis mellifera (L.). The Journal of Neuroscience.* 17: 7148-7156.

Takeuchi, H., Inokuchi, K., Aoki, M., Suto, F., Tsuboi, A., Matsuda, I., Suzuki, M., Aiba, A., Serizawa, S., Yoshihara, Y., Fujisawa, H., Sakano, H. (2010). Sequential arrival and graded secretion of Sem3F by olfactory neuron axons specify map topography at the bulb. *Cell*. 141:1056-1067.

Tilson, M.B., Scholz, A.T., White, R.J., Galloway, H. (1994). Thyroid-induced chemical imprinting in early life stages and assessment of smoltification in kokanee salmon hatcheries. *1993 Annual report.* Prepared for Bonneville Power Administration, Portland, Oregon.

Todrank, J., Heth, G., Restrepo, D. (2011). Effects of in utero odorant exposure on neuroanatomical development of the olfactory bulb and odour preferences. *Proceedings of the Royal Society B: Biological Sciences*. 278(1714):1949–1955.

Ueda, H., Nakamura, S., Nakamura, T., Inada, K., Okubo, T., Furukawa, N., Murakami, R., Tsuchida, S., Zohar, Y., Konno, K., Watanabe, M. (2016). Involvement of hormones in olfactory imprinting and homing in chum salmon. Scientific Reports. 6:21102.

Valle-Leija. P, Blanco-Hernandez, E., Drucker-Colin, R., Guetierrez-Ospina, G., Vidaltamayo, R. (2012). Supernumerary formation of olfactory glomeruli induced by chronic odorant exposure: a constructivist expression of neural plasticity. PLoS One. 7(4):e35358.

Vitebsky, A., Reyes, R., Sanderson, M.J., Michel, W.C., Whitlock, K.E. (2005). Isolation and characterization of the laure olfactory behavioral mutant in the zebrafish, *Danio rerio*. *Developmental Dynamics*. 234: 229-242.

Yamamoto, Y., Ueda, H. (2009). Behavioural responses by migratory chum salmon to amino acids in natal stream water. *Zooligical Science*. 26(11): 778-782.

Yamamoto, Y., Hino, H., Ueda, H. (2010). Olfactory imprinting of amino acids in lacustrine sockeye salmon. *PLOS One*. 5(1): e8633.

Yamamoto, Y., Shibata, H., & Ueda, H. (2013). Olfactory homing of chum salmon to stable compositions of amino acids in natal stream water. *Zoological Science*. *30*(8), 607-612.

Yu, C.R., Power, J., Barnea, G., O'Donnell, S., Brown, H.E., Osborne, J., Axel, R., Gogos, J.A. (2004). Spontaneous neural activity is required for the establishment and maintenance of the olfactory sensory map. *Neuron*. 42: 553-566.

Zeiske, E., Kasumyan, A., Bartsch, P., Hansen, A. (2003). Early development of the olfactory organ in sturgeons of the genus *Acipenser*: a comparative and electron microscopic study. *Journal of Anatomy and Embryology (Berl).* 206 (5): 357-72.

Zheng, C., Feinstein, P., Bozza, T., Rodriguez, I., Mombaerts, P. (2000). Peripheral olfactory projections are differentially affected in mice deficient in a cyclic nucleotide-gated channel subunit. *Neuron*. 26: 81-91.

3.6 Tables and Figures

Table 3.1: Summary of larval stages of Chinook salmon development in 2015 and 2016 for glomerular volume measurement. Age of fish was calculated based on degree days (dd) by summing average daily temperatures (Crisp 1981). Date when fish were collected at each larval stage is also included. Larval stage naming was based on Ochs et al. 2017.

Larval Stage	Description	2015 – Amino acid enrichment		2016 – PEA enrichment	
		Degree days (dd)	Date	Degree days (dd)	Date
Early yolk-sac larvae	Fish have hatched. Large yolk sac weighs fish down within the water.	456	Nov 15	505	Nov 28
Late yolk-sac larvae	Fish have absorbed approximately half their yolk sacs. Have started swimming in Heath trays.	760	Dec 7	714	Dec 26
Fry	Fish have almost completely reabsorbed their yolk sacs. Fish are very active in Heath trays.	869	Dec 21	920	Jan 23

Table 3.2: Average amino acid concentration (nM) over a four month period of water samples from the Credit River and salmon trays housed at the University of Windsor in 2016. Values that are reported as zero represent amino acid concentrations that were too low to be detected. Amino acids coloured orange represent amino acids that were present at relatively high concentrations throughout October to January in both Credit River and salmon tray water. Amino acids highlighted in blue mean they were present in relatively low concentrations. Yellow highlighted entries denote amino acids with concentrations that showed considerable difference over time or between water sources.

Amino acid	Credit River average amino acid concentration (nM)				Salmon tray average amino acid concentration (nM)			
	Oct	Nov	Dec	Jan	Oct	Nov	Dec	Jan
Asn	0.4	0.0	0.3	0.3	0.0	0.0	0.5	0.0
Gln	2.0	0.3	0.6	0.6	1.4	1.5	1.7	0.7
His	0.6	0.3	0.5	0.6	0.6	0.6	1.4	0.7
Arg	2.2	0.3	0.7	2.5	0.5	0.6	2.9	0.8
Tyr	0.9	0.4	0.4	0.4	0.0	0.0	0.6	0.0
Met	0.0	1.8	1.6	0.6	0.3	0.4	0.9	1.1
Asp	1.4	0.7	1.2	0.9	1.1	0.8	1.0	0.6
Ile	2.7	0.3	0.7	1.1	0.7	4.5	2.6	1.1
Leu	2.2	0.4	1.4	1.3	0.8	3.5	3.5	1.2
Glu	2.4	0.9	1.8	2.5	0.8	0.7	1.3	1.2
Trp	2.1	1.9	1.9	2.2	1.2	1.3	1.3	1.3
Thr	3.5	2.0	2.7	2.9	1.9	2.2	4.8	3.0
Ala	8.0	2.2	3.1	5.3	1.9	2.7	6.4	3.8
Pro	3.2	1.2	2.2	10.5	1.9	2.5	5.0	2.4
Ser	3.1	1.8	2.7	1.9	2.6	3.9	6.0	4.0
Lys	4.7	2.2	3.4	3.4	1.8	2.5	4.2	2.6
Val	5.5	6.7	3.9	4.5	3.6	5.8	6.8	5.5
Cys	1.4	18.8	14.0	3.7	4.0	4.5	7.1	4.8
Gly	7.1	3.8	9.2	5.6	3.5	7.5	7.3	6.4
Phe	10.5	10.6	10.8	11.0	12.0	11.0	13.5	12.6

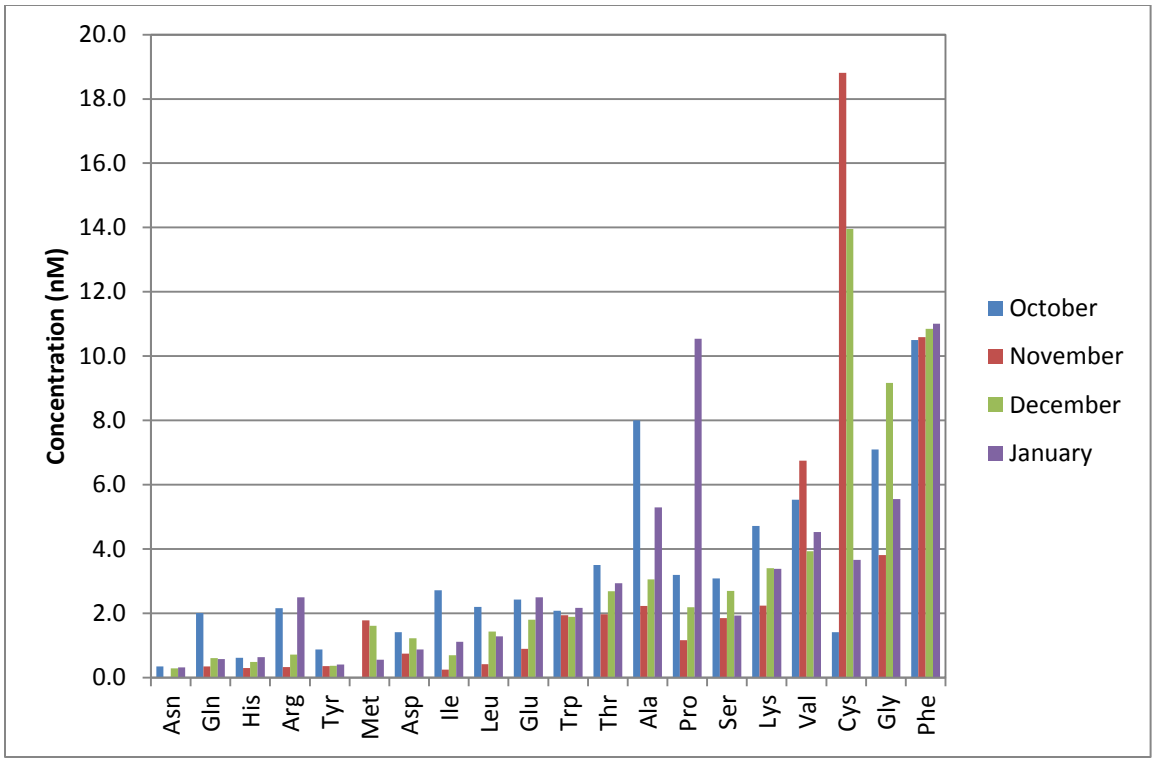

Figure 3.1: Concentration of Credit River water amino acids from October to January. Some fluctuation in amino acid concentrations (nM) occur over the four month period. In particular large fluctuations in concentration is seen for alanine, proline and cysteine while other amino acids like histidine, tryptophan and phenylalanine show relatively stable concentrations from October 2016 to January 2017.

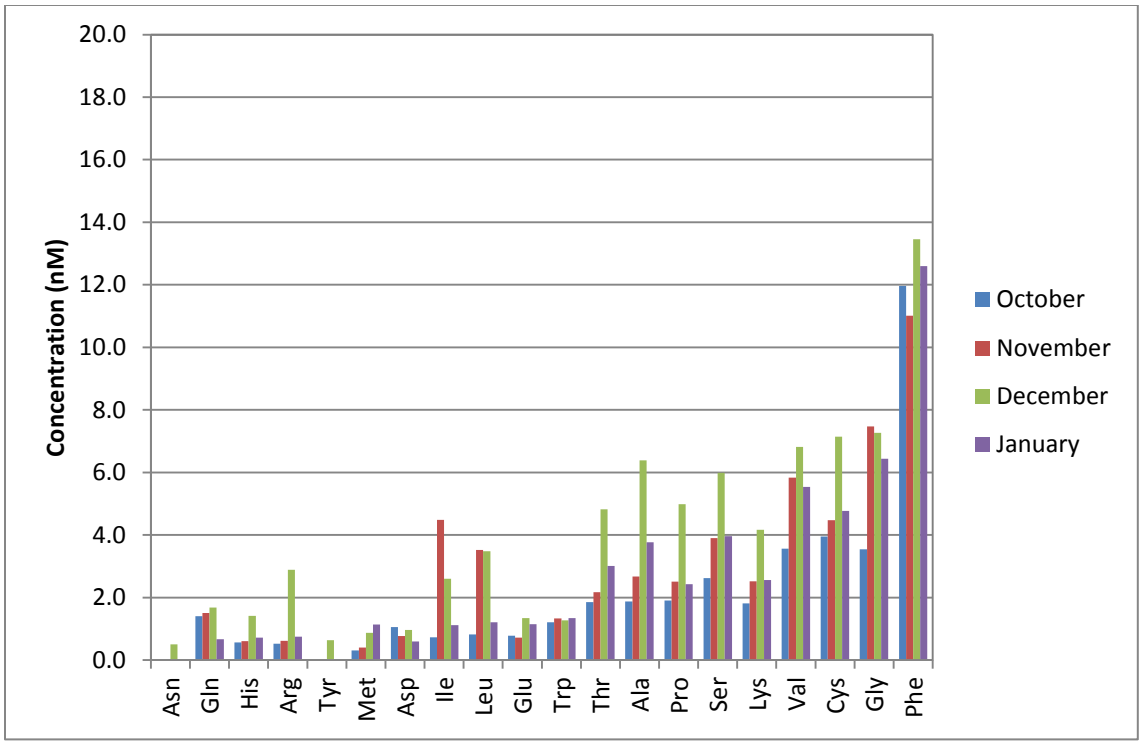

Figure 3.2: Concentration of salmon tray water amino acids from October to January. Some fluctuation in amino acid concentrations (nM) occur over the four month period, but amino acid concentrations were relatively stable over time, particularly for phenylalanine and tryptophan.

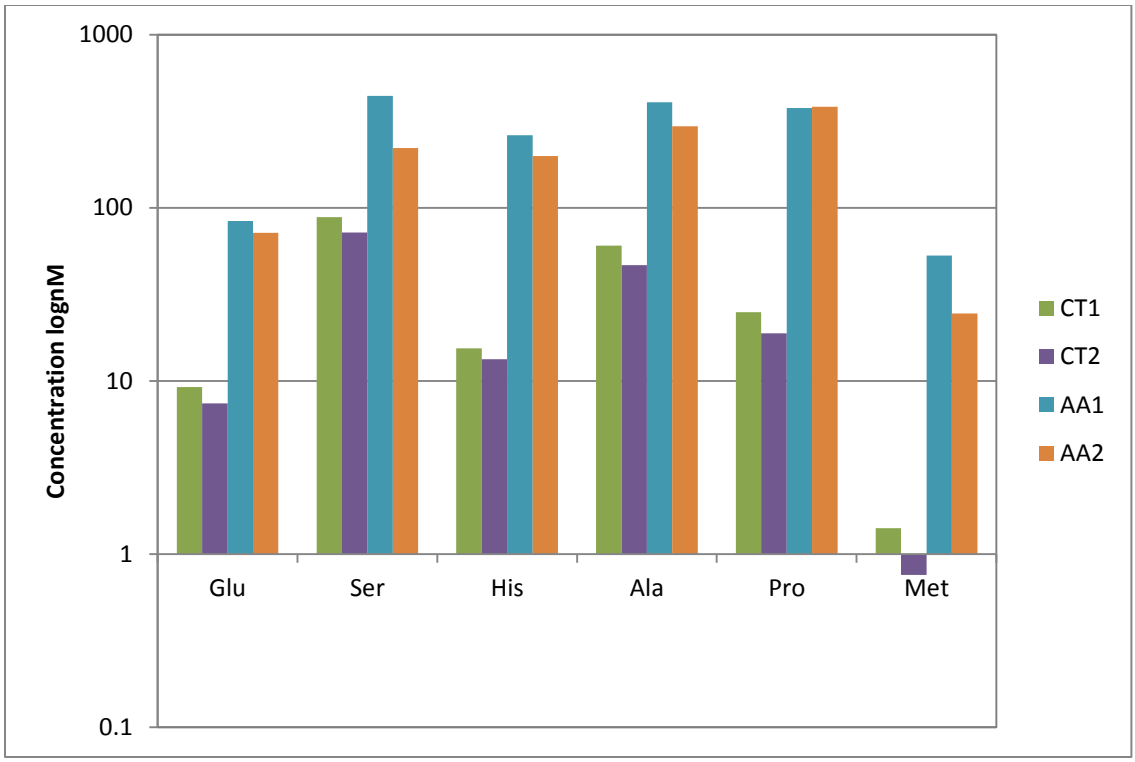

Figure 3.3: lognM concentration of amino acids in water samples from control and amino acid enriched salmon trays. Concentration of the six amino acids (Glu, Ser, His, Ala, Pro and Met) used for the odour enrichment were approximately ten time more concentrated in water samples from the salmon tray that received the amino acid mixture (AA1 and AA2) than water samples from the salmon tray that received dechlorinated water instead (CT1 and CT2).

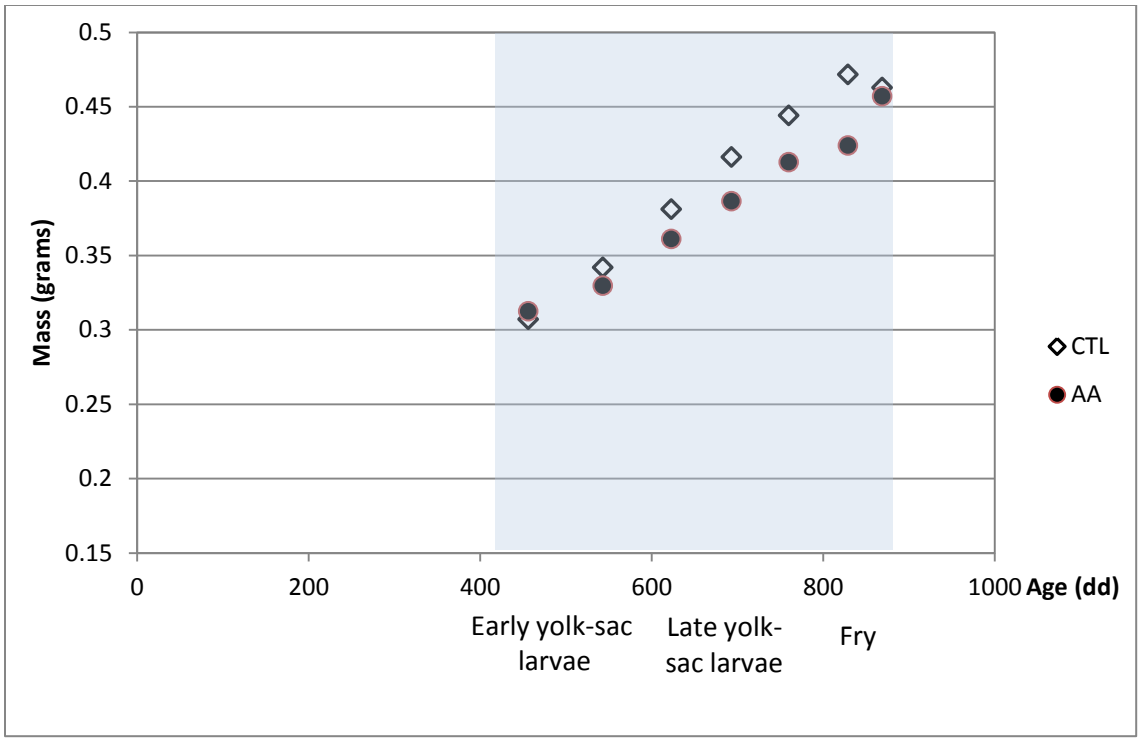

Figure 3.4: Average mass measurements of control and amino acid treated Chinook salmon through larval development. Age of fish is reported in degree days (dd). Shaded region on the graph represents the odour exposure period. Average mass (grams) of control (CTL) animals represented by the outlined diamond while points representing average mass of amino acid exposed (AA) animals are shown by solid black circles. There is a significant increase in mass of fish over time $P(6,276) = 351.472$, $p<0.001$ and a significant difference in mass between CTL and AA fish over the larval period $P(6,276)=11.890$, $p<0.001$.

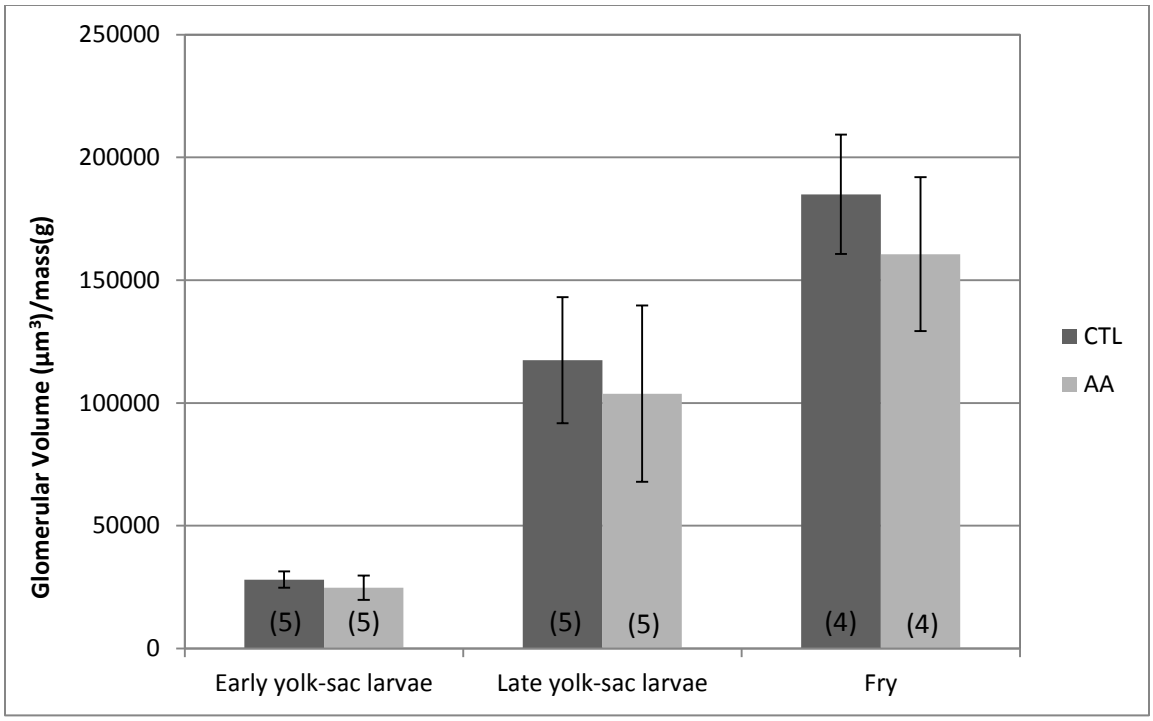

Figure 3.5: Average glomerular volume of calretinin labeled lG_1 in control and amino acid treated Chinook salmon at early yolk-sac larvae, late yolk-sac larvae and fry stages. Glomerular volumes were divided by the average mass of control (CTL) or amino acid (AA) treated fish at each stage. Average glomerular volume of AA fish was lower than in CTL fish at each stage but not statistically significant. Error bars represent standard deviation, N number for each average volume is in the brackets in the bars.

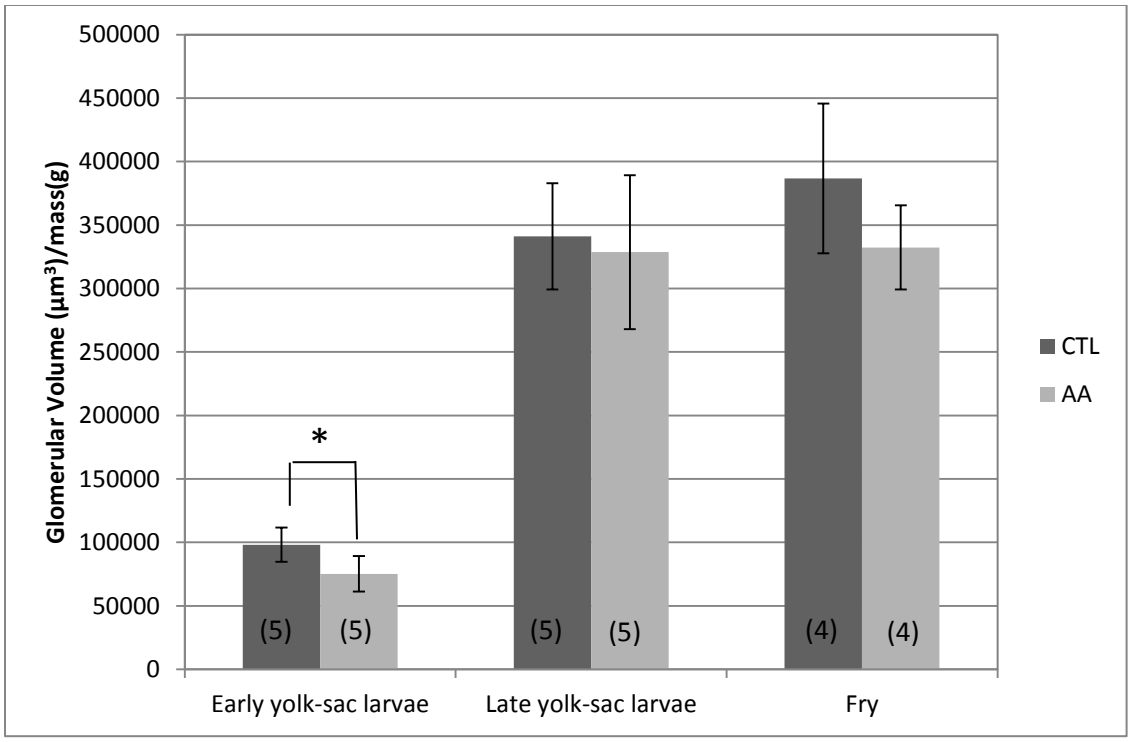

Figure 3.6: Average glomerular volume of calretinin labeled $lG_{3/4}$ in control and amino acid treated Chinook salmon at early yolk-sac larvae, late yolk-sac larvae and fry stages. Glomerular volumes were divided by the average mass of control (CTL) or amino acid (AA) treated fish at each stage. Average glomerular volume in AA fish was lower than in CTL fish at each stage but only statistically significant in the early yolk-sac larvae $t(8)=2.653$, $p=0.029$, denoted by the asterisk. Error bars represent standard deviation, N number for each average volume is in the brackets in the bars.

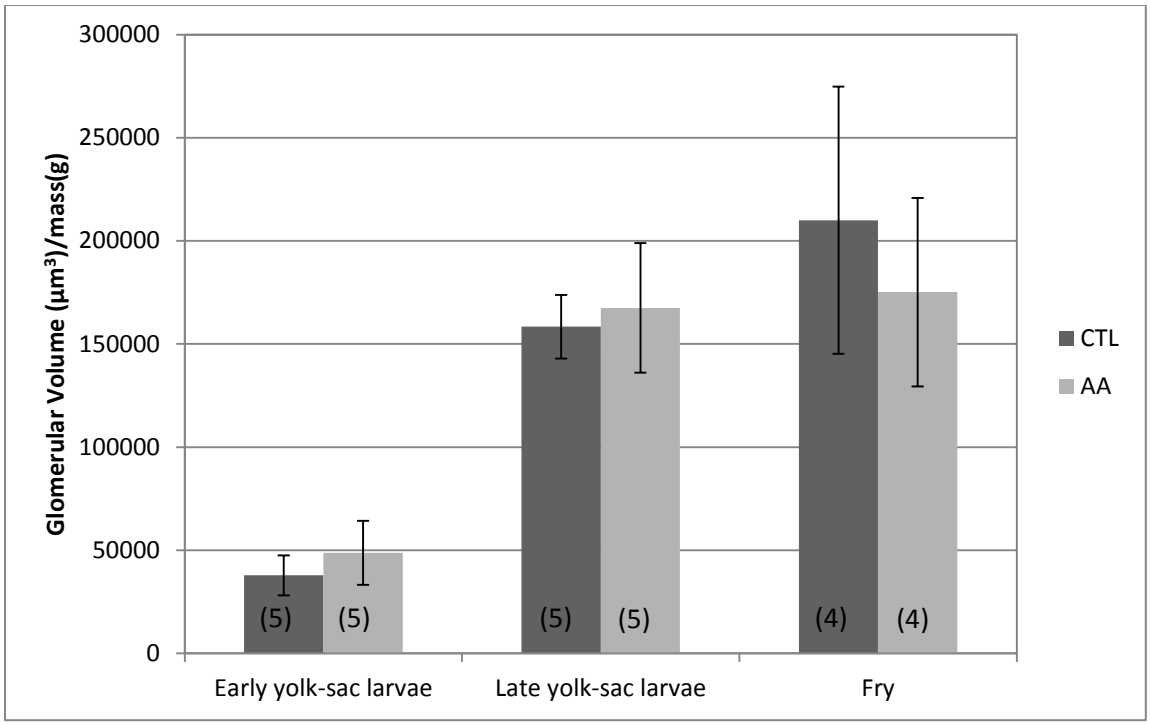

Figure 3.7: Average glomerular volume of calretinin labeled lG_6 in control and amino acid treated Chinook salmon at early yolk-sac larvae, late yolk-sac larvae and fry stages. Glomerular volumes were divided by the average mass of control (CTL) or amino acid (AA) treated fish at each stage. Average glomerular volume of AA fish was higher in early and late yolk-sac larvae but lower in fry than in CTL fish but no statistically significance was determined. Error bars represent standard deviation, N number for each average volume is in the brackets in the bars.

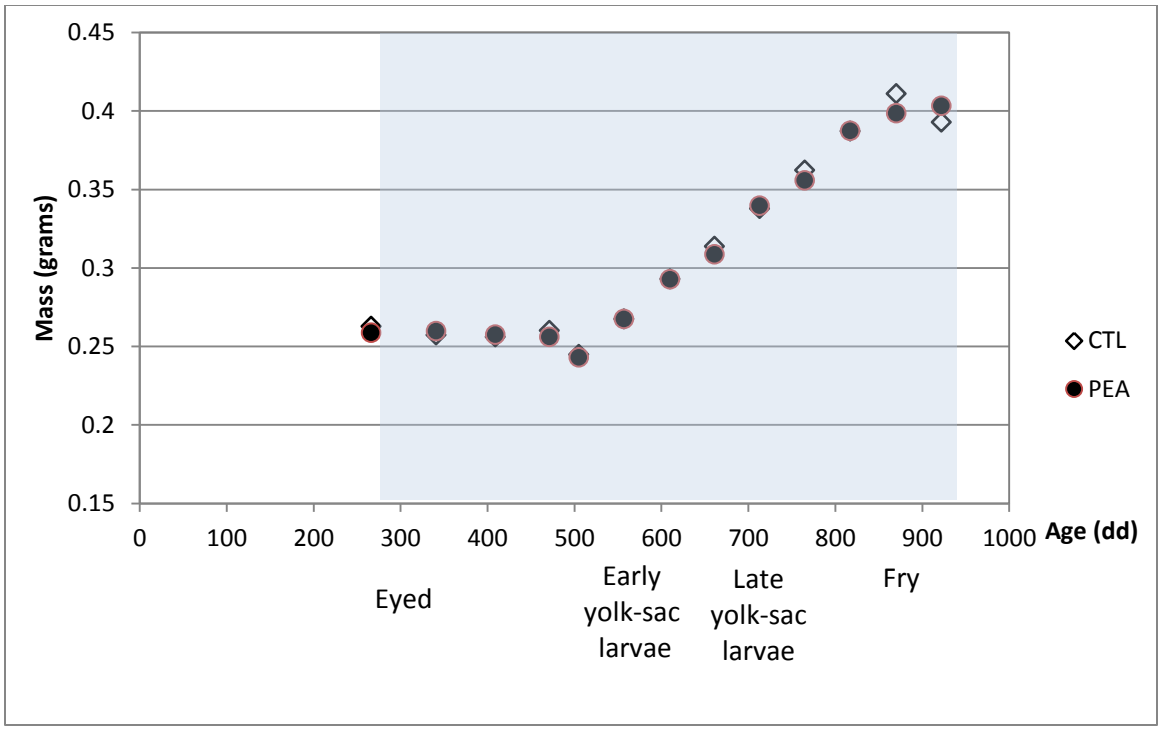

Figure 3.8: Average mass measurements of control and 10^{-7}M PEA treated Chinook salmon through embryonic and larval development. Age of fish is reported as degree days (dd). Shaded region on the graph represents the odour exposure period. Average mass (grams) of control (CTL) animals at is indicated by the outlined diamond while points representing average mass of PEA treated (PEA) animals are shown by solid black circles. There is a small dip in mass at around 500dd where the fish hatched and lost their eggs shells but after this mass appears to increase in a linear fashion. Mass of the fish was statistically different over time $P(12, 456)=724.495$, $p<0.001$ but no difference between masses of CTL and PEA fish was determined.

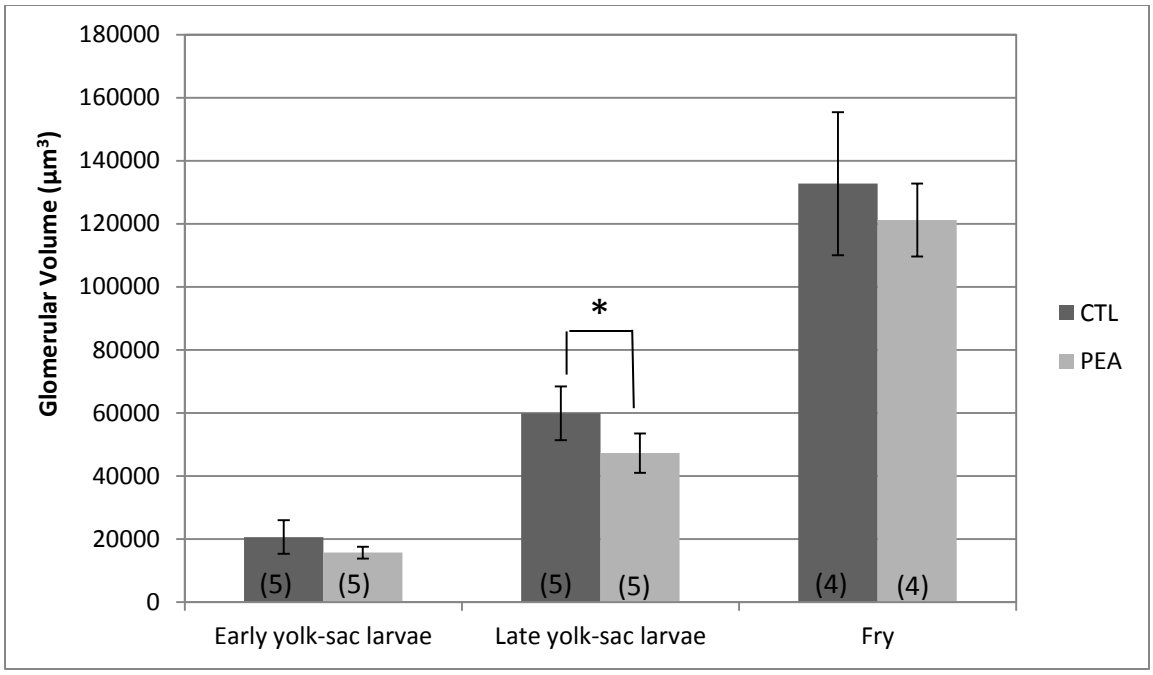

Figure 3.9: Average glomerular volume of vmG_{x+7} in control and PEA treated Chinook salmon at early yolk-sac larvae, late yolk-sac larvae and fry stages. Average glomerular volume of PEA fish was in most cases lower than in CTL fish, however a statistically different glomerular volume of vmGx+7 between CTL and PEA fish was only seen in the late yolk-sac larvae $t(8)=23.682$, $p=0.028$, denoted by the asterisk. Error bars represent standard deviation, N number for each average volume is in the brackets in the bars.

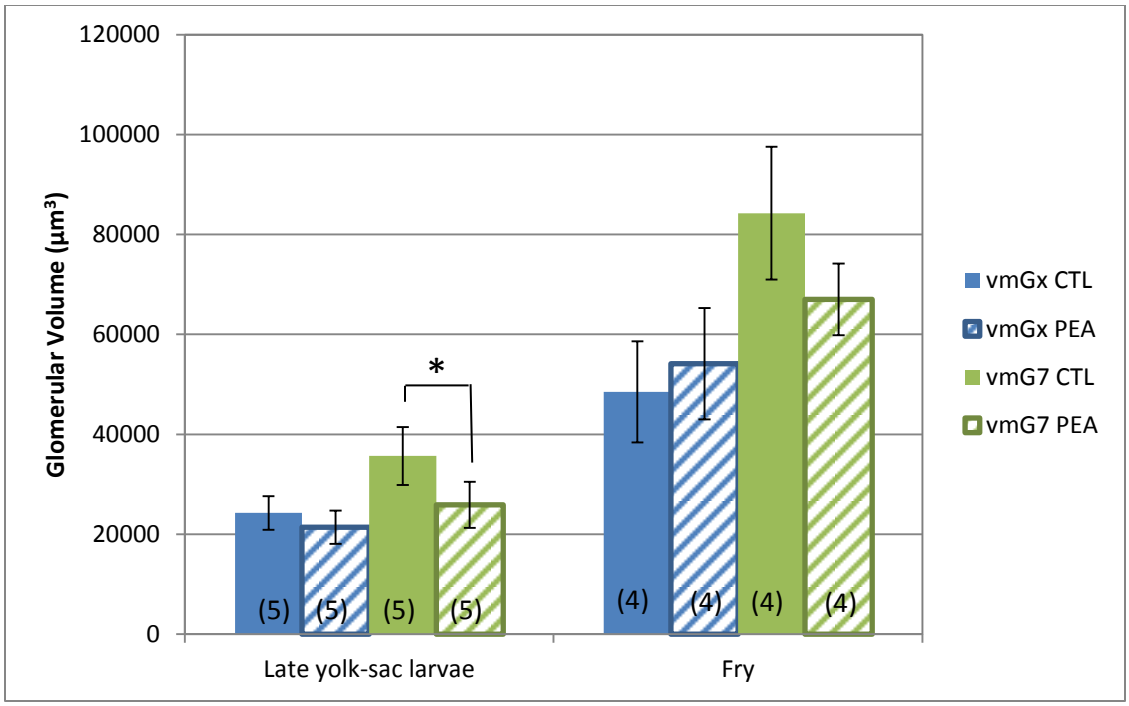

Figure 3.10: Average glomerular volume of vmG_x and vmG_7 in control and PEA treated Chinook salmon at late yolk-sac larvae and fry stages. There was a significant increase in glomerular volume of vmG_x and vmG_7 over larval development: vmG_x: $F(1,6)=56.623$, $p<0.001$; vmG_7: $F(1,6)=103.805$, $p<0.001$. Average glomerular volume of PEA fish was lower than CTL fish, except for vmG_x at the fry stage. A statistically different glomerular volume between CTL and PEA fish was only seen in the late yolk-sac larvae with vmG_7 $t(8)=2.948$, $p=0.018$, denoted by the asterisk. Error bars represent standard deviation, N number for each average volume is in the brackets in the bars.

CHAPTER 4: THESIS SUMMARY

Understanding of the olfactory biology of non-model organisms, such as the Chinook salmon, is important for determining the feasibility of extrapolating discoveries in a "model species" to a whole group of organisms. Additionally, different species may exhibit unique behaviours not typical of model species, such as the long-distance homing migration of salmonids. Hence, research into processes such as olfactory imprinting for homing migration of salmon would benefit from an understanding of the olfactory biology in salmon species.

Olfactory sensory neurons (OSNs) responding to the same odorants project their axons to specific units of odour discrimination within the olfactory bulb called glomeruli (Friedrich and Korsching 1997, Sato et al. 2005). The ability of a species to identify odorants relevant to its life history stage may have implications for differences to the ontogeny of glomerular patterning between species with different life histories. In teleosts, the organization of glomeruli corresponding to different OSN morphotypes has been comprehensively described in zebrafish (Baier and Korching 1994, Gayoso et al. 2011, Braubach et al. 2012, 2013). The course organization of glomerular territories in non-model species, such as the rainbow trout (Riddle and Oakley 1992), brown trout (Castro et al. 2008) and Chinook salmon (Ochs et al. 2017) have revealed comparable glomerular patterning across species in teleosts. Recently, a comprehensive description of lateral glomeruli in the larval Chinook salmon has identified glomeruli specific to a microvillous OSN morphotype (Ochs et al, 2017). However, there are still many gaps in knowledge of the ontogeny of different OSN morphotypes (ciliated and crypt) in the Chinook salmon during early development. As Chinook salmon rely heavily on their

olfactory system, an understanding of the ontogeny of different OSN morphotypes may reveal differences based on presence of relevant odours to the life history stage.

The studies described in the thesis investigate the ontogeny of OSN morphoytpes in the olfactory epithelium and olfactory bulb in Chinook salmon from embryonic to fingerling stages, expanding on a previous study describing course organization of glomerular territories and specific lateral glomeruli in the larval Chinook salmon (Ochs et al. 2017). In Chapter 2 immunohistochemical techniques were used to specifically label OSNs of different morphotypes based on observations with these labels in zebrafish (Gayoso et al. 2011, Braubach et al. 2012, 2013). Both calretinin immunoreactive (microvillous OSNs) and G_{olf} immunoreactive (ciliated OSNs) were present in the olfactory epithelium and in glomeruli within the olfactory bulb in late eyed embryo stages. Specifically, calretinin and G_{olf} labeled lateral glomeruli and ventromedial glomeruli respectively. Calretinin labeled individual distinguishable lateral glomeruli lG_1, $lG_{3/4}$, lG_6 and the dorsal lateral glomerular chain (dlG) from the late eyed embryo stage, while G_{olf} labeling was present in two ventromedial glomeruli vmG_x and vmG_7 and the dlG from late eyed embryo stage. At later developmental stages, labeling with these markers was seen in dorsal glomeruli, medial anterior glomeruli, other ventromedial glomeruli and ventroposterior glomeruli. The ontogeny of some glomeruli showed distinct differences from studies in zebrafish but patterning of glomeruli related to different OSN morphotypes showed consistencies between Chinook salmon and zebrafish.

With the development of some glomeruli during embryonic stages and others at later larval stages it would be important to map the glomerular organization

corresponding to particular OSN mophotypes in adult salmon. This would give a more complete insight into timing of glomerular development which may relate to prevalence of certain odorants relevant for guiding various behvaiours at different life history stages. Additionally, in Chapter 2 S100 antibody was used as a label for crypt OSNs with apical cells in the olfactory epithelium being S100 labeled from embryonic stages in Chinook salmon. However, S100 positive fibers did not reach the olfactory bulb until the fingerling stage and innervated lateral regions of the olfactory bulb typically receiving input from microvillous OSNs (Sato et al. 2005). Hence, using an alternate label for crypt cells such as the anti-Trk-A antibody used in zebrafish (Ahuja et al. 2013) could help elucidate which glomeruli crypt cells project their axons to and the timing of development of these glomeruli. Additionally, a fourth morphotype of OSN has been recently described in zebrafish. The kappe neurons are similar to crypt cells and are labeled with the $G_{\alpha o}$ antibody (Ahuja et al. 2014). Future studies may look into whether kappe cells are present in salmon, when they develop and where their axons project to within the olfactory bulb. Chapter 2 of this thesis gave a description of the development of OSN morphotypes and glomeruli in early developmental stages in Chinook salmon giving a basis for future studies into odour stimulation of various olfactory bulb glomeruli.

In Chapter 3 the effect of olfactory experience during larval stages to the development of individual glomeruli was analyzed through measurements of glomerular volume. Olfactory enrichment to an amino acid mixture showed a decrease in glomerular volume in calretinin positive lateral glomerulus $lG_{3/4}$ at early yolk-sac larvae stage compared to control animals, while olfactory enrichment to PEA odour showed a

decrease in glomerular volume in G_{olf} immunoreactive ventromedial glomerulus vmG_7 during late yolk-sac larvae stage. However, large variations in measured volumes may have distorted results at later larval periods. Overall, through larval development the volumes of lG_1 and $lG_{3/4}$ glomeruli showed a decrease after exposure to an amino acid mixture compared to these glomeruli in fish that received dechlorinated water, and there was a decrease in vmG_7 volume through larval development with exposure to PEA over dechlorinated water. Hence, these findings suggest neuroanatomical evidence for changes in the olfactory bulb to olfactory stimulation during a critical period for olfactory imprinting for homing migration.

However, this study did not test olfactory imprinting directly. A behavioural preference test of the Chinook salmon at older life history stages in a y-maze between a control and treatment odour could be used to determine if the imprinting did occur with the olfactory enrichment during larval development of these Chinook salmon. Additionally, glomerular volume could be measured for adult fish that received olfactory stimulation during larval development to see if any neuroanatomical difference persists throughout the life of the salmon. If difference remains this may indicate that some part of odour memory for imprinting is encoded in glomerular development. Additionally, since amino acid composition was found to be quite similar between water from the river where Chinook salmon spawn and laboratory water raising hatchery fish with an additional odour, such as PEA, and scenting particular natal streams with the PEA odour could be used to determine if these fish have a greater return rate to the stream than fish raised within out the additional odour. This could have implications for restorative biology and perhaps guiding salmon to particular streams most suitable for spawning.

Another interesting factor to look into with regards to the effect of olfactory stimulation during critical periods of early development is changes in expression of NMDA receptors. In adult chum salmon an increase in gene expression of the NR1 subunit of the NMDA receptor was detected in the brain during homing migration (Ueda et al. 2016). As the NMDA receptor has been linked to memory formation (Kinoshita et al. 2004, Cox et al. 2005, Gomez et al, 2006), this increase in gene expression may also be present during larval development when the odour memories are first being formed. If there is an increase in NMDA receptor expression to olfactory stimulation during a critical period related to imprinting, this may suggest an important role of the NMDA receptor in forming olfactory memories and for recognition memory in adult salmon. Hence, looking into the NMDA receptor expression may provide clues to the mechanism of imprinting for homing migration.

Altogether, this thesis gives a description of development of different OSN morphotypes in early life stages of the Chinook salmon olfactory system and highlights differences between wild caught species and model species. Examination of effects of olfactory enrichment revealed a potential for olfactory experience mediated anatomical changes in the olfactory bulb in early larval Chinook salmon. This thesis makes a significant contribution to the field of olfactory biology though better understanding of OSN morphotypes and their patterning in a non-model species that rely on olfactory imprinting for homing, and effects of olfactory stimulation on individual glomeruli identified in developing Chinook salmon.

4.1 References

Ahuja, G., Ivandic, I., Salturk, M., Oka, Y., Nadler, W., Korsching, S. (2013). Zebrafish crypt neurons project to a single, identified mediodorsal glomerulus. *Scientific Reports*. 3: 2063.

Ahuja, G., Bozorg Nia, S., Zapilka, V., Shiriagin, V., Kowatschew, D., Oka, Y., Korsching, S.I. (2014). Kappe neurons, a novel population of olfactory sensory neurons. *Scientific Reports*. 4:4037.

Baier, H., Korsching, S. (1994). Olfactory glomeruli in the zebrafish form an invariant pattern and are identifiable across animal. *Journal of Neuroscience*. 14: 219-230.

Braubach, O., Fine, A., Croll, P. (2012). Distribution and functional organization of glomeruli in the olfactory bulbs of zebrafish (Danio rerio). *Journal of comparative neurology.* 520(11): 2317-39.

Braubach, O., Miyasaka, N., Koide, T., Yoshihara, Y., Croll, R.P., Fine, A. (2013). Experience-dependent versus experience-independent postembryonic development of distinct groups of zebrafish olfactory glomeruli. *The Journal of Neuroscience.* 33(16): 6905-16.

Castro, A., Becerra, M., Anadon, R., Manso, M.J. (2008). Distribution of calretinin during development of olfactory system in the brown trout, *Salmo trutta fario:* comparison with other immunohistochemical markers. *Journal of Chemical Neuroanatomy.* 35(4):306-316.

Cox, J. A., Kucenas, S., Voigt, M. M. (2005). Molecular characterization and embryonic expression of the family of N-methyl-D-aspartate receptor subunit genes in zebrafish. *Developmental Dynamics.* 234**,** 756–766.

Friedrich, R.W., Korsching, S. (1997). Combinatorial and chemotopic odorant coding in the zebrafish olfactory bulb visualized by optical imaging. *Neuron.* 18:737-752.

Gayoso, J.A., Castro, A., Anadon, R., Manso, M.J. (2011). Differential bulbar and extrabulbar projections of diverse olfactory receptor neuron population in the adult zebrafish *(Daniorerio*). *Journal of Comparative Neuroscience.* 519: 247 -276.

Gómez, Y., Vargas, J. P., Portavella, M., López, J. C. (2006). Spatial learning and goldfish telencephalon NMDA receptors. *Neurobiology of Learning and Memory.* 85: 252–262.

Kinoshita, M., Hosokawa, T., Urano, A., Ito, E. (2004). Long-term potentiation in the optic tectum of rainbow trout. *Neuroscience Letters.* 370**:**146–150.

Ngai, J., Chess, A., Dowling, M.M., Necles, N., Macagno, E.R., Axel, R. (1993). Coding of olfactory information: topography of odorant receptor expression in the catfish olfactory epithelium. *Cell*. 72:667-680.

Ochs, C.L., Suntres, T., Zygowska, A., Pitcher, T., Zielinski, B.S. (2017). Organization of glomerular territories in the olfactory bulb of post-embryonic wild Chinook salmon *Oncoryhynchus tshawytscha*. *Journal of Morphology.* 278(4): 464-474.

Riddle, D.R.,Oakley, B. (1992). Immunocytochemical identification of primary olfactory afferents in rainbow trout. *Journal of Comparative Neurology.* 324:575-589.

Sato, Y., Miyasaka, N., Yoshihara, Y. (2005). Mutually exclusive glomerular innervation by two distinct types of olfactory sensory neurons revealed in transgenic zebrafish. *Journal of Neuroscience.* 25: 4889-4897.

Ueda, H., Nakamura, S., Nakamura, T., Inada, K., Okubo, T., Furukawa, N., Murakami, R., Tsuchida, S., Zohar, Y., Konno, K., Watanabe, M. (2016). Involvement of hormones in olfactory imprinting and homing in chum salmon. Scientific Reports. 6:21102.

APPENDIX A: PROTOCOLS AND SOLUTIONS

Immunohistochemistry

Phosphate Buffer (PB) and Phosphate Buffered Saline (PBS) and Phosphate Buffered Saline with Triton X-100 (PBS-T)

0.2 M stock solution of Sodium Phosphate Monobasic (NaH_2PO_4 . H_2O; MW = 137.99):
Dissolve 27.6 g into 1 L of deionized water

0.2 M stock solution of Sodium Phosphate Dibasic (Na_2HPO_4; MW = 141.96):
Dissolve 28.4 g into 1 L of deionized water

0.2 M PB, pH 7.4:
Add 190 mL of sodium phosphate monobasic and 810 mL of sodium phosphate dibasic

0.1 M PB:
Dilute 500mL of 0.2M PB with 500 mL of deionized water

0.1 M PBS, pH 7.4:
Add 8.0g NaCl and 0.2g KCl to 1 L of 0.1 M PB and adjust pH to 7.4

0.1 M PBS-T:
Add 1 mL of Triton X-100 to 1 L of 0.1 M PBS

4% Paraformaldehyde (PFA) for 250 mL

1) Add 10mL of PFA to 55mL of deionized water
2) Heat to approximately 55 °C while stirring. After 10 minutes clear solution by adding approximately 1 NaOH chip and 5M NaOH dropwise as needed
3) Solution is clear when dissolved
4) Let solution cool to 10°C
5) Bring volume up to 125 mL by adding 70 mL of distilled water
6) Bring volume up to the final 250 mL by adding 125 mL of 0.2 M phosphate buffer
7) Check pH using pH strips and adjust to 7.4

Calretinin, G_{olf} and S100 Immunocytochemistry Protocol: Probes to label OSN morphotypes
Adapted from: Braubach et al. 2012; Ochs et al. 2017

1) Rehydrate sections in 0.1M PBS plus 0.1% Triton X-100 3 times over three hours
2) Place slides in antigen retrieval solution HistoVTOne (Nacalai) for 20 minutes in a warm water bath at 70^{o}C
3) Block with 10% Goat Serum in 0.1M PBS-T for 1 hour

4) Incubate with Calretinin monoclonal antibody produced in mouse (6B3, Swant) at 1:500 with wither 1:250 $G_{\alpha olf}$ polyclonal antibody produced in goat and rabbit (Santa Cruz) or 1:250 S100 polyclonal antibody produced in goat and rabbit (Dako) in 0.1 M PBS plus 0.1% triton in a keeper at 4°C on shaker for 3 days
5) Rinse five times (50 min each) in PBS at 4°C on shaker
6) Incubate with 1:250 goat anti-mouse Alexafluor 568 IgG (Sigma-Aldrich Oakville ON, A11004 lot# 1698376) and 1:250 goat anti-rabbit Alexafluor 488 IgG (Sigma-Aldrich Oakville ON, A11008 lot #1829924) diluted in 0.1 M PBS for two days at 4°C in keeper on shaker.
7) Rinse five times (50 min each) in PBS at 4°C on shaker
8) Coverslip with Vectashield Mounting Medium with DAPI to label nuclei.

APPENDIX B: TABLES

Table 1: Chemicals used for olfactory enrichment experiments in 2015, 2016

2015	Supplier	Lot Number	Product
Amino Acids			
L-Histidine	Sigma	SLBL3731V	H8000-100G
L-Serine	Sigma	071M02631V	S4500-100G
L-Proline	Sigma	SLBL4766V	P0380-100G
L-Glutamic acid	Sigma	SLBM4966V	G1251-100G
L-Alanine	Sigma	BCBN6412V	A7627-100G
L-Methionine	Sigma	SLBL7822V	M9625-100G
2016			
Phenethyl alcohol	Sigma	MKBZ9717V	W285803-1KG

Table 2: Mass (grams) measurements of control larval Chinook salmon from 2015 amino acid exposure experiment from hatch to fry stage

Week	0	1	2	3	4	5	6
Degree days	456	543	623	693	760	829	869
Date	10-Nov-15	16-Nov-15	23-Nov-15	30-Nov-15	07-Dec-15	14-Dec-15	21-Dec-15
Mass (grams)	0.31	0.3568	0.3743	0.3928	0.4608	0.4939	0.503
	0.294	0.357	0.3533	0.4316	0.4519	0.446	0.489
	0.332	0.3346	0.3731	0.4486	0.4509	0.4829	0.4674
	0.3	0.3272	0.3818	0.4104	0.4434	0.4867	0.4897
	0.293	0.3631	0.3757	0.4299	0.4563	0.497	0.4806
	0.306	0.3476	0.3872	0.408	0.4282	0.4949	0.4938
	0.286	0.3639	0.3929	0.4028	0.4382	0.4797	0.4889
	0.31	0.3464	0.3861	0.4192	0.4696	0.4766	0.5112
	0.297	0.3174	0.3916	0.4052	0.4331	0.4028	0.4918
	0.287	0.3401	0.3766	0.4036	0.4254	0.4982	0.4836
	0.313	0.3577	0.3667	0.4207	0.4708	0.4705	0.475
	0.312	0.3431	0.3971	0.4131	0.4432	0.5093	0.4819
	0.316	0.3606	0.3834	0.3953	0.4504	0.4769	0.4943
	0.313	0.3232	0.386	0.4384	0.4227	0.4977	0.5137
	0.299	0.3419	0.3887	0.4127	0.4422	0.4864	0.4419
	0.316	0.3524	0.3818	0.4141	0.4697	0.4807	0.4832
	0.305	0.3319	0.3764	0.3951	0.471	0.477	0.4686
	0.311	0.3264	0.3944	0.4109	0.4396	0.491	0.4967
	0.311	0.3232	0.3803	0.4069	0.4536	0.4811	0.3198
	0.32	0.3401	0.3868	0.4224	0.4274	0.4926	0.3257
	0.305	0.3343	0.3719	0.4146	0.4278	0.4748	0.3203
	0.313	0.3561	0.384	0.3959	0.4566	0.5083	
	0.309	0.3616	0.3748	0.4246	0.4859	0.4844	
	0.317	0.3361	0.3793	0.4129	0.4522	0.4529	
	0.295	0.34	0.3694	0.4349	0.4393	0.4661	
	0.311	0.33	0.3864	0.4234	0.4491	0.48	
		0.3208	0.3908	0.4396	0.4736	0.5014	
			0.3851	0.4289	0.4437	0.4777	
			0.3891	0.4142	0.4277	0.4221	
			0.3641	0.4116	0.3179	0.26	
Count	26	27	30	30	30	30	21
Average	0.306962	0.341981	0.38097	0.416077	0.444073	0.471653	0.462862

Table 3: Mass (grams) measurements of amino acid exposed treatment larval Chinook salmon from 2015 amino acid exposure experiment from hatch to fry stage

Week	0	1	2	3	4	5	6
Degree days	456	543	623	693	760	829	869
Date	09-Nov-15	16-Nov-15	23-Nov-15	30-Nov-15	07-Dec-15	14-Dec-15	21-Dec-15
Mass (grams)	0.311	0.3427	0.3663	0.4008	0.4457	0.3976	0.4556
	0.3	0.333	0.3494	0.3906	0.4094	0.4253	0.4466
	0.286	0.3222	0.3594	0.3927	0.4004	0.3957	0.4849
	0.317	0.3194	0.3787	0.4088	0.4129	0.3838	0.4832
	0.308	0.3133	0.3404	0.3819	0.4366	0.4642	0.4677
	0.31	0.3648	0.3713	0.404	0.431	0.4317	0.4802
	0.323	0.3186	0.374	0.3778	0.4011	0.4224	0.494
	0.333	0.361	0.3671	0.3786	0.4238	0.4393	0.4574
	0.314	0.3394	0.357	0.3741	0.4328	0.4246	0.445
	0.314	0.3211	0.3792	0.3762	0.4062	0.4103	0.4569
	0.315	0.3308	0.3727	0.3771	0.3929	0.4552	0.4479
	0.326	0.3289	0.363	0.3644	0.3901	0.4354	0.4823
	0.319	0.3292	0.3752	0.4063	0.4309	0.4256	0.468
	0.31	0.341	0.3638	0.395	0.4088	0.422	0.4758
	0.316	0.3294	0.3403	0.3916	0.4191	0.3911	0.4338
	0.311	0.3254	0.3747	0.3956	0.4379	0.4416	0.4819
	0.295	0.3252	0.3563	0.384	0.4219	0.4281	0.4603
	0.322	0.3468	0.336	0.3802	0.4228	0.4119	0.431
	0.314	0.3153	0.3594	0.403	0.3959	0.4322	0.442
	0.314	0.324	0.3391	0.389	0.3811	0.4282	0.4636
	0.302	0.3374	0.3451	0.389	0.4184	0.4379	0.4384
	0.318	0.3029	0.3563	0.4022	0.4221	0.4227	0.4622
	0.308	0.321	0.3693	0.4004	0.3538	0.4409	0.463
	0.311	0.3244	0.3534	0.3619	0.4158	0.4047	0.4454
	0.32	0.3244	0.3526	0.3908	0.4237	0.4207	0.4074
	0.324	0.323	0.3579	0.3866	0.4254	0.4123	0.4771
	0.297	0.3444	0.3812	0.3954	0.3981	0.3834	0.455
		0.3364	0.3652	0.359	0.4077	0.4412	0.4543
		0.3364	0.3594	0.3542	0.4267	0.4246	0.4328
		0.3076	0.369	0.3804	0.389	0.4638	0.4363
				0.3907			0.4346
Count	27	30	30	31	30	30	31
Average	0.312519	0.329647	0.36109	0.386526	0.412733	0.423947	0.456923

Table 4: Mass (grams) measurements of control (CTL) Chinook salmon from 2016 PEA exposure experiment from early eyed embryo to fry stage. In the top row of the table E__ denotes week during embryonic development and A__ represents the week in larval development. In the first column D__ gives the individual code for the fish.

	E01	E02	E03	E04	A01	A02	A03	A04
Degree days	266	341	409	471	505	557	610	661
D01	0.2457	0.2507	0.2584	0.2627	0.2479	0.256	0.2812	0.3051
D02	0.263	0.2544	0.2476	0.268	0.2433	0.269	0.282	0.3114
D03	0.2613	0.2591	0.2536	0.2574	0.2354	0.2484	0.2998	0.3133
D04	0.26	0.2567	0.2586	0.2554	0.2353	0.277	0.2874	0.302
D05	0.2519	0.2493	0.2558	0.2558	0.2401	0.2774	0.3004	0.3132
D06	0.2562	0.2544	0.2598	0.2707	0.2508	0.2686	0.2976	0.3204
D07	0.255	0.2674	0.2646	0.2646	0.2387	0.2624	0.2978	0.3372
D08	0.2483	0.257	0.2619	0.2604	0.2452	0.263	0.2973	0.3148
D09	0.254	0.2521	0.2677	0.263	0.2493	0.2732	0.2842	0.315
D10	0.2592	0.2542	0.2614	0.2538	0.2362	0.2704	0.3029	0.3099
D11	0.2872	0.2664	0.2511	0.2616	0.2562	0.269	0.2826	0.3157
D12	0.2649	0.2626	0.24	0.257	0.2497	0.2711	0.294	0.3042
D13	0.2749	0.2618	0.2421	0.2611	0.2538	0.2741	0.3004	0.3056
D14	0.275	0.2622	0.259	0.2483	0.2373	0.275	0.2949	0.3061
D15	0.2833	0.2534	0.2566	0.2758	0.2401	0.2686	0.3107	0.3136
D16	0.2641	0.2574	0.2676	0.2554	0.2399	0.2646	0.2878	0.3133
D17	0.2533	0.2593	0.258	0.2516	0.2472	0.2708	0.2953	0.3129
D18	0.2781	0.2566	0.2596	0.2592	0.248	0.2606	0.2866	0.3164
D19	0.2557	0.2581	0.254	0.2629	0.2464	0.276	0.2899	0.3193
D20	0.2663	0.2537	0.2448	0.2587	0.2541	0.256	0.2882	0.3253

Continuation of Table 4

	A05	A06	A07	A08	A09
Degree days	713	765	817	870	922
D01	0.3336	0.3957	0.4836	0.3847	0.3997
D02	0.3329	0.3533	0.3752	0.3962	0.3892
D03	0.3386	0.3489	0.3826	0.394	0.3784
D04	0.3343	0.404	0.3417	0.403	0.4036
D05	0.3376	0.3544	0.3583	0.4424	0.381
D06	0.3604	0.3517	0.4592	0.4121	0.3577
D07	0.3332	0.3664	0.3942	0.4007	0.3759
D08	0.3341	0.3766	0.3851	0.4267	0.4213
D09	0.3383	0.367	0.4052	0.508	0.3759
D10	0.3382	0.384	0.3767	0.3881	0.4206
D11	0.3271	0.3593	0.3774	0.3911	0.3866
D12	0.352	0.3516	0.3691	0.45	0.3928
D13	0.3339	0.3578	0.3464	0.3997	0.4026
D14	0.3269	0.3503	0.3967	0.456	0.3926
D15	0.3461	0.3536	0.4043	0.4516	0.3794
D16	0.3554	0.3402	0.382	0.407	0.424
D17	0.3369	0.3826	0.3744	0.391	0.4316
D18	0.3326	0.3536	0.373	0.4186	0.3871
D19	0.3359	0.3453	0.3676	0.4882	0.4428
D20	0.3277	0.3476	0.3896	0.385	0.3798

Table 5: Mass (grams) measurements of 10^{-7}M PEA treatment Chinook salmon from 2016 PEA exposure experiment from early eyed embryo to fry stage. In the top row of the table E__ denotes week during embryonic development and A__ represents the week in larval development. In the first column D__ gives the individual code for the fish.

	E01	E02	E03	E04	A01	A02	A03	A04
Degree days	266	341	409	471	505	557	610	661
D01	0.2791	0.2511	0.2656	0.2663	0.2419	0.2614	0.2926	0.3213
D02	0.2634	0.2653	0.2593	0.2569	0.2416	0.2651	0.2924	0.2998
D03	0.2592	0.2577	0.2604	0.2461	0.2461	0.2792	0.292	0.3128
D04	0.2499	0.2586	0.2538	0.2504	0.2433	0.2703	0.2977	0.2526
D05	0.2464	0.2552	0.2632	0.2544	0.2577	0.2767	0.2777	0.3066
D06	0.2544	0.2617	0.2506	0.2608	0.2408	0.2647	0.2888	0.3158
D07	0.2677	0.2573	0.256	0.2614	0.2334	0.2684	0.2881	0.312
D08	0.2651	0.2608	0.2549	0.2636	0.245	0.2686	0.2978	0.2994
D09	0.2486	0.2559	0.2654	0.2497	0.2536	0.2683	0.279	0.3401
D10	0.2642	0.2646	0.2498	0.2569	0.2508	0.2618	0.2781	0.3093
D11	0.2688	0.2707	0.2516	0.2609	0.233	0.2616	0.2983	0.3081
D12	0.2503	0.2542	0.2544	0.2671	0.2403	0.2724	0.2919	0.3107
D13	0.2623	0.2617	0.2687	0.2608	0.2434	0.2608	0.2869	0.3014
D14	0.251	0.2579	0.2583	0.2711	0.2486	0.2634	0.2989	0.31
D15	0.2536	0.2629	0.2622	0.258	0.2493	0.2582	0.3013	0.3231
D16	0.2581	0.2517	0.2561	0.2618	0.2437	0.2879	0.3072	0.323
D17	0.2638	0.2577	0.2518	0.2647	0.2308	0.2741	0.2936	0.302
D18	0.2544	0.263	0.2614	0.2526	0.2492	0.2534	0.2958	0.305
D19	0.2571	0.2584	0.2551	0.2573	0.225	0.2669	0.304	0.317
D20	0.257	0.2681	0.2496	0.2	0.2428	0.2673	0.2931	0.3024

Continuation of Table 5

	A05	A06	A07	A08	A09
Degree days	713	765	817	870	922
D01	0.3499	0.3619	0.3739	0.3876	0.4214
D02	0.3391	0.3524	0.3924	0.3958	0.408
D03	0.3501	0.3574	0.3799	0.4231	0.4137
D04	0.3431	0.349	0.4079	0.4238	0.4157
D05	0.3359	0.3684	0.3936	0.3992	0.3977
D06	0.3371	0.356	0.3912	0.4002	0.3743
D07	0.3297	0.3544	0.3953	0.3984	0.415
D08	0.3192	0.36	0.3952	0.3968	0.3896
D09	0.3424	0.3817	0.4026	0.3918	0.4008
D10	0.3381	0.3609	0.3816	0.4038	0.4136
D11	0.3548	0.3648	0.3974	0.3836	0.4122
D12	0.3411	0.365	0.3887	0.3748	0.4144
D13	0.3514	0.3268	0.3794	0.4072	0.4013
D14	0.3438	0.3438	0.3881	0.4011	0.3832
D15	0.3371	0.3516	0.3919	0.417	0.4084
D16	0.3493	0.362	0.3734	0.4007	0.3918
D17	0.3291	0.3494	0.3826	0.3814	0.3853
D18	0.3349	0.3603	0.3843	0.4024	0.4194
D19	0.3238	0.3409	0.3874	0.4013	0.3958
D20	0.3409	0.3492	0.3611	0.3821	0.4076

Table 6: Glomerular volume (μm^3) of lateral glomeruli lG_1, $lG_{3/4}$ and lG_6 for control (CTL) and amino acid exposed (AA) Chinook salmon at early yolk-sac larvae, late yolk-sac larvae and fry stages

Early Yolk-Sac Larvae			
	lG_1	$lG_{3/4}$	lG_6
CTL 1	9119.898	28590.93	13436.55
CTL 2	7290.549	34697.934	15193.827
CTL 3	8429.076	33173.91	8234.127
CTL 4	8232.669	24158.511	8914.563
CTL 5	10001.115	30128.229	12333.744
AA 1	9901.152	21739.032	16605.153
AA 2	6486.633	23723.163	9985.401
AA 3	6944.499	29307.915	22718.934
AA 4	6558.597	17513.424	12495.618
AA 5	8839.08	25291.071	14278.482

Late Yolk-Sac Larvae			
	lG_1	$lG_{3/4}$	lG_6
CTL 1	49131.72	131773.356	60418.404
CTL 2	44297.946	151155.675	67017.492
CTL 3	48454.06	142806.015	71054.892
CTL 4	72227.898	149919.894	76585.005
CTL 5	46622.025	181724.4	76610.268
AA 1	28920.78	171064.206	64310.922
AA 2	38820.429	143319.51	63941.229
AA 3	42935.832	107318.754	66198.663
AA 4	67745.538	140215.77	91855.296
AA 5	35797.437	116406.72	59291.289

Fry			
	IG_1	$lG_{3/4}$	lG_6
CTL 1	102433.45	189443.448	141298.5
CTL 2	78901.326	142559.802	77542.01
CTL 3	80065.476	207181.008	79204.03
CTL 4	81059.67	176808.015	90750.19
CTL 5			
AA 1	90681.642	150607.755	102788.1
AA 2	79527.411	163528.164	88676.33
AA 3	63106.695	130870.863	53742.09
AA 4	60217.299	162416.88	74910.37
AA 5			

Table 7: Glomerular volume (μm^3) divided by average mass (grams) of lateral glomeruli lG_1, $lG_{3/4}$ and lG_6 for control (CTL) and amino acid exposed (AA) Chinook salmon at early yolk-sac larvae, late yolk-sac larvae and fry stages

Early Yolk-Sac Larvae Final			
	lG_1	$lG_{3/4}$	lG_6
CTL 1	29710.18563	93141.59407	43772.68196
CTL 2	23750.65643	113036.5778	49497.41988
CTL 3	27459.67253	108071.7157	26824.58089
CTL 4	26819.83112	78701.95985	29041.25918
CTL 5	32580.95465	98149.70257	40180.03531
Averages	28064.26007	98220.31001	37863.19544
AA 1	31681.76015	69560.67311	53133.2591
AA 2	20755.96364	75909.5063	31951.34056
AA 3	22221.04576	93779.6262	72696.16887
AA 4	20986.23444	56039.5496	39983.5466
AA 5	28283.33637	80926.50687	45688.36455
Averages	24785.66807	75243.17242	48690.53594

Late Yolk-Sac Larvae Final			
	lG_1	$lG_{3/4}$	lG_6
CTL 1	110638.8364	296738.0498	136055.1171
CTL 2	99753.74769	340384.7453	150915.4846
CTL 3	109112.826	321582.2962	160007.233
CTL 4	162648.7042	337601.9123	172460.395
CTL 5	104987.2994	409221.9072	172517.2843
Averages	117428.2827	341105.7822	158391.1028
AA 1	70071.40209	414466.9944	155817.2523
AA 2	94057.00295	347245.0955	154921.5328
AA 3	104028.1053	260019.8046	160391.0107
AA 4	164138.8937	339725.125	222553.7963
AA 5	86732.67463	282038.7999	143655.3147
Averages	103805.6157	328699.1639	167467.7814

Fry Final			
	lG_1	$lG_{3/4}$	lG_6
CTL 1	221304.5054	409287.105	305271.3076
CTL 2	170464.039	307996.3402	167527.2781
CTL 3	172979.1515	447608.5918	171118.0265
CTL 4	175127.0789	381988.6165	196063.1592
CTL 5			
Averages	184968.6937	386720.1634	209994.9429
AA 1	198461.5395	329612.9873	224957.0606
AA 2	174049.9187	357889.9815	194072.7978
AA 3	138112.3187	286417.7619	117617.3819
AA 4	131788.7237	355457.878	163945.2731
AA 5			
Averages	160603.1251	332344.6522	175148.1283

Table 8: Glomerular volume (μm^3) of ventromedial glomeruli vmG_x and vmG_7 and their total for control (CT) and PEA exposed (PE) Chinook salmon at early yolk-sac larvae (A01), late yolk-sac larvae (A05) and fry stages (A09)

Early Yolk-Sac Larvae			
CT	vmG_{X+7}	PE	vmG_{X+7}
A01D01CT	26619.98	A01D01PE	15267.48
A01D02CT	25702.97	A01D02PE	17122.44
A01D08CT	19665.12	A01D08PE	17383.64
A01D05CT	14383.64	A01D05PE	16135.11
A01D10CT	16995.41	A01D10PE	12654.28

Late Yolk-Sac Larvae			
CT	vmG_x	vmG_7	vmG_{X+7}
A05D01CT	19806.17	33473.34	53279.51
A05D03CT	21869.71	27180.32	49050.03
A05D05CT	27916.13	37243.98	65160.11
A05D07CT	24942.82	37601.74	62544.56
A05D10CT	26778.19	42833.83	69612.01
PE	vmG_x	vmG_7	vmG_{X+7}
A05D01PE	18485.98	19464.35	37950.34
A05D03PE	18969.99	26865.17	45835.16
A05D05PE	24811.09	23045.25	47856.34
A05D07PE	25221.36	29688.13	54909.49
A05D10PE	19434.39	30416.36	49850.76

Fry			
CT	vmG_x	vmG_7	vmG_{x+7}
A09D01CT	45305.96	88313.65	133619.6
A09D05CT	51872.49	91277.38	143149.9
A09D24CT	-	-	-
A09D22CT	60396.53	92910.65	153307.2
A09D08CT	36428.15	64504.42	100932.6
PE	vmG_x	vmG_7	vmG_{x+7}
A09D01PE	-	-	-
A09D05PE	41728.91	73100.57	114829.5
A09D07PE	68828.91	67314.11	136143
A09D21PE	52922.04	56888.26	109810.3
A09D08PE	53053.94	70801.45	123855.4

APPENDIX C: SV2 labeling in Chinook salmon olfabtory bulb

SV2 antibody is a synaptic vesicle protein which has been used as a general label of glomeruli within the olfactory bulb of zebrafish (Braubach et al. 2012, Yabuki et al. 2016).

SV2 Immunohistochemistry

Slides of Chinook salmon olfactory bulb were rinsed three times over 3 hours wiht PBS-T to rehydrate tissue sections before being placed in HistoVTOne (06380-05, lot# L6R0020, Nacalai tesque, Kyoto) for 20 minutes at 70^{o}C for antigen retrieval. Slides were rinsed three times with PBS and placed in a blocking solution with 10% goat serum (Sigma-Aldrich, Oakville ON, G9023) in 0.1M PBS with 0.1% Triton-X for an hour. Slides were then labelled with primary antibody 1:250 monoclonal SV2 antibody raised in mouse (developed by Buckley, K.M., Harvard Medical School, and obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH, maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242)for 3 days at 4^{o}C under gentle agitation. The slides were then rinsed with PBS every 50 minutes over 5 hours before being placed in secondary antibody of 1:500 Alexafluor 488 goat anti-mouse IgG (Sigma-Aldrich Oakville ON, A11001) or 1:500 goat anti-mouse Alexafluor 568 IgG (Sigma-Aldrich Oakville ON, A11004 lot# 1698376) for two days at 4^{o}C with gentle agitation. Finally, slides were again rinsed with PBS over 5 hours and coverslipped with VectaShield (H-1200, Vector Laboratories, Burlingame, CA) mounting medium for fluorescence with DAPI counterstain. Pictures were taken using a fluorescence Axio Scan Z1 (Zeiss) microscope at 20x magnification for a final z-stack image of 30µm depth and a 3µm z-step.

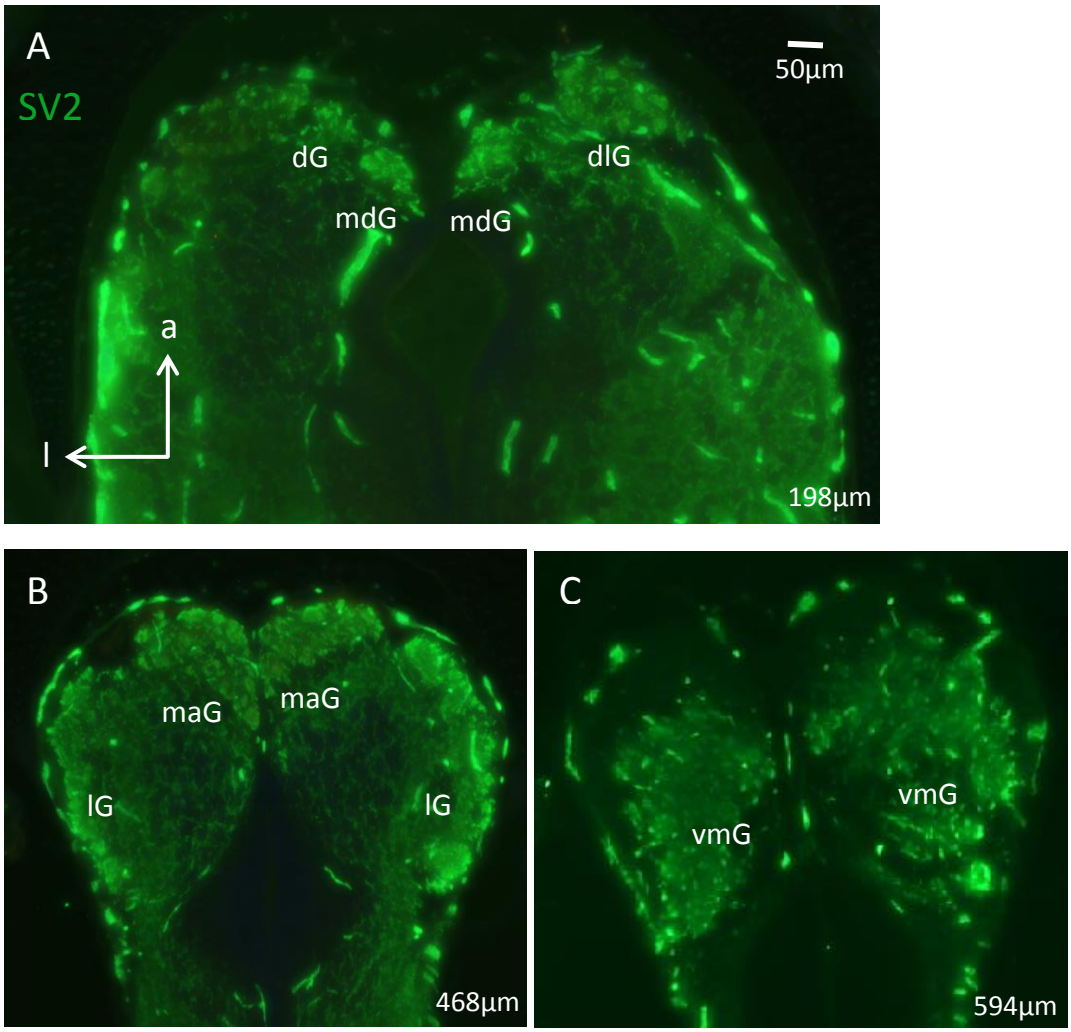

Figure A1: SV2 labeling in olfactory bulb of fry Chinook salmon. A: SV2 labeling is present in medial dorsal glomeruli (mdG), dorsal glomeruli (dG) and dorsal lateral glomerular chain (dlG) in dorsal olfactory bulb sections. B: Midway through the olfactory bulb SV2 labels medial anterior glomeruli (maG) and lateral glomeruli (lG). C: In the ventral olfactory bulb SV2 labeling is present in small ventromedial glomeruli (vmGs). Scale bar of 50µm applies to each micrograph and olfactory bulb depths (measured from dorsal most section) are in the right bottom corner of the micrographs.

References

Braubach, O., Fine, A., Croll, P. (2012). Distribution and functional organization of glomeruli in the olfactory bulbs of zebrafish (Danio rerio). *Journal of comparative neurology.* 520(11): 2317-39.

Yabuki, Y., Koide, T., Miyasaka, N., Wakisaka, N., Masuda, M., Ohkura, M., Nakai, J., Tsuge, K., Tsuchiya, S., Sugimoto, Y., Yoshihara, Y. (2016). Olfactory receptor for prostaglandin F-2alpha mediates courtship behavior of male zebrafish. *Chemical Senses.* 41(9):143.

VITA AUCTORIS

NAME: Alexandra Zygowska

PLACE OF BIRTH: Windsor, Ontario

YEAR OF BIRTH: 1993

EDUCATION:

B.Sc. Biological Sciences
University of Windsor

Windsor, ON

2011-2015

M.Sc. Biology
University of Windsor

Windsor, ON

2015-2017