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Organization of Glomerular Territories in the Olfactory Bulb of Post-Embryonic Wild Chinook Salmon *Oncorhynchus tshawytscha*

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ABSTRACT The post-embryonic odor imprinting paradigm suggests Chinook salmon (*Oncorhynchus tshawytscha*) acquire memory to stream-specific amino acid olfactory odors prior to emergence as fry. Because effects of olfactory experience on development can be examined by mapping olfactory sensory neurons extending into distinct territories of glomerular neuropil in the olfactory bulb, glomerular patterning from early yolk-sac larva to fry was documented in wild salmonids, a temporal scale not yet thoroughly explored. Labeling olfactory sensory neurons with anti-keyhole limpet hemocyanin (anti-KLH) revealed seven spatially conserved glomerular territories visible at hatch and well established by the late yolk-sac larva developmental stage. Because of the responsiveness of microvillous olfactory sensory neurons to amino acids, corresponding glomeruli in the lateral bulbar region were mapped using anti-calretinin. The dorsolateral territory, distinct glomeruli of the lateral glomerular territory and the ventromedial glomeruli were immunoreactive to both KLH and calretinin. This study offers a morphological description of glomerular patterning in post-embryonic stages in wild Chinook salmon, a temporal window previously shown to be significant for olfactory imprinting. J. Morphol. 278:464–474, 2017. © 2017 Wiley Periodicals, Inc.

KEY WORDS: Chinook salmon; olfaction; glomeruli; keyhole limpet hemocyanin; calretinin; alevin

INTRODUCTION

Imprinting is a form of learning and recognition memory that is acquired during a temporal window known as the critical period (Bateson, 1990). The influence of olfactory experience on neuroanatomical development has been explored in vertebrate and invertebrate models (e.g., Devaud et al., 2003; Sachse et al., 2007; Todrank et al., 2011; Arenas et al., 2012; Valle-Leija et al., 2012; Braubach et al., 2013), but the identification of these critical developmental periods is challenging. A hallmark hypothesis of olfactory imprinting is salmon homing migration (Hasler and Wisby, 1951), in which salmonids imprint to stream-specific odor cues during or prior to downstream migration, and, as adults years later, follow these cues to their natal streams to spawn. Stream-specific compositions of dissolved free amino acids have been implicated as the putative olfactory cues that guide Pacific salmonids, *Oncorhynchus* spp, navigating to natal streams to spawn after three to seven years in open waters (Shoji et al., 2000; Ueda, 2012). A critical period for olfactory imprinting in salmonids may occur during post-embryonic development, prior to emergence from gravel on the river bottom (e.g., Tilson et al., 1994; Dittman et al., 2015). Imprinting during this larval period is further supported by isotopic analysis of adult otoliths, which demonstrated salmon occupy an average of four chemically distinct streams between emergence and smoltification, yet home to their chemically distinct natal stream as adults (Shrimpton et al., 2014). This focus on post-embryonic imprinting warrants investigation of olfactory neural components that respond to amino acid odors in wild Pacific salmonids from hatch to emergence from the nests.

Developing olfactory sensory neurons extend an axon from the olfactory epithelium in the nasal cavity, into the olfactory nerve, and onto the olfactory bulb, where the axons of neurons expressing the same odor receptor aggregate into olfactory glomeruli. These glomeruli are regions of high synaptic connectivity and are functional units of odor discrimination (Shepherd et al., 2004). The axons of olfactory sensory neurons responsive to similar odors aggregate, forming consistently patterned glomerular territories (Mombaerts et al., 1996; Dynes and Ngai, 1998; Vosshall et al., 2000; Wang et al., 2003). Physiological studies have shown the functional role of glomerular pattering for odor

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discrimination across taxa (zebrafish, Friedrich and Korsching, 1998; insects, Lei et al., 2004; mice, Marks et al., 2006), and glomerular patterning yields a valuable tool to assess the responsiveness of glomerular territories or individual glomeruli to specific odors (Friedrich and Korsching, 1997, 1998; Mori et al., 2006). Glomerular patterning has also been examined in studies of passive and associative olfactory sensory experience, including tests for critical stages for olfactory imprinting and learning in various animals (Zou et al., 2004; Kerr and Belluscio, 2006; Jones et al., 2008; Todrank et al., 2011; Braubach et al., 2013). Similar glomerular patterning across phylogenetically distantly related rainbow trout (Riddle and Oakley, 1992; Porteros et al., 1997) and zebrafish (Gayoso et al., 2011; Braubach et al., 2012) was revealed by immunolabelling teleost olfactory sensory neurons against the metalloprotein keyhole limpet hemocyanin (KLH). KLH-immunoreactive glomerular territories were mapped in juvenile Chinook salmon *Oncorhynchus tshawytscha* at 0.1-, 2-, and 4-months-post-hatch (Jarrard, 1997), and the organization of separate glomeruli comprehensively described during post-embryonic development of zebrafish (Braubach et al., 2013).

Amino acids are putative homing cues for salmonids (Ueda, 2012). In rainbow trout, amino acid odors stimulate a subset of microvillous olfactory sensory neurons (Sato and Suzuki, 2001). The axons of microvillous olfactory sensory neurons terminate in glomeruli in the lateral region of the olfactory bulb where stimulatory activity by amino acid odors has been observed in both zebrafish (Friedrich and Korsching, 1998; Sato et al., 2005; Koide et al., 2009; Braubach et al., 2012) and salmonids (Doving et al., 1980; Hara and Zhang, 1998; Laberge and Hara, 2003). The mapping of the corresponding glomeruli in Chinook salmon larvae is of particular interest, and requires a label that binds to microvillous olfactory sensory neurons. The immunolabeling of calretinin, a calcium binding protein, targets the dendrites of microvillous olfactory sensory neurons in embryonic, larval, and adult salmonids (Porteros et al., 1997; Castro et al., 2008). In zebrafish adults and larvae, calretinin immunoreactivity labels the dendrites, axons, and lateral glomerular territories of microvillous olfactory sensory neurons (Castro et al., 2006; Germanà et al., 2007; Duggan et al., 2008; Braubach et al., 2012, 2013). Calretinin immunoreacivity has also been seen in zebrafish ciliated and crypt olfactory sensory neuron neurons (Paskin et al., 2011) and in dorsal and posterior glomeruli associated with these morphotypes (Gayoso et al., 2011; Koide et al., 2009). Therefore, the analysis of bulbar KLH and calretinin immunocytochemistry provided a base to develop a comprehensive map of glomerular patterning in post-embryonic Chinook salmon. We hypothesized that a visible template for glomerular territory development was present in early yolk-sac larvae, with refinement in late yolk-sac larvae and during emergence from nesting sites at the fry stage, to facilitate a functioning olfactory system during this early critical period for imprinting to home stream odors.

In this study, the ontogeny of glomerular patterning was examined at three incremental stages: 1–2 weeks early yolk-sac larvae (539–590 degree days), late yolk-sac larvae (643–720 degree days), and at the fry stage (798–996 degree days) during emergence and before the onset of feeding. The glomerular territories and individual lateral glomeruli were mapped using serial sections, and wholemount immunocytochemistry was utilized to visualize the arrangement of individual lateral calretinin immunoreactive glomeruli. This study comprehensively describes glomerular territory patterning, identifies specific microvillous olfactory sensory neuron innervated lateral glomeruli, and provides a framework to further explore the olfactory responsiveness of glomerular territories and individual glomeruli during a developmental stage in Pacific salmonids when sensory learning may be critical for the recognition of imprinted odors.

MATERIALS AND METHODS

Fertilization and Rearing Conditions of Lacustrine Chinook Salmon

Spawning Chinook salmon, *O. tshawytscha* (Walbaum, 1792), were collected using standard electro-shocking techniques (see Pitcher and Neff, 2006; Butts et al., 2012 for details) from the Credit River, Mississauga, Ontario (43° N, 79° W), during the upstream migration from Lake Ontario in October 2012 and 2013. Eggs were collected from females and milt from males within a 1-hour timeframe, and the gametes transferred to the University of Windsor within 4 to 6 h of collection. To accommodate across-family comparison of glomerular patterning in larvae, eggs from each female were fertilized using milt from a unique male, creating seven distinct family groups. Eggs and larvae from each cross were reared in separate incubation trays within a stack sourced by a flow-through of dechlorinated municipal water. Water temperature was monitored throughout this period using HOBO temperature loggers to record temperature every 15 min. The calculation of degree days (dd), an equation incorporating the average daily water temperature from fertilization to each collection date (Crisp, 1981) enabled an accurate assessment of larval age. 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 AUPP 13-01.

Sample Collection and Tissue Preparation

Larvae and fry were euthanized by anaesthetic overdose (1g/L MS-222; pH 7.4), decapitated over the gills, and heads drop-fixed in 4% paraformaldehyde in 0.1 mol l^{-1} phosphate buffer saline (PBS, pH 7.4), then further dissected by removing the mandible, tissue caudal to the eyes, and dorsal skin to expose neural tissue, and post-fixed in fresh fixative. Tissue was cryoprotected by immersion in a 20% and 30% sucrose gradient in 0.1 mol l^{-1} PBS overnight. Horizontal 16–30 μm thick serial sections were sectioned using a cryotome (Leica CM 3050A) and collected onto microscope slides. Sections were left to dry at room temperature before storing at −20°C.

Immunocytochemistry

The organization of the glomerular territories was mapped by applying immunocytochemical labeling techniques adapted from previous protocols. Olfactory sensory neurons were immuno-labelled against KLH (Jarrard, 1997; Gayoso et al., 2011; Braubach et al., 2012) to provide a description of the coarse organization of olfactory glomeruli in the olfactory bulb. The inferred amino acid responding olfactory sensory neurons extending into the lateral glomerular territory (e.g., Sato and Suzuki, 2001; Sato et al., 2007) were identified by labelling against calretinin (Castro et al., 2006; Gayoso et al., 2011; Braubach et al., 2012). Slides containing serial sections of the olfactory bulb were rehydrated in 0.1 mol l^{-1} PBS (pH 7.4) with 0.1% triton X-100 (PBS-T) for 30–40 min to increase the permeability of the tissue before blocking with 10% goat serum (Sigma-Aldrich, Oakville ON, G9023 or) in 0.1 mol l^{-1} PBS for 30 min, then placed in primary antibody (1/500 anti-KLH or 1/200 anti-calretinin in 0.1 mol l^{-1} PBS with 0.1% sodium azide 0.1 mol l^{-1} PBS) for 3 days on a shaker at 4°C. Slides were rinsed six times for 50 min each in 0.1 mol l^{-1} PBS with 0.01% sodium azide prior to transfer to secondary antibody diluted in 0.1 mol l^{-1} PBS (1/250 goat anti-rabbit Alexafluor 568 IgG, Sigma-Aldrich, Oakville ON, A11011 or 1/100 Alexafluor 488 anti-mouse IgG, Sigma-Aldrich, Oakville ON, A11001) for 2 days. Slides were again rinsed in 0.1 mol l^{-1} PBS six times for 50 min each and coverslipped using VectaShield Hardset mounting medium (Vector Labs, Burlington ON, H-1400).

Wholemount Preparations of Calretinin-Immunoreactive Glomeruli

Serial sections convoluted the discernment of adjacently situated glomeruli, specifically within the lateral plexus. Therefore, to better understand the organization of calretinin-immunoreactive glomeruli in addition to identifying these closely-situated lateral glomeruli, confocal 3D-projection analysis of wholemount preparations was conducted at the late yolk-sac larval developmental stage. At this stage, glomerular patterning was well-established, but the olfactory bulb was smaller than in fry. A protocol developed for zebrafish post-embryonic samples by Braubach et al. (2012) was adapted. The olfactory bulb was left attached to the telencephalon, but was excised from the cranium with the olfactory epithelium partially intact. Melanin located in melanophores in the dura surrounding the brain was bleached with hydrogen peroxide to enable visualizing the fluoresecently labeled olfactory sensory neurons (Zukor et al., 2010). The fixed samples were washed with 0.1 mol l^{-1} PBS five times over a 2-h period, followed by immersion in 10% hydrogen peroxide in PBS for approximately 1.5 h at room temperature, then placed in a solution containing 0.25% Triton X-100, 2% dimethyl sulfoxide (DMSO) and 1% normal goat serum in 0.1 mol l^{-1} PBS overnight at 4°C to permeabilize the tissue and to block non-specific binding. The tissue was incubated in a primary antibody solution of 1:200 monoclonal calretinin antibody raised in mouse (Swant, Switzerland, 6B3) for 7 days at 4 °C with low agitation. After a rinse cycle of seven washes with PBS-T over 6 h, the samples were placed into secondary antibody solution of 1:200 488 goat anti-mouse IgG (AlexaFluor, USA, A11001) for 5 days. Another rinse cycle of four washes with PBS-T and PBS each was followed by 24 h immersion in glycerol solution containing *n*-propyl gallate to counteract fading, and mounted with this glycerol mounting medium between a slide and coverslip separated by stacks of coverslip strips sealed with nail polish. Projection images made from stacks of optical section 1-3 μm step-size (z-step) images were collected using an Olympus Confocal FluorView FV1000 Microscope (Olympus, Tokyo, Japan) at 20× magnification. These images were used to visualize the organization of the lateral responsive glomerular chain. The glomeruli were named in accordance to the nomenclature described in zebrafish shape, location and immunoreactivity (Baier and Korsching, 1994; Braubach et al., 2012, 2013).

Assessment of Glomerular Patterning

Glomerular patterning in larvae and fry was qualitatively described, in reference to glomerular territory template establishment and organization, by sampling at three increments: early yolk-sac larva ($n = 18$, 1 family), late yolk-sac larva ($n = 6$, 1 family), and fry ($n = 25$, 7 families). The fry specimens from seven families facilitated a qualitative assessment of across-family consistency in glomerular patterning by comparing KLH labelled-glomerular territories.

The baseline organization of olfactory glomerular territories in the olfactory bulb was established by examining serial sections using epifluorescence microscopy (Nikon Eclipse E800, 20× objective, QImaging Fast1394 camera, Northern Eclipse). A single glomerulus was identified by the projection of olfactory sensory neuron axons into a single unit where the termination of the axons was visible (Allison and Warwick, 1949). Glomerular territories situated in the glomerular layer were comprised of a discreet clustering of glomeruli and partitioned from neighboring territories. 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. 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 sections labelled against KLH. The innervation of each glomerular chain by KLH- and calretinin-immunoreactive olfactory sensory neurons was identified, and lateral territory-specific glomeruli specified.

Olfactory bulb thickness (dorsal-ventral) from a subset of samples of each developmental group 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 axonal endings, while the ventral-most sections were identified by the entry of the calretinin- 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. Increase in mean olfactory bulb thickness from early yolk-sac larvae to fry was quantified (ANOVA, early yolk-sac larvae, $n = 8$, one family); late yolk-sac larvae, $n = 4$, one family; fry, $n = 25$, seven families). Additionally, ANOVA were applied to a sub-sample of these fry to determine whether across-family differences could be detected in either mean olfactory bulb thickness (three families) or mean glomerular territory thickness (four families).

The lateral glomerular territory was examined due to the ecological significance of amino acid detection in salmonids as a putative homing cue (Shoji et al., 2000; Ueda, 2012). These data were compared to patterning in conspecifics and heterospecifics described in alternate studies.

RESULTS

Mean olfactory bulb thickness, calculated by total thickness of serial sections from the dorsal- to ventral-most section, did not differ across three families of Chinook salmon fry (ANOVA, $F_{2,9} = 0.09$, $P = 0.91$). Because of the lack of family differences, data for fry from seven family groups were pooled, whereas early yolk-sac larvae and late yolk-sac larvae contain specimens from the same family. This additional analysis revealed olfactory bulb thickness increased with age (early yolk-sac larvae, $n = 8$; late yolk-sac larvae, $n = 4$; fry, $n = 25$ individuals; ANOVA, $F_{2,34} = 15.98$, $P < 0.01$). A post hoc Tukey multiple comparison test showed a

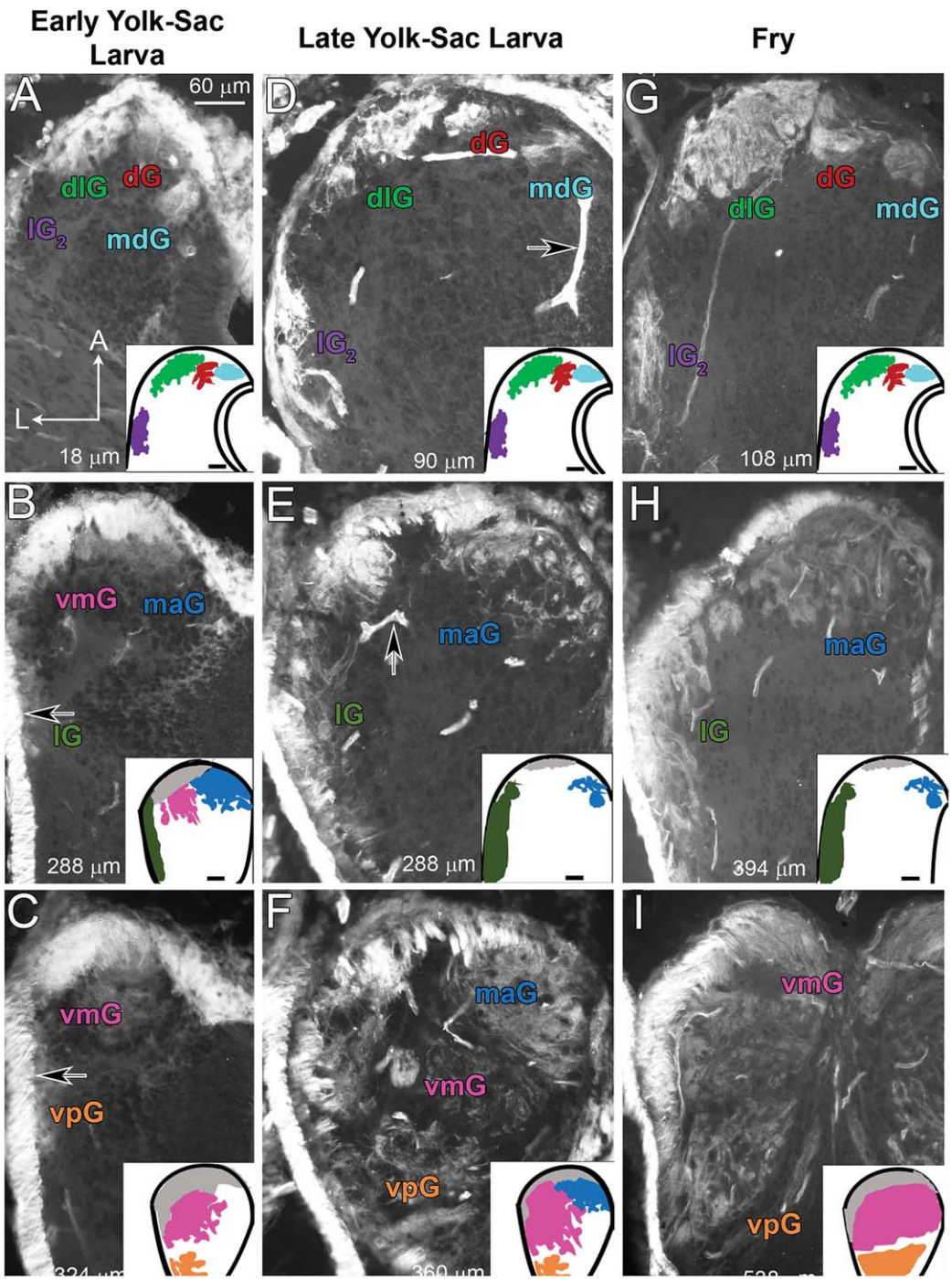

Fig. 1. *Oncorhynchus tshawytscha*, keyhole limpet hemocyanin immunofluorescence (KLH-IR) of glomerular territories in horizontal sections of the olfactory bulb at three ages, early yolk-sac larvae, late yolk-sac larvae, and fry. The dorso-ventral distance for each section is shown at the bottom of each micrograph. Each micrograph is oriented with the anterior at the top and the lateral on the left, and is at the same magnification (scale bar in Micrograph A). The cartoon insets depict color-coded glomerular territories. **A–C**. In early yolk-sac larvae, the seven glomerular territories and lateral glomerulus 2 are recognizable. The glomerular labeling is prominent in the dorsal glomeruli (A) and fainter in the ventral hemisphere (B and C). The olfactory nerve layer is seen as KLH-immunoreactive region at the rostral region of the olfactory bulb. In B and C, there are KLH-IR fibers situated lateral to the brain (arrows). **D–F**. In late yolk-sac larvae, KLH immunoreactivity is clustered in seven glomerular territories and lateral glomerulus 2 and is also seen on capillaries (arrows). The olfactory nerve layer is prominent in the ventral region of the olfactory bulb (F). **G–I**. At fry, the glomerular territories in the dorsal and lateral regions are distinct (G, H). In the ventral region (I), the olfactory nerve layer is prominent, and the ventral glomeruli are clearly labeled. Glomerular territories, dorsal glomeruli (dG, brown), dorsolateral glomeruli (dlG, green), Lateral glomerulus 2 (lG_2, purple), mediodorsal glomeruli (mdG, light blue), lateral glomerular territory (lG, olive green), medial anterior glomerular territory (maG, royal blue) ventromedial glomerular territory (vmG, pink), and ventroposterior glomerular territory (vpG, orange).

TABLE 1. Measured thickness of glomerular territories in fry Chinook salmon O. tshawytscha *innervated by keyhole limpet hemocyanin-immunoreactive olfactory sensory neuron axons.*

Glomerulus	*n*	Thickness (μm) ± SE	
Dorsal (dG)	13	110.77	7.58
Dorsolateral (dlG)	17	174.71	5.73
Mediodorsal (mdG)	14	88.71	5.12
Lateral (lG)	13	276.92	8.78
Medial anterior (maG)	12	222.00	8.68
Ventromedial (vmG)	11	126.00	8.75
Ventroposterior (vpG)	14	99.00	8.39

significant difference between early yolk-sac larvae and fry (mean difference between groups = 99.14 μm, $P < 0.01$).

KLH-Immunoreactive Glomerular Territories

Immunolabeling against KLH revealed the organization of seven glomerular territories, identifiable by shape and location in early yolk-sac larvae, late yolk-sac larvae, and fry (Fig. 1). The dorsal and mid-bulbar glomerular territories (dG, dlG, mdG, lG, maG) were small but detectable in early yolk-sac larvae (Fig.1a,b). Diffusely-organized KLH-immunoreactive fibres persisted in the dG and the mdG territories throughout post-embryonic development (Fig 1 a,d,g). The dlG territory appeared as a diffuse aggregate in early yolk-sac larvae (Fig. 1a), yet individual glomeruli within this territory were clearly distinguishable in late yolk-sac larvae and in fry specimens (Fig. 1d,g). The lateral glomerular territory (lG) commenced slightly caudal to the dlG territory (Fig. 1a,d,g). Although the lateral plexus of the lG was densely matted, separate KLH-immunoreactive glomeruli were seen at all developmental stages (Fig. 1a,b,d,e,g,h). The medial anterior (maG), ventromedial (vmG), and ventroposterior (vpG) glomerular territories were faintly labeled at the early yolk-sac larval stage (Fig. 1b,c) and became more distinct and strongly labeled in the late yolk-sac larval and fry age classes (Fig. 1e,f,h,i). During the early stages (early yolk-sac larva and late yolk-sac larva), KLH immunoreactivity was also seen in fibers surrounding the brain (Fig. 1b,c) and in capillaries (Fig. 1d,e). In fry, the dorsal boundary of the vmG 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 (not currently shown), while the vpG was comprised of two large, complex glomeruli, with the KLH-IR axons terminating on abundant clusters (Fig. 1f,i).

The mean thickness of each of the seven glomerular territories was calculated for fry (Table 1), and did not differ across four families: dlG: ($F_{3,13} = 0.97$, $P = 0.44$), dG: ($F_{3,9} = 2.66$, $P = 0.11$), mdG: ($F_{3,10} = 2.00$, $P = 0.18$), lG: ($F_{3,9} = 1.69$, $P = 0.24$), maG: ($F_{3,8} = 0.93$, $P = 0.47$), vmG: ($F_{3,7} = 2.52$, $P = 0.14$), vpG: ($F_{3,10} = 1.76$, $P = 0.22$). A cartoon summarizes the organization of the seven identified glomerular territories according to approximate depth within the olfactory bulb and innervation by KLH-immunoreactive axons during the three post-embryonic ages (Fig. 2). Throughout larval development the dorsal glomerular territories (dG, dlG, and mdG) occupied the dorsal-most 200 μm of the olfactory bulb (shown by the dashed line in Fig. 2). The depth of the lateral glomerular territory increased from 200 μm in early yolk-sac larvae, to 270 μm in late yolk-sac larvae and to 394 μm in fry, more than half of the total olfactory bulb thickness of each age class (Fig. 2). The ventral territories vmG and vpG remained confined to the ventral edge and were caudal to the olfactory nerve, which entered the ventral bulbar region (Fig. 2).

Calretintin-Immunoreactive Glomeruli

Calretinin-immunoreactive axons projected into two of four glomerular territories in the dorsal region of the olfactory bulb. Glomerular territories dG and mdG were immunoreactive solely to KLH, whereas the dlG and lG territories were immunoreactive to both KLH and calretinin (Fig. 3). Within the lG, only one of five identified glomeruli was not innervated by calretinin-immunoreactive axons. Lateral glomerulus 2 (lG_2), the dorsal-most of the lateral glomeruli, located posterior to dlG, was exclusively immunoreactive to KLH, whereas the olfactory sensory neuron fibers innervating the other lateral glomeruli were both calretinin and KLH-immunoreactive (Figs. (3 and 4)). Lateral glomerulus 1 (lG_1), located ventral and anterior to lG_2, was the dorsal-most calretinin-immunoreactive lateral glomerulus. Glomeruli LG_3 and lG_4 were located rostro-ventral to lG_1, but were not distinguished as separate glomeruli under confocal microscopy of horizontal sections and were consequently designated as $lG_{3/4}$ (Figs. 3e, 4c). However, as early as the early yolk-sac larval stage, separate lG_3 and lG_4 could be resolved in some whole mount preparations (Fig. 4d,e) but not in others.

TABLE 2. A comparison of lateral glomeruli in fry Chinook salmon O. tshawytscha *(this study) and zebrafish* D. rerio *(Braubach et al., 2012, 2013) innervated by keyhole limpet hemocyanin-immunoreactive (KLH-IR) and calretinin-immunoreactive (CR-IR) olfactory sensory neuron axons.*

	Salmon		Zebrafish	
Lateral glomerulus (lG)	KLH-IR	CR-IR	KLH-IR	CR-IR
lG_1	+	+	+	+
lG_2	+	−	+	−
lG_3	+	+	+	+
lG_4	+	+	+	+
lG_5	Absent	Absent	+	+
lG_6	+	+	+	+

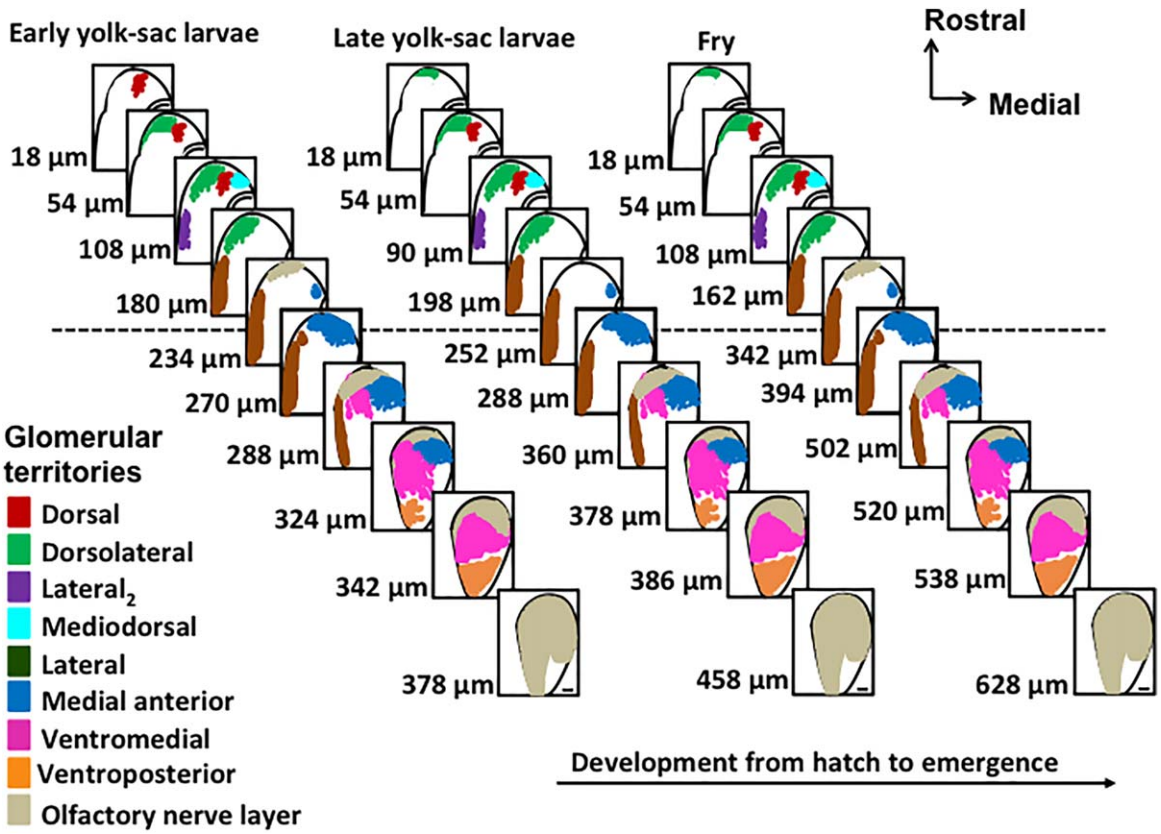

Fig. 2. *Oncorhynchus tshawytscha*, distribution of KLH-IR glomerular territories in the olfactory bulb depicted in horizontal sections from the dorsal to ventral hemispheres. Each glomerular territory is coded by a different color. The olfactory nerve layer is depicted in grey. The dashed line delineates the dorsal most 200 µm span in all three stages. The numerical values of depth show that from a representative specimen for each age.

The dorsal edge of lateral glomerulus 6 (lG_6; the posterior- and ventral-most lateral glomerulus) was at the same dorsal-ventral plane as $lG_{3/4}$. Within lG_6, the labeled axons projected into a number of distinct subclusters of axon endings, creating a bouquet-like appearance (Figs. 3h,i, 4c,d). The dorsal region of lG_6 was calretinin-immunoreactive but not KLH-immunoreactive (Fig. 3d,e) and the ventral region of lG_6 was double labeled (Fig. 3h,i). Of the glomerular territories situated in the medial and ventral bulbar regions, neither maG, vmG, nor vpG were innervated by calretinin-immunoreactive axons (Fig. 3g,h,i).

DISCUSSION

A comprehensive description of glomerular territory patterning was established for wild Chinook salmon from early yolk-sac larvae to fry, a potentially sensitive developmental period for olfactory imprinting (Dittman et al., 2015). Overall stability in glomerular patterning was observed from early yolk-sac larvae to fry and across individuals of unrelated parentage. Moreover, the glomerular pattern is consistent with the segregation of glomerular territories described in zebrafish larvae (Braubach et al., 2013; Miyasaka et al., 2013). The location of the seven described glomerular territories in larvae and fry salmon were also analogous to the description of glomerular territories in adult salmonids (Riddle and Oakley, 1992; Jarrard, 1997), suggesting the observed post-embryonic glomerular organization persists into adulthood, consistent with findings in zebrafish (Braubach et al., 2013). The presence of glomerular territories shortly after hatch, however, does not imply full functionality where olfaction yields a behavioral response. Alarm cues, specifically those belonging to the glucosaminoglycan odor class, stimulate parts of glomeruli mdG and lG in zebrafish (Mathuru et al., 2012), which are discernable at hatch (Braubach et al., 2013), yet zebrafish did not respond behaviorally to alarm cues until 50 days post-hatch (Waldman, 1982). Salmon, however, exhibited chemosensory-mediated anti-predatory behavior prior to emergence in response to olfactory cues released by predators (Louhi et al., 2011).

Apparent spatial asynchrony in glomerular development was observed in this study, with the dorsal glomerular territories more discernable than the ventral glomerular territories in yolk-sac larval specimens. As glomerular territories are

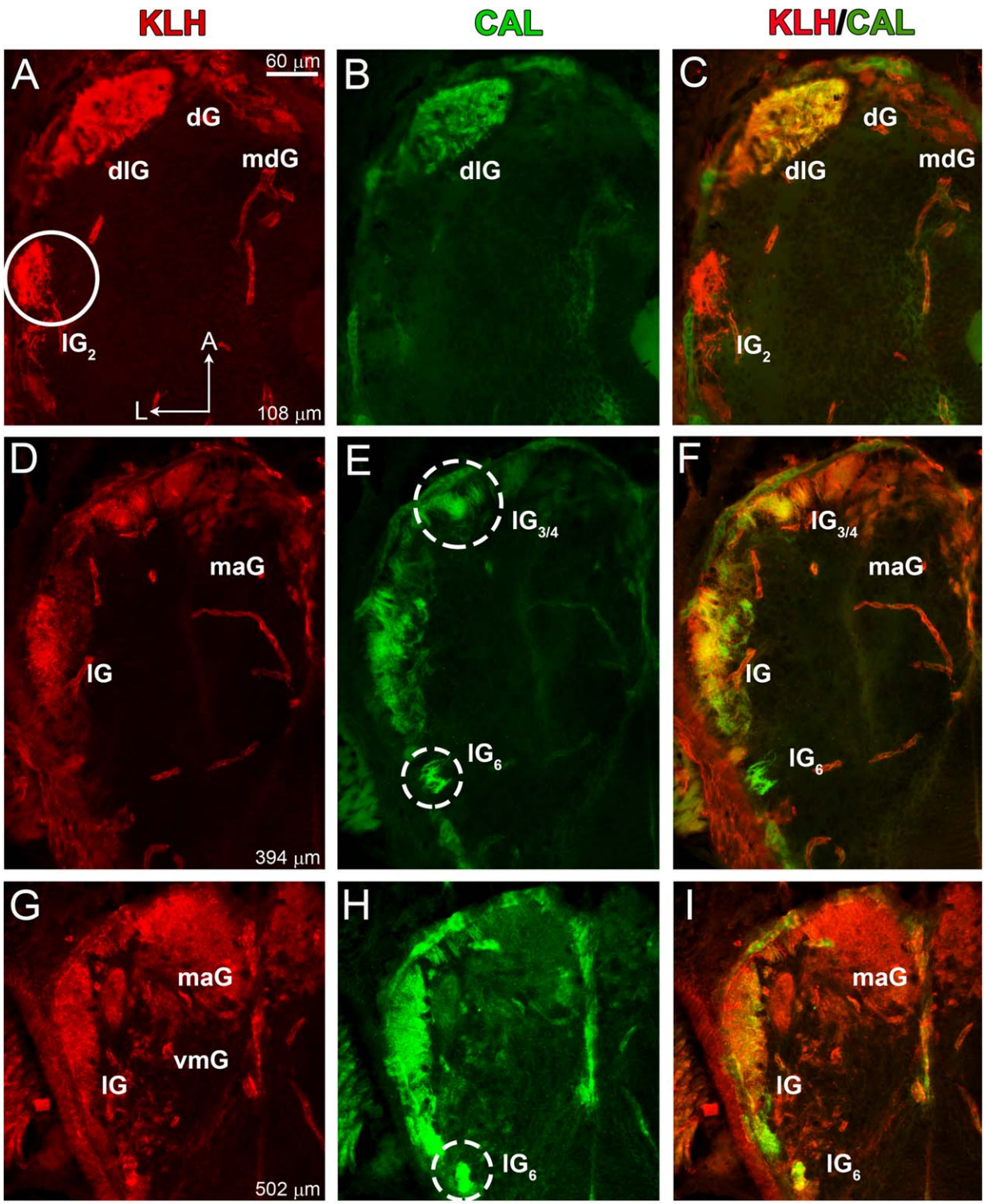

Fig. 3. *Oncorhynchus tshawytscha*, immunofluorescence of double labeling for KLH and calretinin in late yolk-sac larvae. The dorso-ventral distance for each section is shown at the bottom of each micrograph. Each micrograph is oriented with anterior to the top and the lateral to the left, and are at the same magnification (scale bar in Micrograph A). Left column is KLH-IR (red), the middle column is calretinin (CR)(green) immunoreactivity and the right column is merged KLH and CR (red/green). **A–C**: Dorsal hemisphere of the olfactory bulb. The dorsal-lateral glomerulus (dlG) is immunoreactive to both KLH and calretinin. The dorsal glomerulus (dG), the medial dorsal glomerulus (mdG) and lateral glomerulus 2 (lG_2) label for KLH but not for calretinin. **D–F**: Middle region of the olfactory bulb. Most olfactory sensory neuron axons within the lateral glomerular territory (lG) are both calretinin- and KLH- irmmunoreactive. **G–I**: Ventral hemisphere of the olfactory bulb. The medial anterior (maG), ventromedial (vmG), and ventroposterior glomerular territories are KLH immunoreactive, but not calretinin immunoreactive. The lateral glomerular territory (lG) within the ventral hemisphere shows double labeling.

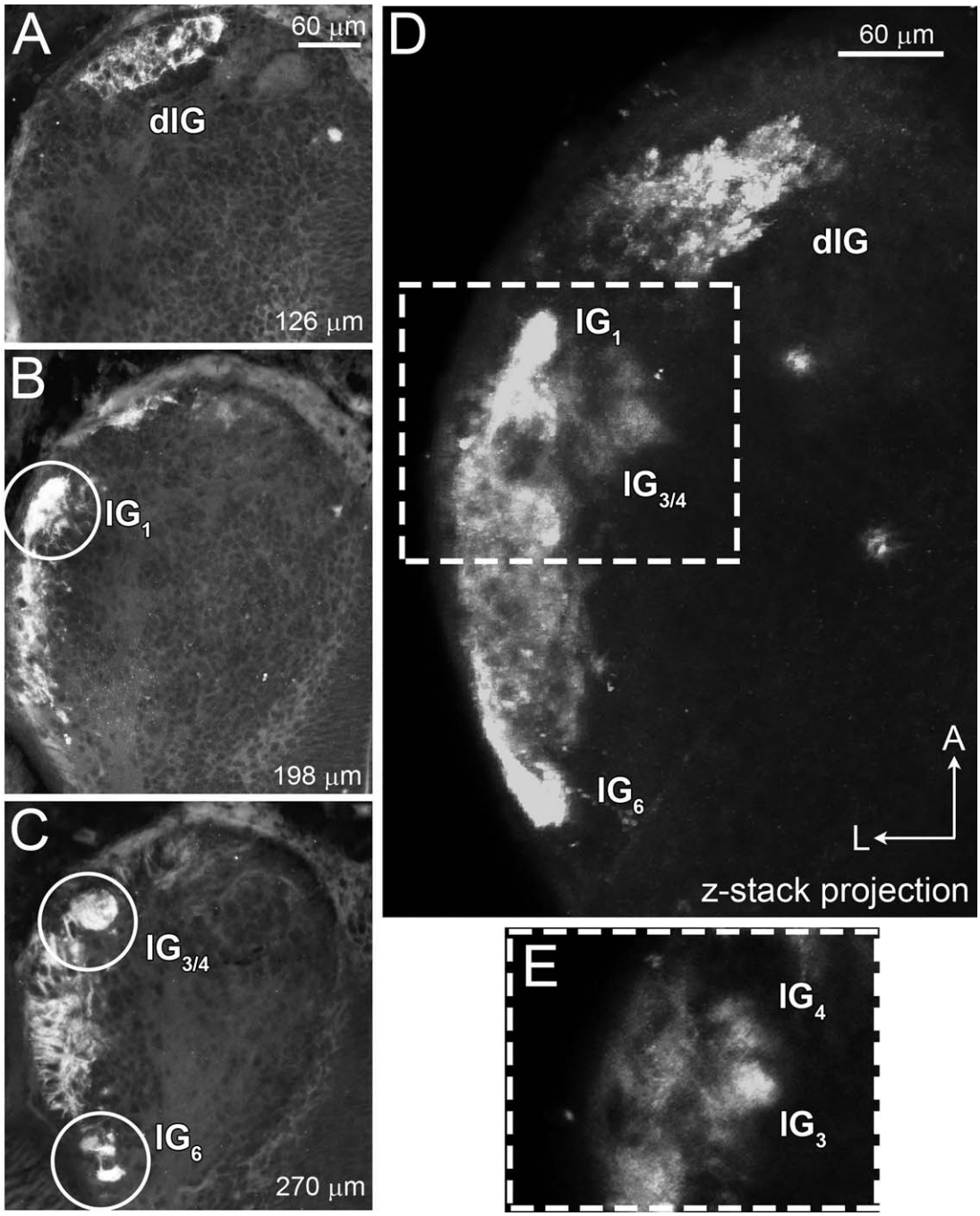

Fig. 4. *Oncorhynchus tshawytscha*, calretinin labeling of glomeruli in the lateral glomerular territory in early yolk-sac larvae. For all images, anterior is at the top and lateral is on the left. Lateral glomerulus lG_2 is not calretinin-immunoreactive (and is not shown) and lateral glomerulus lG_5 is absent in Chinook salmon. **A–C**. Epifluorescent images of horizontal sections. The depth for each section is shown in the bottom right. The micrometer bar shown in A is the same for B and C. A. Fibers are aggregated in the doral lateral glomerular territory (dlG) at a depth of 126 μm. B. Lateral glomerulus 1 (lG_1) is located on the rostral edge of the lateral gomerular territory at a depth of 198 μm. C. Lateral gomerular territories $lG_{3/4}$ and lG_6 are located at a depth of 270 μm. **D** and **E** are confocal images of a whole mount preparation. D is a projection through 90 μm original has 29 sections. The Z step is 3 μm. The projection was collected from a depth of 150 μm to 230 μm The micrometer bar shown in D is the same for E. E. The lateral glomeruli lG_3 and lG_4 are separate in this single plane.

stimulated by different odor classes (reviewed in Kermen et al., 2013), exposure to predominating odorants may influence maturation rates of glomerular territories. In both fishes and mammals, stimulation of a single olfactory receptor may increase gene expression of that receptor in

conjunction with the silencing of expression of alternate olfactory receptor classes, leading to increased recruitment of olfactory sensory neuron axons to corresponding glomeruli and a subsequent deceleration of recruitment to others (e.g., Todrank et al., 2011; Miyasaka et al., 2013).

Similarly, asynchronous glomerular development may reflect the biological relevance of the different odor classes as the animal matures, and early growth of glomeruli has been linked to a critical period for olfactory learning (Valle-Leija et al., 2012). Additionally, change in glomerular size often coincides with aversive or attractive behaviors toward specific olfactory cues, suggesting altered neural circuitry in the olfactory bulb translates to the higher brain structures, altering behavioral responses (Devaud et al., 2001, 2003; Todrank et al., 2011). In zebrafish, a specific lateral glomerulus exhibited plasticity in composition and size in response to amino acid odor exposure during the post-hatch period prior to emergence (Braubach et al., 2013). Thus, the development of distinct lateral glomeruli in Chinook salmon larvae may indicate the importance of amino acids for survival early in development. Indeed, unfed fry exhibit feeding behaviors in response to amino acid odors (Mearns, 1986). Major histocompatibility complex peptides (composed of amino acids) are a major component of kin-specific odors that zebrafish imprint to at 6-days-post-fertilization (Gerlach et al., 2008). These peptides stimulate ventrolateral glomeruli, and do not spatially overlap with glomeruli responding to food odors (Hinz et al., 2013). Biechl et al. (2016) showed kin odor activates crypt cells as well as a subpopulation of microvillous olfactory sensory neurons. Major histocompatibility complex peptide discrimination may also benefit salmon, as evidence suggests juvenile Chinook salmon demonstrate kin recognition behaviors (Henkel et al., 2011).

Post-Embryonic Glomerular Patterning Across Teleosts

The coarse organization of post-embryonic Chinook salmon glomerular territories, in reference to anatomical characteristics and positioning within the olfactory bulb, was almost identical to that in adult rainbow trout (Riddle and Oakley, 1992) and zebrafish (Baier and Korsching, 1994; Braubach et al., 2012), with the seven glomerular territories reliably identified. More specifically, in Chinook salmon larvae and fry the lateral glomerular territory occupied much of the lateral bulbar region, as observed in zebrafish (Baier and Korsching, 1994; Braubach et al., 2012) and rainbow trout (Riddle and Oakley, 1992). Glomeruli of this lG territory were innervated by calretinin-immunoreactive axons as in larval zebrafish (Braubach et al., 2013). The dlG territory also appears to be homologous to structures identified in both zebrafish (Braubach et al., 2012; Braubach et al., 2013) and rainbow trout (Riddle and Oakley, 1992), with KLH-immunoreactive axons terminating on any of dozens of individual glomeruli. Congruent to zebrafish (Braubach et al., 2012), the Chinook salmon mdG was innervated by only KLH-immunoreactive axons that terminated on up to six glomeruli.

In the ventral bulbar region, the maG, vpG, and vmG territories were previously identified in adult rainbow trout (Riddle and Oakley, 1992), adult zebrafish (Baier and Korsching, 1994; Braubach et al., 2012) 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 seen in this study of post-hatch Chinook salmon. 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 larvae and fry, perhaps due to its apparent small size or different developmental stage.

Immunolabelling against calretinin offered further across-species comparisons (Table 2). The calretinin-immureactive laterally projecting axonal endings in Chinook salmon larvae were very similar in number, shape, and location to adult (Baier and Korsching, 1994; Braubach et al., 2012) 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 (Braubach et al., 2012). Of the six lateral glomeruli ($lG_{1–6}$) identified within the lateral glomerular territory in zebrafish, only five were identified in salmon larvae ($lG_{1,2,3,4}$ and $_6$). In zebrafish, lateral glomerulus 5 (lG_5) was located ventro-posteriorly to lG_2 (Braubach et al., 2012; Braubach et al., 2013), and as a calretinin-immunoreactive glomerulus, should have been identifiable in the same serial sections and whole mounts as lG_1 in the Chinook salmon specimens. Without a good understanding of the chemical responsiveness of this particular glomerulus, it is difficult to assess the importance of this anatomical disparity. Unlike the dG identified in zebrafish (Braubach et al., 2012), this dorsal glomerular chain is not calretinin-immunoreactive and was situated directly between the mdG and dorsolateral chains in the Chinook alevin.

In conclusion, this study demonstrates glomerular patterning in Chinook salmon is established shortly after hatch, and is analogous to that of adult salmonids (Rainbow trout,Riddle and Oakley, 1992; Chinook salmon, Jarrard, 1997). Refinement and growth of all glomerular territories, indicated by an observed increase in territory size,

is evident as the larva matures, but specific glomeruli in the lateral territory appear to be already established. Thus, sensory experience may be required to further refine the corresponding synaptic development within the olfactory bulb, as synaptic development and plasticity are adaptive mechanisms that potentially increase the individual animal's sensitivity to cues specific to its environment. Understanding the development of glomerular patterning has implications for understanding neuronal correlates that take place during imprinting. This description of glomerular patterning and the identification of specific lateral glomeruli provide a base for testing neural responsiveness or glomerular growth in response to exposure to important ecological odors, such as natal stream odors. Thus, these findings may enable future studies to elucidate the developmental stage at which Pacific salmon may be sensitive to imprinting.

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