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Response patterns and neurogenesis in the olfactory bulb of Xenopus laevis during metamorphosis

M. Sc. Thesis Defense

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I. Summary

Amphibians possess the ability to smell in water and land. This dual ability requires massive modifications in the aquatic larva to the terrestrial adult life in the olfactory system during its development. In the larva of *Xenopus laevis*, the principal cavity in the olfactory epithelium detects waterborne odors and projects its axons to the ventral olfactory bulb. During metamorphosis the principal cavity is remodeled and transformed into the adult air nose which sends its axons to a newly build enlarged dorsal olfactory bulb. Meanwhile, the development in the olfactory epithelium of a new cavity occurs: the middle cavity and effectuates the role of a water nose. The sensory cells lining in the middle cavity extend their axons into the ventral olfactory bulb. In this project we undertook a stage-by-stage survey of the anatomical changes and odorant response profiles in the olfactory bulb of *Xenopus laevis* during metamorphosis to follow its anatomical changes and its functionality throughout development. To attempt this, functional calcium imaging was performed, neuronal tracing techniques, and immunohistochemistry assays. We found that the ventral bulb remains responsive to waterborne odor all throughout metamorphosis, which suggest a dynamic shift in innervation from the principal to the middle cavity without functional discontinuity. Our results showed that before metamorphosis a neuronal cell layer in the olfactory bulb is already present and enlarges throughout metamorphosis suggesting a higher proliferation rate of the lateral ventricle for the formation of the dorsal olfactory bulb. The increase in number of HuC/D-positive cells implies a growth in the dorsal olfactory bulb starting from stage 52/53. A possible signal, which triggers neurogenesis in the dorsal olfactory bulb could be the projection of olfactory receptor neurons into the olfactory bulb. We observed that prior to metamorphosis Sox2 was expressed in all stem cells and was restricted to the ventricular zone and in early stages of metamorphosis some $Sox2^{+}$ cells began to migrate to become a new neuron in the olfactory bulb. At late metamorphosis, we observed that most of Sox2-positive cells were accumulated in the lateral ventricle and some Sox2-positive cells had reached longer distances from the stem cell niche. These results showed that the ventricles are constrained to neurogenesis and there are cells migrating to differentiate into projection neurons or interneurons.

The undisrupted functionality of the ventral olfactory bulb means that *Xenopus* is capable of smell while it undergoes metamorphosis, which is a relevant behavioral advantage for survival. This means that *Xenopus laevis* can feed, avoid danger and migrate when necessary. Surprisingly, metamorphosis does involve anatomical and physiological changes in the whole olfactory system of *Xenopus laevis* that will allow them to adapt in both environments, water and land. Neurogenesis of the dorsal olfactory bulb, as well as in the olfactory epithelium, is an evolutionary requirement for *Xenopus*, which needs more investigation about the air-nose functionality. The

changes taking place in the olfactory system in amphibians during metamorphosis provide valuable insights into the *de novo* formation and reorganization of functional neuronal networks. It is of great importance to understand the formation of the dorsal olfactory bulb for different reasons: (1) neuronal migration is crucial for brain development, and its migratory defects could lead to neurological diseases, (2) in adulthood, human neuroblasts from the subventricular zone migrate towards striatum, and this process is impaired in some human diseases such as Huntington's disease patients and (3) this continuous supply of new neurons in the OB, is implicated in plasticity and memory regulation.

II. Introduction

Chemosensation is phylogenetically the oldest sensory system, one of the first means of communication between organisms. The chemical senses include olfaction (smell), gustation (taste), and chemesthesis (pain, touch, and thermal dermal sensations [1]. It is essential for the survival of many species, permits to locate nutriments and mating partners and to avoid being eaten by predators or ingesting toxic substances. Smell is distinguished from the rest of the senses by the qualitative ability to detect an immense variety of odorants [2]. Each species is fine-tuned to sense a broad range of chemical stimuli relative to its own evolutionary context. Amphibian species possess a ubiquitous olfactory system that allows them to detect an immense heterogeneity of odorants depending on the environment, air or water.

Despite these variations, basic olfactory anatomy has been conserved throughout millions of years in vertebrate evolution [1]. A phylogenetic analysis of central olfactory projections indicates that at least three distinct olfactory subsystems may be broadly present in vertebrates and that a fourth, the accessory olfactory or vomeronasal system, arose in tetrapods [3]. Like most tetrapods, anuran amphibians have a well elaborated olfactory system that evolves at early stages in developmental events [4]–[8], and is a fully functional system in larvae [9], [10]. Anurans, specifically the African clawed frog *Xenopus laevis*, provide an exceptional model for studying the functionality and structure of the olfactory system since they have a complex life cycle that begins with an aquatic larval stage that eventually, during metamorphosis transforms into a terrestrial juvenile form.

i. The sense of olfaction

The basic olfactory circuit in vertebrates begins with the detection of odorants by olfactory receptor neurons (ORNs) localized in the olfactory epithelium. Each ORN expresses one type of odorant receptor (OR) [11]. Odorants are sensed by OR expressed on the cilia of ORNs. Olfactory receptor molecules are homologous to a large family of other G-protein-linked receptors. G-protein-coupled receptors have 7 transmembrane domains that interact with odorants. Odorant transduction begins with odorant binding to specific receptors on the external surface of cilia. In mammals, the principal pathway involves cyclic nucleotide-gated ion channels. The olfactory receptor neurons contain an olfactory-specific G-protein (G_{olf}), which activates an olfactory-specific adenylate cyclase resulting in an increase of cyclic AMP (cAMP) [12]. The specificity of olfactory signal transduction is presumably the

result of this variety of odorant receptor molecules present in the nasal epithelium. [11], [13].

Olfactory neurons are bipolar neurons that extend a dendrite to the nasal cavity and an axon towards the brain via the olfactory nerve. The ORN axons project into the glomerular layer of the olfactory bulb where they form synapses with the dendrites of mitral/tufted cells (MTCs). The unity of ORN axon terminals and dendritic tufts of MTCs forms the glomeruli (figure 1). In rodents, the axons of the MTCs form the lateral olfactory tract and project to the five different regions in the olfactory cortex: the anterior olfactory nucleus, the olfactory tubercle, the piriform cortex, and parts of the amygdala and enthorinal cortex [2], [14]. In vertebrate species, glomeruli in the olfactory bulb have been described to be morphologically and functionally identifiable invariant structures [15], [16]. Moreover, evidence showed that stimulation with certain odorants induces specific and reproducible patterns of activated glomeruli in distinct species, suggesting that the patterns of glomerular activity contribute to the coding of the odor image [17]–[20].

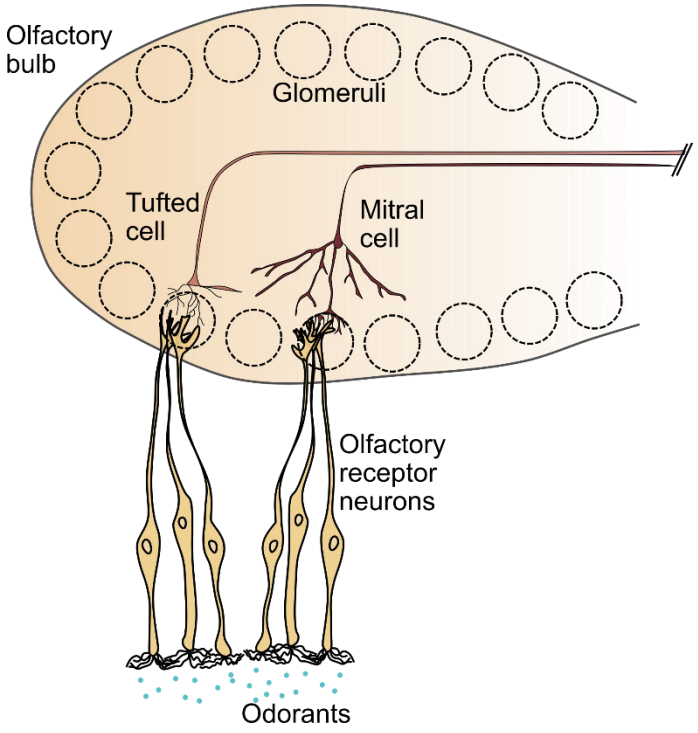


Figure 1. The olfactory system in rodents. Odorants are detected by olfactory receptor neurons which lie in the nasal cavity. The axons of the ORNs project to the olfactory bulb where they form synapses with mitral and tufted cell dendrites forming the glomeruli. MTCs project to higher brain centers. Modified from Kandel, Eric et al., 2000.

ii. Olfaction in *Xenopus laevis*

i. Development and Cellular components of the olfactory epithelium

The sensory circuit and cellular composition of the olfactory system of *Xenopus laevis* is very similar to that of other vertebrates (figure 2B, for an extensive review see [21]). The aquatic larvae of *Xenopus laevis* have an anatomically segregated olfactory system consisting of the main olfactory epithelium (MOE) located in the principal cavity (PC) and the epithelium of the accessory system situated in the vomeronasal organ (VNO). The premetamorphotic MOE is solely exposed to water-borne odorants and contains three kind of cell types: (1) sensory neurons (microvillous and ciliated) that detect odorants and transform this stimuli into an electrical signal and transmit the information along their axons to the OB; (2) two populations of supporting cells (SCs), which have different metabolic functions [22] and (3) basal cells (BCs), the stem cells responsible for the generation of newborn ORNs [22], [23].

To meet the requirements of the adult lifestyle, a third sensory chamber, the middle cavity, arises during stage 52, the beginning of the premetamorphotic phase (figure 2A; [9], [24], [25]. The larval MOE in the PC of *Xenopus* is remodeled into an adult air nose during metamorphosis. During this metamorphotic reorganization, massive apoptotic cell death occurs, former larval ORNs in the PC are replaced and newly generated neurons form the middle cavity [9]. The epithelium of the MC consists of the same cell types as the larval PC. Each cavity in the olfactory epithelium expresses a different type of receptors according to its necessities. The MC expresses receptors for water-sensitive odorants such as V2Rs and TAARs, the same receptor types expressed formerly in the premetamorphotic PC of larvae [26], [27]. Work of Syed and colleagues showed that sensitivity for amino acids shifts from the PC to the MC during metamorphosis [26]. Amino acids are a potent olfactory stimuli used in aquatic species [28]. The reorganized postmetamorphotic PC has only ciliated receptor neurons and microvillar SCs. It expresses air-sensitive olfactory receptors, suggesting that the PC now has become in the air nose although there are not stimuli reported in the literature so far that are detected by the PC as well as the characteristic responses in the PC.

The neuronal representation of odors in *Xenopus laevis* relies on segregation in subsystems within the nasal cavity, and they each make distinct neural connections to regions of the olfactory forebrain [29]. Manzini and colleagues reported for the first time, the existence of two odor-processing streams, spatially segregated in the main olfactory bulb and partially segregated in the olfactory epithelium of pre-metamorphotic larvae [26], [27], [30]. The medial odor-processing

stream is formed by ciliated neurons expressing odorant type I and II receptors (ORs I and ORs II) and exhibits the canonical cAMP-mediated transduction pathway. This stream responds to alcohols, aldehydes, and ketones [27]. The lateral stream contains microvillous ORNs expressing vomeronasal type 2 receptors (V2Rs) and trace amine-associated receptors (TAARs), the lateral stream is characterized by amino acid responses. ORNs in the lateral stream possess a phospholipase C (PLC) and diacylglycerol (DAG)-mediated transduction pathway that is independent of intracellular Ca^{2+} store depletion [31]–[33].

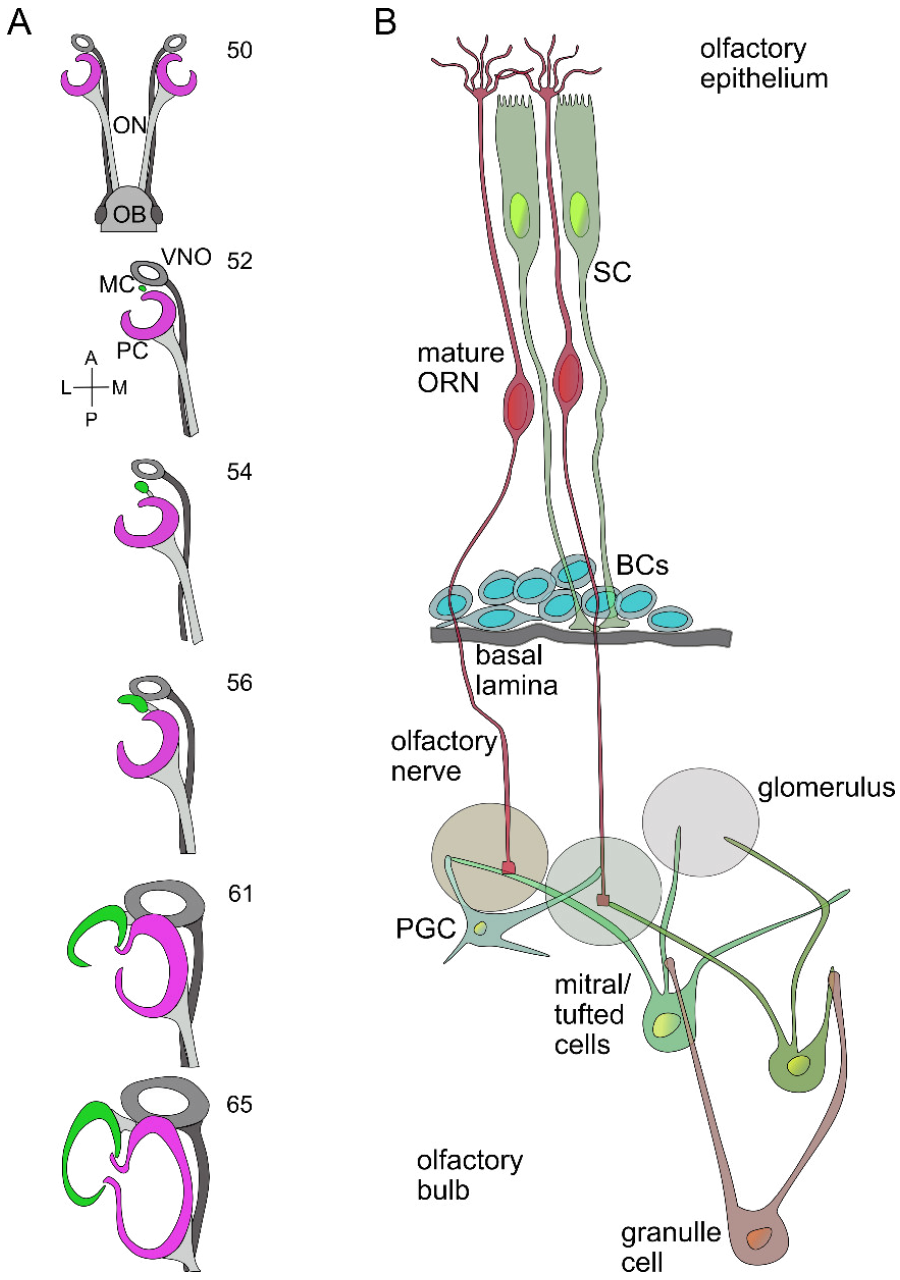


Figure 2. Structure and development of the olfactory system in *Xenopus laevis*. **A**: Development of the olfactory epithelium of the middle (green) and principal (magenta) cavities as well as the VNO (grey) during metamorphosis **B**: Peripheral olfactory system of *Xenopus laevis*. The olfactory epithelium contains three main cell types: the olfactory receptor neurons (ORNs), the supporting cells (SCs), and the basal cells (BCs) responsible of the cell proliferation. The axons of the ORNs penetrate the basal lamina entering the olfactory bulb and coalescing into the glomeruli where they form synapses with mitral/tufted cells and periglomerular cells (PGC). The axons travel to a higher brain center. Modified from Dittrich et al., 2016; Manzini et al., 2015.

ii. Development of the olfactory bulb during metamorphosis

The anatomy of the olfactory bulb consists of various cellular layers: the glomerular layer, the mitral/tufted cell layer and the granule cell layer [34]. Before the beginning of metamorphosis (stage 50/51), the axons of the ORNs originating in the larval PC terminate in the glomerular layer of the ventral side of the olfactory bulb. Anatomically, three large main clusters: the medial cluster, the intermediate cluster, the lateral cluster, and one small ventromedial glomerular cluster can be distinguished in the larval *Xenopus laevis* olfactory bulb (figure 3) [21], [34]–[36].

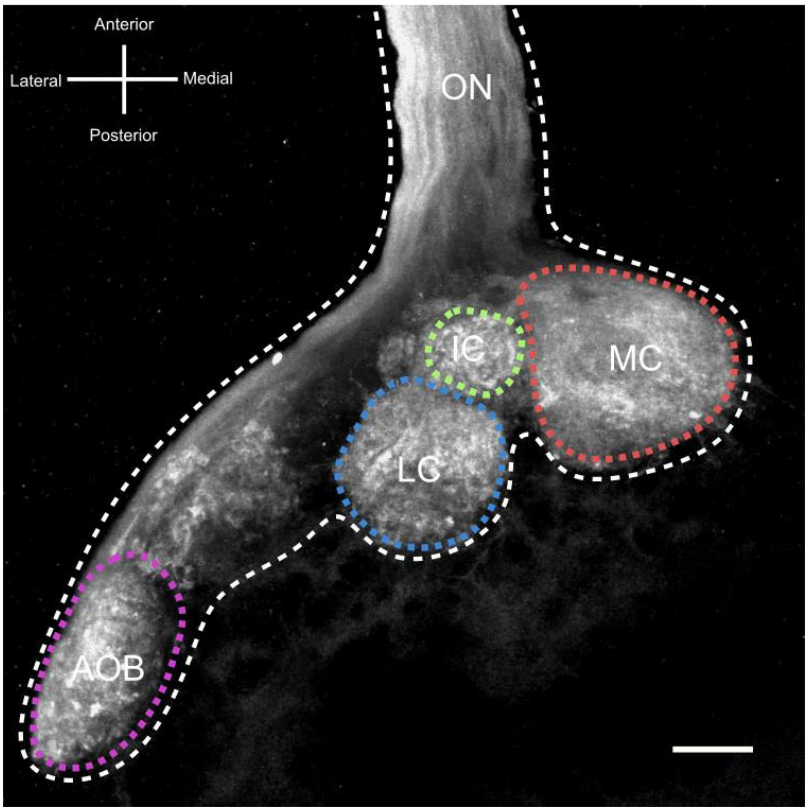


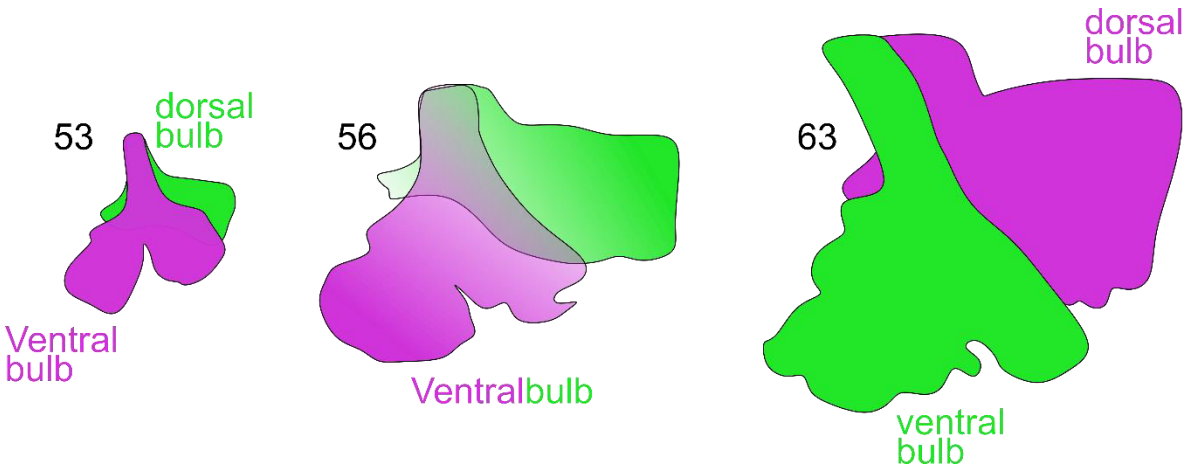
Figure 3. Glomerular clusters in the olfactory bulb. Whole-mount immunostaining against NCam of *Xenopus* at stage 52. The anatomy of the three main glomerular clusters of the main olfactory bulb are shown: LC, IC, and MC. AOB: accessory olfactory bulb, ON: olfactory nerve. Scale bar 20

Like shown for the epithelium of *Xenopus laevis* larvae, the functional and anatomical inputs of the glomeruli in the olfactory bulb are segregated as well [37], [37], [38]. Retrograde labeling experiments in the olfactory nerve showed that the lateral axonal tracts labeled the lateral ORNs whereas the medial axonal tract labeled predominantly medial ORNs. The co-expression of G protein, $G_{\alpha olf/s}$, with tubulin, a marker of ciliated neurons, shows the medial stream to be composed of ciliated ORNs. Correspondingly, the co-expression of G protein, $G_{\alpha i/o}$, with phalloidin, a marker for f-actin, shows microvillous ORNs as the main component of the lateral odor processing stream [27]. These results denote that the stimuli responsible for activating the LC are different from the MeC. These results have also been supported by functional calcium imaging experiments. Amino acids induce responses mainly in the LC, whereas forskolin, an activator of the cAMP pathway, elicits activity predominantly in the MeC [27], [36], [39], [40].

An interesting event that starts at the onset of metamorphosis of *Xenopus laevis* is the development of the dorsomedial olfactory bulb (OB) around stage 51/52. This coincides with the *de novo* formation of the MC. Before stage 52 tadpoles have expression of water-sensitive receptors in the PC and the axons project to the vOB During metamorphosis (from stage 52 to stage 64), the axons of ORNs originating in the newly built MC (adult water nose) project into the vOB, while the new axons of ORNs from the restructured PC start to project into the growing dOB (figure 4). Apoptotic process in the PC leads to a replacement of the soluble odorants-sensitive receptors for a new type of receptors, which are thought to detect air-born odorants [25], [33]. Finally, when metamorphosis is over, all the axons from the PC exclusively project to the dOB and those from the MC exclusively to the vOB (figure 4) [41], [42].

The developmental changes in the olfactory epithelium of *Xenopus laevis* for the olfactory system have been shown in many publications but much less is known about the development and functionality of the glomeruli and the olfactory bulb during developmental stages [9], [34], [36], [39]–[41], [43]. The olfactory bulb, as well as the epithelium, undergoes morphological changes during metamorphosis [44]–[50]. In embryonic mice and rats, the olfactory bulb begins to differentiate at stage E11/12 [51]–[53] and E12/15 [54] respectively. The OB of *Xenopus laevis* begins to differentiate at stage 32 and the first glomerular synapses (from the ORN axons to the MTC dendrites) start to appear around stages 37/38. Suggesting that the olfactory system might be ready to sense water-borne odorants at those stages [8].

Figure 4. Development of the glomerular clusters in the olfactory bulb of *Xenopus laevis*. At stage 53, the PC sends its axons to the glomerular clusters on the ventral side and the glomerular clusters in the dorsal side start to develop. At stage 56, the innervation in both sides of the bulb shifts. The ventral side receives inputs from the PC and the MC, whereas the dorsal side receives input from the newly built ORNs in the PC. The dorsal bulb grows in size. At stage 63, the spatial segregation from the MC and PC is complete; the glomerular clusters on the ventral bulb are innervated by the MC while the axons coming from the PC connect with the dorsal side of the bulb. Modified from Weiss et al., unpublished.



iii. Neurogenesis in the olfactory bulb during metamorphosis

One of the firsts experiments about adult neurogenesis was shown by Kaplan and Hinds when they report that new neurons are being formed in adult rat olfactory bulb [55]. Later, postembryonic mammalian neurogenesis started to take relevance just in the 1990's when a paper described neurogenesis in the anterior portion of the subventricular zone in postnatal mice [56]. The subventricular zone is a paired brain structure situated throughout the lateral walls of the lateral ventricles and represents an important reservoir of dividing cells generating both neurons and glia cells in the adult brain [57], [58]. Neuroblasts, a cell lineage in the SVZ, congregate in the anterior SVZ and start to form the rostral migratory stream leading to increment of neurons in the OB [57], [59]–[61]. The differentiation of neurons to form various cell types in the OB have an important role in shaping the olfactory information that reaches the olfactory cortex and is important for plasticity [59], [62], [63].

In *Xenopus laevis*, neurogenesis in the olfactory bulb begins with cell division of precursors cells that lie in the SVZ and is divided in primary and secondary neurogenesis. Primary neurogenesis occurs in early embryogenesis and the number of progenitor cells increases. The secondary neurogenesis comes as a second wave of proliferation of neural cells that proliferate and an increase in the mitotic rate increases; takes place at stage 52. These two phases of cellular division coincide with a high neuronal production and are interrupted by a period of quiescence, which is thought to be a period of inactivity to enable neural progenitors to slowly migrate to their final destination when metamorphosis is over [41], [64]–[68].

Neurogenesis in *Xenopus laevis* has shown that MTCs in the ventral olfactory bulb and the firsts axonal contacts between the ORNs and the glomeruli take place

at different stages. MTCs are already present in the ventral olfactory bulb before stage 32 then, at stages 36/37 the projections of ORNs innervate the ventral region and interneurons are born. This means that when the metamorphotic process begins there is a network in the ventral region of the bulb already present [69]. Neurogenesis of both MTCs and GCs in the dOB occurs at the developmental stage 52 and coincides with the formation of the MC in the epithelium and the reestablishment of the PC. There is a correlation between the number of axons and mitral/tufted cell number in the dOB during development [8]. The signal for the generation of interneurons or projection neurons in the dOB could be due to mechanisms during the period of metamorphosis such as innervation by afferent fibers from the PC or induction by secretion of thyroid hormone [8], [50], [69].

III. Aim of the project

I) To characterize the response patterns, when present, in the ventral side of the glomerular layer of the OB of *Xenopus laevis* during metamorphosis.

II) To perform immunohistochemistry assays to follow neurogenesis process in the olfactory bulb during metamorphosis.

IV. Materials and methods

All experimental procedures described below were carried out according to the guidelines of the Justus-Liebig University Gießen.

i. Animals

Wild type and albino *Xenopus laevis* used for the experiments were kept in water tanks at room temperature. Water was changed three times a week. Tadpoles were fed with algae (Dose Aquaristik, Bonn, Germany) and postmetamorphotic froglets were fed with Pondstick food (Tetra Pond, Melle, Germany). The animals were staged according to Nieuwkoop & Faber (1994).

ii. Immunohistochemistry

Preparation of the olfactory system

Wild type tadpoles (stage 50-64) [7] were first anesthetized in 0.02% MS-222 (ethyl 3-aminobenzoate methanesulfonate; Sigma, Seelze, Germany) until full unconsciousness and killed by transection of the brain and its transition to the spinal cord. The obtained tissue containing the olfactory organ and the olfactory bulb was cut out with fine scissors. For the froglet preparation (stage 65-66) [7] the skin above the brain was removed with tweezers so that the cartilage was uncovered. Subsequently, a cut through the cartilage was made with a fine scissor and the remains were removed so the brain was exposed and a tissue section with the olfactory system was obtained. The preparations were fixed in 4% paraformaldehyde (Histofix) for 1 hour. Afterwards, they were washed 3 times for 10 minutes in PBS. Tissue sections obtained from froglets were cut transversally into 100-200 µm slices with a vibratome (VT 1200S; Leica, Benshein, Germany). Tissue sections and slices were then washed with PBS containing 0.2% Triton X-100 (PBST) for permeabilization, and nonspecific binding sites were blocked with 2% normal goat serum (NGS; ICN, Aurora, OH) in PBST for 1 hour. The tissue was incubated for 48 hours at 4 °C in the primary antibodies (Table 1) in 2% NGS/PBST.

Primary antibodies were washed off 3 times for 10 minutes in PBS. Secondary antibodies (Table 2) were applied in 1% NGS/PBS for 48 hours at 4 °C. Secondary antibodies were washed 3 times for 10 minutes in PBS.

***Table 1**. Primary antibodies used for immunostaining of the tadpole and froglet brains*

Antibody	Company	Dilution
HuC/HuD 16A11 (mouse)	Thermo Fisher Scientific	1:100
Sox2 (rabbit)	Abcam	1:100
NCam (mouse)	Abcam	1:200

***Table 2**. Secondary antibodies used for immunostaining of the tadpole and froglet brains*

Antibody	Company	Dilution
Alexa Fluor™ 594 goat anti-mouse IgG	Thermo Fisher Scientific	1:200
Alexa Fluor™ 488 goat anti-rabbit IgG	Thermo Fisher Scientific	1:200
Alexa Fluor™ 488 goat anti-mouse IgG	Thermo Fisher Scientific	1:200

iii. Olfactory receptor neuron labeling via electroporation

To visualize calcium transients in the axon terminals of ORNs in the olfactory bulb, fluorescent calcium-sensitive dye Cal 520-dextran conjugate (AAT bioquest) was deposited into sensory neurons via electroporation. *Xenopus laevis* tadpoles (stage 50-64) were anesthetized in a small glass beaker containing 0.02% MS-222 for 5 minutes and (after proper anesthesia was confirmed) transferred into a preparation dish filled with silicone. Dye crystals were placed with fine forceps into both nasal cavities and were dissolved in the residual moisture. Two thin, platinum electrodes were carefully inserted into the nasal cavities. The electrodes were connected to a voltage pulse generator (ELP-01D; npi Electronics), and 12 pulses (15V, 25 ms duration at 2 Hz) with alternating polarity were applied (for more details, see [70]). After electroporation, animals were transferred into a beaker filled with fresh tap water for recovery and killed two days later for functional calcium imaging.

iv. Functional calcium imaging of glomerular clusters

After killing the animals, the palatial tissue covering the ventral side of the telencephalon was removed using fine scissors. The preparation was stabilized using a stringed platinum grid in a recording chamber and constantly perfused with bath solution (standard bath solution consisted of (in millimolar): 98 NaCl, 2 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 5 glucose, 5 Na-pyruvate, 10 hydroxyethyl piperazineethanesulfonic (HEPES), 230 mOsmol/l, pH 7.8). Changes of intracellular calcium concentrations in the ORN axon terminals in the glomerular layers of the OB were monitored using an upright multiphoton microscope (Nikon A1R-MP, Nikon). An eight-channel gravity perfusion system (ALA Scientific Instruments) was connected to an odorant delivery system. Odorants were used at concentrations of 100 µM. The funnel of the perfusion system was directed towards the MOE of the whole olfactory system preparation. To create a constant flow of odorants, a suction syringe driven by a peristaltic pump was positioned caudally to the tissue (for more details, see [70] The region of interest in the bulb was scanned in fast resonant scanning mode. Glomerular clusters of the OB were imaged over time with acquisition rates of 30-40 planes per second at a z-resolution of 3-5 µm. The fluorescent dye was excited at a wavelength of 780 nm. The MOE was stimulated with (in the following order): odorant mixture, Ringer solution, amino acid mixture, bile acids, amines, ATP, and forskolin (see Table 03). After 15 s of recording the baseline fluorescence, a stimulus was applied during 5 s and switched back to Ringer perfusion afterwards. The interval between each different application lasted 1 minute to include the whole calcium transient and let the ORNs recover to avoid olfactory receptor neuron desensitization. Each train of stimulations was repeated at least twice to verify the calcium responses. All experiments were conducted at room temperature.

v. Image acquisition and processing

For immunohistochemistry assays, samples were fixed with a platinum frame stringed with nylon threads and imaged using an upright multi-photon microscope (Nikon A1R-MP; Düsseldorf, Germany). Three-dimensional z-stacks of the olfactory bulb were acquired with a laser wavelength of 780 nm as virtual stacks with a z-resolution of 2 or 3 µm. Image processing was done with ImageJ, contrast and brightness were adjusted. The images presented in this thesis are shown as a maximum projection of z-stacks. Stitching algorithm from ImageJ was used to stitch images together acquired from animals in stages>60 [71].

vi. Analysis of Ca^{2+} imaging and data evaluation

Changes in calcium indicator fluorescence measured in regions of interest are given as $\Delta F/F$ values as a percentage. Each glomerular cluster was subdivided into

4 equally sized ROIs. The Intensity transients are represented as the mean of all pixels in the ROIs according to the following equation: $\Delta F/F = (F-F_0)/F_0$ where F_0 represents an averaged pixel fluorescence value derived from the last five recording frames prior to the stimulus and F is the actual pixel fluorescence value at each recorded time point. A response was assumed if the following criteria were met: (i) the maximum amplitude of the calcium transient had to be higher than the maximum of the five frames before pre-stimulus intensities; (ii) the onset of the response had to be within ten frames after stimulus application. Analyzed regions of interest for the glomerular cluster measurements were calculated from a three-dimensional z-stack. Functional imaging data were analyzed using custom-written programs in Python [72].

We analyzed the responses of the three main clusters to different stimuli application in the olfactory mucosa and compare animal responses between LC, MeC, and IntC at different developmental stages. The response profiles of the glomerular clusters are shown as the percentage of animals responsive to all stimuli.

***Table 3**: Olfactory stimuli used for calcium imaging*

Amines	piperidine, cyclohexylamine, 3-methylbutylamine, 1-ethylpiperidine, ethyl-cyclohexylamine, 2-methylbutylamine, 2-methylpiperidine, 2-phenyl-ethylamine, n,n-dimethylamine, 1-formylpiperidine, tyramine, hexylamine, (n-)butylamine
Amino acid mixture	Proline, valine, leucine, isoleucine, methionine Glycine, alanine, serine, threonine, cysteine, asparagine, glutamine Arginine, lysine, histidine Glutamate, aspartate, Tryptophan, phenylalanine
Bile Acids	Taurocholic acid,

	cholic acid, glycholic acid, deoxycholic acid
Odorant mixture	Amines, Amino Acids and Bile acids

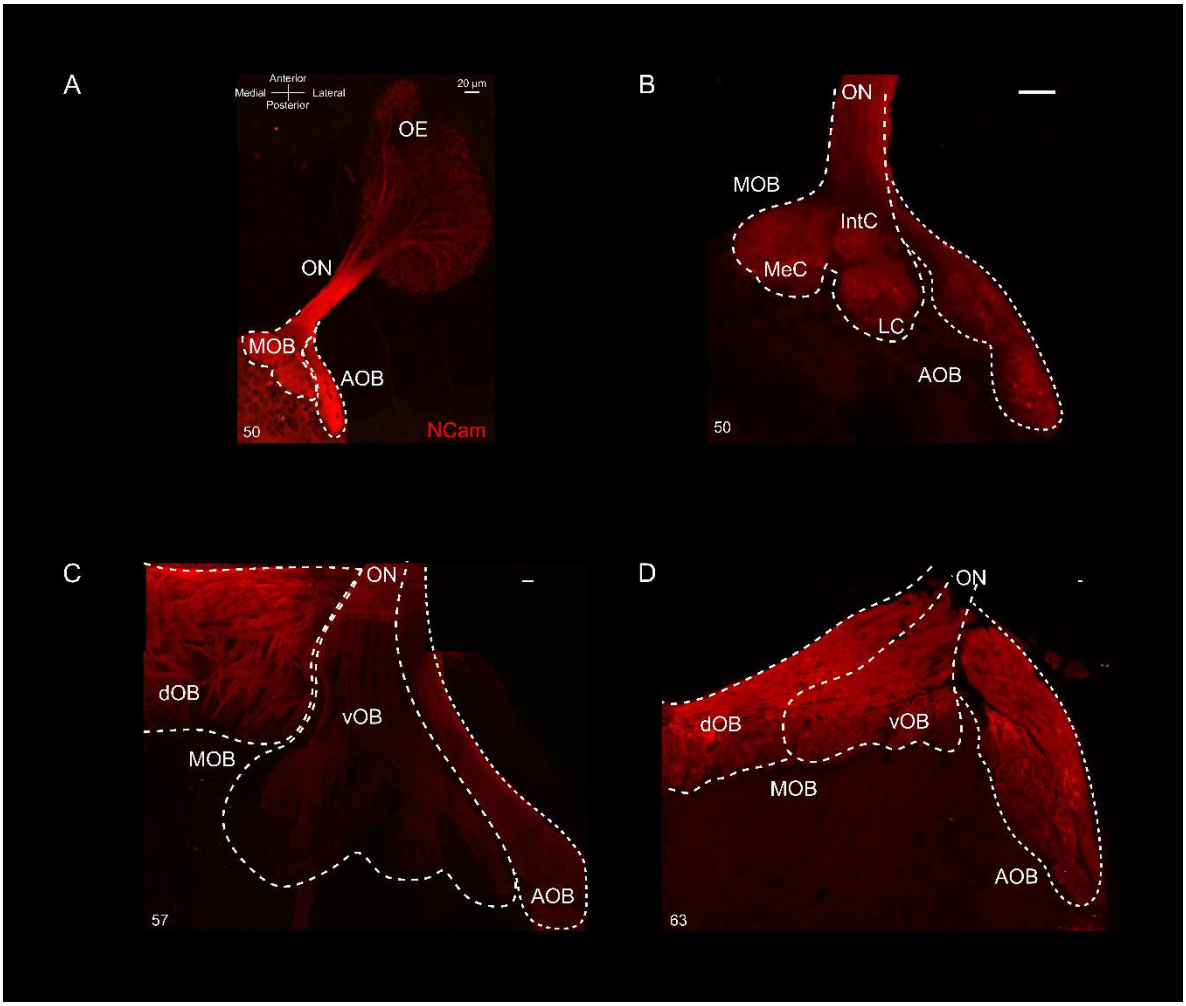
V. Results

i. Development of the olfactory organ and the olfactory bulb during metamorphosis

To visualize the anatomical changes in the olfactory bulb during metamorphosis, immunohistochemical studies were conducted using an antibody against NCam protein. NCam, or neuronal cell adhesion molecule, is a neuronal surface glycoprotein, which binds to a variety of other cell adhesion proteins to mediate adhesion, guidance, and differentiation during neuronal growth and is especially expressed in axonal structures [73].

NCam is visible in axons of ORNs in the olfactory organ that eventually merge to form the olfactory nerve. In the OB, it stains the glomerular clusters of the main olfactory bulb and in the accessory olfactory bulb of *Xenopus laevis* as shown in figure 5. Before stage 52, it can observe that ORNs' axons from the PC converge to form the ON and end up in the glomerular layer of the main olfactory bulb (figure 5A) whereas the axons from the VNO converge into the accessory olfactory bulb (figure 5A). The distance between the OE and the OB is quite substantial in comparison to later stages. The ON has a larger length in early stages (52-59). At this stage the MOB consists only of the ventral glomerular region, the dorsal glomeruli still have not started to form (figure 5B). NCam staining in the glomerular layer shows the anatomical structure of the ventral bulb divided mainly into three clusters; lateral, intermediate, and medial (figure 5B). At stage 57 ORN axons originating in the MC terminate in the ventral glomerular clusters and these maintain the same anatomical structures than before metamorphosis (figure 5C). Meanwhile, the axons coming from the PC converge in the dorsal region, which does not show any sort of clusters but a flat and enlarged layer (figure 5C, D). During the last stages of metamorphosis, the olfactory nerve shortens and now the distance between the MOE and the OB is shorter than in early metamorphosis (figure 5D), the AOB preserves its structure and grows together with the MOB consisting of the dorsal and ventral side (figure 5D).

Figure 5. Anatomical changes in the olfactory bulb of *Xenopus laevis* during metamorphosis. **A**: NCam staining shows in red the anatomical structures in the olfactory bulb of an animal at stage 50. Axons in the olfactory epithelium (OE) merge to form the olfactory nerve (ON) and innervate the glomerular layer in the main olfactory bulb (MOB). Axons coming from the VNO (not shown here) end up in the accessory olfactory bulb (AOB). **B**: NCam staining of an animal at stage 50 showing the three main clusters in the ventral region of the MOB: lateral cluster (LC), intermediate cluster (IntC), and medial cluster (MeC). The AOB is also stained. **C**: Labeling of the olfactory bulb against NCam showing the new formation of the dorsal olfactory bulb (dOB) as a new structure and the grown ventral glomerular clusters in the olfactory bulb (vOB). **D**: At stage 63 the dorsal and ventral regions have bigger dimensions than earlier stages and the dOB possesses a flat and enlarged shape in contrast to the vOB, the ON shortens in length, it is closer to the epithelium, and the vOB remains with the same anatomy. Scale bar: 20 µm.



After following the anatomical modifications in the olfactory bulb during metamorphosis in *Xenopus laevis,* our next question was: what happens with the functionality in the olfactory bulb during metamorphotic remodeling?

ii. Odorant responses in the ventral olfactory bulb during metamorphosis

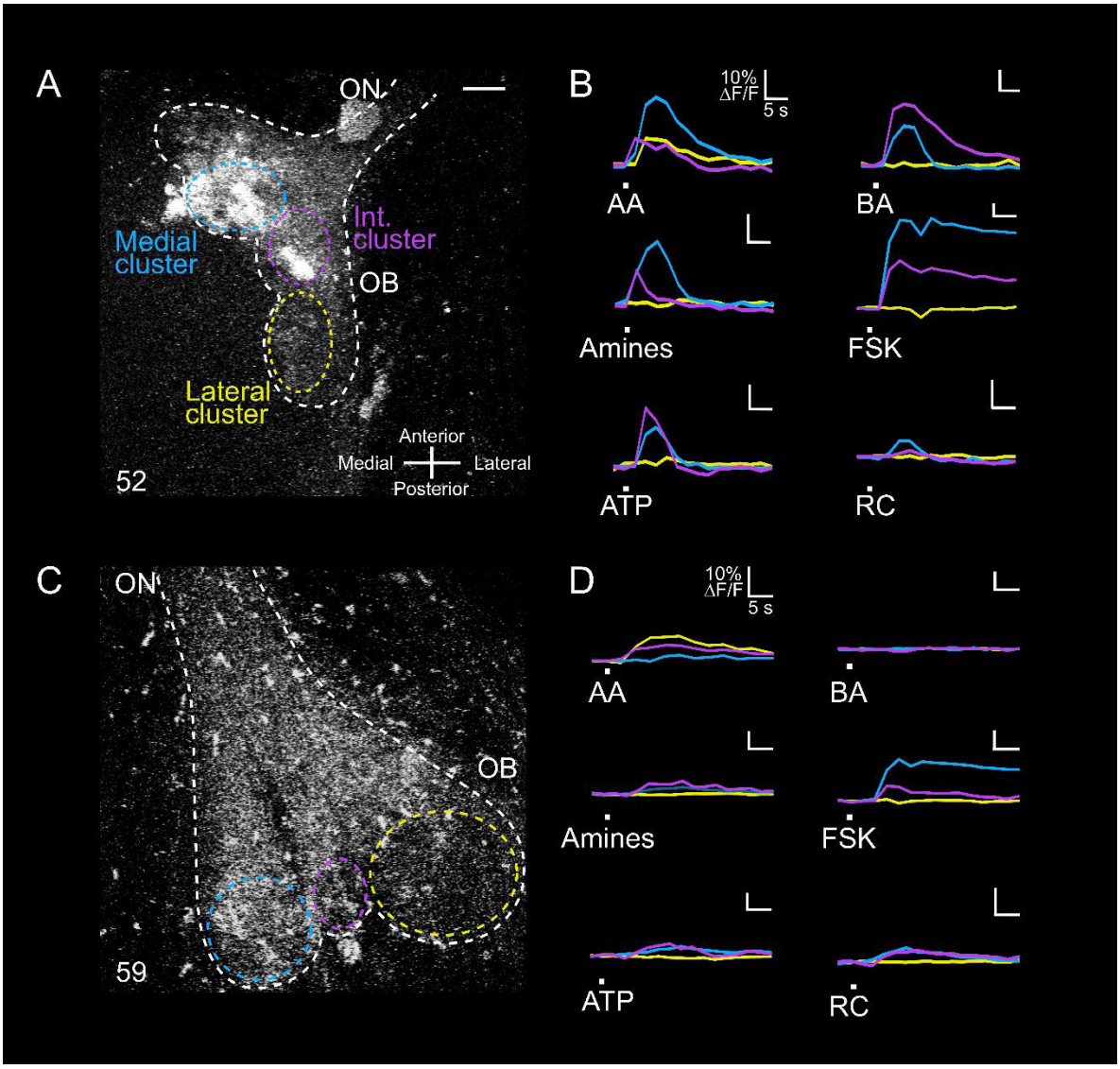
Odorant-induced neuronal activity patterns were recorded in a whole-mount preparation (see materials and methods) of animals during different developmental stages. All animals were divided into two main groups to analyze the responses; premetamorphotic group (stage 52-54) and metamorphotic group (stage 55-63). The applications used were the amino acid mix, known for being a potent stimulus in *Xenopus laevis* [74], Ringer solution as a negative control, odorant mix as a positive control, bile acids, amines, ATP, and forskolin as an activator of adenylate cyclase

which increases intracellular cAMP levels. The odorant mix contains all of the stimuli here mentioned [27], [75].

First, we analyzed responses of the intermediate cluster in animals of the two distinct developmental groups to identify response patterns to different odorant stimuli. Representative traces from the IntC, LC, and MeC are shown in figure 6B, D. The intermediate cluster showed a broad sensitivity range responding to all applications in the premetamorphotic group (purple traces in figure 6B). Responses to all stimuli applications, except to bile acids were present in the intermediate cluster of metamorphotic animals. It seems that the IntC has no preference for a specific odorant application. Representative calcium transient traces of the metamorphotic group showed a smaller change in fluorescence although was a considerable response (figure 6D). Metamorphotic animals had smaller amplitudes in odorant responses (figure 6D). Responses to forskolin remained always in the medial and intermediate clusters of both groups (blue and magenta traces figure 6B, D). The lateral cluster never elicited responses to forskolin in both groups (yellow traces, figure 6B, D).

These representative examples revealed a lateral and medial segregation in responses; especially upon AA and FSK application. AA application elicits responses in the three clusters of animals in the premetamorphotic group and induces calcium transient responses in the LC and IntC of animals in the metamorphotic group (figure 6). FSK application elicited responses on the MeC and Int of both animal groups (figure 6).

Figure 6. Representative calcium transient traces of the clusters in the olfactory bulb of Xenopus laevis during metamorphosis. **A**: Staining of the glomerular clusters of a premetamorphotic animal (stage 52): lateral cluster, medial cluster, and intermediate cluster. **B**: Example traces of each cluster to all applications in the premetamorphotic group. Yellow traces correspond to responses from the lateral cluster, blue traces correspond to responses in the medial cluster and purple traces to responses in the intermediate cluster. Applications used: amino acids AA, Amines, Adenosine triphosphate ATP, bile acids BA, forskolin FSK, and Ringer solution RC. Intermediate cluster exhibits a broad range of responses to all applications. The lateral cluster elicits responses when stimulated with AA. Medial cluster exhibits, as well as the intermediate cluster, a broad range of responses to all applications but the highest response is to FSK. The lateral cluster is not activiated by FSK. **C**: Staining of the glomerular clusters of a metamorphotic animal (stage 59): lateral cluster, medial cluster, and intermediate cluster. **D**: Examples traces of each cluster to all applications in the metamorphotic group. Yellow trace, blue trace and purple trace same as B. Applications used: same in B. Responses are smaller than in the premetamorphotic group. Forskolin application activates the medial and intermediate cluster and not the lateral cluster. Intermediate cluster presents responses to all applications as well as the medial cluster with the exception of BA.



iii. *<u>The intermediate cluster is sensitive to a broad range of stimuli during metamorphosis</u>*

The intermediate cluster of the premetamorphotic group displayed a major sensitivity to forskolin, which elicited responses in 100% of the population (n=6). AA application was the second most sensitive stimulus in the premetamorphotic group (n=6) showing responses in 83 % of the animals (darker magenta, figure 7). Sensitivity to amines showed being the third highest with 75% of the animal responses. ATP, bile acids, and ringer solution showed similar sensitivities in the intermediate cluster, 50 % (darker magenta, figure 7).

The metamorphotic group showed a wide range of responses for stimuli application; 75 % of the population (n=12) responded to AA being the highest percentage followed by a 68 % to forskolin stimulation (lighter magenta, figure 7). The third highest population responses were to amines 50 % in the metamorphotic group and 70 % for the premetamorphotic group. Metamorphotic population elicited responses in the intermediate cluster in 25 % to ATP and Ringer solution (lighter magenta, figure 7) whereas 50 % of premetamorphotic animals responded to ATP and Ringer. The odorant that elicited the smallest responses in the intermediate cluster in metamorphotic stage was bile acids, 18 % (lighter magenta, figure 7). Animal responses to RC might indicate that there are mechanosensitive glomeruli in the intermediate cluster.

Even when example traces in figure 6 showed minimal amplitude in metamorphotic animals, the population analysis showed that still during metamorphosis the intermediate cluster responds to a wide variety of odorant applications (lighter magenta, figure 7).

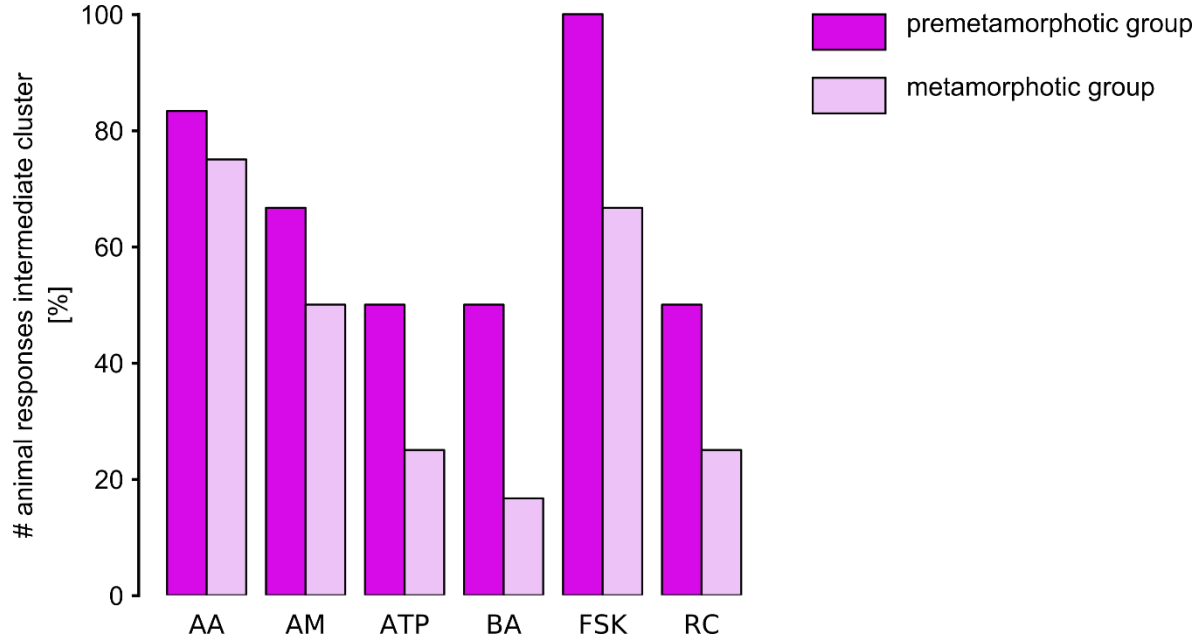


Figure 7. Responses to odorant applications in the intermediate cluster of *Xenopus laevis* during metamorphosis. The premetamorphotic group was broadly tuned to various odors. The odorant with the highest response was forskolin followed by amino acids. The metamorphotic group exhibited also a broad range of odorant responses but it showed a higher sensitivity for amino acids rather than forskolin.

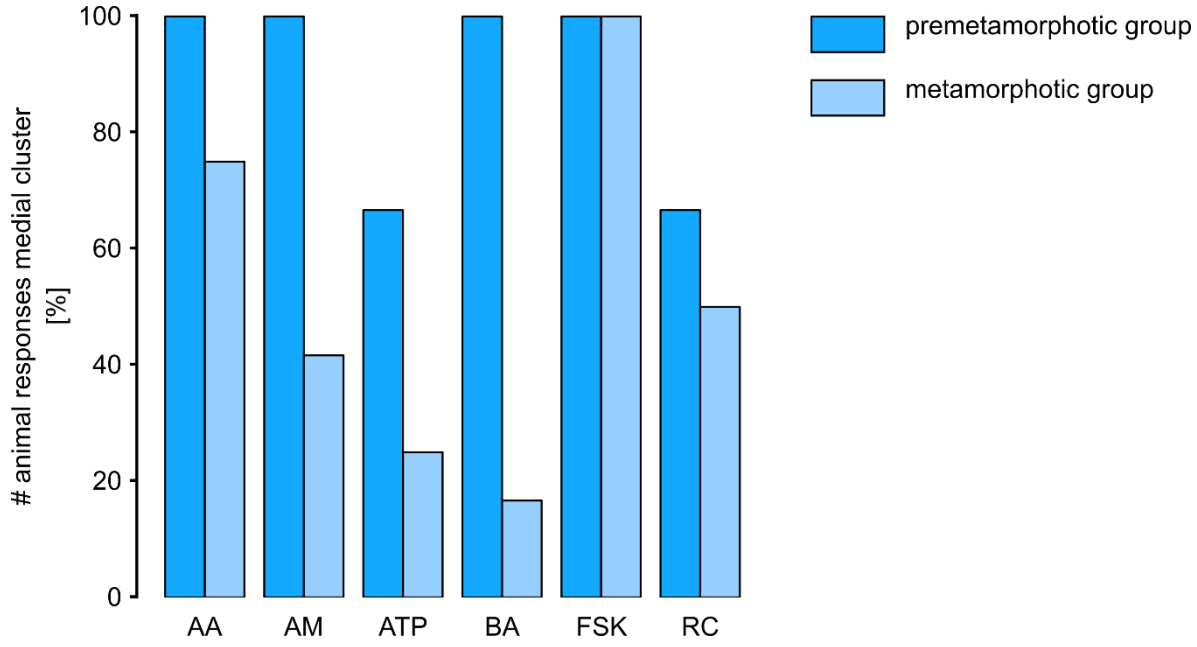
iv. Medial and lateral cluster responses during metamorphosis

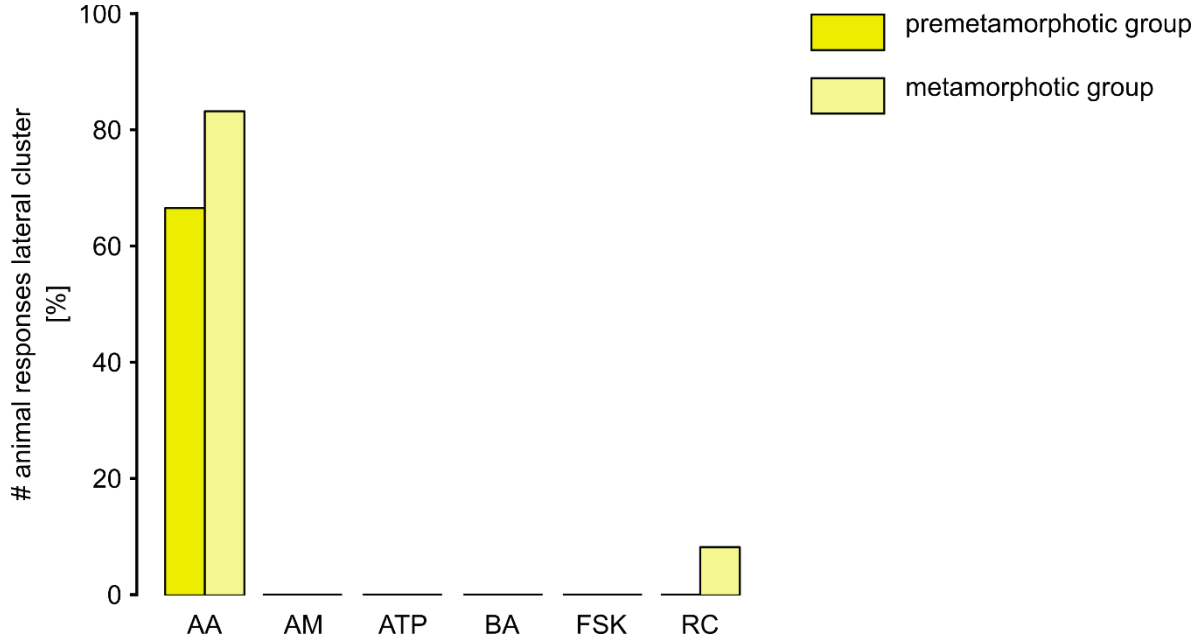
Figure 8 shows animal responses to every odorant application. Results showed that 100% of the population in the pre- and metamorphotic groups responded to forskolin in the MeC (figure 8). Upon FSK application 0% of the pre- and metamorphotic groups were never observed to induce fluorescence changes in the lateral cluster (figure 9). The same pattern was obtained with the metamorphotic group, all odorant applications elicited responses in the medial cluster. The percentage of animals in the premetamorphotic group responding to stimuli application was higher than the percentage of animals in the metamorphotic group. Bile acids and ATP elicited responses on a lower animal percentage in the metamorphotic group than the rest of the odorant applications (darker blue, figure8).

Stimulation of the olfactory mucosa with amino acids clearly affected the lateral cluster in 65 % of the premetamorphotic animals (n=6) and 80 % in the metamorphotic population (n=12) (figure 9). The number of animal percentage responding to AA in the premetamorphotic group was lower than the number of animal percentage of the metamorphotic group (figure 9). Animals responding to RC might be due to a mechanical stimulation of the glomeruli (lighter yellow, figure 9). Comparing between figure 8 and 9 we can observe there is a segregation in responses to a different application in the medial and lateral cluster. The lateral cluster responds to AA stimulus and FSK responses were never present whereas the MeC presents activation to a broader range of stimuli. Medial cluster responded to FSK, AA, AM, BA, RC, and ATP. Certainly, responses are segregated into two streams: medial and lateral cluster.

Figure 8. Responses to odorant applications in the medial cluster of *Xenopus laevis* during metamorphosis. The premetamorphotic group showed a broad range of responses for different odorant applications. The odorant with the highest response was forskolin followed by amino acids with 100% oft he population responding. The metamorphotic group exhibited also a variety for odorant sensitivity although it showed a higher sensitivity for forskolin rather than amino acids. Forskolin stimulation maintain the same responses percentage, 100%, during metamorphosis

Figure 9. Responses to odorant applications in the lateral cluster of *Xenopus laevis* during metamorphosis. The premetamorphotic group showed responses to AA application. The metamorphotic group exhibited also responses to amino acids with an 82% of the population responding. Forskolin application did not elicit responses in the lateral cluster of animals during metamorphosis.





v. *Lateral and medial processing stream remain undisrupted during metamorphosis*

The restructuration of the MOE, the rewiring of the MOB and the new network formation in the dorsal bulb mean there are been changes in the olfactory system

during metamorphosis, which might lead to a disparity in functionality. Reason to answer if the responses in the olfactory bulb remain undisrupted and if so, to what do they respond?

For functional analysis, response patterns of the medial and lateral cluster in different stages of metamorphosis were analyzed. ROIs selected in the lateral (yellow) and medial (blue) cluster of the OB are shown as examples on how areas were selected to analyze the responses in figure 10A, B. Traces in figure 10 show representative calcium transient responses during odorant application of an animal in premetamorphotic (figure10C) and metamorphotic stages (figure10D).

ROIs in the medial cluster of premetamorphotic animals (blue ROIs in the figure 10A) showed a wider range of responses with FSK eliciting the strongest response (blue arrow in figure 10C) followed by the second strongest responses upon AA application (white arrow in figure 10C). In the premetamorphotic group AA application into the olfactory epithelium induced calcium-dependent fluorescence changes in the lateral cluster (yellow arrow in figure 10C) and the medial cluster (white arrow in figure 10C). Forskolin responses were not present in the lateral cluster of the premetamorphotic group (asterisks in figure 10C). The metamorphotic group showed a similar response pattern of the medial and lateral clusters. The MeC showed an increase of intracellular calcium concentration for a wide variety of applications as shown for the premetamorphotic group (figure 10D). Forskolin responses still had the highest amplitude (blue arrow in figure 10D) present in the ROIs of the medial cluster whereas the rest of applications, such as AA, elicited responses in the medial cluster but less strong than forskolin (white arrow in figure 10D). AA application into the olfactory mucosa also induced calcium transients in the lateral cluster (yellow arrow in figure 10D). In accordance with the results obtained in premetamorphotic animals, the metamorphotic group did not show responses to forskolin application in the lateral cluster (asterisks in figure 10D).

It is notable that amplitude in animal responses of the metamorphotic group was smaller than premetamorphotic animals. This decrease in amplitude responses could be due to technical problems during the recordings or the intrinsic responses upon odorant applications.

Figure10 Odorant responses of medial and lateral glomerular cluster in the ventral olfactory bulb of *Xenopus laevis* during metamorphosis. **A**: Whole mount preparation of the olfactory system including the OE and the vOB of a premetamorphotic animal. Staining with Dextran-conjugated Cal520 shows the anatomical structure of the ventral olfactory bulb and its 2 main clusters. Blue and yellow ROIs represent glomeruli in the medial and lateral cluster, respectively. **B**: Whole mount preparation of the olfactory system including the OE and the vOB of a metamorphotic animal. Staining with Dextran-conjugated shows the anatomical structure of the ventral olfactory bulb and its two main clusters. Blue and yellow ROIs represent glomeruli in the medial and lateral cluster, respectively. **C**: Each ROI elicited calcium transient responses after the application of a variety of stimuli to the OE. Upper traces (yellow) are the time courses of $[Ca^{2+}]_i$ transients of each glomerulus in the lateral cluster upon application of seven different stimuli. OD was applied as a positive control. Time courses (blue traces, bottom) of the $[Ca^{2+}]_i$ transients of the 4 ROIs in the medial cluster upon mucosal application of the same stimuli. **D**: Each ROI elicited calcium transient responses after the application of a variety of stimuli to the OE. Upper traces

(yellow) are the time courses of $[Ca^{2+}]_i$ transients of glomeruli responding to AA and OD. OD was applied as a positive control. Time courses (blue traces bottom) of the $[Ca^{2+}]_i$ transients of the 4 ROIs in the medial cluster upon mucosal application of stimuli exhibited responses to AA, OD, AA, BA, and primarily to FSK. Arrows indicate the response to stimuli. AA amino acids, AM amines, ATP adenosine tri-phosphate, BA bile acids, FSK forskolin, OD odorant mix, RC Ringer solution. Scale bar: 50 µm.

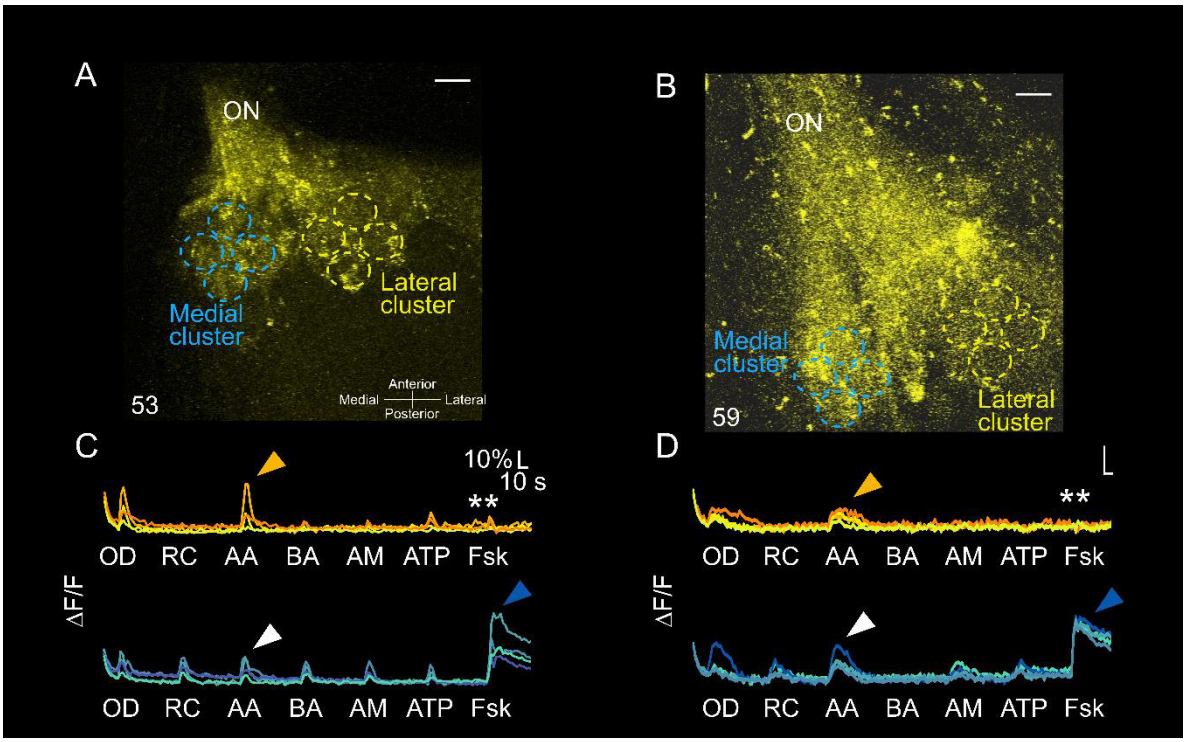


Figure 11 shows the population analysis results. We compared responses in LC with those in the MeC of premetamorphotic and metamorphotic animals to AA and FSK stimulation. 100 % of the animal population (n=6) in the premetamorphotic group showed responses to forskolin in the medial cluster (figure 11A). AA application elicited responses in 100 % of the population in the medial cluster of the premetamorphotic group (figure 11A) whereas for the lateral cluster the responses were present only in 65 of the population. Lateral cluster did not show responses to FSK application in animals of the premetamorphotic group (Figure 11A).

In the metamorphotic group (n=12), forskolin responses in the MeC were present in 100 % of the population however; forskolin, did not elicit any response in the lateral cluster, 0 %, (figure 11B). The LC responses to AA application were present in 85 % of the metamorphotic population, while the premetamorphotic group had just 65 % of the population responding to AA stimulation. 100 % of the premetamorphotic animals responded to AA application in the MeC and the percentage of animals decreased to 76 % upon AA stimulation in the metamorphotic group (figure 11B). Animals in the metamorphotic group did not show responses to

FSK application (figure 11B). Population analysis shows again the lateral and medial streams between the two groups.

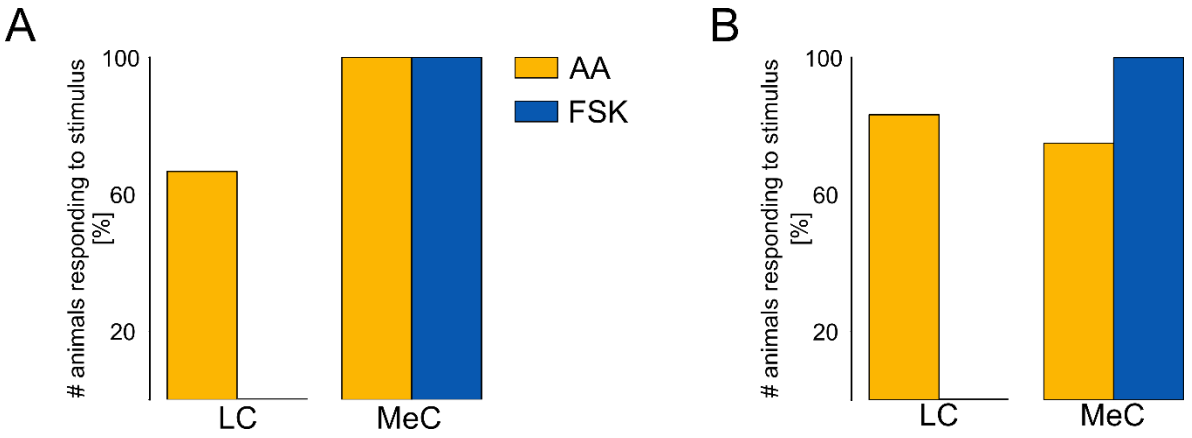


Figure 11 Comparison between responses in the LC and MeC to AA and FSK application in both groups of *Xenopus laevis*. **A**: The premetamorphotic group showed a difference in responses to AA and FSK stimuli in the lateral and medial cluster. Animal responses in the LC were observed upon AA application whereas FSK did not elicit any response in the LC. Responses to AA and FSK in the MeC were observed in 100% of the population. **B**: The metamorphotic group had responses in LC and MeC to both stimuli. A larger number of animals responded to AA application in the LC than in the MeC. FSK application elicited responses in 100% of the population in the MeC and 0% in the LC.

Animals of both groups presented responses in the MeC to all applications. FSK responses were the strongest, followed by AA responses. Both groups also showed sensitivity for AM, BA, RC, and ATP in the MeC. The same percentage was observed on animals of both groups who responded to FSK application while the percentage of animals was different for the rest of the stimuli. Animals of both groups showed responses in the LC only to AA stimulus, with the exception of the metamorphotic group responding to RC. There were not animals responding to FSK application neither on the premetamorphotic nor the metamorphotic group in the LC. Response patterns do not seem to change in the course of metamorphosis development even though there are structural changes going on in both, the MOE and the MOB. Functionality on the vOB does not seem to be interrupted throughout development although rewiring processes are occurring in the MOB and a new network is formed in the dOB. Remodeling in the innervation of the OB does not affect the responses or their mapping into the lateral or medial cluster.

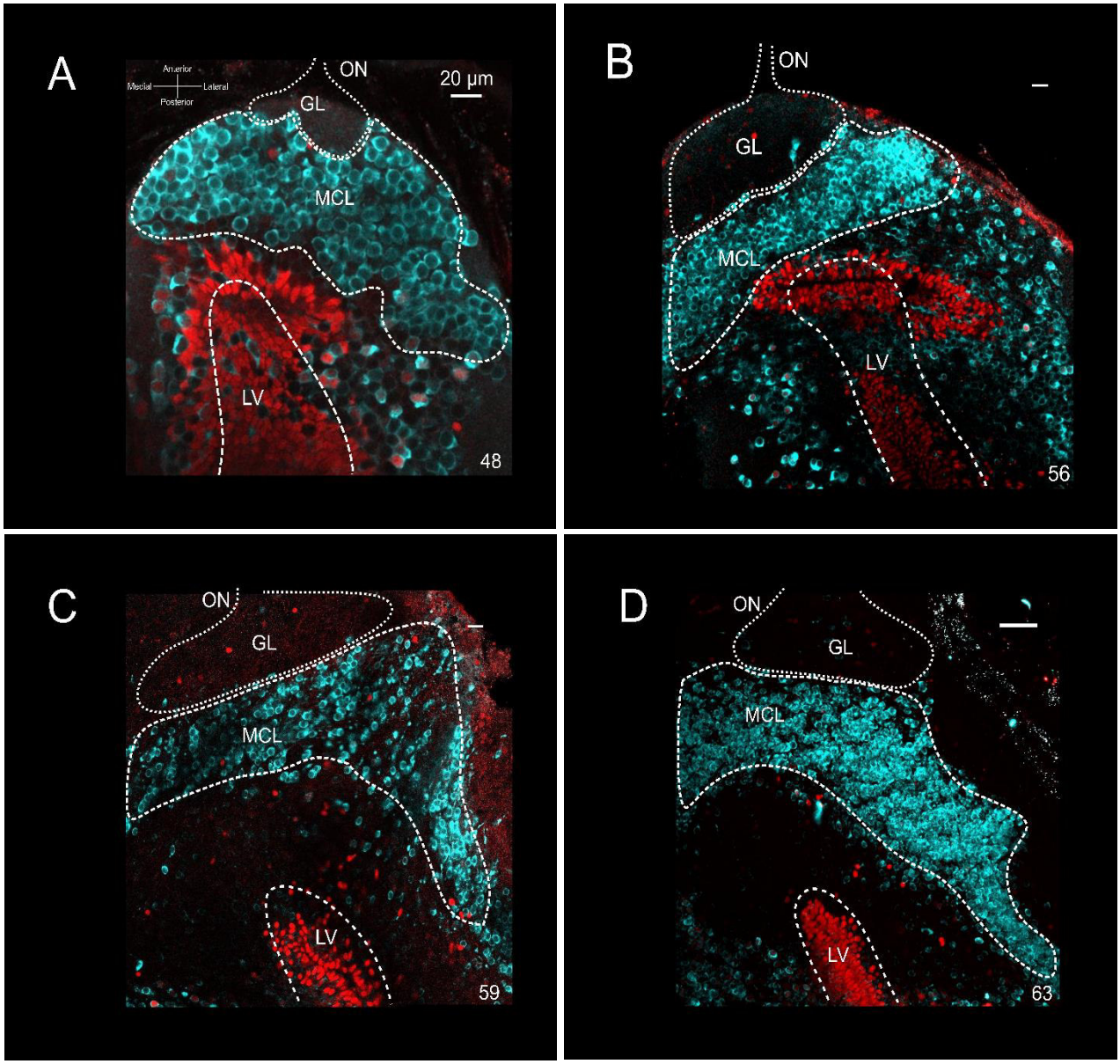
vi. Stem cell niche in the olfactory bulb during metamorphosis

The niche of stem cells during development is located in the wall of the lateral ventricles and the anterior region regulates the migration of progenitor cells, which eventually differentiate into either a projection neuron or an interneuron [69], [76].

To analyze neurogenesis in the olfactory bulb, we performed immunohistochemistry assays in animals at different stages, 48-63, using antibodies against Sox2 as a marker of stem cells and HuC/D as a marker of neuronal cells. Sox2 is involved in stem cell pluripotency maintenance, and cell fate determination. It is also important for the maintenance of stem cells in multiple adult tissues [77]. Sox2 expression after postnatal development is detected in the subventricular zone and the dentate gyrus of the hippocampus [78]. HuC/D is a family member of the Hu proteins. Hu proteins are a group of cytoplasmic RNA-binding proteins that interact with adenine uracil (AU)-rich elements to regulate maturation and function of mRNAs [79]. HuC/D is expressed in neurons and plays an important role in neuronal differentiation, development and neuronal plasticity [80].

Immunostainings showed that at stage 48 Sox2-positive cells were expressed in the subventricular zone of the lateral ventricles (LV; figure 12A). Sox2-positive cells at stage 56 were distributed along the whole LV and few Sox2-positive cells began to migrate to the OB (asterisks in figure 12B). At stage 59, we observed that Sox2-positive cells are accumulated near the LV and SVZ (figure 12C). At stage 63 we found there are Sox2-positive cells migrating and the denser area is located in the LV. Figure 12E is a close up of figure 12D showing the Sox2-positive cells migrating towards the OB (arrowheads). These results show that the ventricles are the primary stem cell niche responsible for neurogenesis in the olfactory bulb and Sox2-positive cells seem to migrate to the OB during metamorphosis.

Figure 12. Neurogenesis of the olfactory bulb during metamorphosis in *Xenopus laevis*. Immunohistochemistry staining using antibodies to label stem cells (Sox2) and a neuronal marker (HuC/D) of the olfactory bulb. **A**: OB of a larva in early premetamorphosis, stage 48, showing a neuronal, HUC/D-positive cell layer already present along the OB and Sox2-positive cells located near the lateral ventricle. **B**: the HuC/D-positive cell layer is enlarged in comparison with A suggesting an increase neuronal proliferation. **C**: Sox2-positive cells are migrating through the RMS and eventually they will differentiate into an olfactory bulb neuron, a projection or interneuron. **D**: it can be appreciated that the HuC/D-positive neuronal cell layer has enlarged as well as stem cells have traveled a longer distance from the ventricle to become a MTC/interneuron. **E**: close-up of ***D*** showing the migration of Sox2-positive cells (arrowheads) traveling from the lateral ventricle. GL glomerular layer, LV lateral ventricle, MCL mitral cell layer, ON olfactory nerve. Scale bar 20 µm.


A
Anterior
Medial
Lateral
Posterior
ON
20 µm
GL
MCL
LV
48
B
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D
ON
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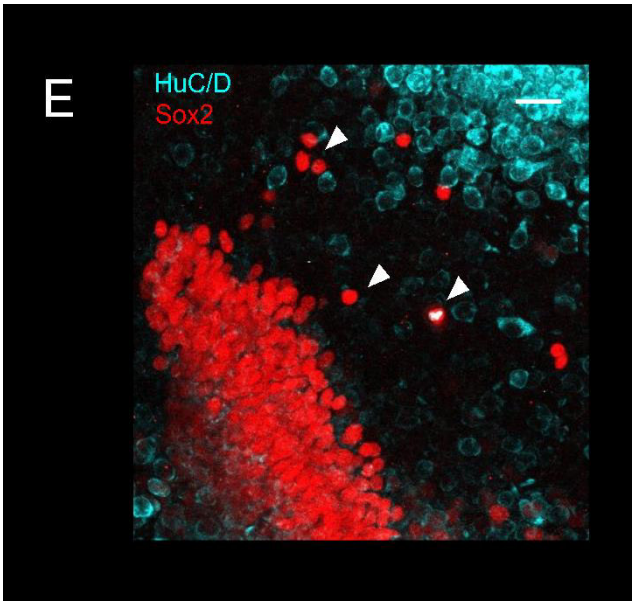

HuC/D stainings show that before the onset of metamorphosis, a HuC/D-positive cell layer is already present in the OB (figure 12A) and might coincide with the location of the mitral cell layer [81]. While metamorphosis continues, the HuC/D-positive cell layer enlarges and an increment in the number of HuC/D-positive cells can be appreciated (figure 12B-D). The enlargement of this neuronal layer could be noticed around stage 54, which coincides with the development of the dorsal olfactory bulb. At stage 63 HuC/D staining is expressed on a bigger area in the MTC layer (figure 12D).

The results suggest that differentiating stem cells from the SVZ of the lateral ventricles migrate into the other bulbar layers throughout metamorphosis. There might be an increase of migrating Sox2-positive cells from the SVZ to the OB starting at stage 54 and there is also an expansion of the HuC/D-positive neuronal cell layer within metamorphosis. We saw that the olfactory bulb had more HuC/D-positive cells in later stages so we asked if the cell bodies increased in size. The increment in body size could be one reason for the MTC layer to have a larger volume within metamorphosis. For that, we selected 20 well defined cell bodies positive to HuC/D protein of 3 different animals at 4 different stages: 48, 56, 59, 63 and measured their cross-sectional area (figure 13). We found that at stage 48 the mean cross-sectional area of the 20 cell bodies was 71.8 $\pm$ 11.4 μm^2 whereas at stage 63 the mean cross-sectional area was 76.9 $\pm$ 18.5 μm^2 (see Table 4). The increment of the cross-sectional area was not very drastic between stage 48 and stage 63 (figure 13) although for stage 56 and 59 the mean cross-sectional areas were 75.1 $\pm$ 24.5 μm^2 and 86.4 $\pm$ 17.1 μm^2 respectively (figure 13). There was a significant difference between stage 59 compared with the rest of the stages. The mean values of the

cross-sectional area of animals are not significantly different among stages 48, 56, and 63 (Table 4).

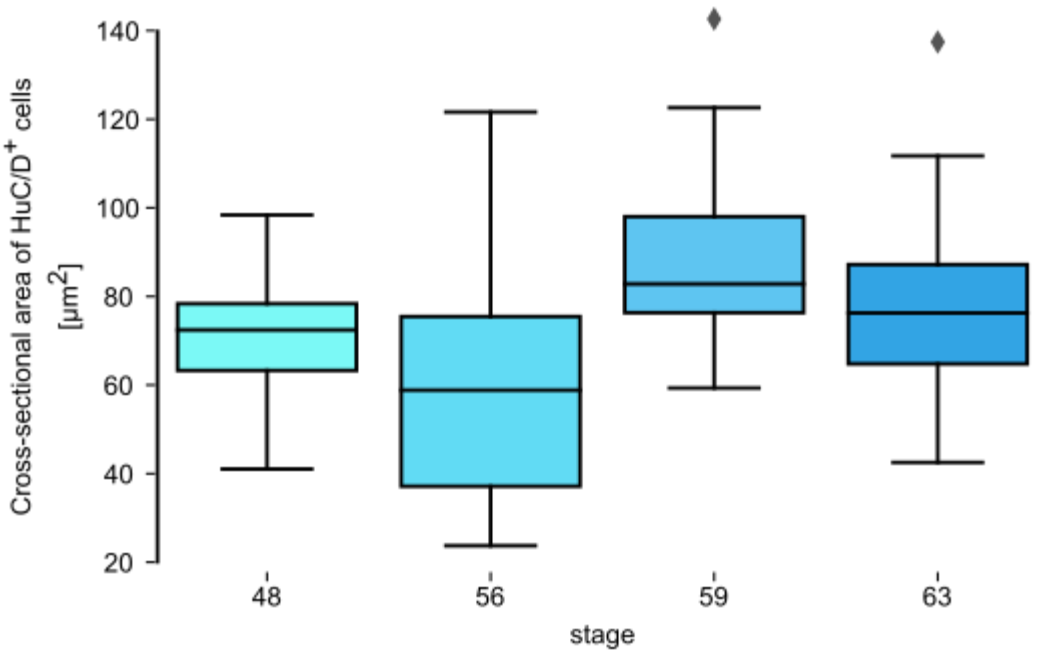

Figure 13. Cross-sectional area of HuC/D-positive cells at different stages of metamorphosis. The mean area from stage 48 to stage 63 did not present a big variation, 71.8 ± 11.4 µm^2 and 76.9 ± 18.5 µm^2 respectively. The cross-sectional area of animals at stage 56 presented a wider range in the cross-sectional area among the 20 cells measured but the mean area was 75.1 ± 24.5 µm^2. In comparison, the bigger cross-sectional area of the HuC/D-positive cells was present in animals at stage 59, 86.4 ± 17.1 µm^2. From stage 59 to stage 63, the mean cross-sectional area decreased almost 10 µm^2.

Table 4: Mean values of cross-sectional area of HuC/D-positive cell bodies and their significance

stage	Mean (µm^2)	S.D. (µm^2)	P-value
48	71.8	11.4	*p>0.05*
56	75.1	24.5	*p>0.05*
59	86.4	17.1	*p<0.05*
63	76.9	18.5	*p>0.05*

VI. Discussion

i. Anatomical changes in the olfactory bulb

The NCam antibody is a good candidate for axonal projections and glomerular clusters staining. Using this antibody, we observed a very well-defined anatomy of the olfactory glomeruli and the olfactory nerve during metamorphosis. The immunostaining assays support what has been shown in the literature ([34], [40], [82], [83]) showing that the glomeruli in the ventral olfactory bulb are mainly composed by three clusters and at stage 52, the development of the dorsal region of the olfactory bulb is observed. At stage 57, the dOB expands and has an enlarged shape. These results show that the dOB is expanding and growing substantially during metamorphosis [23], [84].

ii. Medial and lateral processing stream seem preserved during metamorphotic remodeling

During metamorphosis, an apoptotic process starts to appear in the olfactory epithelium in order to rearrange the olfactory receptor expression in both cavities. ORNs in the PC die and new ORNs are born in the PC and MC expressing different receptor types [37], [85]–[89]. Before metamorphosis, the PC expresses V2R and TAAR receptors, which detect water-born odorants. During metamorphosis, the expression changes for OR-type receptors that sense air-born odorants. Meanwhile, receptors expressed in the PC shift to the MC. Syed et al., has shown that gene expression of TAARs and V2Rs shift from PC to MC [33]. Data has shown that the lateral odor-processing stream is characterized for having microvillous ORNs, responds to AA, and expresses V2R and TAAR receptors. The medial stream is formed by ciliated ORNs, is characterized for its responses to forskolin, alcohols, ketones, and aldehydes; possess OR receptors and uses cAMP as probable signal transducers [27]. While this orchestral protocol occurs, the axonal projections coming from the principal cavity project to the vOB and then shift to the dOB while new axonal projections from the MC project into the ventral olfactory bulb. The OB of larvae is characterized for having two main segregated streams: lateral and medial and they respond to specific sets of odorants [37], [90], [91]. During this shift in innervation, it is unclear whether the functionality of the vOB is preserved or disrupted and there is no evidence about the response patterns of the vOB during metamorphosis. The reason why functional imaging experiments in *Xenopus laevis* during metamorphosis were performed. We wanted to observe the response patterns and if the vOB was responsive throughout metamorphosis.

Our experiments showed that the premetamorphotic group had response patterns segregated into a medial and lateral processing stream, which is consistent with the literature [27], [39], [74], [92]. The same lateral and medial segregated

streams were observed in the metamorphotic group. Throughout metamorphosis remodeling, the lateral cluster remains responsive to amino acids and never responds to forskolin. Forskolin responses were observed only in the MeC and IntC.

Activation of ORNs to forskolin or amino acids applications is projected into medial and lateral streams responses in the glomerular clusters respectively [31], [75], [90]. The amino acid responsive cells projection to the lateral glomerular cluster seem to follow a cAMP-independent pathway. Responses to AA, as well as forskolin, in the medial cluster, seem to have a cAMP-dependent cascade [27], [31], [93]. We show that even though the odorant receptor expression shifts to the MC the responses of the TAAR/V2R-expressing cells remain constant during metamorphosis.

Although many morphological changes are occurring during metamorphosis, we were able to obtain responses in the vOB. These odorant responses were shown to have the same segregation streams; lateral and medial in the glomerular clusters. At the same time, pattern responses observed in metamorphotic animals were constant and remain undisrupted. It is remarkably how the functionality of the glomerular clusters is consistent throughout metamorphosis nonetheless, odorant responses decreased in amplitude in late metamorphosis. Since the same segregation streams are observed in older stages this suggest that the olfactory system is operating while the metamorphotic process takes place.

iii. Intermediate cluster shows a broad range in odorant sensitivity

Response patterns in the intermediate cluster showed activity to most of the applications. The responses to a wide range of stimuli in the intermediate cluster could be due to different signaling pathways or receptor expression coming from the MOE. It has been reported that the IntC is a mechanosensitive and thermosensitive cluster meaning that is capable to respond to mechanical stimuli or changes in temperature [31], [93], [94] [95]. The responses in the intermediate cluster to amino acids and to forskolin could mean they follow the cAMP-dependent cascade although they express different olfactory receptors. The percentage of animal responses decreased in older stages. A limiting factor in the condition of the presented experiments was that using animals in bigger stages the experiments were more difficult to perform. Around stage 59, the olfactory system reduces the length between the nasal cavity and the olfactory bulb, so the tissue preparation is not easy to place under the microscope. This technical issue could be one explanation of the decrease in amplitude response. Possible reasons for the decrease in animal responses could be morphological modifications in the olfactory epithelium, the change in receptor expression, the connections from the receptor neurons to the glomeruli are less active or sensitive, and the rewiring in the ventral olfactory bulb could be the causes of this decrease in animal responses.

iv. Neurogenesis in the OB upon innervation of ORN axons on the dorsal side

In vertebrates, the adult brain is capable to produce new neurons [96]. Adult neurogenesis is important for memory and odor discrimination in rodents, and its alterations are implicated in psychiatric disease in humans [97]. In most species adult neurogenesis mainly occurs in two brain regions, the subventricular zone of the lateral ventricles and the subgranular zone of the hippocampal dentate gyrus. In mammals, neurons born in the SVZ migrate tangentially to the OB through the RMS to become interneurons [96]. Neurogenesis in the olfactory bulb has been studied in several vertebrate species and follows a stereotyped pattern with the major projection neurons, the mitral cells generally born first followed by the birth of various types of interneurons, PGCs and GCs [53], [54], [98]–[102].

Olfactory epithelium of *Xenopus laevis* is not the only one undergoing transformational modifications but a dorsal olfactory bulb also emerges [103]–[109]. The brain of *Xenopus laevis* can produce new neurons during metamorphosis [69] but there is no much evidence about how the dOB is developing during metamorphosis and how is integrated into an already functional olfactory network during metamorphosis in *Xenopus laevis* [48]. During dOB formation, new neurons need to be built in order to make new connections from the OE to the glomerular clusters. A whole new network is assembled with the formation of the dOB although is not known the functionality of this new network. Different timings in olfactory bulb neurogenesis can arise depending on the necessities and the new connections. When the first axon-bulbar connections start to appear in the young larvae a whole network needs to be ready to start detecting signals coming from the exterior corresponding to the aquatic life of *Xenopus laevis*. As soon as the frog undergoes metamorphosis new necessities arise; it requires a whole new network to sense aerial cues.

When does the formation of the dOB begin? There are some studies showing that the formation of the dorsal region starts in line with metamorphosis [69]. From the literature, we know many organs in *Xenopus laevis* undergo changes mainly as a result of the high levels of the thyroid hormone [89]. Results showed that before metamorphosis begins, a neuronal cell layer is already present in the bulb. This neuronal cell layer and according to the literature this must be the ventral olfactory bulb, which is present before axonal innervation; on the contrary, the dorsal region begins to form around stage 52 exactly when the first axons from the newly generated ORNs in the PC penetrate the glomerular layer in the olfactory bulb and make its firsts connections [69]. This suggests the signal for dorsal region neurogenesis could be triggered by axonal innervation.

In our results, stems cells were confined to the SVZ throughout metamorphosis and at stage 59 single Sox2-positive cells were not confined to the SVZ but they were observed outside the SVZ, in a space between the stem cell niche

and the OB, proving this is the niche for neurogenesis and they, at some point, migrate to become a neuron in the OB. Migration of stem cells is important for cell proliferation and differentiation into olfactory neurons considering the bulb needs to recruit more cells throughout metamorphosis for the formation of the dorsal olfactory bulb [78]. The noted cell migration on the immunostainings supports the theory that there is neurogenesis during metamorphosis of *Xenopus laevis* [41], [76].

The formation of the various cell types in the OB take different time points; first, some MTCs are present before metamorphosis begins (stage 48) to form the vOB, even before the olfactory axons of the ORNs make contact with the bulb. Then some interneurons start to be born during development. At stage 56 (figure 12) a gap between the HuC/D cell layer and the SVZ is present and we think might be the granule cell layer. The formation of the dOB starts at stage 52 and might be expressed in the increment of the HuC/D cell layer. Based on our results, the enlargement of the HuC/D-positive cells layer could mean the apparition of newborn neurons in the dOB as well as the vOB. The arrangement of the dOB is suggested to be triggered by the projection of new axons coming from the MC, signal required to increase differentiation of precursor cells from the ventricles [67], [69].

Differentiation of new neurons could be appreciated on the images suggesting there are neurons building up the dorsal region. Our hypothesis was that stem cells are not only intended to differentiate into interneurons but also into projection neurons. The OB might also increase in size during metamorphosis because the mitral cell layer grows. The signal for the generation of neurons in the dOB could be due to mechanisms during the period of metamorphosis such as innervation by afferent fibers from the PC or induction by secretion of thyroid hormone [8], [28], [69]. Here we show that the HuC/D cell layer grows during metamorphosis; the HuC/D cell population might increase as well as the cross-sectional area of single HuC/D cells and we hypothesize that interneurons are not stained by HuC/D antibody meaning that the target cells for the HuC/D antibody might be MTCs. The Sox2 labeling confirms that the niche of stem cells is located in the lateral ventricles and some migration exists to build up new neurons during metamorphosis.

VII. Conclusions

In conclusion, the animals are able to smell under the water during these morphological changes for mere survival. In their natural environment, the tadpoles need to survive, search for food, seek for ponds, and hide from predators even when metamorphosis starts. Their sense of smell is one of the principal senses they need in water and eventually in the land. As expected, the responses to odorant applications remained constant and undisrupted during metamorphosis. The lateral and medial streams presented the same segregation, as reported in literature, especially with the amino acids and forskolin applications. Forskolin responses were never present in the lateral cluster throughout metamorphosis. The intermediate cluster responses were broadly tuned; experiments need to be performed to test the mechanosensation and thermosensation of the glomeruli in the intermediate cluster. It is necessary to apply different mechanical and thermal stimuli beside the common odorants used to test the responses to changes in temperature or mechanical stimuli.

Based on the obtained results we can conclude that, a new dorsal side starts to develop at the same time that metamorphosis begins and might be triggered by the axonal innervation coming from the middle cavity. Our results suggest that stem cells originated in the lateral ventricles appear to migrate to the dorsal region of the olfactory bulb. Stem cells may turn into either a MTC or a GC in the dorsal olfactory bulb. The new formation of the dOB is required for the air nose activity. Neurogenesis is a complementary process together with the metamorphosis and they build the new demands for the olfactory system.

VIII. Perspectives

As future work, to test the hypothesis of which kind of neurons do the stem cells differentiate, immunohistochemistry assays together with dye injection of the subventricular zone will be performed during metamorphosis. The injection of the subventricular zone will trace the migration of stem cells and interneurons or projection neurons will be labeled using HuC/D (for MTCs) and GABA antibody to identify in which kind of neurons the stem cells are differentiating at different stages of metamorphosis. Figure 14 depicts the perspective of the future work.

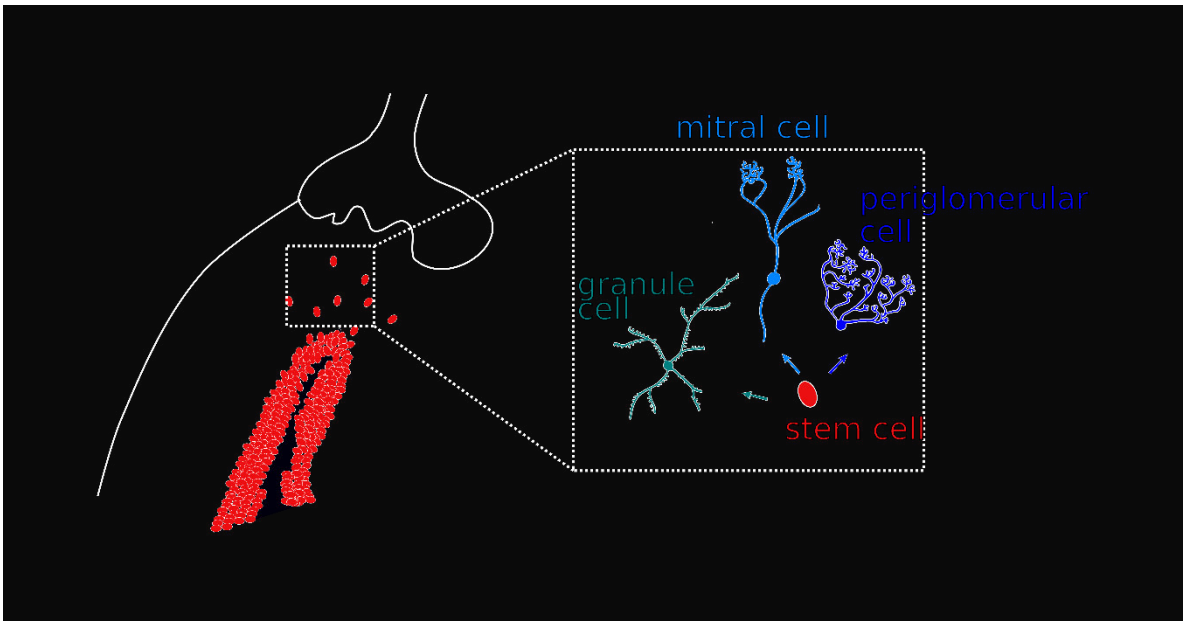

Figure 14. The scheme depicts a perspective to elaborate future experiments to observe neurogenesis in the olfactory bulb. Stem cells might differentiate into either mitral cells, granule cells, or periglomerular cells during metamorphosis of *Xenopus laevis* for the dorsal olfactory bulb development.

IX. List of abbreviations

AA amino acids

AOB accessory olfactory bulb

GCL granule cell layer

GL glomerular layer

MC middle cavity

MCL mitral cell layer

MOB main olfactory bulb

MOE main olfactory epithelium

MTCs mitral/tufted cells

NL nerve layer

OB olfactory bulb

OE olfactory epithelium

OR olfactory receptor

ORN olfactory receptor neuron

PC principal cavity

SVZ subventricular zone

VNO vomeronasal organ

VRN vomeronasal receptor neuron

X. List of figures

XI. References

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Si (como afirma el griego en el Cratilo)
el nombre es arquetipo de la cosa
en las letras de 'rosa' está la rosa
y todo el Nilo en la palabra 'Nilo'.

Y, hecho de consonantes y vocales,
habrá un terrible Nombre, que la esencia
cifre de Dios y que la Omnipotencia
guarde en letras y sílabas cabales.

Adán y las estrellas lo supieron
en el Jardín. La herrumbre del pecado
(dicen los cabalistas) lo ha borrado
y las generaciones lo perdieron.

Los artificios y el candor del hombre
no tienen fin. Sabemos que hubo un día
en que el pueblo de Dios buscaba el Nombre
en las vigilias de la judería.

No a la manera de otras que una vaga
sombra insinúan en la vaga historia,
aún está verde y viva la memoria
de Judá León, que era rabino en Praga.

Sediento de saber lo que Dios sabe,
Judá León se dio a permutaciones
de letras y a complejas variaciones
y al fin pronunció el Nombre que es la Clave,

la Puerta, el Eco, el Huésped y el Palacio,
sobre un muñeco que con torpes manos
labró, para enseñarle los arcanos
de las Letras, del Tiempo y del Espacio.

El simulacro alzó los soñolientos
párpados y vio formas y colores
que no entendió, perdidos en rumores
y ensayó temerosos movimientos.

Gradualmente se vio (como nosotros)
aprisionado en esta red sonora
de Antes, Después, Ayer, Mientras,
Ahora, Derecha, Izquierda, Yo, Tú,
Aquellos, Otros. (El cabalista que ofició de
numen a la vasta criatura apodó Golem;
estas verdades las refiere Scholem
en un docto lugar de su 50opaib.)

El rabí le explicaba el 50opaiba50
"esto es mi pie; esto el tuyo, esto la soga."
Y logró, al cabo de años, que el perverso
barriera bien o mal la sinagoga.

Tal vez hubo un error en la grafía
o en la articulación del Sacro Nombre;
a pesar de tan alta hechicería,
no aprendió a hablar el aprendiz de hombre.

Sus ojos, menos de hombre que de perro
y harto menos de perro que de cosa,
seguían al rabí por la dudosa
penumbra de las piezas del encierro.

Algo anormal y tosco hubo en el Golem,
ya que a su paso el gato del rabino
se escondía. (Ese gato no está en Scholem
pero, a través del tiempo, lo adivino.)

Elevando a su Dios manos filiales,
las devociones de su Dios 50opaiba
o, estúpido y sonriente, se ahuecaba
en cóncavas zalemas orientales.

El rabí lo miraba con ternura
y con algún horror. '¿Cómo' (se dijo)
'pude engendrar este penoso hijo
y la inacción dejé, que es la cordura?'

'¿Por qué di en agregar a la infinita
serie un símbolo más? ¿Por qué a la vana
madeja que en lo eterno se devana,
di otra causa, otro efecto y otra cuita?'

En la hora de angustia y de luz vaga,
en su Golem los ojos detenía.
¿Quién nos dirá las cosas que sentía
Dios, al mirar a su rabino en Praga?

Jorge Luis Borges

Universidad Guanajuato

Asunto: Carta aval de sinodal

León, Gto., Noviembre 21, 2018

Dra. Laura Edith Castellano
Coordinadora de la Maestría en Ciencias Aplicadas
División de Ciencias e Ingenierías

Estimada Dra. Edith Castellano:

Por medio de la presente hago constar que he revisado la tesis titulada: "**Response patterns and neurogenesis in the olfactory bulb of Xenopus laevis during metamorphosis**" que para obtener el grado de Maestría en Ciencias Aplicadas presenta la **Lic. Paola Luz Eréndida Segoviano Arias.**

En dicho trabajo se presenta un estudio muy cuidadoso de la neurogénesis y la respuesta olfatoria de Xenopus. Es importante destacar que en este trabajo la alumna ha hecho una investigación muy completa no sólo de los aspectos teóricos del problema, sino además que ha complementado su investigación con resultados experimentales del espécimen en estado de metamorfosis. El trabajo es una contribución importante en su campo y refleja además un profundo grado de comprensión del problema por parte de la sustentante.

Por todo lo anterior, le comunico que he discutido cuidadosamente dicha tesis con la sustentante, a quien le he hecho llegar mis comentarios y correcciones. Le expreso además que en lo general me parece un buen trabajo por lo que avalo su presentación.

Sin otro particular por el momento, aprovecho para reiterarle las seguridades de mi consideración más distinguida.

Atentamente

DR. MODESTO ANTONIO SOSA AQUINO
PROFESOR TITULAR "C"
Sinodal

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UNIVERSIDAD DE
GUANAJUATO

León, Gto a 3 de diciembre de 2018

Dr. David Yves Ghislain Delepine
Director de la División de Ciencias e Ingenierías
Campus León-UG
Presente

Por este medio me permito informar que he revisado y discutido el documento escrito de la tesis de Maestría de la Ing. Biomédica Paola Luz Eréndira Segoviano Arias, del programa de la Maestría en Ciencias Aplicadas de la División de Ciencias e Ingenierías, Campus León, cuyo título es "**Response patterns and neurogenesis in the olfatory bulb of *Xenopus laevis* during metamorphosis**". Manifiesto que estoy de acuerdo con el documento y que la defensa de la tesis se pueda programar.

Agradezco de antemano sus atenciones a la presente y me despido cordinalmente.

Atentamente

Laura E. Castellano

Dra. Laura Edith Castellano Torres
Profesor Investigador

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UNIVERSIDAD DE GUANAJUATO
CAMPUS LEÓN
DIVISIÓN DE CIENCIAS E INGENIERÍAS

Universidad Guanajuato

León, Gto., 19 de sept-18

Asunto: Aprobación de tesis de maestría

Dr. David Ghislain Delepine
Director de la División de Ciencias e Ingenierías
Universidad de Guanajuato

Por medio de la presente me permito informar que he revisado el trabajo titulado **"Response patterns and neurogenesis in the olfactory bulb of Xenopus laevis during metamorphosis"** que para obtener el grado de **"Maestría en Ciencias Aplicadas"** presenta la **Ing. Paola Luz Eréndida Segoviano Arias**

Considero que el trabajo es de gran relevancia y con la calidad suficiente para que la **Ing. Paola** obtenga al grado, por lo tanto, no tengo inconveniente en que se realicen los trámites necesarios para su presentación ante el comité.

Agradezco de antemano su atención y quedo a sus órdenes para atender cualquier duda o aclaración.

Saludos cordiales,

Dr. Gustavo Basurto Islas
Profesor Asociado C
Departamento de Ingenierías Química,
Electrónica y Biomédica
División de Ciencias e Ingenierías
Universidad de Guanajuato Campus León

UNIVERSIDAD DE GUANAJUATO

León, Guanajuato a 30 de noviembre de 2018

Dr. David Yves Ghislain Delepine
Director de la División de Ciencias e Ingenierías, Campus León
Universidad de Guanajuato
PRESENTE

Por medio de la presente le comunico que he revisado la tesis titulada **"Response patterns and neurogenesis in the olfactory bulb of Xenopus laevis during metamorphosis"**, que presentó la estudiante **Paola Luz Eréndida Segoviano Arias,** estudiante de la Maestría en Ciencias Aplicadas de la Universidad de Guanajuato. Derivado de esta revisión otorgo mi voto aprobatorio para que se lleve a cabo la defensa de su trabajo de tesis a fin de obtener su grado de Maestra en Ciencias Aplicadas.

Sin más por el momento, le envió un cordial saludo.

Atentamente

Dra. Argelia Rosillo de la Torre.
Profesor Asociado B
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División de Ciencias e Ingenierías.
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