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Ontogeny of glomerular territory patterning in the olfactory bulb of juvenile Chinook salmon (Oncorhynchus tshawytscha) and exploring for potential effects of sensory experience on glomerular development

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Ontogeny of glomerular territory patterning in the olfactory bulb of juvenile Chinook salmon (*Oncorhynchus tshawytscha)* and exploring for potential effects of sensory experience on glomerular development

By

Cory L. Ochs

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Declaration of Co-Authorship

I hereby certify that this thesis incorporates material that is the result of joint research as follows: My second chapter was co-authored with my supervisors, Dr. Barbara Zielinski and Dr. Trevor Pitcher, as well as Tina Suntres. My third chapter was co-authored with my supervisors, Dr. Barbara Zielinski and Dr. Trevor Pitcher. My collaborators contributed to experimental design, provided technical support that directly contributed to the dataset, and provided editorial input. I am the primary contributor to each chapter of this thesis. No part of this thesis has been published or submitted for publication.

I am aware of the University of Windsor Senate policy on authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained written permission from my co-authors to include the above materials in my thesis.

I certify that, with the above qualification, this thesis, and the research to which it refers, is the product of my own work, completed during my registration as a graduate student at the University of Windsor.

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Abstract

Axon terminals of olfactory sensory neurons (OSNs) aggregate into glomeruli, functional units of odour discrimination within the olfactory bulb. Glomerular patterning facilitates assessment of olfactory stimulation-induced changes to neural circuitry. Contemporary studies indicate Chinook salmon (*Oncorhynchus tshawytscha*) alevin imprint to olfactory cues, purportedly amino acids, prior to emergence. In this study, OSNs were labelled against keyhole limpet hemocyanin to characterize the development of glomerular territories from hatch to emergence, focusing on calretinin-immunoreactive OSNs. Glomerular territories were distinguishable at hatch, and showed expansion and moderate refinement with maturation into emergent fry. Calretinin-immunoreactive OSNs innervated the dorsolateral and lateral glomerular territories, and four lateral glomeruli, lG_1, lG_2, $lG_{3/4}$, and lG_6. Neither amino acid exposure nor somatic growth influenced glomerular volume of one glomerulus, lG_1. The establishment of glomerular territories at hatch infers a functional olfactory system, but a sensitive stage for visible effects of olfactory experience on glomerular volume requires further investigation.

Dedicated to Pierre-Paul and Sawyer Bitton, Karen Ochs, and Cameron Beck

"A simple intuition, a single observation, can open vistas of unimagined potential. Once caught in the web of an idea, the researcher is happily doomed, for the outcome is always uncertain, and the resolution of the mystery may take years to unfold."

— Wade Davis, *Shadows in the Sun: Travels to Landscapes of Spirit and Desire*

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List of Abbreviations

Abbreviation	Description
OB	Olfactory bulb
OE	Olfactory epithelium
ON	Olfactory nerve
OR	Olfactory receptor
OSN	Olfactory sensory neuron
dd	Degree days
CAL	Calretinin
ICC	Immunocytochemistry
IgG	Immunoglobulin G
IR	Immunoreactive
KLH	Keyhole limpet hemocyanin
dG	Dorsal glomerular territory
dlG	Dorsolateral glomerular territory
lG	Lateral glomerular territory
maG	Medial anterior glomerular territory
mdG	Mediodorsal glomerular territory
vmG	Ventromedial glomerular territory
vpG	Ventroposterior glomerular territory
Ala	Alanine
Glu	Glutamic acid
His	Histidine
Pro	Proline
Ser	Serine
Trp	Tryptophan

Chapter 1: General Introduction

Ecological significance of olfaction in Pacific salmonids

Chemosensation, specifically olfaction, is key to inter- and intraspecific interactions among fishes. Teleosts utilize olfactory cues for breeding (Stacey et al., 2003), feeding (Løkkeborg, 1998), kin recognition (Olsen and Winberg, 1996), predator avoidance (Chivers and Smith, 1998), and homing (Hasler and Wisby, 1951); ultimately, these factors are vital to the fitness of the fish (i. e. survival and reproductive success). Of the multi-modal sensory mechanisms used to map and orient to natal waters by Pacific salmonids (*Oncorhynchus* spp), olfaction appears to be the most critical navigational mechanism, supported by the successful navigation of sockeye salmon to natal streams despite impaired visual and magnetic sensory systems (Ueda et al., 1998). Behavioural evidence supports salmonids respond to olfactory cues early in development, discriminating odours as embryos (Bodznick, 1978), and responding to feeding-specific chemosensory cues as emergent fry (Mearns, 1986). A contemporary review encourages fisheries managers to focus on the life history stage prior to exogenous feeding (emergence) as a probable developmental stage for imprinting to natal stream odours (Dittman et al., 2015), a passive and permanent learning process in which an organism learns to recognize a cue within a specific timeframe (Lorenz, 1935). A better understanding of olfactory system ontogeny from hatch to emergence may help elucidate the olfactory discriminatory abilities present at this early life stage.

Olfactory development from embryo to emergence in salmonids

Olfactory development commences very early during salmon embryogenesis. As water

temperature is highly correlated with development in salmon (Crisp, 1981), degree days (dd), the sum of the average daily water temperature from fertilization to each collection date, reflects a broadly-accepted assessment of age. Prior to the eyed-egg stage and to the first heartbeat, salmonid olfactory epithelia, consisting of a rosette-shaped pseudostratified epithelium that supports OSNs, are observable (150-200 dd; Ballard, 1973). Olfactory nerves projecting from the olfactory placodes to the olfactory bulbs develop shortly afterwards, at around 220 and 280 dd, commencing the development of the olfactory bulb. Specific olfactory sensory neuron (OSN) morphotypes, such as the ciliated OSNs, are identifiable shortly after the eyed-egg stage (340-430 dd; Yanagi et al., 2004). Spontaneous firing of these OSNs is detectable well before hatch and prior to the development of the embryo's fins (Zielinski and Hara 1988).

Corresponding with the period close to hatching, the olfactory pits fully develop into the two nares that facilitate the intake and output of water through the olfactory pit (Kunz, 2004), as the connective tissue around the olfactory pits thicken (460-580 dd; Yanagi et al., 2004). The pseudostratified epithelia within the olfactory pits reflect those of adult olfactory epithelia (Yanagi et al., 2004). The development of the olfactory system continues after hatch as the larval fish grows and experiences sensory induced activity. After hatch, the olfactory epithelial cell-types are distinct, and can be classified into four types (olfactory sensory neurons, basal cells, sustentacular cells, and goblet cells; Yanagi et al., 2004). Although the olfactory pits and nasal cavities are developed, nostril formation continues during this post-hatch stage (Yanagi et al., 2004). Indeed, alevin and fry Chinook possess only 4-11 olfactory lamellae, whereas about 18 lamellae are possessed at maturity (Yanagi et al., 2004). The number of OSNs appears to be

allometric with fork length, and rather than increasing in density, appear to increase in number as the development of additional olfactory lamellae increase surface area in the olfactory rosette (Kudo et al., 2009).

Detection and distinction of odours molecules

In teleosts, detection of water-soluble odours including amino acids, bile acids, prostaglandins, steroids, and nucleotides is initiated by the binding of an odour molecule to the G-protein coupled receptor located on the apical surface of OSNs (Hino et al., 2009) found within the olfactory epithelium (OE) of the nares. The depolarization and subsequent excitatory response is conducted by OSN axons along the olfactory nerve (cranial nerve 1). The OSN axons terminate in the olfactory bulb (OB; Friedrich and Korsching, 1997) the most rostral structure in the teleost brain (Fig. 1.1). OSN axons synapse onto output neurons (mitral cells), further transmitting odour responses to higher brain centres including the dorsal-posterior region and ventral nucleus of the telencephalon, in addition to the habenula and hypothalamus (Zebrafish, *Danio rerio*, reviewed in Kermen et al., 2013). In teleosts, the olfactory bulb is divided into four layers in accordance to the characteristic cellular organization. The outermost layer of the olfactory bulb, the glomerular layer, houses the axonal endings of OSNs, which terminate on highly organized neuropil called glomeruli, which are distinguishable by olfactory receptor (OR) type (Mombaerts, 1996). Glomeruli vary neuroanatomically (Gayoso et al., 2011), physiologically (Friedrich and Korsching, 1997), and according to outputs (Miyasaka et al., 2009).

The coarse organization of glomerular patterning is facilitated by the targeted migration of OSN axons from the OE to the OB, which is controlled by a number of factors including guidance by pioneer axons, intercellular signaling systems, axon guidance receptors, and OR expression (Miyasaka et al., 2013; Mombaerts, 2006; Miller et al., 2010). Pioneer axons originate from the olfactory placode, and extend from the OE to the OB, creating a framework for following OSN axons. The pioneer axons are guided by a combination of chemokine signaling from the olfactory placode and expression of axon guidance receptors from the Robo family. OSN axons extend from the OE to a targeted glomerulus in a direct, uninterrupted manner assisted by Slit/Robo signalling (Dynes and Ngai, 1998).

Odour discrimination is facilitated, in part, by OSN morphotype, the OR expressed by the OSN and the organization of the OSN axons in the OB. OSNs are represented by three morphotypes, and are highly discriminative, where one of the approximately three hundred ORs is stimulated by a particular odour molecule (Zebrafish, Shi and Zhang, 2009), thus following the one neuron- one receptor rule. The subfamily Olfactory Receptor (OR) is expressed in ciliated OSNs, which are broadly responsive to bile acids (Miyasaka, 2013). Crypt OSNs, responsive to steroids, express Vomeronasal 1 Receptors (V1Rs), whereas microvillous OSNs, responsive to amino acids, express both V1R and V2R subfamilies (Miyasaka, 2013). Olfactory discrimination is further facilitated by the organization of the OSN axons in the glomerular layer of the OB, where axons from OSNs expressing the same OR converge upon one glomerulus. Crypt OSN axons project into the dorsomedial region of the olfactory bulb (Gayoso et al., 2011; Ahuja et al., 2013), whereas the axons of ciliated

OSNs terminate predominantly in the dorsomedial region of the OB. Microvillous OSN axonal projections terminate in the lateral region of the OB. The clusters of axons expressing a specific OR converge upon a single glomerulus, respecting the one receptor-one glomerulus rule evident in adults (Mice: Mori and Sakano, 2011; Zebrafish: Sato et al., 2007). Glomerular patterning in the teleost olfactory bulb is highly stereotyped, where groups of glomeruli congregate into distinctive glomerular territories that are predictably situated throughout the glomerular layer (Friedrich and Korsching, 1997; Gayoso et al., 2011; Braubach et al., 2012, 2013).

Olfactory glomerular patterning as a measure of olfactory experience

The consistent organization of the aforementioned olfactory glomeruli within species has led to the generation of anatomical maps depicting glomerular patterning, which are strengthened by physiological studies that match the stimulation of particular glomerular regions to the corresponding odour classes. Odotopic maps have been generated for invertebrates and vertebrates, including fishes (Zebrafish: Baier and Korsching, 1994; Braubach et al., 2012), and provide a tool to determine factors that may influence an animal's ability to learn to detect and discern odourants, especially when combined with behavioural or physiological assays.

Genetic and environmental influences both appear to contribute to glomerular maturation. Because the coarse organization of olfactory glomeruli is apparent at hatch in some teleosts, it is surmised to be genetically predetermined (Zebrafish: Braubach et al., 2013). However, a particular olfactory glomerulus may initially be innervated by more than one receptor type in animals that are still developing or lacking olfactory stimulation

(Zebrafish, Braubach et al., 2013; Mice, Zou et al., 2004). Olfactory glomerular refinement may reflect the developmental patterning of neurons observed in the human cerebral cortex, where synaptic connectivity and synaptic number peaks at an early developmental stage before declining due to the pruning of non-stimulated connections (reviewed in Huttenlocher, 1985). Exposure to odourants, and the subsequent excitation of the corresponding OSNs, can refine the organization of axons within glomeruli, leading to changes in either glomerular volume, the number of supernumerary glomeruli, or the number of OSN axons projecting to a specific glomerulus (Braubach et al., 2013). Thus, sensory experience may be a second factor that further refines glomerular development (Zebrafish: Braubach et al., 2013; Mice: Todrank et al., 2011; Drosophila: Devaud et al., 2003). Furthermore, there appears to be a sensitive period for the refinement of glomerular development which varies with olfactory receptor type (Zou et al., 2004). Glomeruli are responsive to olfactory enrichment very early in development; an increase in glomerular volume was found in mice exposed to odours in utero and as pups (Todrank et al., 2011), but a decrease in lateral glomerular volume was found when zebrafish were exposed to amino acids during the larval developmental stage (Braubach et al., 2013). The neuroanatomical plasticity of olfactory glomeruli in response to sensory stimuli may improve the animal's ability to detect and respond to olfactory cues it frequently encounters in its environment, inferring neural plasticity is an adaptive mechanism. Although the patterning of glomerular territories has been described in adult salmonid species, rainbow trout, *Oncorhynchus mykiss* (Riddle and Oakley, 1992), individual glomeruli have not been specified, specifically in the amino acid-stimulated lateral region of the olfactory bulb during the alevin developmental stage.

Potential impact of hatchery environment on olfaction

Supportive breeding programs using hatchery-reared fish to supplement salmon populations are of high socio-economic value, yet there is little understanding of the effects of rearing environment on development and behaviour of released salmon. Studies exploring the effects of environmental enrichment on the development of juvenile fishes have focused primarily on the structural component of the captive environment. Physical complexity of the rearing environment has been positively correlated with neurodevelopment in fishes (Salvanes et al., 2013; Kihslinger and Nevitt, 2006). Sensory experience, however, further impacts the development of an animal. Behaviourally, foraging success is inhibited in hatchery reared fish (Orlov et al., 2006; Larsson et al., 2011), while brain structures, including the olfactory bulb, telencephalon and cerebellum, are larger in salmonids raised in rivers compared to those raised in hatcheries (Kihslinger et al., 2006; Kihslinger and Nevitt, 2006). Together, these findings suggest early rearing environment is important for behavioural and neural development. The question whether the olfactory system of a younger salmon (alevin) is sufficiently mature to detect and discern odours, and whether the system is plastic to neuro-stimuli caused by olfaction, can be evaluated using neuro-anatomical metrics.

Chinook salmon as a model species

Relationships between neuroanatomy and environment and/or behaviour are more evident in teleosts than in other taxa (Ito et al., 2006). Chinook salmon (*O. tshawytscha*) are one of five species of anadromous Pacific salmon, hatching in fresh water before migrating to

open waters. Originally sourced from a Pacific population in Washington State, they were introduced to the Laurentian Great Lakes in the 1960's (Crawford, 2001). Natural populations of Chinook salmon are now well established in the Great Lakes system, including Lake Ontario (Smith et al., 2006), Lake Huron, and Lake Michigan (Dettmers et al., 2012), all of which continue to be augmented by hatchery-reared parr that exhibit homing behaviour. The socioeconomic and ecological importance of both the Pacific and Laurentian Great Lakes populations of Chinook salmon warrants a better understanding of the mechanisms that are involved in their homeward migration to spawn.

Lake Ontario Chinook salmon spawn after upstream migration from open waters to their natal streams in October. The eggs hatch during the winter months, and alevin remain in redds until the yolk-sac is absorbed between April and May, at which point the fry emerge and disperse. Juvenile dispersal rate varies with differences in population density, food availability, and habitat quality (Grant and Noakes, 1987; Gowan et al., 1994; Achord et al., 2003), and isotopic analysis of otoliths have revealed that juvenile Chinook salmon may occupy a number of different streams prior to smoltification (Shrimpton et al., 2014). Smoltification occurs in the early summer, and Chinook salmon mature from smolts into adults after migrating from the river to open waters (Quinn, 2005). Individuals from the Lake Ontario populations exhibit ocean-type migratory behaviour, migrating downstream after inhabiting estuaries for at least a few weeks Crawford, 2001). Once in open waters, they feed for three to four years where they gain more than 90% of their biomass (Quinn, 2005). Once they reach sexual maturity, most populations migrate upstream in the fall, returning to their natal streams to spawn. All Chinook adults are semelparous, reaching the termination of their lifecycle after

spawning (Quinn, 2005).

American waters alone account for the introduction of hundreds of millions of salmon raised in hatcheries to supplement natural or naturalized populations (Rand et al., 2012), and current management guidelines suggest straying rates, where the adult salmon fails to return to its natal stream to spawn (Dittman et al., 2015), should not exceed 10% (Paquet et al., 2011). The benefit of natal stream fidelity is two-fold: to mitigate potentially detrimental interactions between hatchery-reared fish and natural populations, and to re-establish threatened or extirpated populations. Consequentially, this socioeconomic importance of both the Pacific and Laurentian Great Lakes populations of Chinook salmon, in addition to their ecological relevance as top predators, warrants a better understanding of the mechanisms involved in their homeward migration to spawn.

Olfactory imprinting paradigms

Two explanations for salmonid homing behaviour have been proposed: the pheromone hypothesis (Nordeng, 1971) and the olfactory hypothesis (Hasler and Scholz, 1983). The pheromone hypothesis suggests juvenile salmon imprint to pheromones, species-specific chemical signals (Wyatt, 2003). Released by conspecifics upstream, these pheromones may serve as migratory cues for spawning adults during upstream migration (Nordeng, 1971). However, during the timeframe in which Pacific salmon migrate to their home streams to spawn, there are not necessarily juvenile salmon upstream releasing pheromones to guide the migrating spawning adults.

The olfactory hypothesis suggests salmon imprint to the chemical cues that are distinct to each stream and show constancy across years (Hasler and Scholtz, 1983). This

hypothesis stems from a preceding study showing salmonids can discriminate between stream odours (Hasler and Wisby, 1951). Additionally, behavioural experiments demonstrated the imprinting and homing abilities of salmonids when exposed to low concentrations of the artificial odours phenylethyl alcohol and morpholine during parr-to-smolt transformation (Scholz et al., 1976; Dittman et al. 1996). Support of the broadly-accepted olfactory hypothesis was further garnered by electro-physiological and behavioural evidence supporting the responsiveness of *Oncorhynchus* sp. to dissolved free amino acids (DFAA), naturally occurring odours released by a stream's unique biofilm composition (reviewed in Ueda, 2012). Application of artificial stream water reflecting the DFAA composition of the natural waters to the olfactory epithelium elicited a larger electro-physiological response than bile acids, and yields similar response levels to those stimulated by natural water application (Shoji et al., 2000). Additionally, chum salmon, *O. keta*, introduced to a y-maze showed higher attraction to artificial water reflecting the DFAA composition of their natal stream than artificial water reflecting the DFAA composition of an alternate stream (Yamamoto and Ueda, 2009). Together, these results introduce DFAA as a possible homing odour candidate recognized by Pacific salmonids.

Studies support that olfaction appears to be a major driver of successful homing behaviour in Pacific salmonids (Ueda, 1998), yet there is little understanding of the ontogeny of the olfactory system in salmonids from hatch to emergence. This developmental stage is potentially important for olfactory imprinting to natal water odourants (Tilson et al., 1994), which largely facilitates the spawning migration (reviewed in Ueda, 2012). Indeed, the embryonic imprinting paradigm (Dittman et al.,

2015) is again re-averting attention to this possible critical stage for olfactory learning in Pacific salmonids. The ability to discern and recognize requires a well-developed olfactory system, where the organization of the sensory cells responsive to olfactory cues is presumably established. Thus, a description of the organization of OSN axonal projections in the olfactory bulb from hatch to emergence may provide an inference of the odour discriminatory abilities of these juvenile salmon.

Thesis Objectives

The establishment of a map characterizing glomerular patterning in Chinook salmon alevin could be used to assess whether this glomerular patterning persists into adulthood (Adult rainbow trout, Riddle and Oakley, 1992), as it does in zebrafish (Braubach et al., 2013). Well-developed glomerular patterning early in development would imply the salmon is able to discern odours, an imperative function to allow the fish to learn to respond appropriately to olfactory cues prior to emergence and exogenous feeding. As amino acid-derived olfactory cues are particularly important to stimulate feeding behaviours and potentially for imprinting, the lateral region of the olfactory bulb where the microvillous OSNs project is of particular interest.

In the first data chapter of this thesis, I will describe the immunocytochemical techniques that were applied to generate maps of the ontogenic progression of glomerular patterning in Chinook salmon ranging from posthatch (1- to 2-weeks-posthatch) to emergence (9- to 13-weeks-posthatch), a potentially sensitive period for imprinting. Because DFAAs have been implicated as possible olfactory imprinting cues in salmonids, amino acid-stimulated OSNs were targeted, facilitating the identification of

distinguishable glomeruli. Secondly, glomerular patterning was compared across different families to establish the stability of the system. Lastly, the glomerular patterning observed in Chinook salmon alevin was compared to that of alternate teleosts (Rainbow trout, *O. mykiss*: Riddle and Oakley, 1992; Brown trout, *Salmo trutta*: Castro et al., 2008; Zebrafish: Baier and Korsching, 1994; Braubach et al., 2012) to determine the extent to which glomerular patterning is evolutionarily conserved.

My second data chapter experimentally tests whether olfactory stimulation refines neural circuitry in Chinook salmon alevin glomeruli, measured as a change in glomerular volume. Chinook salmon eggs from one family were equally subdivided and assigned to one of two environments, olfactory-enriched with amino acid odours or the control environment. Throughout the duration of the treatment, from hatch to emergence, alevin from both groups were sacrificed weekly, facilitating a two-by-two factor design with treatment as the between factor and age as the within factor. Amino acid-responsive OSNs were labelled in a pair-wise comparison (amino acid-enriched versus control) for each age group, allowing for a direct comparison of whether amino acid exposure influenced glomerular size. Glomerular volume was expected to increase with somatic growth of the alevin and olfactory bulb, and were expected to decrease in volume in response to olfactory enrichment as observed in a previous teleost study (Zebrafish; Braubach et al., 2013).

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Figures

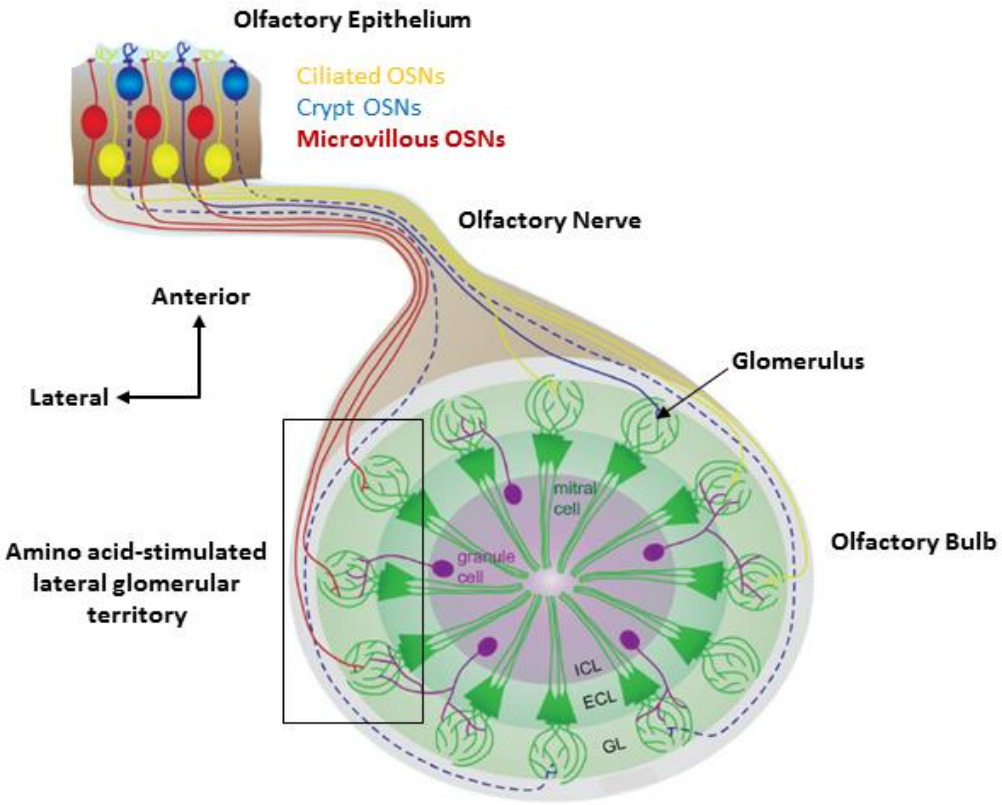

Figure 1.1. Olfactory sensory neuron (OSN) pathway from the olfactory epithelium to olfactory bulb. OSN axons coalesce into glomeruli. Amino acid-stimulated glomeruli are found in the lateral region of the olfactory bulb in teleosts. Image modified from Kermen et al., 2013.

Chapter 2: Ontogeny of glomerular territory patterning in the olfactory bulb of juvenile Chinook salmon Oncorhynchus tshawytscha

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Introduction

Olfaction is induced by the binding of an odour molecule to a corresponding receptor on olfactory sensory neurons (OSNs) in the peripheral organ. Fish utilize olfaction for assessing mate quality (Stacey et al., 2003), kin recognition (Olsen and Winberg, 1996), predator avoidance (Chivers and Smith, 1998), foraging (Løkkeborg, 1998), and homing (Hasler and Wisby, 1951). The precocial development of the teleost olfactory system is exhibited by salmonid species which show responsiveness to olfactory cues by distinguishing different water sources as embryos (Bodznick, 1978), and behaviourally responding to feeding-specific chemosensory cues at emergence (Mearns, 1986).

The sensory pathway for olfaction is well-documented in fishes (reviewed in Laberge and Hara, 2001; Miyasaka et al., 2013). Three main features of the olfactory system contribute to odour discrimination in fishes: olfactory sensory neuron (OSN) morphotype, the receptor type expressed by the OSN, and the organization of OSN axonal endings in the olfactory bulb. Approximately 300 genes code for different receptor classes (Zebrafish, *Danio rerio*, Shi and Zhang, 2009) and allow teleosts to detect water-soluble odours including amino acids, bile acids, prostaglandins, steroids, and nucleotides (Sorenson and Caprio, 1998).

Across taxa, only one receptor type is found on a single OSN (one neuron – one receptor rule). These receptors are found on one of three distinct OSN morphotypes. Microvillous OSNs that bind amino acids predominantly express V2R-like *olfcs* olfactory receptors (Sato et al., 2005). Ciliated OSNs are generalists that bind bile acids, steroids, and some amino acids. This OSN morphotype expresses OR-class (Sato et al., 2005) and

trace amine associated receptors (TAARs; Hussain et al., 2009; Korsching, 2009). Crypt OSNs, responsive to steroid odours (Rainbow trout, *Oncorhynchus mykiss*, Bazaes and Schmachtenberg, 2012), express V1R-likes *oras* odourant receptors (Zebrafish, Oka et al., 2011). Once the odour molecule binds to its corresponding receptor, the subsequent neuron depolarization and excitatory response is conducted via the OSN axon towards the olfactory bulb. OSN axons terminate in the glomerular layer of the bilaterally symmetrical olfactory bulbs (Friedrich and Korsching, 1997), situated rostroventrally to the telencephalon.

Functional units of odour discrimination are formed by the coalescence of OSN axon endings within the glomerular layer of the olfactory bulb. These OSN axon endings terminate in discrete clusters of axons defined as olfactory glomeruli, regions of high synaptic connectivity with mitral cells (Shepherd, 2004). Olfactory glomeruli were initially identified by Ramon y Cajal (1891), and have since been described in insects (Rospars and Chambille, 1981; Rospars, 1983; Stocker et al., 1983; Rospars and Hildebrand, 1992), fish (Baier and Korsching, 1994; Hamdani and Doving, 2007), and mammals (Vasser et al., 1994; Mombaerts et al., 1996).

Generally comprised of OSN axons from a single OSN morphotype, glomeruli are consequently activated by chemically similar odours (Mori et al., 2006; Friedrich and Korsching, 1997). Glomeruli responsive to similar odours aggregate, forming glomerular territories patterned consistently throughout the olfactory bulb glomerular layer. In zebrafish, axons of ciliated OSNs, responsive to bile acid odours, terminate in the anterior medial region of the olfactory bulb (Sato et al., 2005), and to a lesser extent, the posterior lateral olfactory bulb (Friedrich and Korsching, 1998). Consistent with zebrafish, bile

acids stimulate neurons in the medial dorsal region of the olfactory bulb in salmonids (Doving et al., 1980; Hara and Zhang, 1998; Laberge and Hara, 2003). Axons of crypt OSNs, responsive to sex pheromones (Rainbow trout, Bazaes and Schmachtenberg, 2012), project to the dorsomedial region in the zebrafish olfactory bulb (Gayoso et al., 2011; Ahuja et al., 2013). Physiological recordings indicate the responsiveness of ventral medial glomeruli to prostaglandin and saponin extract in zebrafish (Friedrich and Korsching, 1998), but not in salmonids (Laberge and Hara, 2003). Microvillous OSNs are specialists in rainbow trout, responding almost exclusively to amino acids (Sato and Suzuki, 2001), and terminate in the lateral region of the olfactory bulb in zebrafish (Hamdani and Doving, 2007; Sato et al., 2005). Amino acid odours stimulate the anterior lateral region of the OB in zebrafish (Friedrich and Korsching, 1998) and the lateral OB region in salmonids (Doving et al., 1980; Hara and Zhang, 1998). Alternatively, amino acid odours stimulate the lateral posterior region of the mid-bulb in brown and rainbow trout (Laberge and Hara, 2003). MHC peptides are comprised of amino acids, and have been implicated as kin odours in zebrafish (Hinz et al., 2013). These odours also stimulate the lateral region of the olfactory bulb. Nucleotides are odours that may indicate the freshness of food, and stimulate glomeruli in the lateral posterior OB (Zebrafish, Friedrich and Korsching, 1998).

Socio-economic initiatives rely on the release of millions of juvenile salmon to supplement natural populations (Rand et al., 2012). These juvenile salmon depend on a functional olfactory system for survival, but little is known about the ontogeny of the glomerular territories that largely contribute to odour discrimination. Pacific salmonids, *Oncorhynchus* spp, navigate to natal streams to spawn after three to seven years in open

waters, guided by natal stream-specific olfactory cues, purportedly composed of dissolved free amino acids (Shoji et al., 2000; Ueda, 2012). The identification of a critical period for olfactory imprinting in salmonids remains ambiguous, but can occur as early as the alevin developmental stage prior to emergence from the redd (Sockeye salmon, *O. nerka*, Tilson et al., 1994). Early imprinting is further supported by isotopic analysis of otoliths that suggests parr occupy an average of four chemically distinct streams prior to smoltification (Shrimpton et al., 2014). This redirection of focus on embryonic imprinting as a fish management strategy (Dittman et al., 2015) warrants characterization of glomerular patterning from hatch to emergence in a Pacific salmon species to infer the olfactory-discriminatory abilities of this age group.

Immunolabelling OSNs against the metalloprotein keyhole limpet hemocyanin (KLH) revealed distinct glomerular territories in the lateral region of the olfactory bulb of juvenile Chinook salmon *O. tshawytscha* at 0.1, 2 and 4-months-posthatch (Jarrard, 1997). These territories morphologically correspond with KLH-immunoreactive glomerular territories identified in adult rainbow trout (Riddle and Oakley, 1992; Porteros et al., 1997), and unspecified lateral glomerular territories stained with cobalt-lysine in emergent Chinook salmon (Bazer et al., 1987). As KLH binds to an unknown epitope general to all OSN morphotypes, this immunocytochemical (ICC) technique has also been applied to the zebrafish olfactory bulb to comprehensively map glomerular patterning (Baier and Korsching, 1994; Braubach et al., 2012, 2013, White et al., 2015).

Because of the responsiveness of microvillous OSNs to amino acids, the putative homing cue for salmonids, the identification of the corresponding glomeruli in alevin Chinook salmon are of particular interest, and requires OSN morphotype-specific

labelling for identification. Calretinin, a calcium binding protein, successfully targets microvillous OSNs in zebrafish adults (Castro et al., 2006, Germana et al., 2007; Duggan et al. 2008, Braubach et al., 2012); zebrafish larvae (Braubach et al., 2013); and embryo, larval, and adult salmonids (Rainbow trout, Porteros et al., 1997; Brown trout, Castro et al., 2008). The general description of calretinin-immunoreactive OSN axons in juvenile teleosts (Rainbow trout, Porteros et al., 1997; Brown trout, Castro et al., 2008; Zebrafish, Braubach et al., 2013) in conjunction with a description of glomerular territories in adults (Rainbow trout, Riddle and Oakley, 1992, Porteros et al. 1997; Chinook salmon, Jarrard, 1997; Zebrafish, Baier and Korsching, 1994; Braubach et al., 2012) provide a base to develop a comprehensive map of glomerular pattering in developing Chinook salmon (Table 2.1).

ICC techniques were modified and applied to map the patterning of OSN axonal endings in the olfactory bulb of juvenile Chinook salmon. Specifically, patterning of glomerular territories was described comprehensively by labelling against KLH. Additional labelling against calretinin facilitated the identification of glomerular territories and individual glomeruli innervated by amino acid-responsive microvillous OSNs. The ontogeny of glomerular patterning at three incremental stages from hatch to emergence was described and included posthatch alevin (one- to two-weeks posthatch; 539-590 dd), midalevin (four- to six-weeks posthatch; 643-720 dd), and emergent fry (nine-weeks plus posthatch, and absorption of yolk-sac; 798-996 dd). The structural maturity of this system was further inferred by comparing the glomerular patterning to that in adult salmonids (Chinook salmon, Jarrard, 1997; Rainbow trout, Riddle and Oakley, 1992). The stability of this glomerular patterning was determined by applying

across-family and across-species comparisons.

I anticipated consistent glomerular patterning throughout the alevin developmental stage from hatch to emergence for two reasons: the coarse organization of glomerular territories is determined during larval development in zebrafish (Braubach et al., 2013), and important behaviours exhibited by juvenile teleosts are olfactory-mediated, including those used for kin recognition (Zebrafish: Hinz et al., 2013) and feeding (Salmonids: Mearns, 1986). Amino acid-responsive lateral calretinin-immunoreactive glomeruli should be consistently identifiable no later than emergence, as predicted by the sensitivity of this stage to this odour class (Mearns, 1986; Tilson et al., 1994). Physiological studies support the functional role of glomerular pattering for odour discrimination across taxa (Fish: Friedrich and Korsching, 1998; Mice: Marks, 2006; Insects: Lei et al., 2004). Because the stimulation of specific regions of the OB with specific odour classes yields similar results within the subclass teleostei, consistency in glomerular patterning should also be observed. Additionally, there are observable consistencies in patterning of glomerular territory patterning between phylogenetically distantly related rainbow trout (Riddle and Oakley, 1992) and zebrafish (Baier and Korsching, 1994; Gayoso et al., 2011; Braubach et al., 2012; Braubach et al., 2013).

Materials and Methods

Fertilization and rearing conditions of lacustrine Chinook salmon

All animal handling and care was conducted with approval by the University of Windsor Animal Care Committee and in compliance to the Canada Council of Animal Care.

Spawning Chinook salmon were electro-shocked from the Credit River, Mississauga, Ontario (43° N, 79° W), during the upstream migration from Lake Ontario in October 2012 and 2013. Adult fish were euthanized by percussive stunning prior to gamete collection.

Eggs were collected from females and milt from males within a one-hour timeframe. The eggs and milt were transferred to the University of Windsor Animal Care Facilities, where the eggs were fertilized using Detroit River water to activate the milt. To accommodate across-family comparison of glomerular patterning in alevin, eggs from each female were fertilized using milt from a unique male, creating seven different family groups. Eggs and alevin were reared in incubation trays sourced by a flow-through of dechlorinated municipal water. Water temperature is an important determinant of development in salmonids, and was monitored throughout this period using HOBO temperature loggers to record temperature every 15 minutes. Thus degree days (dd), the sum of the average daily water temperature from fertilization to each collection date (Crisp, 1981), were presented with developmental stage to provide a more accurate assessment of age.

Sample collection and tissue preparation

The ontogeny of glomerular patterning in alevin was determined by sampling alevin at three increments (Fig. 2.1). Glomerular patterning was formulated for 18 posthatch alevin (2013 animals, one family), six mid-alevin individuals (2013 animals, one family), and more than 25 emergent fry (2012 animals, six families, 996 dd; 2013 animals, one family). All ICC techniques were established using emergent fry from the 2012 group

prior to application to the 2013 group.

Alevin were euthanized by anaesthetic overdose (1g/L MS-222; pH 7.4), decapitated over the gills, and heads dropped-fixed in 4% paraformaldehyde (PFA) in 0.1M PBS. A few days prior to sectioning, heads were further dissected by removing the mandible, tissue caudal to the eyes and dorsal skin to expose neural tissue, and post-fixed in fresh 4% PFA in 0.1 M phosphate buffer saline (PBS). Tissue was cryoprotected by immersion in a 20% and 30% sucrose gradient in 0.1 M PBS overnight. Horizontal 16-30 µm thick serial sections were sectioned from the olfactory bulb to the olfactory epithelium using a cryotome (Leica CM 3050A) and collected onto Fisherbrand Superfrost Plus microscope slides (Fisher Scientific, Waltham MA, 12-550-15). Sections were left to dry at room temperature before storing at -20 °C.

Immunocytochemistry techniques

The application of several immunocytochemical labels to the OSN pathway from the origin in the olfactory epithelium to the termination in the olfactory bulb (Fig. 2.2), facilitated the adaptation of several ICC protocols. Serial tissue sections from the olfactory epithelium to the olfactory bulb were double-labelled against KLH produced in rabbit (Sigma-Aldrich, Oakville ON, H0892), and monoclonal acetylated tubulin produced in mouse (AT; Sigma-Aldrich, Oakville ON, T7451), a marker for an α-tubulin epitope on microtubules that is used as a probe for vertebrate neurons. OSN axons are KLH-immunoreactive, and are easily traced from the OE to the OB, where they fasciculate onto olfactory glomeruli, using fluorescence microscopy. The KLH antibody, however, also binds to non-neural tissue, including muscle fibers and capillaries;

therefore, AT was used to confirm the identification of labelled axonal projections (see Fig. 2.4). The visualization of cell bodies within the olfactory bulb was facilitated by applying a mounting medium including a nuclear label to coverslip the sections (VectaShield Hardset mounting medium with DAPI, Vector Labs, Burlington ON, H-1500).

Slides were rehydrated in 0.1M PBS (pH 7.4) with 0.1% triton X-100 (PBS-T) for 30-40 minutes to increase the permeability of the tissue before blocking with 10% goat serum (Sigma-Aldrich, Oakville ON, G9023) in 0.1M PBS for 30 minutes. Slides were placed in primary antibody (1/500 KLH and 1/1000 AT in 0.1M PBS) for 16-20 hours on a shaker at 4 ºC. Slides were rinsed three times for 20 minutes each in 0.1M PBS with 0.01% sodium azide prior to transfer to secondary antibody diluted in 0.1M PBS (1/250 goat antirabbit Alexafluor 568 IgG, Sigma-Aldrich, Oakville ON, A11011; 1/100 Alexafluor 488 antimouse IgG, Sigma-Aldrich, Oakville ON, A11001) for one to two hours. Slides were again rinsed in 0.1M PBS three times for 20 minutes each and coverslipped using VectaShield Hardset mounting medium (Vector Labs, Burlington ON, H-1400).

Once the successful labeling of OSNs was established, a more rigorous protocol was adapted to increase resolution of the individual axonal fibers, better defining the different glomerular territories (refined from Braubach et al., 2012, 2013 and Castro et al., 2008). These techniques were used to determine the consistency of glomerular patterning across individuals. The olfactory pathway of emergent fry was immuno-labelled against KLH to provide a description of the coarse organization of olfactory glomeruli in the olfactory bulb. Because of the nonspecificity of KLH labelling, amino

acid-sensitive lateral glomeruli were concealed within a lateral plexus, and could only be identified by labelling against calretinin. Therefore, the innervation of microvillous OSNs was concurrently targeted with anti-calretinin (produced in mice, Swant, Switzerland, 6B3). Slides were rehydrated in PBS-T for one to two hours before being transferred into a 10% goat serum blocking solution. Slides were transferred into primary antibody 1/500 KLH and/or 1/200 calretinin in 0.1M PBS with 0.1% sodium azide) for three to five days, then rinsed six times for 50 minutes per cycle. Slides were incubated in secondary antibody (1/250 goat antirabbit Alexafluor 568 IgG and/or 1/200 goat antimouse Alexafluor 488 IgG) for three to five days before being rinsed six times for 50 min each and coverslipped using VectaShield HardSet mounting medium. Unless otherwise indicated, 0.1M PBS with 0.1% sodium azide was used for all washes and as a vehicle for the probes. All incubations and rinses occurred on a shaker at 4 °C.

Analysis

The baseline organization of olfactory glomerular territories in the olfactory bulb was established by imaging OB serial sections using epifluorescence microscopy (Nikon Eclipse E800, 20x objective, QImaging Fast1394 camera, Northern Eclipse). A single glomerulus was identified by the projection of OSN axons into a single unit where the termination of the axons were visible. Glomerular territories situated in the glomerular layer of the OB were comprised of a discreet clustering of glomeruli and partitioned from neighbouring territories by DAPI-IR cell bodies. Horizontal serial sections further delineated the glomerular territories, as the dorsal and ventral boundaries could be identified due to the visibility or absence of KLH-immunoreactive axons innervating the

territories. These techniques may have confounded the identification of adjacent glomerular territories, such as the medial anterior and ventroanterior glomerular territories identified in zebrafish (Braubach et al., 2012). The positioning of each glomerular territory from anterior, posterior, medial and lateral perspectives was also recorded, as was its depth within the bulb, with the dorsal-most section at plane zero. Glomerular territory thickness (dorsal-ventral) was calculated from a subset of emergent fry sections labelled against KLH.

The innervation of each glomerular chain by KLH- and calretinin-immunoreactive OSNs was identified, and lateral territory-specific glomeruli specified. Each territory and lateral glomerular was imaged (60x oil immersion objective) under confocal microscopy (Olympus fluoview FV1000, Fluoview version 2.1c), eliminating the haze associated with epifluoresence microscopy caused by the scattering of photons, to further visualize the unique anatomical traits that were consistent across individuals without encountering out-of-focus blur.

Baseline maps of glomerular patterning was established for alevin at three specific developmental stages, posthatch (1- to 2-weeks posthatch), mid-alevin (5- to 6-weeks-posthatch) and emergence (9- to 13-weeks-posthatch). Not all tissue from posthatch and mid-alevin samples were labelled against KLH, but subtle autofluorescence of all glomerular neuropil in sections labelled against calretinin facilitated identification of otherwise KLH-immunoreactive glomerular territories. Olfactory bulb thickness (dorsal-ventral) from a subset of samples of each developmental group investigated in this study was calculated by multiplying the total number of olfactory bulb sections by thickness. The dorsal-most olfactory bulb section was identified by the presence of KLH-

immunoreacitve OSN axonal endings, while the ventral-most sections were identified by the entry of the CAL- and/or KLH-immunoreactive olfactory nerve into the bulb. Maps depicting glomerular patterning include the boundaries of each glomerular territory, and its innervation and depth within the olfactory bulb, facilitating comparison to glomerular patterning across the seven families of emergent fry incorporated into this study. These data were then compared to glomerular patterning in con- and heterospecifics described in alternate studies.

Results

Labelling efficacy was determined by double-labelling the olfactory epithelium of emergent fry against KLH and calretinin to target all OSN morphotypes and amino acid-stimulated microvillous OSNs respectively (Fig. 2.3). Triple-labelling 18 µm horizontal sections against KLH, acetylated tubulin, and DAPI revealed the different olfactory bulb layers. The glomerular layer was identifiable by the uneven clustering of OSN axon endings immunoreactive to both KLH and acetylated tubulin, separated by glial and periglomerular cells, which contain nuclei immunoreactive to DAPI. The glomerular layer was situated around the periphery of the olfactory bulb (Fig. 2.4). However, because horizontal sections were collected, the dorsal- and ventral-most olfactory bulb contain the glomerular layer in the centre of the section, evident by the presence of KLH- and acetylated tubulin-IR OSN fibers. OSN axons that coalesce in glomeruli synapse onto the mitral cells of the mitral cell layer, which was readily identifiable by the dense cluster of acetylated tubulin-immunoreactive nerve fibers (Fig. 2.4). The granule cell layer was

identifiable by the high concentration of cell nuclei labelled against DAPI (Fig. 2.4). Labelling against either KLH or acetylated tubulin further showed the olfactory nerve projecting into the olfactory bulb ventro-anteriorly. Therefore, the nerve layer was highly visible only in this region of the olfactory bulb.

Patterning of KLH-IR glomerular territories was revealed by the distinguishable gaps between territories occupied by supporting periglomerular and glial cells. Eight distinct glomerular territories (Fig. 2.5), distinguishable by shape, size, and location, were consistently situated throughout the glomerular layers of the olfactory bulb in posthatch alevin (KLH-IR, n=2; CAL-IR, n=16), mid-alevin (KLH- and CAL-IR, n=8), and emergent fry (KLH-IR, n=25). Labelling against calretinin revealed conspicuous innervation of microvillous OSNs into two glomerular territories (Fig. 2.5). The glomerular territories identified in this study were further described according to location within the OB (Dorsal, Lateral or Ventral), and in context of innervation, morphology, and ontogeny. Mean thickness of glomerular territories were reported exclusively for emergent fry (µm +/- SE).

Dorsal glomerular territories

The dorsal region olfactory bulb of posthatch alevin, mid-alevin individuals and emergent fry contained three distinct glomerular territories, all anteriorly situated and detectable within the first 18-100 µm from the dorsal-most section of the olfactory bulb (Fig. 2.5). The dorsal-most glomerular territories were identified as the adjacently situated dorsal and dorsolateral glomerular territories (dG and dlG respectively). The largest of the dorsal territories, dlG (Emergent fry: thickness = 174.71 µm, SE = 5.73 µm, n = 17), was

innervated by KLH- and CAL-IR OSN fibers clustering into dozens of small glomeruli (Fig. 2.6), except for the lateral-most region, which formed four to six glomeruli immunoreactive to KLH only (Fig. 2.7). The dG territory (Fig. 1.6; Emergent fry: thickness = 110.77 µm, SE = 7.58 µm, n = 13), located medial to dlG, was innervated solely by diffusely-organized KLH-IR OSN fibers (Fig. 2.7), confounding the identification of individual glomeruli. The mediodorsal territory (mdG; Fig. 2.6) was the smallest and medial-most territory (Emergent fry: thickness = 88.71 µm, SE = 5.12 µm, n = 14), and situated slightly ventral to the aforementioned territories. Roughly spherical in shape, mdG was innervated by KLH-immunoreactive OSN projections that terminated at one of roughly six glomeruli (Fig. 2.6). Lateral and slightly ventral to dlG, KLH-IR OSN axons endings coalesce within the largest and most distinct glomerulus in the Chinook salmon olfactory bulb, lateral glomerulus$_2$ (Fig. 2.6; lG_2, Emergent fry: thickness = 102.00 µm, SE = 3.56 µm, n = 18). Contradictorily to alternate lateral glomeruli, lG_2 lacks microvillous OSN axon innervation, and instead shows intense immunoreactivity to KLH. Situated dorsal of the lateral plexus in the lateral olfactory bulb, its visual plane overlaps with the ventral segments of the dlG, dG and mdG territories (Fig. 2.7).

Lateral glomerular territory

Emphasis was placed on identifying specific lateral glomeruli, due to the ecological significance of amino acid detection in salmonids as a putative homing cue. A lateral plexus composed of a dense innervation of KLH-IR OSN axons that terminated in the lateral region of the OB was the most prominent of glomerular territories (Fig. 2.5, lG, Emergent fry: thickness = 276.92 µm, SE = 8.78 µm, n = 13). Individual glomeruli were

difficult to discern within the plexus of KLH-IR OSN axons (Fig. 2.8); therefore, the amino acid-responsive microvillous OSN axons were targeted by labelling against calretinin (Figs. 2.9, 2.10).

Calretinin labelling revealed three consistently identifiable glomeruli in addition to lG_2 (Fig. 2.10). Lateral glomerulus 1 (lG_1) was located directly ventral to lG_2. The OSN axons project into the glomerulus from the posterior, and the axons terminate in a diffuse medial-anterior direction. Lateral glomerulus 3/4 ($lG_{3/4}$), an irregularly spherical glomerulus larger than lG_1, was located medial-anteriorly and ventral to lG_1. The dorsal-most region of the final identifiable lateral glomerulus, lateral glomerulus 6 (lG_6) was found at the same dorsal-ventral plane as $lG_{3/4}$. This posterior- and ventral-most lateral glomerulus, lG_6, was consistently identifiable, as the OSN axons endings project into a number of distinct microglomeruli, subclustering of axon endings within a glomerulus, generating a bouquet-like appearance.

Ventral glomerular territories

Large complexes of glomerular territories occupied much of the ventral olfactory bulb. The medial anterior glomerular chain (maG; Emergent fry: thickness = 222.00 µm, SE = 8.68 µm, n = 12) extended from the anterior nerve layer and occupying much of the ventral half of the olfactory bulb (Fig. 2.5). Anteriorly situated, the KLH-IR OSN axons enter this territory in a diffuse pattern, confounding consistent identification of specific glomeruli (Fig. 2.8).

The KLH-IR ventromedial (vmG) and ventroposterior (vpG) were identified in the ventral-most region of olfactory bulb (Fig. 2.5), occupying the medial and posterior

sections of the glomerular layer from the olfactory nerve to about 100 µm into the ventral region of the olfactory bulb (Fig. 2.8). The dorsal boundary of vmG (Emergent fry: thickness = 126.00 µm, SE = 8.75 µm, n = 11) exceeded that of vpG, noticeable by the distinct projection of two to four distinct glomeruli just medial to the anterior region of the lateral glomerular territory. The vpG (Emergent fry: thickness = 99.00 µm, SE = 8.39 µm, n = 14) appeared to be comprised of two large, complex glomeruli, with the OSN axons terminating upon a high number of microglomeruli. Neither vmG nor vpG appeared to be innervated by calretinin-IR OSN axons (Fig. 2.9). A caricature denoting a double-labeled emergent fry olfactory bulb summarizes the eight identified glomerular territories according to approximate depth and innervation by KLH- and calretinin-IR OSN axons (Fig. 2.11).

Ontogeny of glomerular territories

Olfactory bulb thickness increased with development from posthatch to emergence (Posthatch alevin: 384.75 µm, SE = 5.83 µm, n = 8; Mid-alevin: 468.00, SE = 22.05, n = 4; Emergent fry from 2013 (798-834 dd): 490.40 µm, SE = 15.02 µm, n = 10; Emergent fry from 2012 (996 dd): 672. 43 µm, SE = 16.73 µm, n = 14). The coarse organization of all seven glomerular territories, in respect to patterning within the olfactory bulb, was visible at hatch and persisted into emergence (Posthatch: Fig. 2.12; Mid-alevin: Fig. 2.13; Emergent fry: Figs. 2.14, 2.15). At posthatch, the OSN axons projecting into the glomerular territories were diffusely organized and showed weak immunoreactivity against KLH. Small and diffusely organized, but detectable, dorsal and mid-bulbar glomerular territories (dG, dlG, mdG, lG, maG) were visible at posthatch and persisted as

larger, more complex units in the emergent fry developmental stage. The ventral glomerular territories (vmG and vpG), meanwhile, were very difficult to discern in posthatch alevin due to very weak immunoreactivity against KLH, but were clearly visible at the mid-alevin stage. The shape of these ventral territories at posthatch reflected that of the more mature counterparts, and the dorso-anterior complex of glomeruli belonging to the vmG was identifiable throughout development.

Visually, the smaller olfactory bulb size of the posthatch alevin corresponded with smaller glomerular territory size relative to emergent fry; however, the proportion of olfactory bulb occupied by some territories remained consistent across age. Specifically, the lateral glomerular territory extended anterio-posteriorly across the OB and occupied up to half of the total olfactory bulb thickness throughout all three alevin developmental stages. The dorsal glomerular territories (dG, dlG, and mdG) showed stable dorsal-ventral distribution across the three stages, occupying the dorsal-most 200 µms of the olfactory bulb in all developmental stages (Fig. 2.16). However, the ventral territories vmG and vpG expanded with the increase in olfactory bulb thickness from the posthatch to emergent fry developmental stages (Fig. 2.16). Although such glomerular territory growth with alevin development was observable, size of some identifiable glomeruli within the lateral glomerular chain appeared to remain constant. The three calretinin-IR lateral glomeruli, lG_1, $lG_{3/4}$, and lG_6, did not appear to grow or change shape across the three alevin developmental stages (Fig. 2.17).

Discussion

Glomerular patterning yields a valuable tool to assess the physiological responsiveness of glomerular territories or individual glomeruli to specific odours, and to test the effect of sensory experience on the neuro-circuitry. Integrated with behavioural assays, glomerular patterning can be further applied to test for critical stages for olfactory learning. Techniques to establish glomerular maps in salmonids have been explored since the early 1990s, before the physiological implications were unravelled. Here a comprehensive description of glomerular territory patterning was established for Chinook salmon alevin from posthatch to emergence, a potentially sensitive developmental period for olfactory imprinting (Dittman et al., 2015), by labelling OSNs against KLH and calretinin.

Ontogeny of glomerular patterning and functional implications

Overall stability in glomerular patterning was observed in Chinook salmon from hatch to emergence, consistent with the segregation of glomerular territories at hatch in zebrafish (Braubach et al., 2013; Miyasaka et al., 2013). The location of the seven described glomerular territories in mid-alevin and emergent salmon were also analogous to the description of glomerular territories in an adult salmonid species (Rainbow trout, Riddle and Oakley, 1992; Chinook salmon, Jarrard, 1997), suggesting the observed glomerular organization that is detectable at hatch persists into adulthood, consistent with findings in zebrafish (Braubach et al., 2013). This presence of glomerular precursors, however, does not imply full functionality of the olfactory system. Alarm cues, specifically those belonging to the glucosaminoglycan odour class, stimulate parts of mdG and lG in zebrafish (Mathuru et al., 2012), which are discernable at hatch (Braubach et al., 2013),

yet zebrafish do not respond behaviourally to alarm cues until 50 days posthatch (Waldman, 1982). However, caution must be applied when comparing olfactory-mediated behaviours of laboratory-raised zebrafish to salmonids, as salmon exhibit chemosensory-mediated anti-predatory behaviour prior to emergence in response to olfactory cues released by the predatory burbot, *Lota lota* (Louhi et al., 2011).

Apparent asynchrony in glomerular development was observed in this study, as the dorsal glomerular territories were more discernable than the ventral glomerular territories at the posthatch developmental stage. Additionally, the dorsal glomerular territories occupied the same spatial depth of the olfactory bulb across the examined developmental stages, whereas the ventral glomerular territories expanded with the expanding olfactory bulb. Glomerular territories are stimulated by different odour classes (reviewed in Kermen et al., 2013); therefore, exposure to predominating odourants that stimulate specific OSN morphotypes may influence different rates of glomerular territory maturation. Specifically, stimulation of a single olfactory receptor may lead to an increase in gene expression of that receptor in conjunction with the silencing of expression of alternate olfactory receptor classes (Miyasaka et al., 2013), leading to increased OSN axon recruitment to corresponding glomeruli and a subsequent deceleration of OSN axon recruitment to others.

Asynchronous glomerular development may reflect the biological relevance of the different odour classes as the animal matures. Thus, the early establishment of lateral glomeruli suggests sensory receptivity to the corresponding olfactory cues, likely amino acids, is important for survival early in development. Indeed, unfed emergent fry exhibit feeding behaviours in response to amino acid odours (Atlantic salmon *Salmo salar*,

Brown trout, Mearns, 1986). Early maturation of these glomeruli may also indicate an early critical period for olfactory learning. Lateral glomeruli in zebrafish are stimulated by major histocompatibility complex (MHC) peptides composed of amino acids (Hinz et al., 2013), which appear to be a major component of kin-specific odours that zebrafish imprint to at six-days-post-fertilization, as demonstrated by behavioural experiments (Gerlach et al., 2008). Discrimination of MHC peptides may also be important for salmon, as evidence suggests juvenile Chinook salmon demonstrate kin recognition behaviours (Henkel et al., 2011).

Sensory experience may also affect the rate of axon recruitment. Crypt OSN axons project to the ventral region of the olfactory bulb (Catfish *Ictalurus punctatus*, Hansen et al., 2003; Crucian carp *Carassius carassius*, Hamdani and Doving, 2007), and their responsiveness to different odour classes varies with sexual maturity (Rainbow trout; Bazaes and Schmachtenberg, 2012). During early life stages, these crypt OSNs respond to some bile acids and amino acids, but show higher responsiveness to gonadal extracts and hormones during maturity (Bazaes and Schmachtenberg, 2012). The ventral glomeruli in post-hatch Chinook salmon alevin, therefore, may not show refinement until the olfactory receptors are responding to social cues released by conspecifics after hatch.

The established but generally underdeveloped glomerular patterning observed in Chinook salmon alevin at posthatch, as indicated by the smaller glomerular territories, diminished immunoreactivity to KLH, and decreased aggregation of OSN axons into discreet glomeruli, corresponds with the developmental patterning of glomerular territories described in zebrafish models. As in zebrafish larvae (Braubach et al., 2013), OSN axons in Chinook salmon alevin reorganize, as demonstrated by higher complexity

of glomerular territories which exhibit a higher number of glomeruli and increased immunoreactivity to KLH by the mid-alevin developmental stage. Glomerular territories in zebrafish during early larval development are innervated by more than one OSN morphotype (Braubach et al., 2013), suggesting that the pruning of unstimulated OSN axons occurs during early development. At all three developmental stages in Chinook salmon alevin, calretinin-immunoreactive OSN axons innervate two glomerular territories, dlG and lG, which does not elucidate whether dissimilar OSN morphotypes originally innervate glomerular territories. Application of labeling techniques targeting crypt and ciliated OSNs would test whether this finding can be extrapolated to the salmonid model.

Glomerular patterning across teleosts

The glomerular patterning established in this study was compared to previously established maps of glomerular patterning in closely phylogenetically related species, adult salmonids (Rainbow trout, Riddle and Oakley, 1992; Chinook salmon, Jarrard, 1997), and to that of a distantly related cyrpinid species, larval and adult zebrafish (Baier and Korsching, 1994; Braubach et al., 2012, 2013). The coarse organization of Chinook salmon alevin glomerular territories, in reference to anatomical characteristics and positioning within the OB, was almost identical to that defined in adult rainbow trout (Riddle and Oakley, 1992) and zebrafish (Braubach et al., 2012; Baier and Korsching, 1994), with seven glomerular territories reliably identified and outlined below (also see Table 2.2). Additionally, the organization of individual glomeruli innervated by microvillous OSN axons in the lateral OB identified by labelling against calretinin

antibody revealed consistencies in glomerular patterning and number of glomeruli when compared to larval zebrafish (Braubach et al., 2013).

The dlG territory appears to be homologous to structures identified in zebrafish and rainbow trout. The OSN axons that terminate on this large, irregularly-shaped chain are both KLH- and calretinin-immunoreactive, and terminate upon any of dozens of glomeruli that comprise this chain. Congruent to zebrafish, the mdG was innervated by only KLH-IR OSN axons that terminated upon up to six glomeruli (Braubach et al., 2012). Additionally, the Chinook salmon alevin lateral glomerular territory occupied much of the lateral glomerular layer in the olfactory bulb, as observed in zebrafish (Braubach et al., 2012; Baier and Korsching, 1994) and rainbow trout (Riddle and Oakley, 1992).

In the ventral region of the olfactory bulb, the maG, vpG and vmG territories were previously identified in adult rainbow trout (Riddle and Oakley, 1992), adult zebrafish (Braubach et al., 2012; Baier and Korsching, 1994) and larval zebrafish (Braubach et al. 2013). However, a fourth ventral glomerular territory, the ventroanterior glomerular chain, was described in adult (Braubach et al., 2012) and larval zebrafish (Braubach et al., 2013), but was not discernable in this study. A posteriorly-situated ventral lateral glomerulus has been identified in adult rainbow trout (Riddle and Oakley, 1992), but could not be reliably identified in Chinook salmon alevin, perhaps due to its apparent small size.

In respect to focus on a specific OSN morphotypes the coarse glomerular organization and innervation by calretinin-immunoreactive laterally projecting OSN axonal endings in Chinook salmon alevin was found to be very similar in reference to

number, shape and location of glomerular chains compared to descriptions provided for adult (Braubach et al., 2012; Baier and Korsching, 1994) and larval zebrafish (Braubach et al. 2013). A stretch of about five glomeruli ventrolaterally located in the dlG in addition to lateral glomerulus lG_2 were immunoreactive to only KLH and not calretinin, analogous with zebrafish OSN immunoreactivity (Braubach et al., 2012). Identification of these glomerular landmarks allowed for the identification of distinctly calretinin-immunoreactive glomeruli in the lateral glomerular chain, which are also homogenous to zebrafish lateral chain glomeruli (Table 2.2; lateral glomeruli lG_1, lG_2, $lG_{3/4}$, lG_6). Perhaps due to the sectioning technique applied in this study, the lG_3 and lG_4 identified in zebrafish could not be differentiated. If situated immediately adjacently, horizontal sections would not provide the resolution to distinguish a boundary between the two glomeruli. Despite the across-species similarities in glomerular patterning, differences were observed in this study. Unlike the dG identified in zebrafish (Braubach et al., 2012), this chain is not calretinin-immunoreactive and is situated directly between the mdG and dorsolateral chains in the Chinook alevin. Discrepencies in the situation of glomerular territories may also be attributed to differences in ICC techniques. Specifically, the collection of horizontal serial sections at the same angle between specimens was not possible, and confounds the description of the situation of glomerular territories in the OB. Whole-mount preparations applied by Braubach et al. (2012) allow for comprehensive imaging of the olfactory bulb, although any compression of the olfactory bulb may distort the perceived organization of glomerular territories.

This within- and across-species constancy in glomerular patterning suggests potential variation in coarse organization would be deleterious to the animal, such that

mutations leading to the misguiding of OSN axons from the olfactory epithelium to their respective glomeruli would likely lead to olfactory impairment, preventing the propagation of the mutated gene. These across-species analogies in odour-evoked glomerular activity again supports that glomerular patterning is a conserved trait, allowing different fish species to use the same proximate mechanism to identify different odour classes. Thus physiological studies associating glomerular responsiveness to certain odours in alternate teleost species, namely the transgenic zebrafish, may be applicable to the glomeruli identified in this study and have been outlined in Table 2.2.

In conclusion this study demonstrates glomerular patterning in Chinook salmon alevin appears to be established at hatch, and is analogous to that of adult salmonids (Rainbow trout, Riddle and Oakley, 1992; Chinook salmon, Jarrard, 1997). Refinement of all glomerular territories, indicated by an observed increase in territory size and number of microglomeruli, is evident as the alevin matures, but specific glomeruli in the lateral territory appear to be already established. Thus, at hatch, alevin are likely capable of odour discrimination, but sensory experience may be required to further refine the corresponding glomerular regions, an adaptive mechanism that potentially increases the individual's sensitivity to cues specific to its environment.

Understanding the effect of olfactory experience on the development of glomerular patterning in developing Chinook salmon may have implications at the hatchery level, where fish are often exposed to filtered municipal water rather than to the array of odours present in natural water systems. Thus, this baseline qualitative study provides a stepping stone to experimentally test the factors that influence the neurodevelopment and learning in a socio-economically important species.

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Tables and Figures

Table 2.1. Nomenclature of terminal fields of olfactory sensory neuron (OSN) axonal projections in the teleost olfactory bulb in across-species comparison of olfactory glomerular patterning. "Unidentified" in brown trout indicate innervation of OSN axons without identification by authors. Dot represent unaccounted glomerular territory or glomerulus.

Zebrafish Mature Probe: DiI **Baier and Korsching, 1994**	**Zebrafish** mature/larval Probe: KLH, CAL, GFP **Braubach et al., 2012/2013**	**Rainbow trout** Mature Probe: KLH **Riddle and Oakley, 1992 and Porteros et al., 1997**	**Chinook salmon** hatch to adult Probe: KLH **Jarrard, 1997**	**Brown trout** hatch to adult Probe: KLH, CAL **Castro et al., 2008**
Mediodorsal posterior	Dorsal	Dorsal posterior medial	.	unidentified
Anterior plexus	Mediodorsal	Anterior medial	Anterior medial	.
Dorsal cluster	Dorsolateral	Dorsal lateral	.	unidentified
Dorsal cluster (1-5)	Dorsolateral $G_{1\text{-}5}$	Dorsal posterior lateral	.	.
Lateral Chain	Lateral Plexus	Lateral/ Posterior lateral	Lateral/ Posterior lateral	.
Lateral chain 1	Lateral G_1	.	.	.
Lateral chain 2	Lateral G_2	.	.	.
Lateral chain 3	Lateral G_3	.	.	.
Lateral chain 4	Lateral G_4	.	.	.
Lateral chain 5	Lateral G_6 Lateral G_5	. .	.	unidentified
.		Ventral posterior lateral	.	.
Medioanterior	Medial Anterior	Anterior Medial	.	.
Medial elongated	Medial Posterior	.	.	.
Ventral triplet	Ventromedial G6 and Gx	.	.	.
Ventroanterior	Ventroanterior	.	.	.
Ventromedial	Ventromedial	Ventral medial	.	.
Ventroposterior.	Ventroposterior G1	Ventral posterior	.	unidentified

Ventroposterior.	Ventroposterior G2			

Table 2.2. Glomerular territories and glomeruli identified in the Chinook salmon alevin olfactory bulb analogous to those identified in zebrafish larvae and mature rainbow trout. Function is inferred by physiological studies that have linked odour stimulant with glomerular territory activity. Dots represent unaccounted glomerular territory or glomerulus.

Zebrafish	**Rainbow trout**	**Chinook salmon**	**Putative odour stimulant**
larval	mature	Posthatch to emergence	
Braubach et al., 2013	**Riddle and Oakley, 1992**	**Ochs et al., unpublished**	
Dorsal	Dorsal posterior medial	Dorsal	Bile acids (Friedrich and Korsching, 1998)
Mediodorsal	Anterior medial	Mediodorsal	Bile and amino acids acids in juvenile trout and sex hormones in mature rainbow trout (Bazáes and Schmachtenberg, 2012)
Dorsolateral	Dorsal lateral	Dorsolateral	
Dorsolateral G_{1-5}	Dorsal posterior lateral	Dorsolateral (presumably 1-5)	Bile acids (Friedrich and Korsching, 1998)
Lateral Plexus	Lateral/ Posterior lateral	Lateral Plexus	Amino acids (Friedrich and Korsching 1998; Braubach et al., 2013; Hinz et al., 2013)
Lateral G_2	.	Lateral G_2	
Lateral G_1	.	Lateral G_1	
Lateral G_3	.	Lateral $G_{3/4}$	
Lateral G_4	.	Lateral $G_{3/4}$	
Lateral G_5	.	unaccounted	
Lateral G_6	.	Lateral G_6	Nucleotides (ATP, IMP; Friedrich and Korsching, 1998)
Medial Anterior	Anterior Medial	Medial anterior	Bile acids (Friedrich and Korsching, 1998)
Medial Posterior	.	.	
Ventroanterior	.	.	
Ventromedial	Ventral medial	Ventromedial	
Ventromedial G6 and Gx	.	Ventromedial G_x	Prostaglandin and maybe 17,20P-S (Friedrich and Korsching, 1998)

Ventroposterior $G_{1,2}$	Ventral posterior	Ventroposterior	

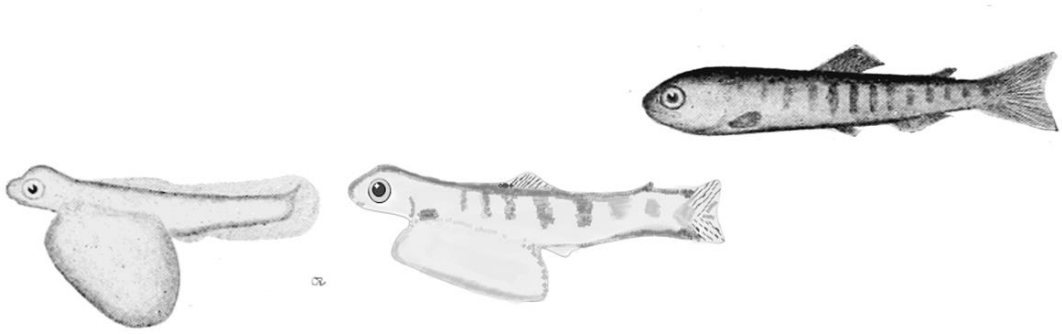

Figure 2.1. Comparison of glomerular patterning at three increments in Chinook salmon to assess ontogeny of these structures from posthatch to emergence. Salmon at these early developmental stages are situated within the gravel of the spawning stream, or just exiting in the case of emergent fry, and are thus exposed to natal stream odours.

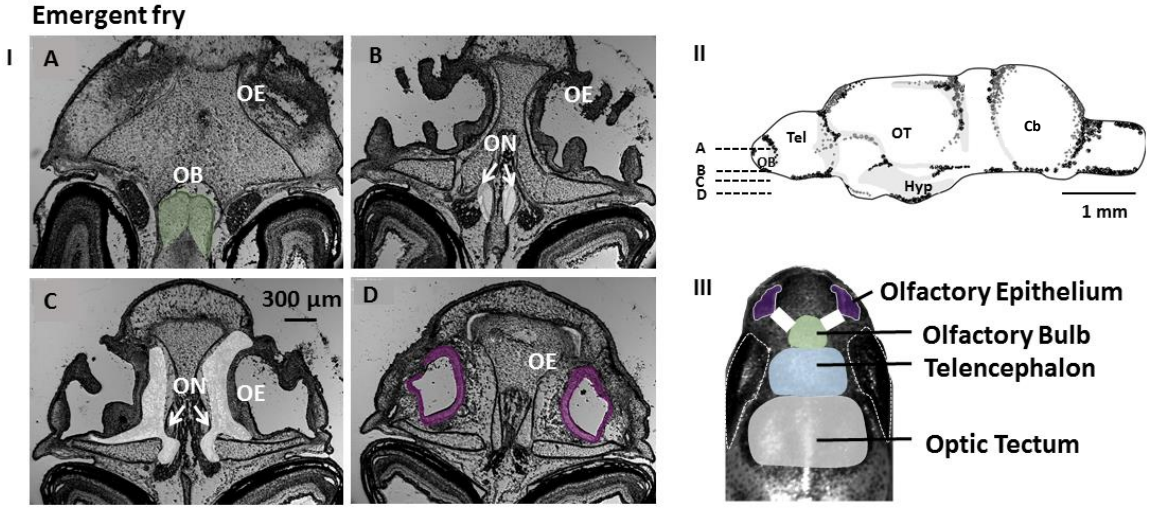

Figure 2.2. Chinook salmon emergent fry olfactory bulb situated dorsal to the olfactory nerve and olfactory epithelium. I: Brightfield micrographs of horizontal sections of the olfactory system from the dorsal to ventral regions. A, B: The olfactory bulbs (green) innervated by the olfactory nerves (ON, white). C: Extension of olfactory sensory neuron axons from the olfactory epithelium to the ON (white). D: The dendritic endings of the OSNs are situated in the olfactory epithelium (OE; purple). II) Saggital view of Chinook salmon brain. Inset letters correspond with the approximate depth of the horizontal sections. OB = Olfactory Bulb, Tel = telencephalon, OT = Optic Tectum, Cb = cerebellum. III) A two dimensional horizontal view of the olfactory system.

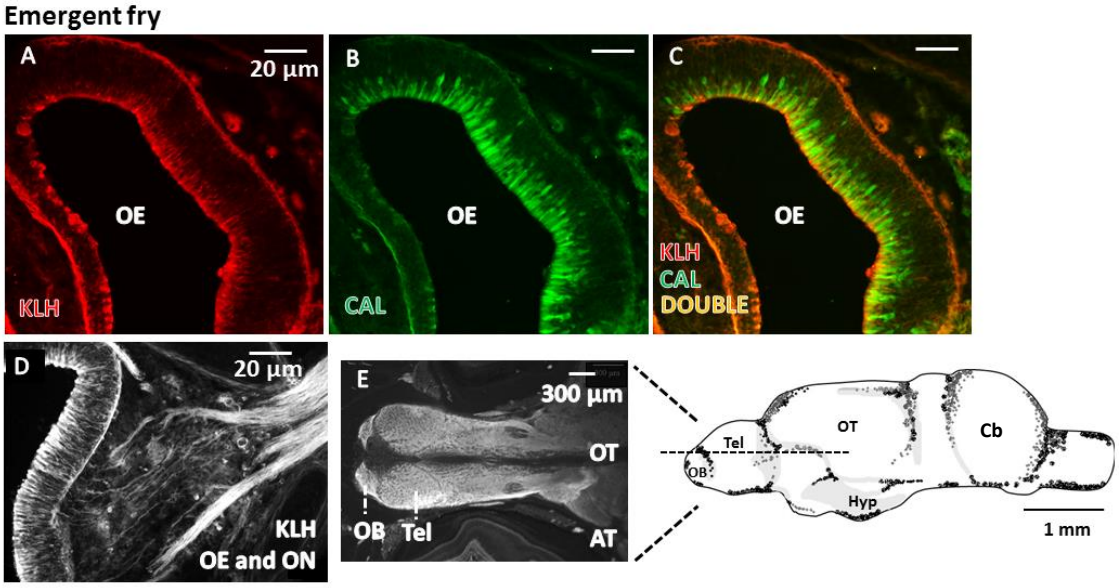

Figure 2.3. KLH and Calretinin immunolabeled horizontal serial sections of the olfactory epithelium, olfactory nerve and olfactory bulb of Chinook salmon emergent fry. A-C: OSN axons in the olfactory epithelium labelled against keyhole limpet hemocyanin (KLH) and calretinin (CAL). D: KLH-immunoreactive olfactory sensory neuron axons project away from the olfactory epithelium towards the sessile olfactory bulbs (E), labelled against acetylated tubulin (AT). E: Also includes saggital view of Chinook salmon alevin brain. OB = Olfactory Bulb, Tel = telencephalon, OT = Optic Tectum, Cb = cerebellum.

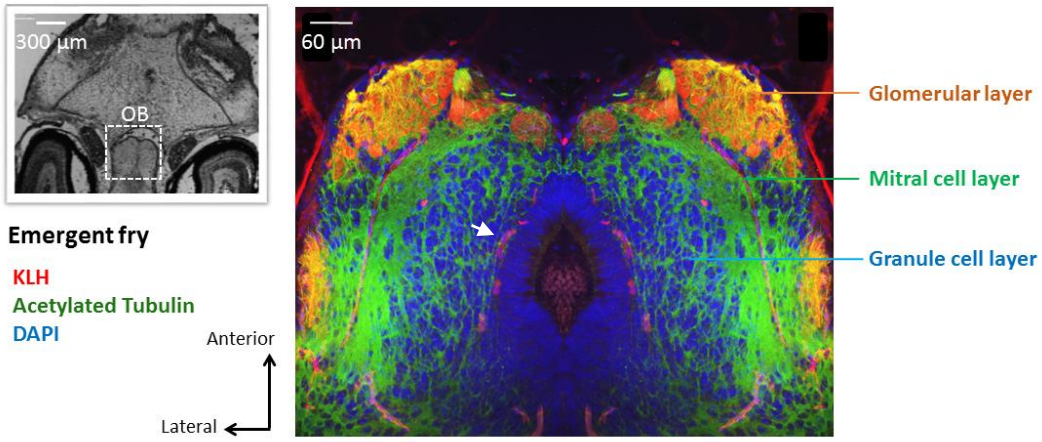

Figure 2.4. Horizontal section of an emergent Chinook salmon olfactory bulb triple labelled against KLH, DAPI, and AT to identify the different layers. The outermost glomerular layer is comprised of OSN axons (immunoreactive to KLH and AT). The mitral cell layer is characterized by dense AT-immunoreactive nerve fibers, while the innermost granule cell layer is denoted by a high concentration of DAPI-immunoreactive cell bodies. Artifacts (labelled with arrow) such as blood vessels are immunoreactive to the KLH antibody, but are identifiable due to the tubular shape and extension beyond the glomerular layer.

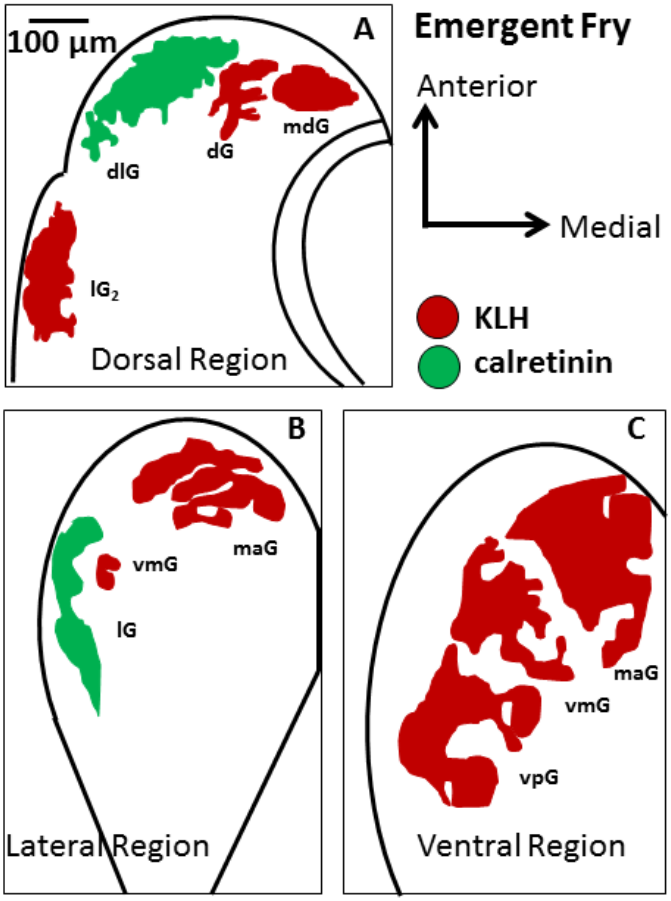

Figure 2.5. Organization of glomerular territories in an emergent fry, innervated by KLH- and calretinin-immunoreactive OSN axons. All OSN morphotypes were immunoreactive to KLH, but only microvillous OSNs were immunoreactive to calretinin (highlighted in green). A: The dorsal region of the olfactory bulb contains the mediodorsal (mdG), dorsal (dG), and dorsolateral (dlG) glomerular territories. The dorsal-most lateral glomerulus, lateral glomerulus 2 (lG_2) is also situated in the dorsal bulb. B: The lateral glomerular (lG) territory is innervated by calretinin-IR fibers. C: Ventral glomerular territories are organized in three distinct groups, the medial anterior (maG), ventromedial (vmG) and ventroposterior (vpG).

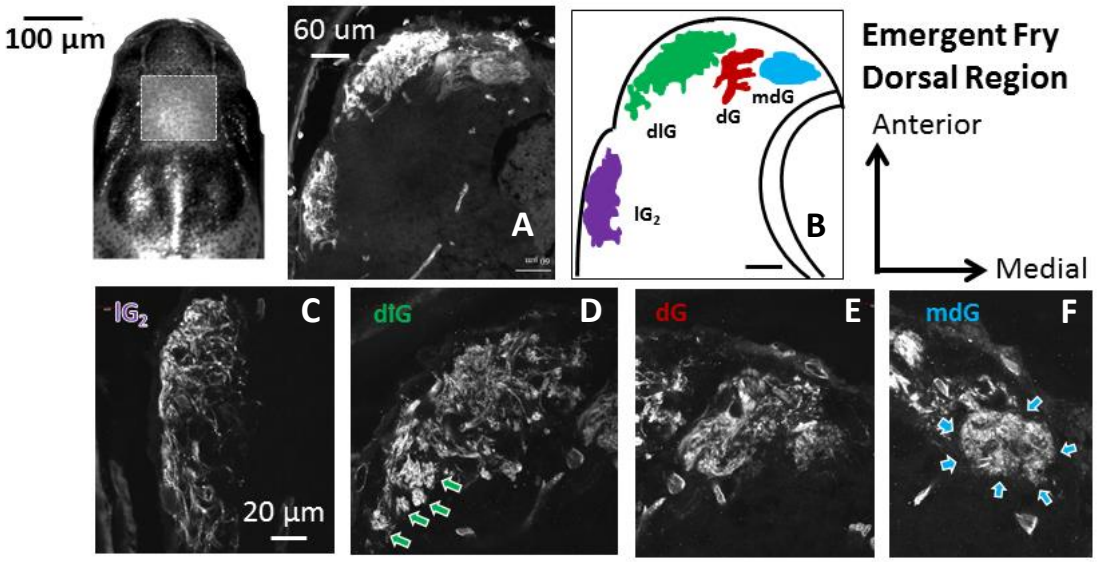

Figure 2.6. A,B: Confocal acquired micrographs of KLH-immunoreactive glomerular territories in the dorsal region of the emergent Chinook salmon olfactory bulb (horizontal sections). C: lG_2 is a large glomerulus composed of a diffuse arrangement of OSN axon endings. D: The dorsolateral glomerular chain (dlG) is a large chain comprised of a high density of small glomeruli, and contains a chain of four to five glomeruli projecting from the lateral-most region. E: The dorsal glomerular territory (dG) is an irregularly-shaped chain, whereas the mdG (F) is comprised of about six glomeruli in a spherical configuration.

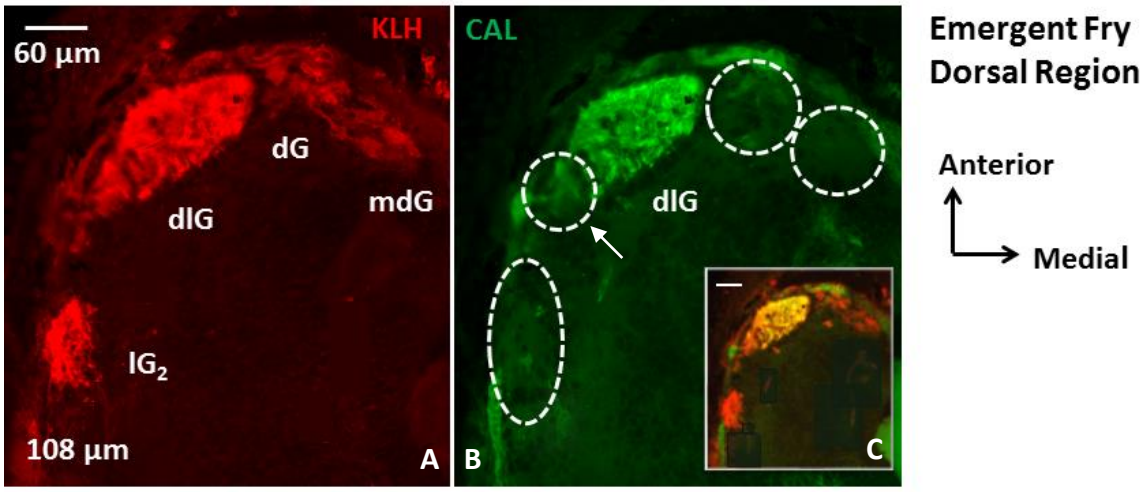

Figure 2.7. Dorsal glomerular territories (108 µm from the dorsal-most section) in emergent Chinook salmon double-labelled against KLH (A) and calretinin (B). C: Inset shows a merge of the two labels. Immunoreactivity to calretinin is indicative of innervation by microvillous OSNs. A and B: The dorsolateral (dlG) and lateral glomerular (lG) territories are innervated by calretinin-immunoreactive axonal projections (dashed circles indicated lack of immunoreactivity), but four to five glomeruli extending from the lateral-most region of dlG and lG_2, the dorsal-most glomerulus of lG, are immunoreactive to only KLH (arrow).

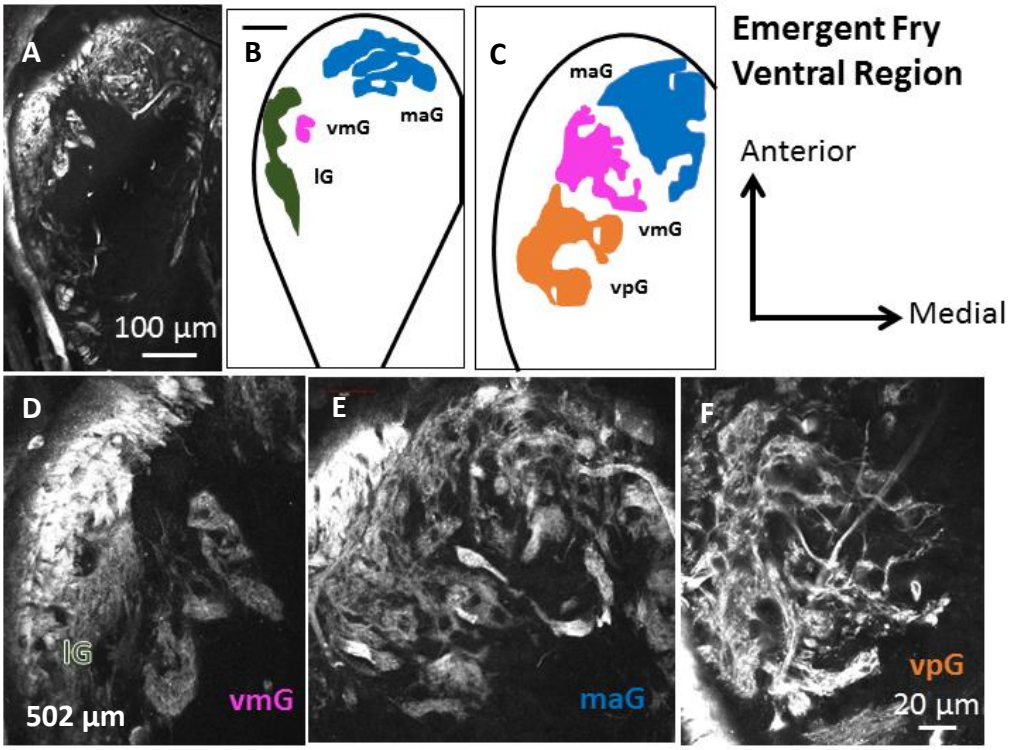

Figure 2.8. A, D, E, F: Confocal acquired micrographs of KLH-immunoreactive glomerular territories in the ventral region of the emergent Chinook salmon olfactory bulb (502 µm from dorsal-most section). A, B: The lateral glomerular territory (lG) terminates in the ventral region of the olfactory bulb. C, D: The ventromedial glomerular territory (vmG) projects ventral-medially from the lateral plexus as an array of three to four distinct glomerular groups before expanding to occupy about half of remaining area of the olfactory bulb adjacent to the olfactory nerve. C, E, F: The medial anterior glomerular chain (maG), the dorsal-most ventral glomerular territory, is composed of a diffuse arrangement of olfactory sensory nerve axon endings, whereas the ventroposterior glomerular chain (vpG) is the ventral-most glomerular territory.

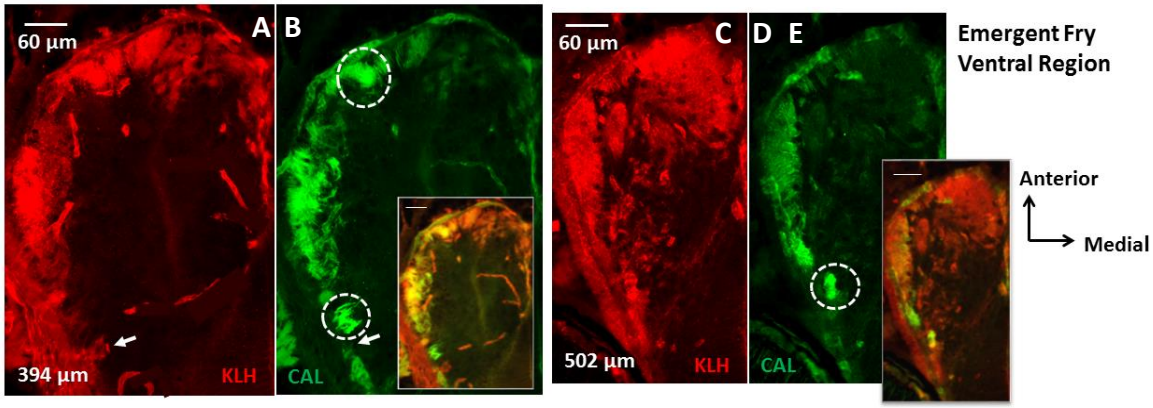

Figure 2.9. Ventral glomerular territories in emergent Chinook salmon double-labelled against KLH (A, D), calretinin (B, E) and merged labelling (C, F). Immunoreactivity to calretinin, inferring innervation by microvillous OSNs, is only visible in the lateral glomerular territory (lG). B, E: Calretinin-immunoreactive glomeruli belonging to lG (dashed circles). A, B: The arrow points to a small glomerulus that is immunoreactive to KLH only, and may reflect a posterior lateral glomerulus observed in adult salmonids (Rainbow trout; Riddle and Oakley, 1992). D, F: The ventral-most glomerular territories are innervated by KLH-IR axons.

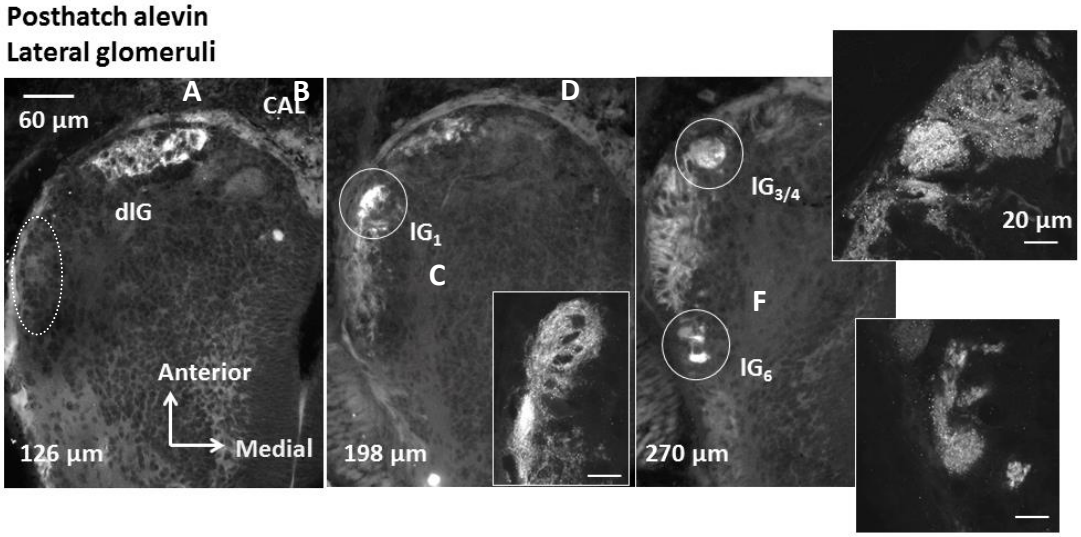

Figure 2.10. A-F: Confocal projections of calretinin-IR lateral glomeruli in horizontal sections of the posthatch Chinook salmon olfactory bulb (olfactory bulb depth from dorsal-most section noted). A: The dorsal-most lateral glomerulus, lG_2, is not calretinin-immunoreactive, and not visible (dashed circle). B, C: lG_1 is comprised of a diffuse arrangement of OSN axonal endings projecting towards the anterior region of the olfactory bulb. D, E: Anteriorly situated $lG_{3/4}$ is an irregularly shaped glomerulus. D, F: Posterior-most lateral glomerulus (lG_6), is comprised of a number of small OSN bundles terminating on unevenly distributed boutons.

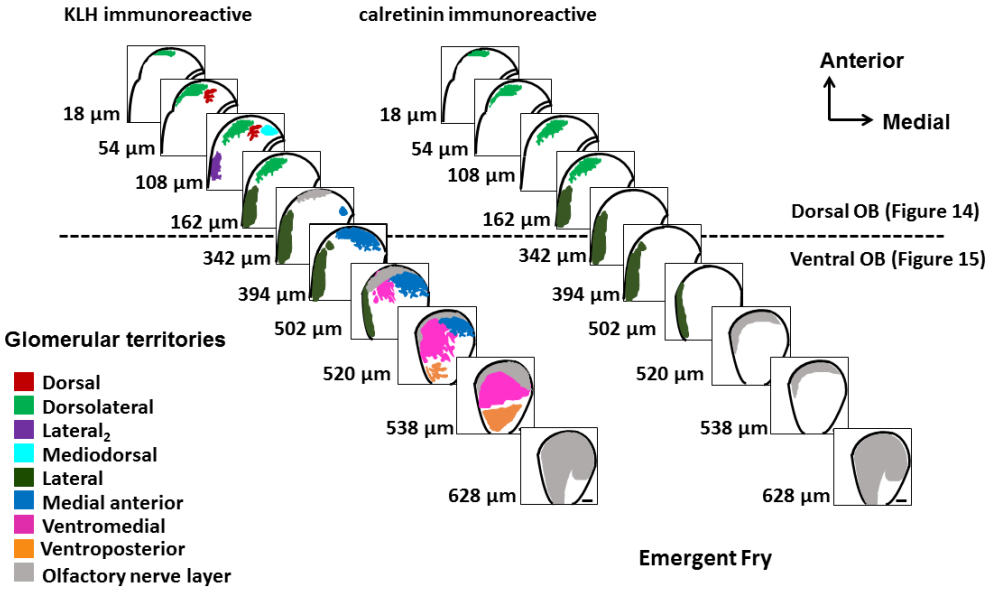

Figure 2.11. Coarse patterning of glomerular territories innervated by KLH-immunoreactive (left) and calretinin-immunoreactive (right) olfactory sensory neurons in emergent Chinook salmon. Only two of the seven KLH-IR glomerular chains, the dorsolateral and lateral glomerular territories, were immuno-reactive to calretinin.

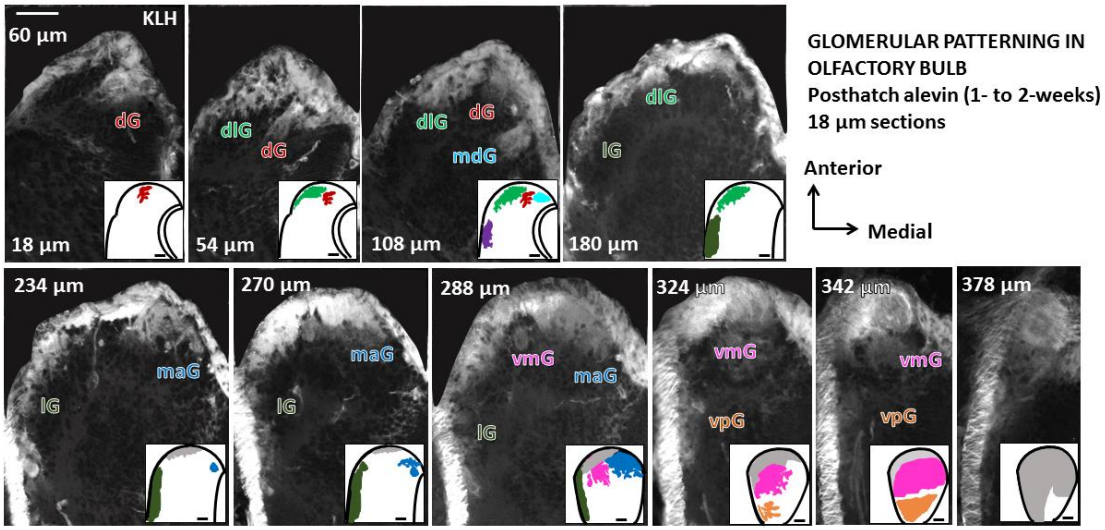

Figure 2.12. Horizontal micrographs of KLH-IR glomerular patterning in posthatch (2-week-old) Chinook salmon alevin. Eight KLH-immunoreactive glomerular territories were located dorsal-ventrally throughout the olfactory bulb and were diffusely innervated by olfactory sensory neurons. Diffuse but discernable OSN axons project into the ventral olfactory bulb (324-378 µm).

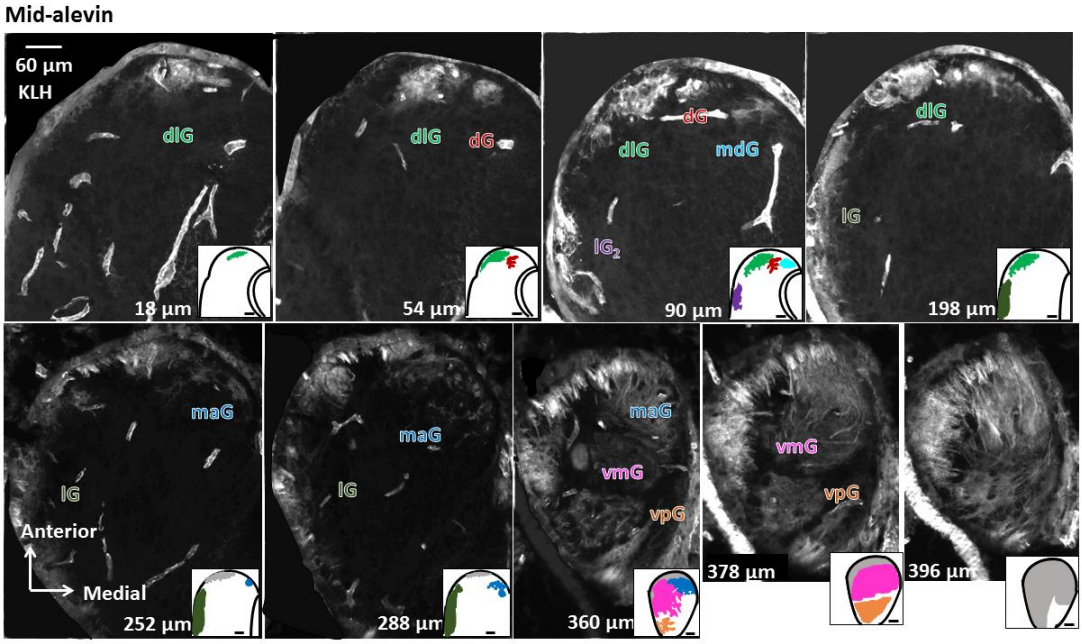

Figure 2.13. Horizontal sections showing glomerular patterning in mid-alevin (5-week-old) Chinook salmon. All eight KLH-immunoreactive glomerular territories were clearly identifiable throughout the olfactory bulb.

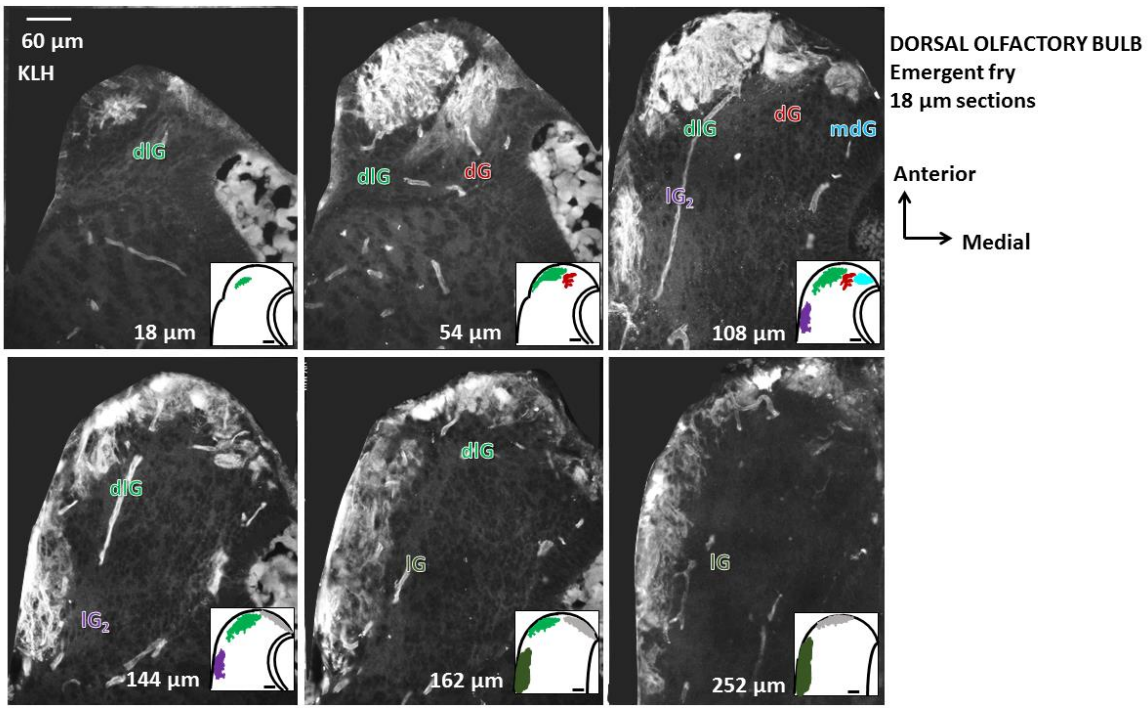

Figure 2.14. Low power fluorescence microscopy of horizontal sections of the dorsal olfactory bulb of Chinook salmon emergent fry, distinctly occupied by KLH-immunoreactive dorsolateral (dlG), dorsal (dG), mediodorsal (mdG), and lateral (lG) glomerular territories. The dorsal-most lateral glomerulus, lG_2, is visible.

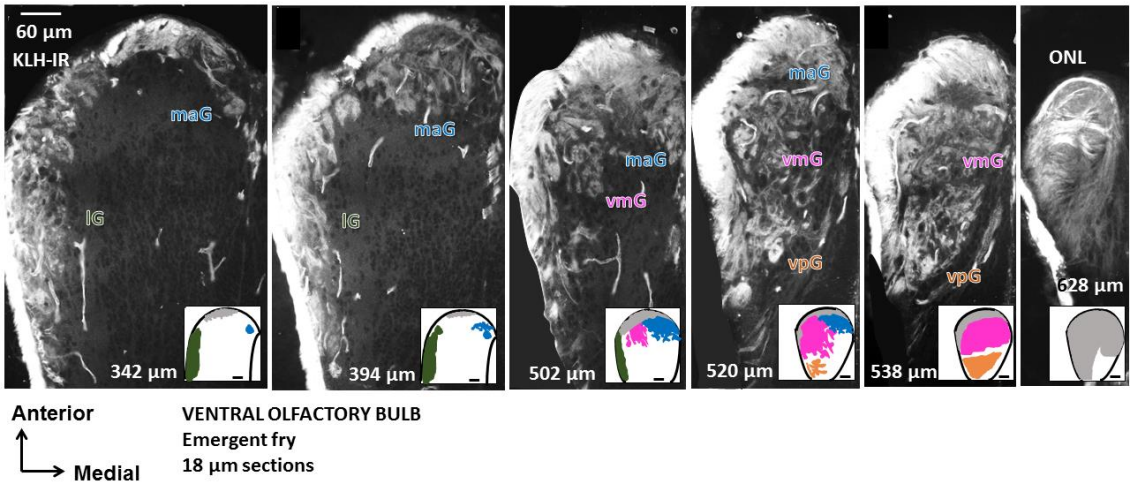

Figure 2.15. Low power fluorescence microscopy of horizontal sections of the ventral olfactory bulb of Chinook salmon emergent fry labelled against KLH. The lateral glomerular territory (lG) extends from the dorsal to ventral olfactory bulb, overlapping with the dorsal projections of the medial anterior glomerular territory (maG). Together with maG, the ventromedial (vmG) and ventroposterior (vpG) occupy much of the ventral region of the olfactory bulb.

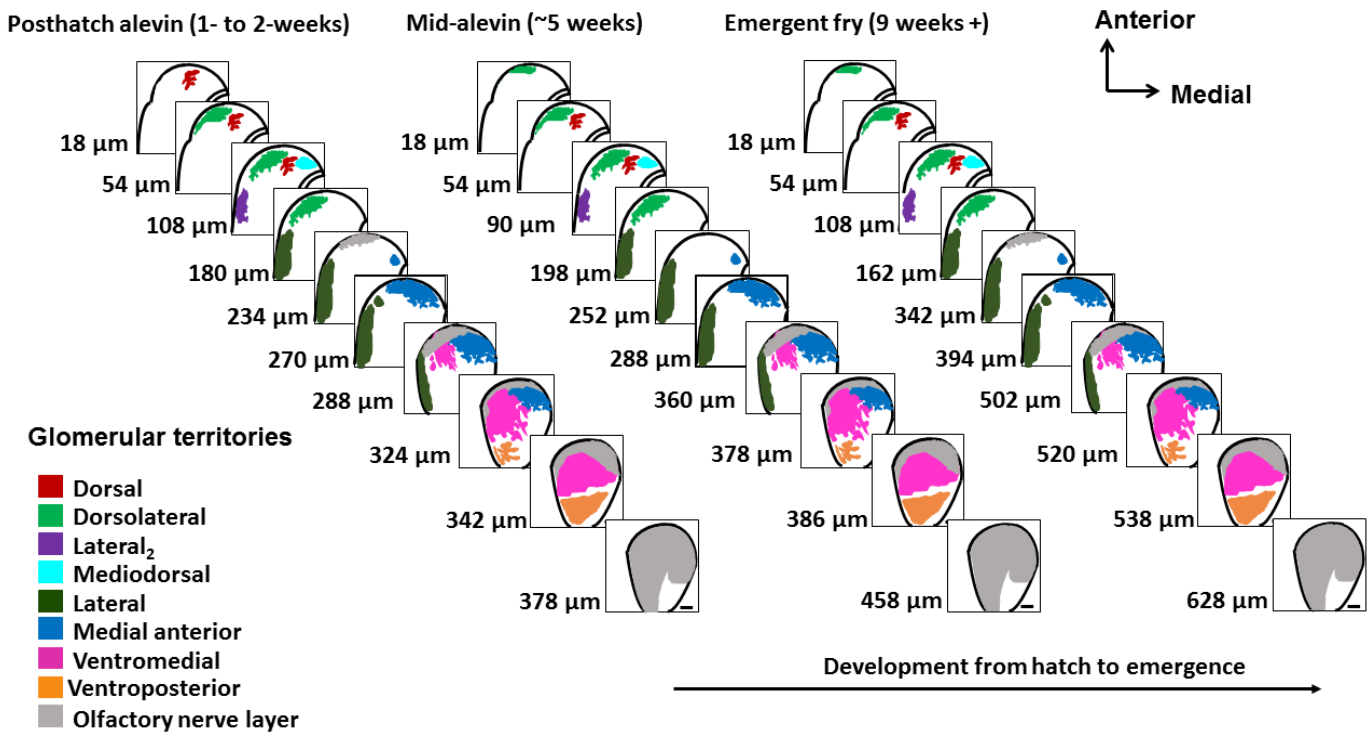

Figure 2.16. Distribution of KLH-immunoreactive glomerular territories in the glomerular layer of the olfactory bulb depicted with horizontal sections of the olfactory bulb from the dorsal- to ventral-most regions. All seven glomerular territories, depicted by a different colour, occurred at each developmental stage, but the ventral glomerular territories expanded with the growth of the olfactory bulb from posthatch to emergence. Lateral glomerulus 2 was readily identifiable when labelled against KLH, whereas the remaining lateral glomeruli were found in the lateral glomerular territory.

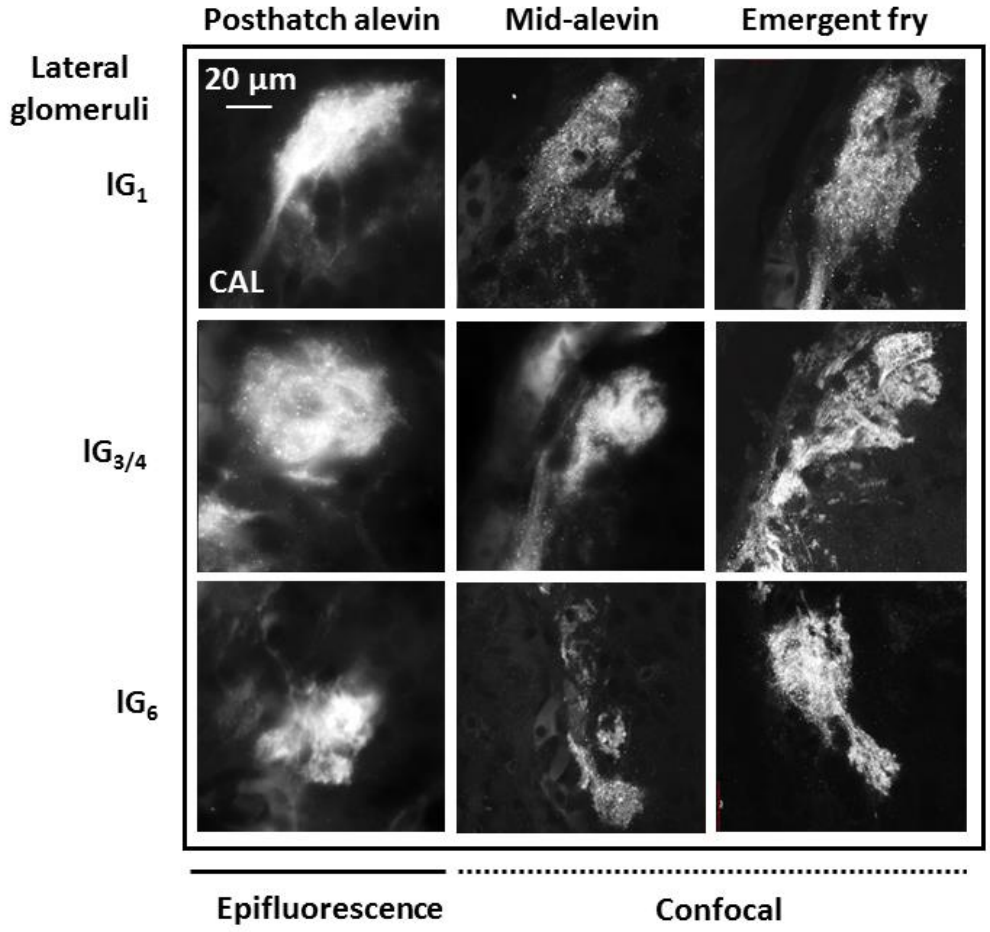

Figure 2.17. Calretinin-immunoreactive lateral glomeruli are visible from hatch to emergence in Chinook salmon alevin. Imaging using fluorescence (left column) and confocal (middle and right columns). These calretinin-immunoreactive glomeruli are consistent in shape and somatotopic positioning within the olfactory bulb, and show little difference in size.

Chapter 3: Exploring for effects of olfactory enrichment on volume of lateral glomerulus 1 in Chinook salmon *Oncorhynchus tshawytscha* alevin

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Introduction

Animals are exposed to an array of sensory experiences that influence the circuitry of the central nervous system. Hubel and Wiesel's seminal study (1962) demonstrated visual deprivation in kittens inhibits activity-dependent survival of neural connections in a brain region receiving visual sensory input, and has inspired a plethora of studies investigating the plasticity of sensory systems across taxa. Animals are exposed to an array of sensory stimuli that may alter neural circuitry as early as embryogenesis (e.g., Todrank et al., 2011), but identification of these potentially critical developmental periods is challenging. Plasticity of the developing human brain has been attributed to the overproduction of neurons and synaptic connections during prenatal and the first year of development followed by a rapid decline during the preschool years (reviewed in Huttenlocher, 1985). Pacific salmonids learn to recognize natal stream-specific odours early in life, with a critical period for learning purportedly occuring prior to emergence (Dittman et al., 2015), rather than exclusively during the parr-to-smolt transformation as previously thought (Hasler and Scholz, 1983; Dittman et al., 1996). As such, evidence of neural-plasticity in Pacific salmonid alevin in response to olfactory stimulation, specifically the reorganization of olfactory sensory neurons in the central nervous system, could provide anatomical evidence to support this paradigm.

A basic understanding of the teleost olfactory system elucidates the importance of olfactory sensory neuron (OSN) axonal organization for odour discrimination. The olfactory system is stimulated by the ligand binding of an odour molecule to an olfactory receptor (OR) situated on the dendritic end of an OSN positioned in the olfactory

epithelium (Ngai et al., 1993; Dulac and Axel, 1995). The subsequent depolarization of the OSN (Firestein, 2001) stimulates an excitatory response that is conducted along the OSN axon, which projects into the olfactory bulb (Ngai et al., 1993; Hara and Zhang, 1998). OSN axons terminate on glomeruli, discrete regions of high OSN axon synaptic connectivity with output neurons (mitral cells). ORs bind only one odourant due to a self-regulation process where a single OR gene is expressed in conjunction with the silencing of hundreds of alternates, facilitating the one odour, one receptor rule (reviewed in DeMaria and Ngai, 2010). Because a glomerulus is comprised of the coalescence of OSN axons expressing the same OR, glomeruli are also stimulated by a single odour molecule (reviewed in DeMaria and Ngai, 2010). Thus, glomeruli are functional units that facilitate the identification and response to distinct odourants (Friedrich and Korsching, 1997).

The topographical organization of olfactory glomeruli are highly standardized in zebrafish, *Danio rerio*, (Baier and Korsching, 1994; Gayoso et al., 2011; Braubach et al., 2012), such that glomeruli congregate as distinguishable chains that are predictably situated throughout the olfactory bulb, a pattern that emerges as early as hatch (Zebrafish: Braubach et al., 2013; Chinook salmon, *Oncorhynchus tshawytscha*: see Chapter 2). This functional coding of the olfactory bulb is facilitated by the innervation of glomerular territories by axon endings predominantly from one of three OSN morphotypes. Bile acid, steroid, and some amino acid odours stimulate the generalist ciliated OSNs, which project to the dorsomedial region of the olfactory bulb (Zebrafish: Friedrich and Korsching, 1998), and pheromones stimulate crypt OSNs which extend to the dorsal medial region of the olfactory bulb (Gayoso et al., 2012). The lateral region of the olfactory bulb receives input from microvillous OSNs, a specialist OSN morphotype

which are responsive to amino acid odours (Sato and Suzuki, 2001; Koide et al., 2009).

The influence of olfactory experience on neural circuitry has been explored using transgenic lines of laboratory animals as models. Studies investigating specific changes in glomerular size expressed in volume or area were summarized for mammalian, insect, and teleost models (Tables 3.1-3.3). Briefly, studies support sensory experience induces a change in glomerular area or volume across taxa, but these changes may only occur at a specific life stage. In conjunction with behavioural trials, change in glomerular size often coincides with aversive or attractive behaviours towards the specific olfactory cues, suggesting the altered neural circuitry in the olfactory bulb translates to the higher brain structures, altering behavioural responses (Devaud et al., 2001 & 2003; Todrank et al., 2011). For example, mouse pups exposed to a specific odourant *in utero* and while nursing show an increase in corresponding glomerular volume, and show preference to the odour (Todrank et al., 2011). Glomerular size and structure have also been applied as a metric to determine critical periods for glomerular development. Valle-Leija et al. (2012) demonstrated that odour exposure during the first 20 postnatal days of life, but not during adulthood, resulted in the formation of microglomeruli in mice, caused by the sub-clustering of axons within a single glomerulus into additional boutons.

Behavioural and neurological studies support larval teleosts are also receptive to sensory experience early in life. Teleosts can discriminate odours immediately posthatch (Zebrafish, Li et al., 2005), at which point the OSNs are stimulated by environmental olfactory cues. Larval zebrafish respond to kin odours only when exposed to a combination of olfactory and visual cues during a 24 hour developmental period between five and six days post fertilization, or two to three days after hatch (Gerlach et al., 2008;

Hinz et al., 2013). Braubach et al. (2013) identified a glomerulus, lG_x, in the lateral glomerular chain responsive to olfactory stimuli during the first two weeks posthatch, as evident by decreased glomerular area and increased number of supernumerary glomeruli. Substantial differences in the structural and olfactory environment between fish hatcheries and natural waterways are thus expected to lead to neural differences between fishes from the two groups. Gross comparisons of brain structures has shown discrepancies between hatchery and wild salmonids, with the optic tectum and telencephalon (Rainbow trout, *O. mykiss*, one- to two-year-old fish, Marchetti and Nevitt, 2003), and olfactory bulb and telencephalon volumes (Rainbow trout smolts, Kihslinger et al., 2006) smaller in hatchery-reared salmonids, although the reciprocal was found in a Laurentian Great Lakes population of introduced Chinook salmon (adult, Wiper et al., 2014). Finer scale analyses have shown that proliferation rates of cells in the telencephalon is higher in salmonid parr reared in more structurally complex systems (Coho salmon, *O. kisutch*, Lema et al., 2005), again suggesting an environmental effect on neurodevelopment.

Salmonid receptivity to olfactory cues is presumably important during early development. Eggs from Laurentian Great Lakes Chinook salmon hatch in the late fall months, and alevin remain situated within the redd, or gravel nest, until the yolk-sac is absorbed. As emergent fry exhibit feeding behaviour in response to food cues, namely amino acids odours (Mearns, 1986), they must learn to recognize these olfactory cues sometime prior to emergence. Additionally, spawning Pacific salmon predominantly use olfactory cues to home to natal streams to reproduce (Ueda, 2012), with dissolved free amino acids supported as the most probable guidance cue (Shoji et al., 2003). Successful

homing requires the salmon to imprint to these olfactory cues sometime during or prior to the downstream migration towards open waters where the salmon will mature. Isotope analyses revealed that juvenile salmon can occupy a number of different streams prior to smoltification (Shrimpton et al., 2014), supporting olfactory imprinting may occur prior to this developmental stage. Few studies have investigated the implications of chemosensory experience on neural circuitry in the sensory regions of the brain, specifically the effect of sourcing salmon incubation trays with filtered well water as opposed to natural water. The chemistry of water can change drastically as a result of chlorination, where amino acids may be converted into aldehydes (Trehy et al., 1986). Additionally, many filters that are used to dechlorinate water contain activated carbon, which can adsorb amino acids (Dusart et al., 1991).

The somatotopic organization of glomerular territories in Chinook salmon alevin are analogous to that in zebrafish (see Chapter 2). Anti-calretinin labels amino acid-responsive microvillous OSN endings in the olfactory bulbs of zebrafish (Castro et al., 2006; Germana et al., 2007; Duggan et al., 2008; Braubach et al., 2012, 2013) and salmonid alevin (Brown trout, *Salmo trutta*: Castro et al., 2008; Chinook salmon: see Chapter 2). Of the six lateral glomeruli identified in zebrafish larvae (Braubach et al., 2013), four are distinguishable in Chinook salmon alevin (lG_1, lG_2, $lG_{3/4}$, lG_6; Fig. 3.1), three of which are immunoreactive to calretinin (lG_1, $lG_{3/4}$, lG_6), indicating amino acid-responsive microvillous OSNs also project into these glomeruli (see Chapter 2).

Emergent rainbow trout behaviourally respond preferentially to creek water to which they were exposed during embryonic and alevin development; subsequently embryonic imprinting is already being applied as a management strategy by the Lake

Sammamish Kokanee Workgroup 2012 (reviewed in Dittman et al., 2015). Testing for the effect of olfactory experience on glomerular development in salmonid alevin is therefore a timely contribution to research supporting the embryonic imprinting paradigm. In this study, we experimentally tested whether exposing Chinook salmon alevin to amino acid odours from hatch to emergence results in noticeable differences in olfactory sensory neuron axonal endings, determined by measuring glomerular volume of calretinin-immunoreactive lateral glomeruli (lG_1, lG_2, $lG_{3/4}$, lG_6). Glomerular volume of lateral glomeruli were also compared between posthatch (1- to 2-week-old) and emergent (9- to 10-week-old) individuals. Because water temperature is a strong predictor of development in Chinook salmon, the degree days (dd), or sum of mean daily water temperature, more accurately represents age (Crisp, 1981). Thus, the posthatch stage represents 530-590 dd and emergent fry represent 798-834 dd. lG_1, the dorsal-most glomerulus, was selected to assess the effect of olfactory experience and age on glomerular volume, as it was the most readily identifiable glomerulus with strong immunoreactivity and the most distinct borders. Additionally, amino acids stimulate the anterior region of the lateral territory (Friedrich and Korsching, 1997), the location of this particular glomerulus. Glomerular volume was expected to decrease with olfactory stimulation at both developmental stages, as was observed in zebrafish (Braubach et al., 2013). This decrease may be attributed to the pruning of unstimulated OSN morphotypes in conjunction with increased synaptic connectivity of amino acid- stimulated OSN axons with mitral cells, thus condensing the organization of OSN axons in the glomerulus. Comprehensively this study offers a description of the development of identifiable glomeruli in the lateral chain on Chinook salmon from posthatch to emergence, with

further exploration into the possible influence of sensory experience on glomerular volume.

Materials and Methods

Experimental animals

Eggs and milt were collected from Chinook salmon harvested from the Credit River, ON, during the first week of October, 2013. Eggs were fertilized by Ontario Ministry of Natural Resources and Forestry personnel and maintained at the Normendale Fish Culture Station in incubation trays at 9 °C. Roughly 350 eggs from a single family group were transferred during the eyed-up stage of development to the University of Windsor Animal Care Facilities on November 8th, where they were randomly divided between two incubation stacks, containing either the control or amino acid group, sourced by a flow-through of dechlorinated municipal water (3 litres/ minute) maintained at an average temperature of 10.5 °C. All fish collection, care and treatments are in accordance to University of Windsor Animal Care, and thus Canada Council on Animal Care, specifications.

Experimental design

Immediately post-hatch (November 25th), the incubation stack containing the treatment group was injected every 15 minutes with 2 mL of an amino acid stock solution (22.5 mM of each L-isomer proline, glutamic acid, tryptophan, alanine, histidine, and serine (Tables 3.4 and 3.5) dissolved in ultrapure water (deionized water filtered through a 0.2

μm filter), allowing for a brief, physiologically relevant, peak concentration of 1 μM (Table 3.6). This concentration has been effective for olfactory memory establishment in salmonids (Yamamoto et al., 2010; Dittman et al., 1996), and has resulted in significant anatomical changes to olfactory glomerular organization in the olfactory bulbs of zebrafish- namely an increase in number of smaller glomeruli in the lateral glomerular chain and a decrease in glomerular area (Braubach et al., 2013). Furthermore, this concentration exceeds the proposed response thresholds of salmonid olfactory receptors to amino acids (0.1 μM), according to electro-olfactogram recordings (Laberge and Hara, 2003). The incubation stack containing the control group was injected with 2 mL of ultrapure water every 15 minutes. The amino acid solution and control were delivered into the top trays of their respective incubation stacks using a Cole Parmer L/S digital drive with Masterflex pump heads to allow the solutions to mix with the down-flow of water to the trays below where the alevin were situated. Preceding dye trials confirmed the solutions would be well-mixed, and would remain within the rearing tray for at least 20 minutes, exceeding the allotted interval for odour delivery.

Sample collection and preparation

At 50% hatch (Week 0, Day 1), 10-15 alevin from each treatment group were euthanized by overdose of buffered MS-222 (1 g/L), forklength measured to the nearest millimeter, and mass measured to the nearest milligram. The head was decapitated posterior to the gills and was drop-fixed in a fresh solution of 4% PFA in 0.1M phosphate buffer solution (PBS). This collection was replicated weekly from hatch to emergence (0-10 weeks) until the alevin absorbed their yolk sacs, but only individuals representing the pothatch, (1- to

2-weeks-posthatch), mid-alevin (5- to 6-weeks-posthatch), and emergent (9- to 10-weeks-posthatch) developmental stages were included in these analyses (Table 3.7).

At least three days prior to sectioning, neural tissue was further exposed by removing the dorsal dermal layer covering the brain from the optic tectum to the olfactory epithelium, revealing the paired olfactory bulbs. Any tissue posterior to the optic tectum was also removed, and the sample was post-fixed in fresh 4% PFA in 0.1M PBS. The sample was cryoprotected in a 20% followed by 30% sucrose gradient in 0.1 M PBS overnight. Samples were embedded in Shandon M-1 Embedding Matrix (Thermo Scientific, Kalamazoo, 1310), and 18-30 µm serial sections throughout the olfactory bulbs were collected at a horizontal plane using a Leica cryotome (Leica Biosystems, Concord, model CM 3050A), which were mounted on Fisherbrand Superfrost Plus microscope slides (Fisher Scientific, Ottawa, 12-550-15). Sections were left to dry at room temperature before storing at -20 °C.

Immunocytochemistry and microscopy

Lateral glomeruli innervated by amino acid-sensitive microvillous OSNs were targeted with anti-calretinin, a calcium binding protein. All solutions were prepared using 0.1M PBS buffered to pH 7.4 preserved with 0.1% sodium azide as a vehicle for the blockers and probes, and all incubations and rinses occurred on a shaker at 4 °C.

Slides were rehydrated in 0.1M PBS with 0.1% triton X-100 for 30-40 minutes to increase tissue permeability. Slides were transferred to 10% goat serum (Sigma-Aldrich, Oakville ON, G9023) in 0.1M PBS for 30 minutes to block nonspecific binding to proteins. Sections were then incubated in primary antibody, a solution of 1/200 monoclonal anti-calretinin (produced in mice, Swant, Switzerland, 6B3) for five to seven

days, followed by a series of six rinses at 50 minute increments. To permit visualization of the calretinin-labelled OSN axons, slides were transferred to secondary antibody (1/200 goat anti-mouse Alexafluor 488 IgG, Life Technologies Inc, Burlington ON, A11001) for three to five days and again rinsed six times for 50 min each. Slides were coverslipped using VectaShield HardSet mounting medium (Vector Labs, Burlington ON, H-1400).

Once labelled, high power micrographs (60x oil immersion objective) of the serial sections containing lG_1, lG_2, and lG_3 were captured using fluorescence (Nikon Eclipse E800, QImaging Fast1394 camera, Northern Eclipse) or confocal (Olympus fluoview FV1000) microscopy, including sections with the dorsal- and ventral-most regions of the glomerulus. These micrographs were assigned random values to facilitate blind analysis. Glomerular area was calculated three times using ImageJ (http://rsb.info.nih.gov/ij) with threshold adjustment, where the freehand selection allowed for exclusion of OSN axons entering the glomerulus (Todrank et al., 2011); the calculated mean area was multiplied by section thickness (Fig. 3.3). Measurement error associated with mean area was conservatively calculated: $(Area_{Max} - Area_{Min})$/ Mean Area. Average measurement error of sectional glomerular area for all glomeruli was low (average measurement error = 8.8%, n = 134). Total glomerular volume was calculated by summing the volume of each section containing the glomerulus. All reported glomerular volumes were absolute measures.

Volumetric and statistical analyses

To test whether stimulation of microvillous OSNs by amino acid odours influences

glomerular volume, salmon eggs were randomly assigned to the amino acid-enriched or control incubation tray. The target glomerular volume was then measured across age from posthatch to emergence. Descriptive statistics report mean glomerular volume with corresponding standard error were calculated.

Paired samples from the olfactory enriched and control groups were processed concurrently to control for variables that may have been introduced during tissue preparation and ICC application. As a measure of the paired design, the ratio of amino acid to control glomerular volume was calculated. The grouping of data to represent the three developmental stages (posthatch, mid-alevin and emergent fry) was supported by lack of statistical difference in glomerular volume ratios: posthatch alevin (1- to 2-weeks-posthatch: Welch's two sample t-test: $t_3 = 0.091$, $p = 0.09$), and emergent fry (9- to 10-weeks: Wilcoxon rank sum test: $W_3 = 2$, $p = 1$, true location shift not equal to 0). However, the sample size was too limited to confirm the mid-alevin grouping (5- to 6-weeks-posthatch).

Paired t-tests were applied to test for the effect of olfactory enrichment on mean glomerular volumes of lG_1, $lG_{3/4}$ and lG_6 at the posthatch developmental stage, reported in millimetres cubed. I conducted a linear regression analysis on pooled glomerular volume of lG_1 to test if glomerular volume changed with age. Response magnitude for lG_1 (lG_1 $volume_{olfactory\ enriched}$ / lG_1 $volume_{control}$) was further calculated and a one-sample t-test conducted to test whether the ratio of glomerular volumes significantly differ from one to test for an effect of olfactory enrichment on glomerular volume.

The assumption of normality was assessed using the Shapiro-Wilk analysis, which confirmed normal distribution of all but one group. The glomerular volume of lG_1

from the control group violated this assumption with the distribution skewed right (W_{14} = 0.85, p = 0.02). A log transformation of the data did not correct the distribution and caused a non-normal distribution of glomerular volumes in the olfactory enriched group. Thus a Wilcoxon ranked test was applied to any comparisons including the glomerular volume of lG_1 from the control group, with a null hypothesis of true location shift should equal zero. Homogeneity of variance was satisfied according to the Levene's test for equal variance ($p > 0.05$ for all groups). All statistical analyses were conducted using SPSS (Version 20.0; IBM Corp, 2011) or the programing language R (R Core Team, 2014). Effect size, ω^2, was calculated for all paired t-tests: $\omega^2 = (t^2 - 1)/(t^2 + 2n - 1)$ (Sheskin, 2004).

Results

Consistent growth patterns as measured in length and mass (Fig. 3.2) between the treatment and control groups inferred there was no observable stack effect. Noticeable difference in mass measurements at Weeks 0 and 1 were due to refinement of measurement technique, which was not consistent with subsequent weekly measures, and resulted in an outlier.

At posthatch, mean absolute glomerular volume was largest for $lG_{3/4}$ (mean = 133.03 x 10^{-6} mm^3, SE = 23.71 x 10^{-6} mm^3, n = 15), followed by lG_1 (mean = 94.79 x 10^{-6} mm^3, SE = 9.01 x 10^{-6} mm^3, n = 17) and smallest for lG_6 (mean = 70.80 x 10^{-6} mm^3, SE = 6.31 x 10^{-6} mm^3, n = 10). Paired t-tests indicated olfactory enrichment did not affect glomerular volume for any of the three identified glomeruli at posthatch, as there was no

significant differences between paired means calculated for the treatment and control groups: lG_1 ($W_3 = 70$, $p = 0.20$), $lG_{3/4}$ ($t_6 = -1.69$, $p = 0.14$, $\omega^2 = 0.06$), or lG_6 ($t_4 = 0.05$, $p = 0.96$, $\omega^2 = 0.04$).

lG_1 did not increase in size with age nor with treatment in alevin (Fig. 3.4); the mean glomerular volume pooled across age was almost identical between the olfactory enriched group (mean = 88.14 x 10^{-6} mm^3, SE = 7.77 x 10^{-6} mm^3, n = 14) and control group (mean = 88.17 x 10^{-6} mm^3, SE = 10.28 x 10^{-6} mm^3, n = 14). A Wilcoxon signed rank test further confirmed there is no difference in mean glomerular volume of lG_1 pooled across age between the olfactory enriched and control groups ($Z_{13} = 134$, $p = 0.30$). lG1 glomerular volume, pooled to include both olfactory enriched and control glomerular volumes, did not increase from posthatch to emergence, as indicated by a linear regression analysis (Adj $r^2 = -0.04$, $t_{27} = -0.16$, $p = 0.87$, $\omega^2 = -0.02$).

Response magnitude (lG_1 $volume_{olfactory\ enriched}$ / lG_1 $volume_{control}$) was further calculated for lG_1 (Fig. 2.5). A one-sample t-test was applied to the response magnitude ratios pooled across age, and indicated the mean ratio of olfactory enriched glomerular volume to control glomerular volume, 1.10, did not significantly differ from one ($t_{13} = 0.94$, $p = 0.37$, 95% CI = 0.87, 1.33, $\omega^2 = < 0.01$), again showing no effect of olfactory enrichment on glomerular volume. Although visual assessment of the response magnitude at Week 1 suggested glomerular volume may have been larger in olfactory enriched individuals, statistical power was too low to support this observation ($t_3 = 1.38$, $p = 0.26$, 95% CI = 0.56, 2.12, $\omega^2 = 0.10$). Alternately, glomerular volume decreased with olfactory enrichment at Week 2, but also was not supported statistically ($t_3 = -2.47$, $p = 0.09$, 95% CI = 0.43, 1.07, $\omega^2 = 0.39$).

Discussion

The influence of sensory experience on neural circuitry in the central nervous system offers an anatomical metric to assess whether olfactory experience can be evaluated by notable changes in glomerular volume. Although such experiments have been applied to laboratory organisms, these studies have broader ecological implications, as this neural plasticity allows fish to adapt to their diverse environments (Gonda et al., 2011). Salmonids passively occupy redds during the first post-hatch developmental stage as alevin where they are stimulated by an array of olfactory cues that are important for survival once the fish emerge. Glomeruli, therefore, were expected to undergo a corresponding period of refinement due to the excitation of OSNs by environment-specific odour molecules. In this experimental study, however, we did not measure a noticeable difference in glomerular volume in Chinook salmon exposed to odours at any period during the alevin developmental stage. Additionally, the absolute volume of lG_1 did not change with age between hatch and emergence, despite the increase in body mass and length. These results contradict with previous findings, as the absolute glomerular area of three lateral glomeruli steadily increased after hatch in zebrafish (Braubach et al., 2013). However, other glomeruli did not exhibit a change in area until 21 days posthatch, well beyond emergence in this species (Braubach et al., 2013).

Noticeable trends in lG_1 glomerular volume, with larger glomerular volume with olfactory enrichment at Week 1 and smaller glomerular volume with olfactory enrichment at Week 2, alludes to a possible sensitive period to olfactory sensory

stimulation. The increase in glomerular volume may be attributable to OSN axon recruitment, corroborated by an increase in OSN number in the nose of mice in response to olfactory stimulation (Jones et al., 2008). The subsequent observed decline in glomerular volume at Week 2 may be due to the pruning of unstimulated OSN axons, or tighter synaptic connections with output neurons. Decrease in glomerular volume in response to olfactory enrichment has been observed in previous studies (Devaud et al., 2003, Braubach et al., 2013). However, without previous knowledge about development of glomerular territories and their corresponding glomeruli in Chinook salmon, it is difficult to predict a potentially critical stage when sensory experience will manifest at the glomerular level. Different developmental patterns are observable in zebrafish glomerular territories, showing one of three patterns: a sudden decrease after hatch and little subsequent growth, steady growth followed by abrupt increase in size at the mid-larval stage, or steady growth from hatch (Braubach et al., 2013). Of the glomeruli targeted by Braubach et al. (2013), the area of lG_3 and lG_4 did not differ between the control and amino acid enriched group, whereas the area of lG_x was smaller in the olfactory enriched group. The glomerulus monitored in this study, lG_1, followed the growth patterns observed in the former group with a decline in size at the mid-alevin stage, followed by a slight increase at emergence, which may account for the overall lack of growth observed in this study.

Potential differences in glomerular volume between the amino acid-exposed and control groups may have been convoluted by the large variation in interspecific mean glomerular volume. Although the general shape of each of the four lateral glomeruli identified in Chinook salmon alevin was consistent across individuals, the high variation

in volume is consistent with previous findings where prominent differences in both shape and size of a specific glomerulus, the ventroposterior glomerulus, were observed (Zebrafish, Baier and Korsching, 1994). Variation in glomerular volume may also be attributed to sex, a potentially important variable that was excluded from this study. Measureable differences in pheromone-responsive glomeruli between males and females has been observed in insects, where the glomeruli were larger, and presumably more developed, in males (found in Baier and Korsching, 1994). Polymerase chain reaction could be applied as a tool for sex identification by sourcing DNA from fin clips in future studies. The prior sensory experience of an animal may also contribute to the influence of olfactory stimulation on glomerular volume, as shown by an increase in glomerular volume in male moths pre-exposed versus naive to female pheromones (*Spodoptera littoralis,* Guerrieri et al., 2012). However, the alevin reared in this experiment were full siblings, and raised in identical environments, consequently exposing them to the same array of sensory experiences.

Transgenic lines of laboratory animals have advanced the study of sensory-induced neural plasticity, as these organisms facilitate the visualization of target OSN axon projections from the olfactory epithelium to corresponding glomeruli (Mice, Mombaerts et al., 1996; Insects, Galizia et al., 1999; Zebrafish, Braubach et al., 2013). These studies compliment physiological recordings that have already matched an odour molecule to its corresponding olfactory receptor. Although physiological studies support salmonids respond to the amino acids that were used to enrich the olfactory environment of the alevin in this study (Yamamoto et al., 2010; Laberge and Hara, 2003), neither specific amino acids nor other odours have been linked to corresponding glomeruli.

Amino acids do indeed target specific glomeruli in teleosts, as observed by Braubach et al. (2013), where glomerular volume changed in response to olfactory stimuli in only one of three focal lateral olfactory glomeruli, lG_x, of which an analogous glomerulus has not yet been identified in Chinook salmon (see Chapter 2). Thus, a different glomerulus may have been a more appropriate target to explore for effects of neuroplasticity in this study.

Background levels of amino acids in both incubation trays was an important variable also excluded in this study. Because the alevin were reared until emergence, feeding was not required, eliminating the need to introduce amino acids into the system as food. However, microbes constituting a biofilm may release amino acid odours into the systems, while the fish release peptides composed of amino acids (Hinz et al. 2013), possibly diluting the olfactory effect of amino acid enrichment. Future studies may benefit by applying artificial odours that would not occur in the background water, such as morpholine or phenylethyl alcohol, both of which have been successfully applied in behavioural studies investigating imprinting (Coho salmon, Dittman et al., 1995).

The effect of olfactory experience on the developing olfactory system can manifest in a number of ways not explored in this study. Sensory-dependent neural plasticity can promote neurogenesis (Cline et al., 2008), increasing gene expression of stimulated OSN morphotypes, identified by an increased OSN number in the olfactory epithelium (Jones et al., 2008), an increase of axons innervating the glomeruli, or by the application of assays to determine the level of gene expression of different olfactory receptor families. The ICC and microscopy techniques applied in this study did not offer the resolution required to record OSN axon density of the glomeruli, but alternate studies did not observe a change in OSN axon number with olfactory stimulation of target

glomeruli (Zebrafish, Braubach et al., 2013; Mice, Kerr and Belluscio, 2006). OSN axons can also coalesce upon boutons within a glomerulus, creating microglomeruli, of which some studies have reported an increase (Hourcade et al., 2010; Valle-Leija et al., 2012; Braubach et al., 2013) or decrease (Kerr and Belluscio, 2006) in number in response to olfactory enrichment. Anatomical characteristics vary with glomeruli, and the OSN axons of the focal glomerulus in this study did not appear to terminate upon boutons, but instead appeared diffusely organized. Thus, microglomeruli count was not possible for this particular analysis.

Braubach et al. (2013) postulated that lateral glomeruli develop via two processes, by increasing in size and/or by increasing number of supernumerary glomeruli. Although all lateral glomeruli investigated in this study were identifiable at hatch and persisted throughout the alevin developmental stage, the olfactory bulb and ventral glomerular territories experience noticeable growth between the hatch and mid-alevin developmental stages (See Chapter 2). OSN axons recruited due to olfactory enrichment enter the olfactory bulb anterioventrally, and may have not yet migrated to their respective glomeruli, further confounding the results.

To our knowledge, this study was the first attempt to explore the effects of olfactory experience on the central nervous system in a non-model species. The inability to control for gene expression of non-target OSN morphotypes limits the resolution of the ICC assay, inhibiting the ability to quantify subtle neural differences. Despite lack of evidence supporting the influence of sensory experience on glomerular refinement in the alevin developmental stage of Chinook salmon, it is improbable that refinement is not occurring at this stage. The olfactory system is one of the most plastic sensory systems

due to continuous neurogenesis; young organisms appear to be especially susceptible to neural changes caused by sensory system, and many exhibit critical developmental stages early in life (see Tables 3.1-3.3 for references). Aside from the aforementioned alternative metrics that can be used to quantify changes in neural circuitry, gross metrics, such as olfactory bulb size were not quantified in this study. Trade-offs between somatic growth of different sensory systems may also convolute results, and could be further explored. These results suggest the first few weeks posthatch deserves particular attention, with emphasis on increasing sample size.

This study provided the first insight into the development of specific glomeruli in a salmonid species, namely the amino acid-responsive lateral glomeruli, and created a base to further explore mechanisms underlying sensory-induced development. Factors involved in development of the olfactory system remain ambiguous (review, Valle-Leija, 2015), and integrative approaches applying anatomical, physiological and behavioural assays create ample opportunity to better understand how sensory environment affects neural plasticity, whether there exists critical stages for olfactory learning, and how this plasticity influences olfactory perception in salmonids.

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Tables and Figures

Table 3.1. Effects of sensory experience on neural circuitry of corresponding glomeruli in the antennal lobe of insects in a laboratory setting. Both passive and associative methods of odour exposure results in a change in glomerular volume, with greater influence in insects under eight days after emergence (D8).

References	Species (insects)	Developmental Stage	Method	Olfactory Trait	Results
Devaud et al. 2003	*Drosophila*	D2-5 and D8-11	Passive exposure	Antennal lobe and Glomeruli volume	AL volume increased before D12; Decrease on glomerular volume at D5, but not if exposed at D8-11
Sachse et al. 2007	*Drosophila*	D1-D12	Passive exposure	Glomerular volume	Increase with TRT
Hourcade et al. 2009	*Apis*	Adult *unspecified*	Associative exposure	Glomerular volume	Increase with TRT
Hourcade et al. 2010	*Apis*	D7+	Associative exposure	Microglomerular density	Increase with TRT
Arenas et al. 2012	*Apis*	D5-D8	Associative exposure	Glomerular volume	Increase with TRT
Sigg et al. 1997	*Apis*	D1-D10	Passive exposure	Glomerular volume	Increase with TRT

Table 3.2. Effects of passive and associative olfactory sensory experience on neural circuitry of corresponding glomeruli in the laboratory mouse olfactory bulb. Comprehensively, glomerular development has been explored in specimens ranging in development from embryo (E0-21) to postnatal day (PD) 90. Weaning occurs at around PD 21 and mice are considered adults at around PD 60.

References	Species (mammals)	Developmental Stage	Method	Olfactory Trait	Results
Zou et al. 2004	Mus	PD 0-90	Unilateral naris closure	Heterogeneity of glomerulus	Persists into adulthood with occlusion
Kerr and Belluscio 2006	Mus	PD 7-60	Aversive conditioning and passive exposure	Number of supernumerary glomeruli, OSN axon number between TRT group or across age	Fewer supernumerary glomeruli by PD13 in TRT group; No difference in OSN axon numbers
Todrank et al. 2011	Mus	Embryological to pre-weaning	Passive (in utero) Associative (nursing)	Glomerular Volume	Increase with TRT No difference in gestation vs nursing exposure
Jones et al. 2008	Mus	Adult	Passive and Associative	Glomerular volume, OSN number in nose	Increase glomerular volume with associative TRT only; increase in OSN number
Valle-Leija et al. 2012	Mus	PD 0-20	Associative	Glomerular Volume Number of supernumerary glomeruli	Increase glomerular volume with TRT; More supernumerary glomeruli

Table 3.3. Influence of passive olfactory sensory experience on neural circuitry of corresponding glomeruli in the zebrafish olfactory bulb. Zebrafish hatch at about 72 hours post fertilization (hpf) and typically remain in the larval developmental stages until about Day 45 (D45). Zebrafish larvae retain a yolk-sac until about D4 and emergence at around D9.

References	Species (fish)	Developmental Stage	Method	Olfactory Trait	Results
Braubach et al. 2013	*D. rerio*	72 hpf to D14	Passive exposure	Glomerular Area; Number of supernumerary glomeruli; OSN axon number	Glom Area: Decrease with TRT; More supernumerary glomeruli; No difference in OSN number

Table 3.4. L-amino acids and concentrations used in previous studies to investigate the effects of amino acid exposure on the olfactory responses of teleosts.

Reference	Species	Life History Stage	Observation technique	Olfactory Stimulants	Odour Concentration (µM)
Braubach et al., 2013	*D. rerio*	Larval	Anatomical (glomerular volume)	Ala, His, Lys, Met, Phe, Trp, Val	1 µM
Yamamoto et al., 2010	*O. nerka*	1-year-old	Behavioural (imprinting)	Pro, Glu	1µM
Li et al., 2005	*D. rerio*	2.5-3 DPF	Physiological (OB Ca^{2+} imaging)	Phe, Lys, Val	1000 µM
Laberge and Hara, 2003	*Salmo trutta* and *O. mykiss*	1-to 4-years- old	Physiological (EOG)	Cys, Arg, Ser, Glu, His, Ala, Lys, Asp, Gln, Met, Trp and Tyr	100 µM
Friedrich and Korsching, 1997	*D. rerio*	Adult	Physiological	Met, Ala, Lys, Asp	100 µM
Dittman et al., 1996	*O. kisutch*	Parr-to-smolt	Behavioural (imprinting)	PEA	1 µM

Table 3.5. Properties of stock solution amino acids to test whether odours affect the early olfactory development of juvenile Chinook salmon.

Amino Acid	Characteristic	Molecular Weight (g/mol)	Product Number (Sigma-Aldrich)
L-Proline	Hydrophobic	115.13	P0380
L-Histidine	Basic	155.15	H8000
L-Glutamic Acid	Acidic	147.13	G1251
L-Tryptophan	Aromatic	204.23	T0254
L-Alanine	Small Chain	89.09	A7627
L-Serine	Nucleophilic	105.09	S4500

Table 3.6. Mass of test L-amino acids required for 500 mL stock solution to inject into the amino acid incubation tray for a duration of two days ((2 mL * 4 injections / hr)*24*2).

Amino Acid	Molecular Weight	g/L	g/500mL (stock solution for two days)
L-Pro	115.13	2.60	1.298
L-His	155.15	3.50	1.750
L-Glu	147.13	3.32	1.659
L-Trp	204.23	4.61	2.303
L-Ala	89.09	2.01	1.004
L-Ser	105.09	2.37	1.185

Concentration in Stock Solution (A) = (XYMF)/Z (g/L)
X = total outflow of water from incubation tray (3 L/min)
Y = Desired conc. of amino acid in incubation tray (0.001 mmol/L)
M = Molecular Wt of amino acid used (g)
F = Molar fraction of amino acid in salt (1 because almost pure)
Z = Drip rate of amino acid solution from stock solution bottle (0.133 mL/min)

Table 3.7. Collection of Chinook salmon reared in control and amino acid treatment conditions for comparison of olfactory bulb glomerular volume. All specimens belong to one family with same parentage, facilitating a repeated-measures design despite destructive sampling.

Age (weeks)	Developmental Stage	# Samples / Control	# Samples / Treatment	Samples successfully labelled
0	50% Hatch	6	7	0
1	Posthatch Alevin	15	15	18
2	Posthatch Alevin	15	10	16
3	Alevin	15	15	0
4	Alevin	15	15	0
5	Mid-Alevin	15	15	2
6	Mid-Alevin	15	15	2
7	Alevin	15	15	0
8	Alevin	15	15	0
9	Emergent fry	15	15	4
10	Emergent fry	15	15	4
	SUB TOTAL	**156**	**152**	
		TOTAL	**308**	**46**

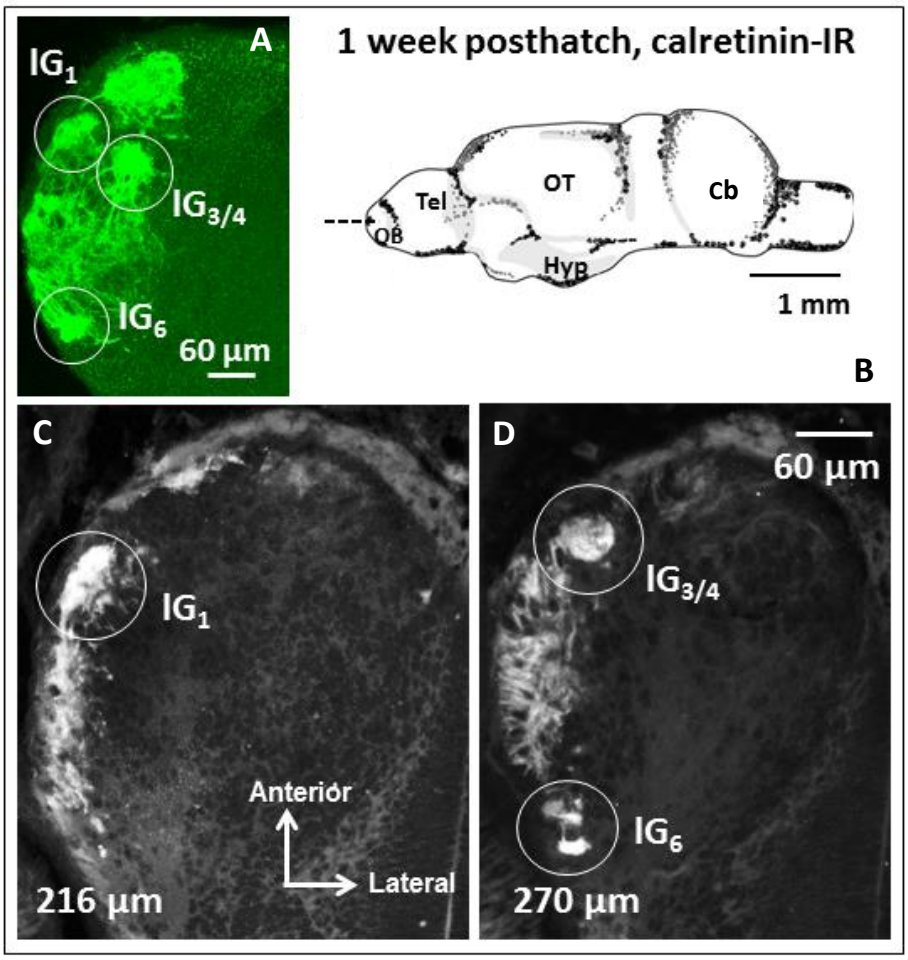

Figure 3.1. Somatotopic organization of lateral glomeruli in Chinook salmon posthatch alevin. A: Lateral view of confocal z-stack (57 sections with a 1 µm z step) showing identifiable calretinin-immunoreactive lateral glomeruli. B: Horizontal sections of olfactory bulb labelled in C and D. OB = Olfactory Bulb, Tel = telencephalon, OT = Optic Tectum, Cb = cerebellum. C,D: Calretinin- immunoreactive lateral glomeruli (lG_1, $lG_{3/4}$, lG_6) represented at actual bulbar depth from dorsal (C) to ventral (D). All glomeruli are identifiable throughout the alevin developmental stage.

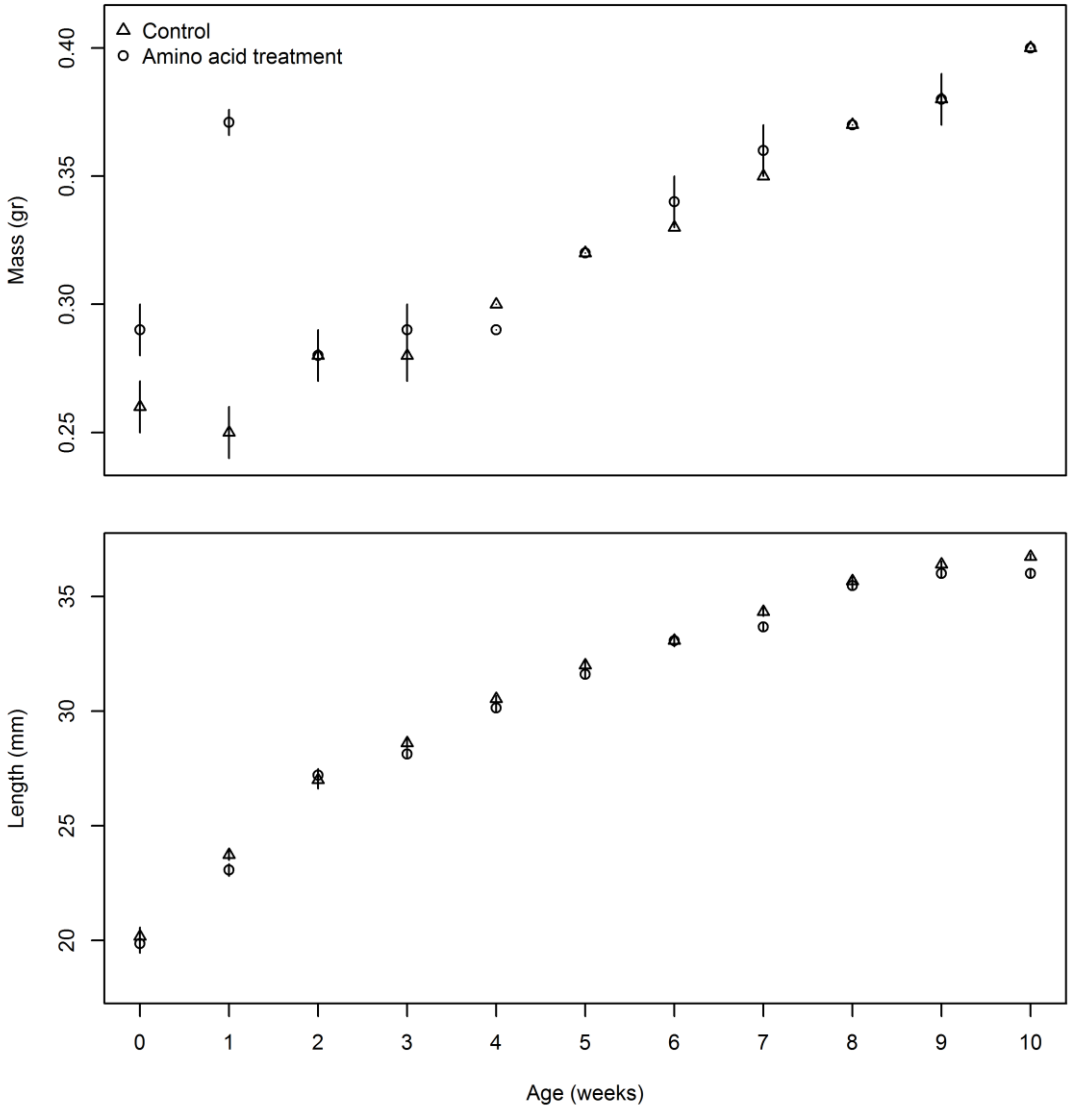

Figure 3.2. Growth charts (mass and length) for Chinook salmon alevin collected from hatch to emergence. Length tapers between weeks eight and ten, inferring entry into emergence. The apparent variability is mass at Weeks 1 and 2 are due to inconsistencies in measurement techniques, and thus measurement error, accounting for the outlier at Week 1.

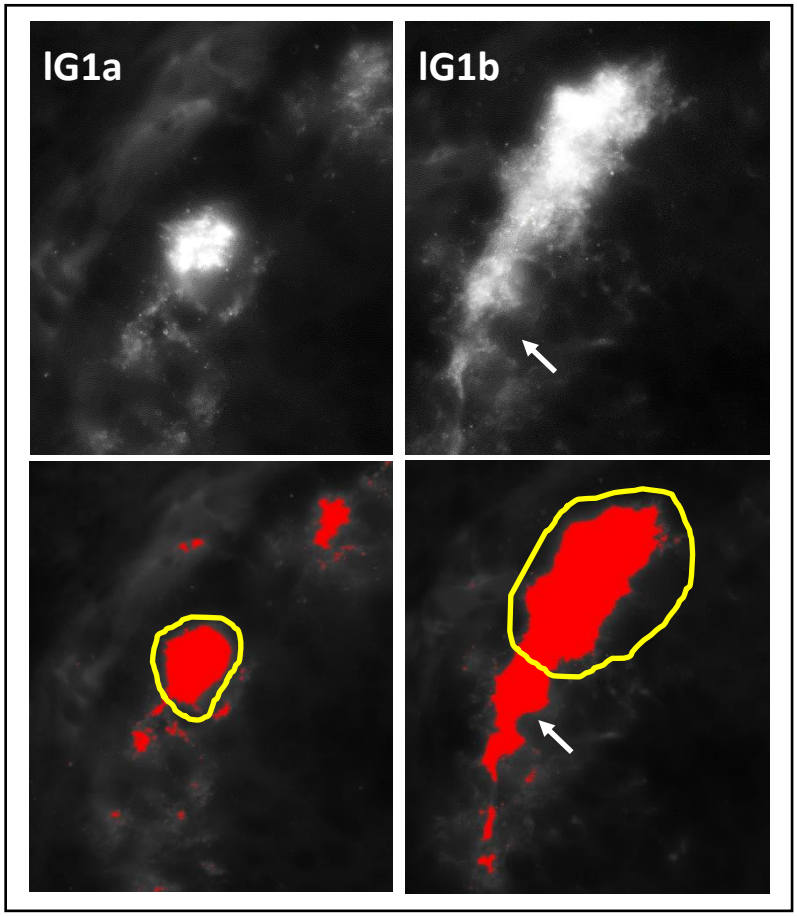

Figure 3.3. Micrographs of serial sections of a single posthatch Chinook salmon alevin lateral glomerulus (lG1a and lG1b) labelled against calretinin allowed for calculation of area using ImageJ with colour threshold adjustment to outline glomerulus. Area was calculated for coloured image within yellow line. OSN axons projecting into the glomerulus were excluded from the analysis (arrows).

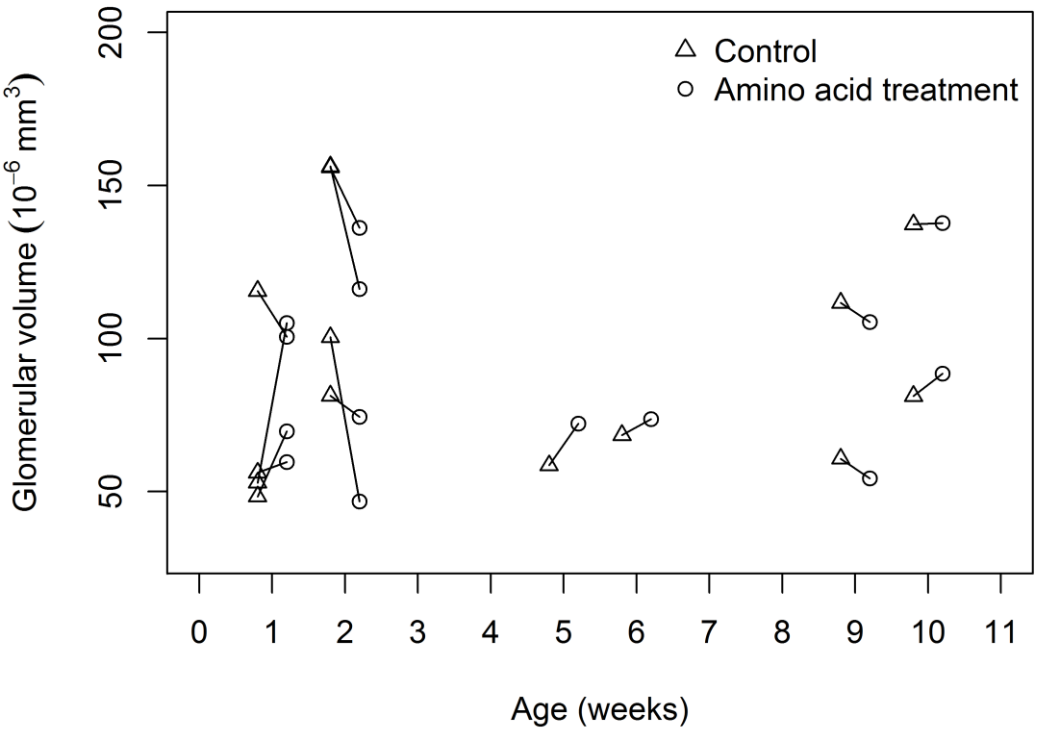

Figure 3.4. Glomerular volume of lG_1 in 29 paired samples from hatch to emergence. Glomerular volume was not different between olfactory enriched and control groups ranging in age from 1-week (posthatch) to 10-weeks (emergence). Samples that are connected with a solid line were paired to control for the effect of tissue handling associated with immunocytochemistry techniques. Pooled glomerular volume inclusive of all samples did not increase with age (Adj $r^2 = -0.04$, $t_{27} = -0.16$, $p = 0.87$, $\omega^2 = -0.02$).

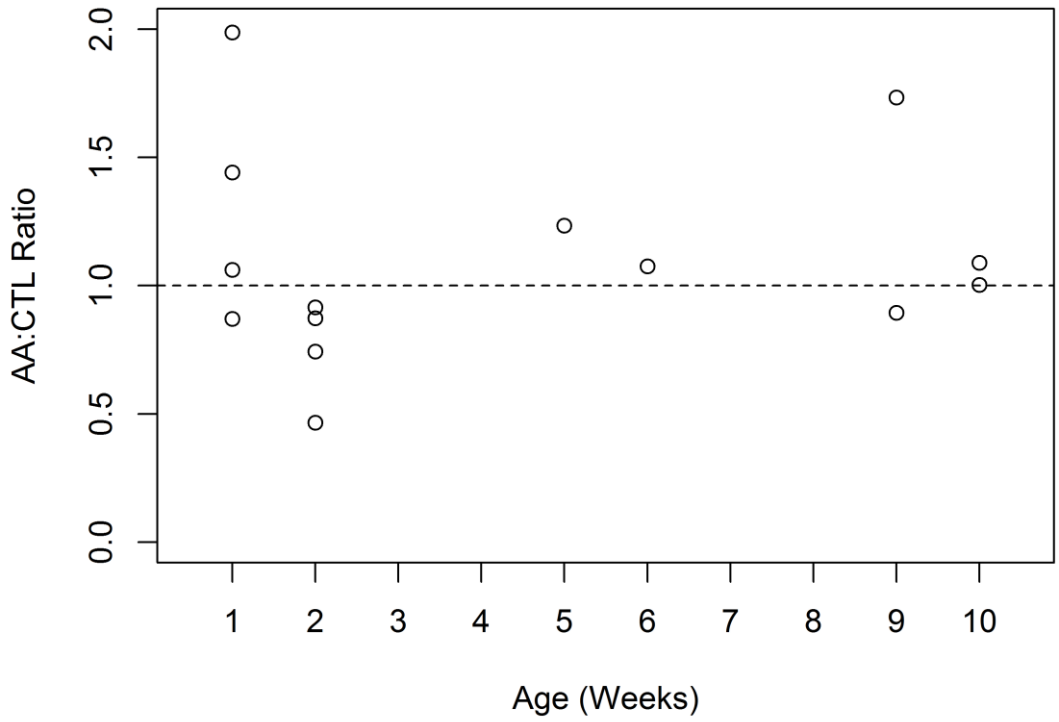

Figure 3.5. Response magnitude calculating the glomerular volume ratio of olfactory enrichment to control groups for lG_1. Each ratio reflects a paired sample that was run concurrently to standardize the effect of tissue processing on glomerular volume. Any value that exceeds one indicates the glomerular volume was higher for the olfactory enrichment group than the control group. No effect of olfactory enrichment on glomerular volume is observed ($t_{13} = 0.94$, $p = 0.37$, 95% CI = 0.87, 1.33, $\omega^2 = < 0.01$).

Chapter 4: General Discussion

Summary

Axons from a single olfactory sensory neuron (OSN) morphotype that express a distinct olfactory receptor coalesce upon a single glomerulus (Mombaerts et al., 1996), which is consequently activated by a single odour cue (Friedrich and Korsching, 1997; Mori et al., 2006). A species-specific description of glomerular patterning, conglomerates of OSN axons in the olfactory bulb glomerular layer (reviewed in Firestein, 2001), is a baseline tool that can be applied to link an olfactory stimulus to a discreet glomerulus. Integrated with behavioural or physiological studies, this proximate mechanistic approach can be applied to determine whether sensory experiences, especially those associated with learning and experience, are manifested at the neural level.

Sensitivity to environmental olfactory stimuli should increase expression of the stimulated olfactory receptor expression, consequently altering the glomerular territories to which the corresponding OSN axon is recruited (See Tables 3.1-3.3 for references). This adaptive mechanism increases the animal's acuity to environment-specific olfactory cues. Glomerular patterning is well established in insect (Vosshall and Wong, 2000) and mouse, *Mus*, (Mombaerts et al., 1996) models, as well as in zebrafish, *Danio rerio*, (Baier and Korsching, 1994; Gayoso et al., 2011; Braubach et al., 2012, 2013). The coarse special organization of glomerular territories has been described also in non-model species, including rainbow trout, *Oncorhynchus mykiss*, (Riddle and Oakley, 1992), but has not comprehensively identified the innervation by different OSN morphotypes nor specific glomeruli. The objectives of this thesis were to describe the glomerular patterning in a socio-economically and ecologically important species, Chinook salmon,

O. tshawytscha, which rely heavily on chemosensory cues for survival and reproduction. In my first data chapter (Chapter 2), I applied immunocytochemistry techniques to identify distinct glomerular territories and define the territories innervated by amino acid-stimulated microvillous OSNs in emergent fry (9- to 13-weeks-posthatch). Additionally, the ontogeny of the coarse organization of glomerular territories was described from posthatch (1- to 2-weeks posthatch) to emergence, a proposed sensitive period for olfactory learning (Dittman et al., 2015). All glomerular territories previously identified in adult rainbow trout (Riddle and Oakley, 1992) were visible at the earliest stage investigated and persisted into emergence, with no observable family effect. The glomerular territories, however, showed signs of refinement by expanding in size and showing increased coalescence of axon endings into glomeruli. Conversely, lateral glomeruli analogous in location to zebrafish amino acid-responsive glomeruli (Braubach et al., 2013) did not show any modification with respect to size nor organization from hatch to emergence. This glomerular map was applied in the second data chapter (Chapter 3) to experimentally test whether sensory experience, specifically exposure to amino acid odours, affected the development of the putative amino acid-responsive glomeruli found in the lateral glomerular territory. The lateral glomeruli of Chinook salmon alevin exposed to amino acid odours from hatch to emergence did not show a volumetric difference to those in alevin not exposed to any odours. The outcomes of this research are summarized in more detail in this chapter, and possible future research initiatives deriving from the results are outlined.

Overview of Chapter 2

Odour discriminatory abilities may vary with species and life history (reviewed in Kasumyan, 2011), and should correlate with ontogenic differences of glomerular organization. The ontogeny of glomerular patterning has been described in zebrafish only recently (Braubach et al., 2013), and although the coarse organization of glomerular territories has been described in adult rainbow trout (Riddle and Oakley, 1992; Porteros et al., 1997), little is known about the ontogeny of glomerular patterning during the alevin developmental stage in salmonids. Consequently, the aim of this study was to outline the progression of glomerular organization from posthatch to emergence using a general label, keyhole limpet hemocyanin (KLH), that targets all olfactory sensory neuron morphotypes and calretinin, a calcium-binding protein that specifically targets amino acid-responsive microvillous olfactory sensory neurons.

Seven of nine consistently identifiable KLH-immunoreactive glomerular territories described in adult rainbow trout (Riddle and Oakley, 1992) were found in the Chinook salmon emergent fry olfactory bulb. The two remaining glomerular territories not identified in the Chinook salmon emergent fry may reflect discrepencies in glomerular territory identification, where the ventroposterior glomerular territory identified in Chinook salmon may be analogous to two ventral territories identified in rainbow trout. Additionally, the lateral glomerulus 2 identified in Chinook salmon emergent fry may be analogous to the dorsal posterior lateral territory identified in rainbow trout (Riddle and Oakley, 1992). Calretinin-immunoreactive olfactory sensory neurons innervated two territories: the dorsolateral and lateral glomerular territories. Four specific glomeruli belonging to lateral glomerular territory were identified, and include lG_1, lG_2, $lG_{3/4}$, and lG_6. As in zebrafish (Braubach et al., 2013), lG_2 was immunoreactive

solely to KLH, while the remaining were immunoreactive to both KLH and calretinin. The seven KLH-immunoreactive glomerular territories appear to expand with the somatic growth of the olfactory bulbs from the posthatch to emergent fry developmental stages, as characterized by comparing micrographs set to the same scale between the two developmental stages. The calretinin-immunoreactive glomeruli identified in the lateral glomerular territory, however, appeared to remain constant in size and organization from posthatch to emergence, visual observations that were quantitatively confirmed in the third chapter of this thesis. These findings suggest that although there must be changes in glomerular number or size with an increase in glomerular territory size, not all glomeruli grow in conjunction with the territory. There did not appear be any across-family differences in glomerular organization in emergent fry.

Numerous studies have investigated glomerular patterning across taxa, creating a tool that can be applied to test the effects of sensory experience on neurodevelopment. This study revealed arguably the most comprehensive description of the ontogeny of glomerular organization during the alevin developmental stage in a salmonid species. Glomerular patterning observed in this study was consistent with that described in adult zebrafish (Baier and Korsching, 1994; Gayoso et al., 2011; Braubach et al., 2012), larval zebrafish (Braubach et al., 2013), and adult rainbow trout (Riddle and Oakley, 1992). These findings suggest glomerular patterning is determined sometime prior to hatch, and persists into adulthood. The growth and increased organization within glomerular territories during the alevin developmental stage implies the earliest patterning may provide a template for OSN recruitment and for refinement of synaptic connections with

the output neurons, mitral cells, as has been suggested for zebrafish (Miyasaka et al., 2013).

Across-species analogies in glomerular patterning was highlighted by the homologous organization and innervation of lateral glomeruli between Chinook salmon alevin and larval zebrafish. However, species-specific discrepancies in overall organization were observed. Only four of the six distinct lateral glomeruli identified in larval zebrafish (Braubach et al., 2013) were recognizable in Chinook salmon alevin. However, $lG_{3/4}$ may indeed be two separate glomeruli, but could not be differentiated using horizontal sections. Furthermore, calretinin-immunoreactive OSN axons did not innervate ventral glomerular territories as observed in zebrafish (Braubach et al., 2012) and brown trout (Castro et al., 2008), nor did they innervate the dorsal glomerular territory as observed in zebrafish (Braubach et al., 2012). Species-specific organization may allow populations to be more receptive to olfactory cues specific to their environment.

Although labelling against calretinin facilitated the targeting of amino-acid responsive microvillous OSNs in this thesis, the expansion of ICC techniques could further identify specific OSN morphotypes innervating each glomerular territory, better linking odour class with corresponding glomerular fields. Ciliated OSNs are immunoreactive to Gα s/olf, a calcium-binding protein, and have been successfully targeted in sea lamprey (Frontini et al., 2003), channel catfish, *Ictalurus punctatus*, (Hansen et al., 2003), and zebrafish (Koide et al., 2009, Gayoso et al., 2011, Braubach et al., 2012, 2013). Crypt cells have been identified by labelling against the calcium-binding proteins S100 (zebrafish, Braubach et al., 2012, 2013). Establishment of whole-mount

immunocytochemistry techniques (Mollusks, *Placopecten magellanicus*, Too and Croll, 1995; Zebrafish, Braubach et al., 2012, 2013) negates sectioning of the bulb, as confocal microscopy allows for the visualization of glomeruli at different bulbar depths. Whole-mount preparations thus facilitate more comprehensive description of OSN innervation into glomeruli. Additionally, this method allows for improved quantitative descriptions of glomerular patterning, such as determining glomerular volume contextual to total bulbar volume. Such techniques have been applied to map glomerular organization in zebrafish (Gayoso et al., 2011; Braubach et al., 2012, 2013; White et al., 2015).

The presence of glomerular territories posthatch does not imply the alevin are responsive to odours, and different glomeruli appear to mature at different stages in zebrafish (Braubach et al., 2013). Physiological assays may assist in identifying the developmental stages at which odours are identifiable. Electro-olfactograms applied to an *in vivo* preparation would indicate whether OSNs in the epithelium are stimulated by odour application (eg. Sveinsson and Hara, 2000). Physiological techniques including single-unit (eg. Friedrich and Laurent, 2004) or multi-unit electrode (eg. Christensen et al., 2000) recordings in the olfactory bulb may reveal which odour molecule stimulates which glomerulus, or which odour ensemble stimulates a glomerular array.

As sensory stimulation does not imply behavioural responses, as inferred by glomerular stimulation of glomeruli by alarm cues (Mathuru et al., 2012) without behavioural response until 50 days posthatch (Waldman, 1982), behavioural assays would elucidate whether physiological and behavioural responses occur in conjunction during development.

The congruency in glomerular patterning between salmonids and zebrafish that

has been revealed in this study may allow findings pertinent to the zebrafish model to be extrapolated and applied to salmonid studies, accelerating the understanding of this system. For example, ICC techniques applied to target OSN morphotypes should be transferable. Physiological assays that link an odour molecule to a glomerulus in zebrafish may also be transferable should the salmon olfactory bulb contain an analogous glomerulus. The ontogeny of glomerular patterning in alevin has ecological implications, as olfactory stimulation may be required to allow glomeruli to fully develop and be functional, an especially relevant matter for fish reared with the intent to augment natural populations.

Overview of Chapter 3

Environmental enhancement in hatcheries has been correlated with differences in brain structures and behaviour of salmonids reared in enhanced versus non-enhanced environments (Lema et al., 2005). These studies have focused on physical, rather than sensory enrichment, which is arguably more relevant to brain development. Olfactory experience has been associated with changes in glomerular volume (Mice: Todrank et al., 2012, Valle-Leija et al., 2012; Insects: Devaud et al., 2003; Hourcade et al., 2010; Arenas et al., 2012; Fish: Braubach et al., 2013), likely in response to recruitment of olfactory sensory neurons of the stimulated morphotype, or tighter synaptic connectivity of already established olfactory sensory neurons with output neurons. Chinook salmon rely on a functional olfactory system to identify odourants, such as amino acids, which are important for feeding (Mearns, 1986) and arguably kin recognition (Hinz et al., 2013) in addition to homing (Shoji et al., 2000), warranting investigation of the effect of olfactory

experience during the alevin development stage on neurocircuitry within the olfactory bulb. Specifically, I experimentally tested whether exposure of alevin from hatch to emergence to a 0.1 µM solution of amino acids resulted in a difference in corresponding calretinin-immunoreactive glomerular volume between the treatment and control (no amino acid exposure) group. Glomerular volume did not significantly differ between the treatment and control groups at any point from posthatch to emergence, nor did absolute glomerular volume increase with the increase in olfactory bulb thickness corresponding with alevin maturation. However, glomerular volume of lG1 trended towards a larger size with olfactory enrichment in week 1 posthatch followed by a decrease in size with enrichment at week 2 posthatch. An increased sample size in conjunction with increased resolution may determine whether these trends are indicative of OSN axonal recruitment at week 1 posthatch, followed by OSN axon pairing and/or tighter synaptic connections at week 2 posthatch, the latter which was observed by Braubach et al. (2013).

Contradictory to my findings, zebrafish exposure to 0.1 µM amino acid odours for two weeks after hatch resulted in a steady decrease in glomerular volume (Braubach et al., 2013). Although my study targeted three putatively amino acid-stimulated lateral glomeruli that correspond with those identified in zebrafish, the appropriate glomerulus may not have been identified. Transgenic lines expressing green fluorescence protein in amino acid responding OSNs was applied to the zebrafish model, which provide for clear resolution GFP expressing neurons. Thus future studies can better target amino acid-stimulated glomeruli by applying electro-physiological techniques to an *ex vivo* preparation. Insertion of electrodes into targeted olfactory bulbar regions can detect neural activity associated with specific olfactory stimuli. Such physiological studies have

been used to link odours with neural activity in the subclass teleostei olfactory bulb (Døving et al., 1980; Hara and Zhang, 1998; Friedrich and Korsching, 1998; Laberge and Hara, 2003). Combined with whole-mount ICC techniques, target glomeruli may be identified. The lack of overall growth of the target glomeruli may also suggest that either a critical stage for sensory-induced refinement was exceeded (prehatch) or has not yet occurred (post-emergence). Therefore, embryos could also be targeted in future studies to investigate whether a critical stage for sensory-refinement exists. Inclusion of behavioural assays to test whether developing salmon are attracted to the corresponding odour would further support any findings.

The plasticity of the central nervous system in fishes is assumed to be adaptive, allowing individuals to show increased receptivity to environmentally relevant chemosensory cues, and thus likely increasing likelihood of survival. Pursuit of this research topic should be of great interest to fish husbandry practices with the objective of improving survivorship of released fry and increasing return rates of spawning salmon. However, the lack of observable effect of amino acid enrichment on volume of lateral glomeruli found in this study suggests artificial amino acid solutions may not be sufficient for imprinting in Chinook salmon alevin. In light of these findings, alternative approaches to olfactory imprinting hatchery-reared salmonids should be further explored and compared for efficacy. (Dittman et al., 2015)

Conclusions

My research identified distinct glomerular territories in conjunction with the ontogeny of their organization within the olfactory bulb glomerular layer in Chinook salmon alevin from hatch to emergence. Techniques targeting lateral glomeruli innervated by the microvillous OSN morphotype facilitated the identification of putatively amino acid-stimulated glomeruli. Although an effect of olfactory enrichment on glomerular volume was not observed, the lack of increase in absolute glomerular volume with age is contradictory with previous findings in zebrafish. Together, my results demonstrate that glomerular patterning is established as early as hatch and persists at least until emergence. The consistency in glomerular patterning observed between Chinook salmon alevin, rainbow trout and zebrafish suggests this patterning is a highly conserved trait in teleosts. Further studies exploring the effect of sensory experience on glomerular organization and olfactory-mediated behaviour is important to test whether critical periods for olfactory learning or imprinting exists in this species, particularly during the first few weeks posthatch.

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Chapter 2

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Best, Trevor

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Barbara Zielinski <zielin1@uwindsor.ca> Wed, Apr 22, 2015 at 11:15 PM To:
Courtney Ochs <ochs3@uwindsor.ca>, Trevor Pitcher <tpitcher@uwindsor.ca>

I agree with the use of this material for your thesis

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Chapter 3

Courtney Ochs <ochs3@uwindsor.ca> Wed, Apr 22, 2015 at 2:41 PM To:
Trevor Pitcher <tpitcher@uwindsor.ca>, Barbara Zielinski <zielin1@uwindsor.ca>

Hello Barb and Trevor,

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Thank-you,
Cory

Trevor Pitcher <tpitcher@uwindsor.ca> Wed, Apr 22, 2015 at 4:55 PM To:
Courtney Ochs <ochs3@uwindsor.ca>, Barbara Zielinski <zielin1@uwindsor.ca>

Cory,
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Barbara Zielinski <zielin1@uwindsor.ca> Thurs, Apr 23, 2015 at 6:39 AM To:
Courtney Ochs <ochs3@uwindsor.ca>, Trevor Pitcher <tpitcher@uwindsor.ca>

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