1 **Developmental neuroanatomy of the Rosy Bitterling**

2 ***Rhodeus ocellatus* (Teleostei: Cypriniformes)—A microCT**

3 **study**

4 *Running title*

5 Neuroanatomy of the Rosy Bitterling

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1 AUTHOR CONTRIBUTIONS

2 WY and MKR conceived the study. WY performed all neuroembryology studies,
3 including microCT analysis. MR helped with microCT studies. All authors helped WY
4 with interpretation of the microCT data. WY prepared the manuscript and figures.
5 MKR and TM helped edit the manuscript.

6 CONFLICT OF INTEREST

7 The authors declare that they have no competing interests.

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DATA AVAILABILITY STATEMENT

2 The data that support the findings of this study are available from the corresponding
3 author upon reasonable request.

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ABSTRACT

Bitterlings are a group of teleost fish (Cypriniformes: Acheilanthidae) notable for their brood parasitic lifestyle. Bitterling embryos develop as parasites inside the gill chamber of their freshwater mussel hosts. However, little is known about brain development in this species. Here, we have imaged the development of the brain of the Rosy Bitterling (*Rhodeus ocellatus*) at four embryonic stages (165, 185, 210, 235 hours post-fertilization) using micro-computed tomography (microCT) with special emphasis on developmental regionalization and brain ventricular organization. We provide a detailed neuroanatomical account of the development of the brain divisions with reference to The *Atlas of Early zebrafish Brain Development* and the updated prosomeric model. Segmentation and three-dimensional visualization of the ventricular system were performed in order to clarify changes in the longitudinal brain axis as a result of cephalic flexure during development. During early embryonic and larval development, we find that histological differentiation, tissue boundaries, periventricular proliferation zones, and ventricular spaces are all recognizable using microCT. Importantly, our approach is validated by the fact that the profile of CT values displayed here in the bitterling brain are consistent with genoarchitecture identified in previous studies. We also find developmental heterochrony of the inferior lobe in the Rosy Bitterling compared to the zebrafish. Our study provides a foundation for future studies of the brain development in the Rosy Bitterling, a valuable model species for studying the evolutionary adaptations associated with brood parasitism.

1 **Keywords:** proliferation zone, prosomeric model, neuroembryology, brain
2 development, brain ventricle, bitterling.

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4

5 1. INTRODUCTION

6 Bitterlings, a group of freshwater teleosts, are valuable model species in behavioral,
7 population and evolutionary ecology due to their brood parasitic life history. Their
8 peculiar life style involves the laying of eggs by the bitterling in a host mussel, a
9 phenomenon that has been recognized for more than a century (Boeseman et al.,
10 1938; Chang, 1948; Duyvené de Wit, 1955; Kitamura et al., 2012; Mills and
11 Reynolds, 2003; Olt, 1893; Reichard et al., 2007; Rouchet et al., 2017; Smith, 2016;
12 Wiepkema, 1962). Noll (1877) was the first to show that the embryos of European
13 bitterling (*Rhodeus amarus*) develop in the gill chamber of their host mussel. This
14 location provides a sheltered environment which may protect the developing
15 embryos from predators (Aldridge, 1999; Liu et al., 2006; Reichard et al., 2007;
16 Smith et al., 2004). In a recent study (Yi et al., 2021) we compared developmental
17 sequences of the Rosy Bitterling (*Rhodeus ocellatus*) to the zebrafish (*Danio rerio*);
18 the latter is a non-parasitic teleost which lays its eggs into the open water (Kimmel et
19 al., 1995; Lawrence, 2007). Our study identified a number of heterochronic (timing)
20 shifts, including the relative pre-displacement of hatching, and the relative delay of
21 development of the pectoral fins, in the bitterling.

22 The specialized development of the bitterling, in relation to its brood parasitic
23 lifestyle, make it a potentially interesting model for the study of developmental
24 mechanisms underlying brain evolution. In this study, we generated an atlas of the

1 developing bitterling brain as a reference for cross-species comparisons. To this end,
 2 we compared bitterling brain development with published data for the zebrafish. The
 3 comparison to the zebrafish is useful for two reasons. First, the zebrafish is firmly
 4 established as a genetic model system that has been thoroughly investigated with
 5 regard to its embryonic, postembryonic, and larval development (Mueller and
 6 Wullimann, 2003; Mueller and Wullimann, 2016; Mueller et al., 2006; Wullimann,
 7 2009; Wullimann and Knipp, 2000; Wullimann and Mueller, 2004). Second, both, the
 8 bitterling and the zebrafish belong to the group of carp-like (cyprinid) teleosts and
 9 have similar adult brain anatomy. However, as we show, these two species show
 10 distinct developmental timing differences (heterochronies). The developmental
 11 staging atlas of the bitterling brain, that we describe here, provides a foundation for
 12 future studies on the molecular mechanisms underlying these heterochronies.

13 In the vertebrates, the central nervous system, including its anterior part, the
 14 brain, develops from the neural tube (Richardson and Wright, 2003; Schmitz et al.,
 15 1993; Wullimann, 2009). In teleosts, including bitterlings, the development of the
 16 neural tube involves secondary neurulation (Schmitz et al., 1993). The lumen of the
 17 definitive central nervous system contains cerebrospinal fluid (Lowery and Sive,
 18 2009). The spinal cord has a narrow lumen, the central canal. The brain, by contrast,
 19 has an inflated, irregular lumen which form a series of brain ‘ventricles’ (Korzh, 2018).
 20 The four main divisions of the vertebrate are: (i) the secondary prosencephalon; (ii)
 21 the diencephalon; (iii) the mesencephalon and (iv) the rhombencephalon.
 22 Accordingly, the brain ventricular system has been divided into the prosencephalic
 23 ventricle (including the telencephalic ventricle and the hypothalamic part of the
 24 classic diencephalic ventricle; Nieuwenhuys and Puelles, 2016); the 3rd ventricle (the

1 lumen of diencephalon proper); the mesencephalic ventricle; and the 4th ventricle
2 (the lumen of the rhombencephalon).

3 In this study we use the updated prosomeric model of Puelles and Rubenstein
4 (2003) for dividing neuromeres in the secondary prosencephalon as well as for
5 defining other brain divisions. The prosomeric model recognizes longitudinal zones
6 and transverse zones (corresponding to the neuromeres) is a powerful paradigm for
7 vertebrate cross-species comparisons. The model is based on the conserved
8 expression patterns of developmental genes and on morphological characters.
9 These data permit the demarcation of the CNS into developmental units
10 (morphogenetic entities) along the neuraxis of a range of vertebrate species. In
11 teleosts, patterns of cell migration and differentiation also identify prosomeric units
12 and can be used to map them (Mueller and Wullimann, 2003; Mueller and Wullimann,
13 2016; Mueller et al., 2006; Wullimann, 2009; Wullimann and Knipp, 2000; Wullimann
14 and Mueller, 2004).

15 The prosomeric model analyzes brain regions along the general brain axis
16 and according to the mediolateral extent of brain regions. Longitudinally, the neural
17 tube has four compartments divided by the *sulcus limitans* of Wilhelm His, Sr. (His,
18 1895); reviewed by Puelles (2019). The four compartments are: the roof plate
19 dorsally, the alar plate dorsolaterally, the basal plate ventrolaterally, and the floor
20 plate ventrally.

21 According to Nieuwenhuys and Puelles (2016), the rhombencephalon
22 consists of twelve neuromeres (rhombomeres) whereby the isthmus is designated r0,
23 and the remaining rhombomeres r1–r11) in longitudinal series. In cyprinids such as
24 the Goldfish (*Carassius auratus*), rhombomeres r2–r6 correspond to the mammalian
25 pons, whereas the rhombomeres 7-8 correspond to the mammalian medulla

1 oblongata (Gilland et al., 2014; Rahmat and Gilland, 2019). Rhombomeres r2–r6
2 form clearly segmented neural clusters while rhombomeres r7–r8 are only poorly
3 delimited (Ma et al., 2009).

4 In the zebrafish, r8 is twice as large as the rostral rhombomeres; the latter are
5 composed by multiple crypto- or pseudo-rhombomeres that can only be clearly by
6 molecular markers, as is the case in their avian homologs (Cambronero and Puelles,
7 2000). The rostral subdivision of neuromeres is more complex, because bending of
8 the neural tube (the cephalic flexure, Hauptmann and Gerster, 2000; Mueller and
9 Wullimann, 2009) has obscured the delineation of the longitudinal neuraxis (Mueller
10 and Wullimann, 2009; Puelles, 2019; Vernier, 2017; Wullimann and Rink, 2002).

11 Here, we visualize the development of the Rosy Bitterling (*Rhodeus ocellatus*)
12 brain using micro-computed tomography (microCT, x-ray microscopy or μ CT) with a
13 specific focus on developmental regionalization and brain ventricular organization.
14 MicroCT is a high-resolution, non-destructive, three-dimensional (3D) imaging
15 technique (Babaei et al., 2016; Metscher, 2009a) that has recently begun to be used
16 in developmental studies (Wong et al., 2015). The contrast of CT scans is based on
17 the absorption of x-ray radiation passed through the sample. Highly mineralized
18 structures such as bones and teeth have a higher attenuation coefficient (CT value;
19 Hounsfield units). This makes them appear brighter in the final image, and easier to
20 recognize than soft tissues. For the visualization of the latter, a treatment with a
21 contrast agent is needed. In this work, we used phosphotungstic acid (Metscher,
22 2009b), which enhances the visualization of soft tissues such as muscles, nerves,
23 and blood vessels.

24 In this study, we extend MicroCT techniques to the developing bitterling brain.
25 Our goal is to establish microCT and 3D visualization as complementary methods for

1 cross-species comparisons of the morphology of both the developing and the mature
2 brain. Our results indicate that microCT is a valuable modality for the study of the
3 ventricles, and for discriminating white matter from proliferative and postmitotic cell
4 masses.

5 **2. MATERIALS AND METHODS**

6 *Sample preparation*

7 Embryos of six developmental stages (135, 150, 165, 185, 210, 235 hours post
8 fertilization (hpf); Table 1) were obtained by *in vitro* fertilization following the method
9 of Nagata and Miyabe (1978). Embryos were staged according to Yi et al. (2021).
10 Embryos were incubated at 22.5 ± 1 °C and fixed in 3% buffered paraformaldehyde
11 (pFA) and 1% glutaraldehyde (GA). Digital microphotographs of fixed samples were
12 obtained with a CCD (charge-coupled device) camera connected to stereo
13 microscope (Nikon SMZ1500). For x-ray contrast enhancement, embryos were
14 stained for at least 24 h in 0.3% phosphotungstic acid (PTA) dissolved in 70%
15 ethanol. Samples were then brought back to 70% ethanol without PTA and mounted
16 in low-melting point agarose for non-shift scanning in Gilson® Pipetman® tips.

17 *MicroCT scanning*

18 Attenuation-based microtomographic images were acquired using an Xradia 520
19 Versa 3D X-ray microscope (Zeiss), with the x-ray tube voltages source set at 80/7
20 or 40/3 keV/W (keV: kiloelectron volts). A thin LE1 filter was used to avoid beam-
21 hardening artifacts. During the CT scanning, the sample was placed on a rotation
22 table and projection images were acquired over an angular range of 180 degrees. To
23 obtain high resolution images, a 4x CCD (charge-coupled device) optical objective

1 was used. Images were acquired with voxel sizes (volumetric 3D pixels after
2 reconstruction) of 1-1.5 μm , and tomographic reconstructions were made with the
3 resident software (XMReconstructor). Reconstructed images were exported as TIFF
4 files and loaded into Avizo version 9.5 (Thermo Fisher Scientific) for 3D visualization.

5 *3D visualization*

6 The reconstructed volume was viewed slice-by-slice as virtual sections using the
7 *Slice* module in the Avizo software (Version: 9.5; Thermo Fisher Scientific). A
8 computational module *Resample transformed image* was applied to register images
9 in the orthogonal direction of the anatomical axis. *Ortho View* was used for
10 interactive orthogonal views in the *xy*, *yz* and *xz* axes simultaneously. The *Volume*
11 *rendering* module was used for 3D viewing. Colormap and opacity were optimized in
12 the rendering settings. Scalebars were added using the *Scalebars* module.

13 *Annotations on 2D slices*

14 To generate a developmental atlas, serial virtual transverse sections were taken in
15 rostrocaudal sequence from the olfactory bulb to the medulla oblongata. The section
16 plane was parallel to the deep ventricular sulcus (the anterior intraencephalic sulcus
17 or AIS) between the telencephalon and diencephalon. To keep a consistent
18 prosomeric axis annotation, we visualized the transverse sections from the rostral to
19 caudal, the coronals section from dorsal to ventral, and the sagittal sections from
20 medial to lateral along the primary body axis (Figure 1f). Labels were added to each
21 section in using Adobe InDesign® software (Version: 15.0.2; Adobe Systems Inc.,
22 San José, California). For anatomical terms see the list of abbreviations (Figure xx).
23 We mainly used the anatomical terms in the *Atlas of Early zebrafish Brain*
24 *Development* (Mueller and Wullimann, 2005, 2016).

1 *A. Development of the brain ventricular system of the Rosy Bitterling*

2 General description

3 The greyscale values observed on virtual microCT sections are influenced by PTA
4 staining, tissue density, and cell type. In the brain, the gray matter generally
5 appeared brighter than the white matter (for example, compare the telencephalon
6 and anterior commissure in Figure 1, Tel and ac). The white matter with its
7 myelinated axons and tracts yielded greyscale values typical of low-density soft
8 tissues (Figure 1, ac, poc, pc). In contrast, the brain ventricle, essentially a hollow
9 space filled with cerebrospinal fluid (CSF), showed a lower density than the nervous
10 tissue of the brain. It was visible in CT scans as the darkest part (Figure 1a-f,
11 rendered in yellow color in Figure 1a-e). Therefore, the radiological appearance of
12 the brain encompasses, broadly speaking, three greyscale values: (i) a black lumen:
13 the ventricular system; (ii) a bright white periventricular layer of gray matter, and (iii)
14 a gray-coloured peripheral layer of white matter (Figure 1f).

15 The forebrain ventricle is divided dorsally by the anterior intraencephalic
16 sulcus (AIS) into the anterior telencephalic ventricle (TVe) and the posterior
17 diencephalic ventricle proper (DVe or the 3rd ventricle; Figure 1). During the eversion
18 of the telencephalic ventricle, the dorsal part of the AIS shows concomitant
19 enlargement (Figure 1). Around the preoptic recess (PoR), the optic recess region
20 (ORR) was recognizable, bordered by the anterior commissure (ac) and postoptic
21 (poc) commissures (see locations of ac, poc and PoR in Figure 1c). The
22 hypothalamic ventricle topographically locates caudal (topologically basal) to the
23 optic recess region, and topologically anterior to the 3rd ventricle, including the lateral
24 recess (LR) and the posterior recess (PR; Figure 2).

1 The mesencephalic ventricle encompasses the paired tectal ventricle (TeVe),
 2 the median ventricle (MV), and the median ventricular sulcus (MVS) (Puelles, 2019).
 3 The TeVe projects dorsolaterally from the median ventricle (Figure 3a), covered by
 4 the subarea of the alar plate of the midbrain (optic tectum). A transient median
 5 ventricular sulcus (MVS) branches off from the ventral bottom of the median ventricle
 6 and shows separately at the floor plate area (Figure 3b, inset in the upper right
 7 corner). The median ventricle extends caudally to the rhombencephalic ventricle
 8 (RVe or the 4th ventricle; Figure 3; García-Lecea et al., 2017; Korzh, 2018).

9 STAGE 1-ovl/pec-bud (165 hpf).

10 In the Rosy Bitterling, the ventricular expansion is completed at stage 165 hpf
 11 (compare Figure 1a, b). The rhombencephalic ventricle (RVe) is diamond-shaped
 12 (rhombic) in dorsal and rostral aspects (Figure 3a and c). The bilateral tectal
 13 ventricles (TeVe) resemble scallop shells (Figure 3a). The telencephalic ventricle is
 14 triangular in rostral aspect (Figure 3d). A pair of oval fossae appear caudal-ventral to
 15 the midbrain (Figure 3c, of). These fossae are occupied by the rostral cerebellar
 16 thickenings, which develop into the valvula cerebelli of the adult (Wullimann and
 17 Knipp, 2000). The lateral recess (LR) and the posterior recess (PR) of the
 18 hypothalamic ventricle are in shallow groves (Figure 2a).

19 STAGE pec-bud (185 hpf).

20 The width of the rhombencephalic ventricle (RVe) decreases to less than half that of
 21 the tectal ventricles (TeVe) (Figure 4a). The RVe gradually flattens along its dorsal-
 22 ventral axis (compare Figures 3b and 4b). The dorsal surface of the TeVe appears
 23 smoother due to the further development of the optic tectum (mammalian superior
 24 colliculus) and torus semicircularis (mammalian inferior colliculus; Figure 4a). There

1 is a deep midline ridge separating the left and right parts of the TeVe (Figure 4a, d).
 2 The preoptic recess (PoR) is compressed, and reduced in width (compare Figures
 3 3c and 4c). The oval fossae enlarge with the growth of the rostral cerebellar
 4 thickening (compare Figures 3c and 4c). The lateral recess and posterior recess
 5 become visible and extend outward (Figure 2b).

6 STAGE high-pec (210 hpf).

7 Compared to earlier stages, the brain ventricles appear compressed (compare
 8 Figures 4a and 5a). However, the dorsal ventricle of the anterior intraencephalic
 9 sulcus (AIS) appears is expanded posteriorly, probably due to the eversion of the
 10 telencephalic ventricle (Tve). The tectal lobes grow larger and appear to fuse in the
 11 midline; as a result, the deep midline ridge of the TeVe appears compressed rostrally
 12 (Figure 5b). The lateral recess (LR) extends basalward to form a flange at the
 13 posterior recess (PR). The inferior lobe forms around the lateral recess (IL, Figure 2c;
 14 Bloch et al. 2019).

15 STAGE long-pec (235 hpf).

16 The rhombencephalic ventricle (Rve) can be divided into two parts, a rostral
 17 rhomboid opening with very thin roof plate over it, and a caudal, elongated ventricle
 18 between the rhombic lips (RL; Figure 6a). The rostral aspect of the telencephalic
 19 ventricle (Tve) gradually deepens from a triangle into a T-shape (Figure 6d).
 20 Compared to earlier stages, the tectal ventricles (TeVe) are further compressed and
 21 separated from the median ventricle (Figure 6b). The lateral recess (LR) extends
 22 more basalward (compare Figures 2c and d), and the inferior lobe (IL) is
 23 considerably enlarged. The posterior recess (PR) extends alarward (Figure 2d).

1 *B. Developmental atlas of the Rosy Bitterling brain*

2 We observed cell clusters in periventricular locations that appeared brighter than the
3 adjacent gray matter (eg. Figure 7j). The distribution of these cell clusters is highly
4 consistent with the distribution of proliferation zones detected during neurogenesis in
5 the zebrafish (Mueller and Wullmann, 2005, 2016). This fact has helped us to map
6 putative proliferation zones of the developing bitterling brain and delineate brain
7 territories. As a consequence, our delineation of anatomical structures is based on
8 three types of observation: (i) their topological relationship to proliferation zones; (ii)
9 their relative location to brain ventricles (see previous section) and other landmarks
10 such as commissures and fiber tracts; (iii) their grayscale values in virtual microCT
11 sections. To take an example, the zona limitans intrathalamica (Zli) can be
12 demarcated by its dark appearance in contrast to the surrounding, bright-white
13 thalamic tissues (Figure 7g, h).

14 Secondary prosencephalon

15 Rostral to the telencephalic region, the olfactory epithelium (OE) is visible as a very
16 bright region from the 1-ovl/pec fin stage (165 hpf, Figure 7a). At the long-pec stage
17 (235 hpf) the olfactory epithelium develops into a bow-shaped structure surrounding
18 the lumina of the olfactory pits (Figure 10a). The olfactory epithelium (OE) is
19 connected to the olfactory bulb (OB) through the easily-recognizable olfactory nerve
20 (ON; Figures 7c, 9b and 10b). The olfactory bulb (OB) is characterized by its
21 glomerular structure (Figure 7a, 8a, 9a and 10a; Dynes and Ngai 1998).

22 The telencephalic ventricle is located at the dorsum of the pallium and the tela
23 choroidea is visible in its roof. The periventricular proliferation zones of the
24 subpallium (S) and pallium (P) appear bright in virtual transverse sections. At the

1 long-pec stage (235 hpf), the bright subpallial cell clusters were separated by distinct,
2 dark boundaries (dashed line in Figure 10d), corresponding to the dorsal and ventral
3 subdivisions of the subpallium (Sd and Sv). The zones of pallial proliferation consist
4 of a few cell rows, and are visible as a pair of arches flanking the dorsal subpallium
5 (Figure 7c, 8c, 9d, 10c). The telencephalic migrated area (M4) was recognizable in
6 the form of cell clusters at the margin of the lateral subpallium (Figure 10e).

7 Immediately caudal to the subpallium, the anterior commissure (ac) and lateral
8 forebrain bundle (lfb) appeared as dark fiber bundles crossing the rostral end of the
9 forebrain (Figure 7e,f, 8d,e, 9d,e, 10f). We shall define the anterior commissure (ac)
10 and the postoptic commissure (poc) as the boundaries of the preoptic region (Po).
11 This region recently has also been designated as the alar hypothalamus (aHyp) or
12 the optic recess region (ORR) (Affaticati et al., 2015; Schredelseker and Driever,
13 2020; Slack, 2005). The proliferation zone of the preoptic region (Po) was easy to
14 identify as a triangular region surrounding the optic recess. The Po proliferation zone
15 is broad on its ventral side and thins out dorsally (Figure 7f, 8e, 9e, 10g). The optic
16 chiasma (oc) which has a bright, thick appearance, decussates in the midline (Figure
17 7h, 8h, 9h, 10j); it marks the anterior end of the neural axis.

18 In the basal hypothalamic region, the hypophysis is located topologically
19 acroterminal (anterior) to the hypothalamus (Figure 2). It projects form the ventral
20 midline of the brain (Figure 2). As in the zebrafish developmental brain atlas
21 (reference?), we divide the basal hypothalamus into the following regions (i) the
22 rostral hypothalamus (Hr); (ii) the caudal hypothalamus (Hc); and between them: (iii)
23 the intermediate hypothalamus (Hi), located near the hypophysis, and including the
24 inferior lobe (IL). The hypothalamus encloses the hypothalamic ventricles, including
25 the lateral recess (LR) in the Hi, and the posterior recess (PR) in the Hc. This

1 division is consistent with those classically used for describing the zebrafish
 2 hypothalamus (Wullimann et al., 1996; Manoli and Driever, 2014; Biran et al., 2015;
 3 Mueller and Wullimann, 2016; Muthu et al., 2016).

4 Recent molecular studies in the zebrafish have improved the interpretation of the
 5 teleostean hypothalamus and its evolutionary relationships with the mammalian
 6 hypothalamus (Baeuml et al., 2019; Herget et al., 2014). Likewise, gene expression
 7 in the PR tuberal region of the embryonic zebrafish has revealed homology with two
 8 domains of the mammalian hypothalamus: the TuV (tuberal region, ventral part) and
 9 Tul (tuberal region, pars intermedia). Therefore, Schredelseker and Driever (2020)
 10 proposed to refer to this region as PRR (posterior recess region) to set it apart as a
 11 teleost-specific entity. Because our analysis of the Rosy Bitterling is based on
 12 anatomical microCT data, are not able to confidently relate our findings those just
 13 cited. We therefore look forward to molecular studies in bitterlings.

14 Diencephalon

15 The prosomeric model divides the diencephalon into alar plate and basal plate
 16 derivatives. In this model, the pretectum (aP1), the thalamus proper (aP2), and the
 17 prethalamus (aP3) form the alar plate portions of the diencephalon (Lauter et al.,
 18 2013). In contrast, the proliferation zones of the nucleus of the medial longitudinal
 19 fasciculus (N; bP1), and the dorsal (bP2) and ventral (bP3) posterior tegmentum (PT;
 20 Mueller and Wullimann, 2005, 2016) form the corresponding basal plate derivatives.

21 The most prominent structure in the diencephalic roof plate is the epiphysis (E), a
 22 swelling in the midline (Figure 7h, 8g, 9j, 10i). The habenular nuclei (Ha) are located
 23 one on each side of it (Figure 7h, 8g, 9i, 10i) and show discrete habenular cell
 24 clusters from the pec-bud stage (185 hpf) onwards (Figure 8g). Notice that the

1 fasciculus retroflexus (fr) appeared in our microCT photographs in the form of
2 distinctive, dark fiber-bundles in the gray matter (Figure 10k, i). The fasciculus
3 originates from the habenular nuclei and connects to the interpeduncular nucleus
4 across the isthmus (r0) and rhombomere 1 (Akle et al., 2012). In the prosomeric
5 model, the fasciculus retroflexus is used to delimit pretectum (P1) and thalamus (P2;
6 Akle et al., 2012; Lauter et al., 2013; Puelles, 2019). We use the caudal end of the
7 posterior commissure as the caudal boundary of the pretectum (P1), which divides
8 the diencephalic and mesencephalic areas (Figures 7j, 8h, 9l, 10k).

9 In the thalamic region, we identified the zona limitans intrathalamica (Zli) as a dark
10 band (Figure 7i, 8f, 9i, 10h) that marks out the boundary between the prethalamus
11 (P3) and thalamus (P2). Therefore, we annotated the separate periventricular
12 proliferation zones of the prethalamus (PTh) and thalamus (Th) based on their
13 topological relationships (anterior vs. posterior) and Zli landmark (e.g. Figure 10j).

14 The thalamic eminence (EmT) is a relatively complex region of the diencephalon and
15 is viewed in the prosomeric model as the anterior portion of the prethalamus (and is
16 therefore often termed the “prethalamic eminence”, PThE). However, while the EmT
17 (or PThE) generates glutamatergic derivatives, the prethalamus proper mostly
18 contains GABAergic neurons. In addition, some recent studies in the zebrafish and
19 tetrapods indicated that the Emt/PThE contributes to telencephalic territories such as
20 the medial extended amygdala and the newly-identified nucleus of the lateral
21 olfactory tract (Alonso et al., 2020; Porter and Mueller, 2020; Vicario et al., 2017).

22 Due to the lack of gene expression data in bitterlings, we are conservative in our
23 analyses and place the EmT topologically anterior to the prethalamus, and posterior
24 to the preoptic region, similar to the condition described for larval zebrafish
25 (Wullimann and Mueller, 2004). It abuts the lateral forebrain bundle (Mueller, 2012)

1 which is identifiable in our CT scans (Figure 7f, 8e, 9e, 10f). The use of molecular
2 markers such as Tbr-1 (Wullimann, 2009; Wullimann and Mueller, 2004) would help
3 validate the anatomical hypotheses in the present study.

4 We conclude this section by noting that the PT has distinct PTd and PTV proliferation
5 zones (eg. Figure 10k). Because of the cephalic flexure, the dorsal-ventral topology
6 in the virtual transverse sections, corresponds to the anterior-posterior axis of the
7 neural tube.

8 Mesencephalon and rhombencephalon

9 The boundary between the diencephalon and mesencephalon is defined dorsally by
10 the posterior end of the posterior commissure, and ventrally by the anterior margin of
11 the oculomotor nerve root (Moreno et al., 2016). In the tegmentum, we were able to
12 identify the oculomotor nerve (III), which projects ventrolaterally from the oculomotor
13 nucleus (NIII) and exits from the ventral surface of the brain (e.g., Figure 7n and o).
14 The course of the oculomotor nerve is a useful landmark for helping to identify the
15 oculomotor nucleus (NIII) and the basal plate of the mesencephalon.

16 In the prosomeric model, the mesencephalon contains two mesomeres, m1 and m2
17 from anterior to posterior. The tectal gray, optic tectum (mammalian superior
18 colliculus), and torus semicircularis (mammalian inferior colliculus) constitute the alar
19 plate of m1. The oculomotor nucleus (NIII) represents the basal plate of m1.

20 We noticed two pairs of tectal membrane thickenings which invaginate into the tectal
21 ventricle, toward the tegmentum, at the 1-ovl/pec-bud stage (165 hpf; Figures 7q and
22 r). During development, the boundaries between these thickenings gradually
23 disappears as they growth together (Figure 8o and 9o). The tectal proliferation zones
24 are distinct. At the 1-ovl/pec-bud stage (165 hpf) and the pec-bud stage (185 hpf),

1 the tectal region appears as a large, bright field with microCT (Figure 7o and 8l).
2 Beginning at the high-pec stage (210 hpf), the rostral tectal proliferation becomes
3 restricted to one mediodorsal cluster and two bilateral clusters (Figure 9o and 10n).
4 These lateral and medial proliferation zones merge in the midline at caudal levels
5 and form a continuous cap of tectal proliferation (e.g., Figure 9t).
6 The torus longitudinalis (TL) is a specialized brain region exclusive to ray-finned fish
7 (Folgueira et al., 2020; Wullimann, 1994). We identified the TL from rostral to caudal
8 along the medial margins of the optic tectum. Virtual horizontal sections through the
9 optic tectum horizontally from the level of the epiphysis and the rostral cerebellar
10 thickening, showed the TL at the top of the mesencephalic ventricle (Figure 11).
11 The r0, or isthmus, is coincident with the midbrain-hindbrain boundary (MHB). The
12 MHB is composed of the posterior tectal membrane and the rostral cerebellar
13 thickenings (the valvula cerebelli; Wullimann and Knipp, 2000). The rostral cerebellar
14 thickenings appeared bright throughout the developmental period in this study
15 (Figure 7r, 8q, 9r, 10q). The cerebellar plate appeared bright only at its basal and
16 medial aspects (e.g., Figure 10t and u). The trochlear nucleus (NIV) topologically
17 belongs to r0. It is easier to identify the trochlear decussation (DIV) and the
18 commissura cerebelli (Ccer) in the midsagittal plane (Figure 12a-d) in the valvula
19 cerebelli (Va) than in the horizontal or the transverse planes. Then follow the
20 caudolateral projection of the trochlear axon in the horizontal plane (Figure 12e) until
21 it exits the brain as the trochlear nerve (IV, Figure 12f) between the torus
22 semicircularis and rhombencephalon. The axonal tract of the trochlear nucleus
23 delineates the boundary between r0 and r1.
24 The fasciculus retroflexus (fr) of teleosts projected to the interpeduncular nucleus
25 (NIn) and so we used it to identify the NIn in the bitterling embryo (Figure 13a).

1 According to Lorente-Cánovas et al. (2012), the interpeduncular nucleus is at the
2 basal plate at the level of the isthmus (r0) and r1. Rhombomere r1 is devoid of
3 cranial motor neurons (Nieuwenhuys and Puelles, 2016), so we used the posterior
4 margin of the interpeduncular nucleus as a landmark for the boundary between r1
5 and r2.

6 In the more caudal rhombencephalic region, the roof plate is a thin layer of tela
7 choroidea, which is visible in microCT scans because it remains intact during the
8 fixation and staining procedures (e.g., Figure 7x). The dorsal medullary proliferation
9 zone is broad, and expands ventrally up to the high-pec stage (210 hpf; (Figure 7v,
10 8v, and 9v). At the long-pec stage (235 hpf) it becomes more spatially restricted,
11 forming the rhombic lip proliferation zone (Figure 10w).

12 The boundaries between rhombomeres from r2 to r8 are visible in early bitterling
13 embryos at 135 hpf (Figure 14), but soon become less visible from the 1-ovl/pec fin
14 stage (165 hpf). Teleosts retain a segmented pattern of reticulospinal neurons
15 through embryonic life to adulthood (Gilland et al., 2014). For example, the large
16 bilateral Mauthner neurons (M) are markers of r4 (Eaton and Farley, 1973; Moens
17 and Prince, 2002). By virtually sectioning the 135 hpf embryo at the axial level of r4,
18 we determine that the Mauthner cell reside near the center of rhombomere 4, rather
19 than at the segmental boundaries (Figure 14). The rhombomeric segments of the
20 older embryo (235 hpf, Figure 13a to k) were thereby identified, by assuming that
21 each reticulospinal neuronal cluster is located in the center of each rhombomeres.

22 The nerve roots of the cranial nerves are also reliable landmarks of rhombomeres.
23 By tracing the projection of cranial nerves, we identified the trigeminal nerve root (V)
24 in r2 (Figure 13e), the facial root (VII) and the accompanying vestibulo-cochlear
25 nerves associated with r4 (Figure 13g), the abducens root (VI) in r5 (Figure 13h), the

1 glossopharyngeal root (IX) in r7 (Figure 13j), and the vagus root (X) in r8 (Figure
2 13k). Rhombomere r2 was also indicated by cell bodies of the anterior trigeminal
3 motor neurons (NVa, Figure 13e) and r3 by the posterior trigeminal motor neurons
4 (NVp, Figure 13f). Facial nerve motor neurons (NVII) are distinct in r6 (Figure 13i).

5 **DISCUSSION**

6 *Brain imaging and the 3D visualization of neuroanatomy*

7 To gain insight into the complex morphogenesis of the Rosy Bitterling brain, we
8 analyzed the formation of the brain ventricular system in 3D from the 1-ovl (150 hpf)
9 stage to the long-pec (235 hpf) stage. We combined annotations of brain functional
10 subdivisions and morphological landmarks, based on microCT scanning results and
11 on the literature for the zebrafish embryo. We found that 3D visualization with
12 microCT scanning protocols were extremely useful for the study of the bitterling brain.
13 They provide an updating of imaging modalities for morphological and anatomical
14 analyses in this non-model organism. This systematic application of microCT may
15 help provide an essential foundation for future comparative studies of the teleost
16 brain.

17 Our study provides 3D reconstructions of the brain ventricles in the bitterling; they
18 show a similar organization to the zebrafish larval ventricular system, as visualized
19 by dye-injection into the hindbrain ventricle (Lowery and Sive, 2005). We have also
20 defined boundaries of brain ventricular subdivisions, based on published anatomical
21 studies (Turner et al., 2012). Furthermore, we found that the flexure of the neuraxis
22 is easily visualized continuously from the rostral tip of the brain to the spinal cord.
23 Understanding the cephalic flexure is crucial, due to its profound effect on the

1 topology of the brain, especially in the highly complex secondary prosencephalon
2 (Hauptmann and Gerster, 2000; Puelles, 2019). It has been shown that the
3 mechanism of brain ventricle development is highly conserved among vertebrates
4 (Lowery and Sive, 2009). The cerebrospinal fluid in the brain ventricular system
5 could contribute to specialization of the early brain, because the production and
6 circulation of cerebrospinal fluid performs an important role in the homeostasis of the
7 central nervous system (Fame et al., 2016).

8 In contrast to other vertebrates, teleosts and other actinopterygian fish, lack a pair of
9 lateral ventricles in the telencephalon (Wullimann and Rink, 2002). Instead, they
10 show a T-shaped midline telencephalic ventricle that is the result of a morphogenetic
11 process called 'eversion' (Mueller and Wullimann, 2009). Our 3D models of the Rosy
12 Bitterling brain show that the anterior intraencephalic sulcus (AIS) develops much
13 earlier than the eversion of the telencephalic ventricle, whereas the expansion of the
14 dorsal ventricular surface of the AIS is synchronous with the eversion process. Our
15 results are consistent with the pattern of telencephalic ventricle morphogenesis
16 described in the zebrafish (Folgueira et al., 2012). We were able to visualize the
17 development of the deep ventricular sulcus (AIS), followed by an anterolateral
18 eversion of the telencephalic neuroepithelium.

19 We found distinct periventricular cell clusters in the gray matter, with higher
20 greyscale values than the surrounding tissue. Their distribution pattern was
21 consistent with the distribution of proliferation zones described during neurogenesis
22 of zebrafish (Mueller and Wullimann, 2003; Mueller and Wullimann, 2016; Mueller et
23 al., 2006; Wullimann, 2009; Wullimann and Knipp, 2000; Wullimann and Mueller,
24 2004). It is possible that newly postmitotic neurons appear brighter than most of the
25 postmitotic cell masses in more peripheral positions, remote from the periventricular

1 proliferation zones. The results of immunohistochemistry in the zebrafish for the
2 neurotransmitter GABA (γ -aminobutyric acid) in zebrafish (Mueller and Wullmann,
3 2016; Mueller et al., 2006; Panganiban and Rubenstein, 2002), and PCNA
4 (proliferation cell nuclear antigen) for proliferation patterns (Wullmann and Knipp,
5 2000; Wullmann and Mueller, 2004; Wullmann and Puelles, 1999) are consistent
6 with our interpretation of the proliferation zones.

7 Our study will hopefully pave the way for more detailed analyses of the bitterling
8 brain. We hope that it will also prove valuable in studies using the growing number of
9 fish models (and even non-fish models). Application of microCT 3D imaging provides
10 a heuristic model of the brain, an extremely complex anatomical region. Importantly,
11 our approach is validated by the fact that the profile of CT values displayed here in
12 the bitterling brain are consistent with genoarchitecture identified in previous
13 neurodevelopmental studies. For example, our annotation of the zona limitans
14 intrathalamica (ZLI) is extremely close to the highly conserved expression pattern of
15 the gene sonic hedgehog (*shh*; Vieira et al., 2005; Mueller et al., 2006; Scholpp et al.,
16 2006) in the zebrafish. In addition, microCT allows for the time-efficient imaging of
17 intact brains, while providing a resolution (micron level) sufficient for displaying
18 critical landmarks, groups of neurons such as proliferation zones versus postmitotic
19 cell masses, and white matter tracts. These histological characteristics are critical for
20 detecting landmarks and visualizing structural features as a means to describe
21 developmental neuroanatomy.

22 The resolution of microCT is inferior to light microscopy, whether it is paraffin
23 histology, or the light sheet microscopy-based imaging of fluorescence-stained intact
24 brains. It is also lower in resolution than Synchrotron imaging, whose resolution
25 reaches the sub-micron level, and therefore permits quantitative histological

1 phenotyping (Ding et al., 2019). Neurons and neuropils in the central nervous system
2 can be selectively stained with the salts of metallic elements including gold, silver,
3 platinum and mercury (Keklikoglou et al., 2019; Mizutani and Suzuki, 2012), which
4 can compensate for the inability to use antibodies in combination with microCT. In
5 summary, MicroCT imaging produces lower resolution than some imaging modalities,
6 but has the special advantage that complex 3D models can be produced without the
7 need for time-consuming reconstruction from histological material. The specimens
8 imaged with microCT can be subsequently run through paraffin histology if needed.

9 *Comparison of developmental stages between bitterling and zebrafish*

10 The phylogenetic relationship of the Rosy Bitterling and the zebrafish is a very close
11 one (they are both Cypriniformes: Cyprinidae; Mayden et al., 2009). This closeness
12 allows for a relatively straight forward interspecies comparison of their brain
13 development. The process of brain ventricle inflation, flexion of the neuroaxis, and
14 establishment of prosomeric units, happens in bitterling in much the same way as in
15 the zebrafish. Specifically, the 165 hpf bitterling brain and 30 hpf zebrafish brain are
16 in the same process of brain ventricle inflation and have a similar degree of flexion of
17 the neuroaxis. We also found that the stratification of the proliferation zones of the
18 diencephalic region (Pr, Th, PTd, PTv) is similar in the 185 hpf bitterling brain and 36
19 hpf zebrafish brain.

20 We have detected distinct developmental heterochronies between bitterling and
21 zebrafish brains. The bitterling shows precocious development of the inferior lobe
22 and basalward extension of the lateral recess in the hypothalamic region (Figure 2):
23 the inferior lobe is visible as early as the long-pec stage (235 hpf in the bitterling
24 (Figure 15); but not until 48 hpf in the zebrafish. The long-pec stage is defined by the

1 development of the pectoral fin bud, when the pectoral fin bud in both species grows
2 significantly, and its height grows to twice the the width at its base (Kimmel et al.,
3 1995; Yi et al., 2021). In the zebrafish, the inferior lobe develops relatively late, at
4 around five days post fertilization (Bloch et al., 2019). In teleost fish, the inferior lobe
5 is known as a multisensory integration center that is involved in gustatory (Rink and
6 Wullimann, 1998; Wullimann, 2020), visual (Butler et al., 1991), and octavolateralis
7 system (Yang et al., 2007). Further studies can use the detailed descriptions in our
8 bitterling brain developmental atlas to uncover the underlying regulatory mechanisms
9 that control brain development.

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20

21

22 TABLES

23 **Table 1 Sample information and microCT scanning parameters.**

Age (hpf)	Stage name	Pixel size (µm)	Voltage (keV/W)	Exp. time (sec.)	Intensity
135	2-ovl	2.19	40/3	4	5000-9000
150	1-ovl	2.0615	40/3	4.5	5000-10000
165	1-ovl/pec-bud	0.9765	40/3	17	5000-8500
185	pec-bud	0.9989	80/7	3	5000-6300

210	high-pec	1.4582	40/3	9.5	5000-10000
235	long-pec	1.4299	40/3	8.5	5000-10000

1 Note: for each stage, we scanned at least two specimens. The stage names follow Yi et al.
2 (2021). For the pec-bud stage (185 hpf), we also tried a lower resolution scan with the
3 voltage set to 40/3 keV/W. We found no practical difference in image quality between scans
4 at 80/7 keV/W and 40/3 keV/W. Therefore, we decided to use the less time-consuming
5 option for this stage, namely scanning with the higher voltage but shorter exposure time.
6 Key: Dev, developmental; hpf, hours post fertilization; Exp, exposure; keV, kiloelectron volt.
7

8 FIGURE LEGENDS

9 **Figure 1** *Rhodeus ocellatus*, development of the brain ventricular system, microCT images, dorsal to
10 the top, head to the left. (a-e) virtual midsagittal sections overlayed with surface view of manually-
11 segmented brain ventricles, and showing the distinct ridge of the anterior intraencephalic sulcus (AIS)
12 and progressive compartmentalization of the ventricular system (a-e). (f) virtual midsagittal section
13 illustrating the black lumen of the brain ventricle, a bright white periventricular layer of gray matter
14 (GM), and a gray peripheral layer of white matter (WM). The red dotted line indicates the neuraxis,
15 with anterior, posterior, alar and basal topological direction for the curved axis. The green axes refer
16 to the linear axial system apply to the brain parts with the terms rostral, caudal, dorsal, and ventral.
17 The (a) stage 1-ovl, 150 hpf. (b) stage 1-ovl/pec-bud, 165 hpf. (c) stage pec-bud, 185 hpf. (d, f) stage
18 high-pec, 210 hpf. (e) stage long-pec, 235 hpf. For annotations, see List of Abbreviations. Scale bars
19 = 100 μ m (a, b, d, e, f); 80 μ m (c).
20

21 **Figure 2** *Rhodeus ocellatus*, development of the lateral recess and posterior recess in the
22 hypothalamus region. Virtual coronal sections, head to the left. At the right upper corner is the 3D
23 volume rendering of the hypothalamus region seen from the dorsal side. (a) stage 1-ovl/pec-bud, 165
24 hpf. (b) stage pec-bud, 185 hpf. (c) stage high-pec, 210 hpf. (d) stage long-pec, 235 hpf. For
25 annotations, see List of Abbreviations. Scale bars = 100 μ m.

1

2 **Figure 3** *Rhodeus ocellatus*, brain ventricular system at the stage 1-ovl/pec-bud, 165 hpf. (a-d)
3 microCT images, the pseudo-colour volume rendering of the head region overlayed with a surface
4 view of the manually-segmented brain ventricles. (a-d) dorsal, lateral, ventral, and rostral views,
5 respectively. The inset in the upper right corner in (b) illustrates the median ventricular sulcus (MVS)
6 branches off from the ventral bottom of the median ventricle of the mesencephalon. For annotations,
7 see List of Abbreviations. Scale bars = 100 µm.

8

9 **Figure 4** *Rhodeus ocellatus*, brain ventricular system at the stage pec-bud, 185 hpf. (a-d) microCT
10 images, the pseudo-colour volume rendering of the head region overlayed with a surface view of the
11 manually-segmented brain ventricles. (a-d) dorsal, lateral, ventral, and rostral views, respectively. For
12 annotations, see List of Abbreviations. Scale bars = 80 µm.

13

14 **Figure 5** *Rhodeus ocellatus*, brain ventricular system at the stage high-pec, 210 hpf. (a-d) microCT
15 images, the pseudo-colour volume rendering of the head region is overlayed with a surface view of
16 the manually-segmented brain ventricles. (a-d) dorsal, lateral, ventral, and rostral views, respectively.
17 For annotations, see List of Abbreviations. Scale bars = 100 µm.

18

19 **Figure 6** *Rhodeus ocellatus*, brain ventricular system at the stage long-pec, 235 hpf. (a-d) microCT
20 images, the pseudo-colour volume rendering of the head region is overlayed with a surface view of
21 the manually-segmented brain ventricles. (a-d) dorsal, lateral, ventral, and rostral views respectively.
22 For annotations, see List of Abbreviations. Scale bars = 100 µm.

23

24 **Figure 7** *Rhodeus ocellatus*, brain cross-sectional anatomy, stage 1-ovl/pec-bud, 165 hpf. (a-x)
25 microCT images, virtual sections, transverse plane, dorsal towards the top, sections from rostral to
26 caudal, direction of section plane indicated in inset in (a). For annotations, see List of Abbreviations
27 Scale bars = 100 µm.

1

2 **Figure 8** *Rhodeus ocellatus*, brain cross-sectional anatomy, stage pec-bud, 185 hpf. (a-x) microCT
3 images, virtual sections, transverse plane, dorsal towards the top, sections from rostral to caudal,
4 direction of section plane indicated in inset in (a). For annotations, see List of Abbreviations. Scale
5 bars = 100 μ m.

6

7 **Figure 9** *Rhodeus ocellatus*, brain cross-sectional anatomy, stage high-pec, 210 hpf. (a-x) microCT
8 images, virtual sections, transverse plane, dorsal towards the top, sections go from rostral to caudal,
9 direction of section plane indicated in inset in (a). For annotations, see List of Abbreviations. Scale
10 bars = 100 μ m.

11

12 **Figure 10** *Rhodeus ocellatus*, brain cross-sectional anatomy, stage long-pec, 235 hpf. (a-x) microCT
13 images, virtual sections, transverse plane, dorsal towards the top, sections go from rostral to caudal,
14 direction of section plane indicated in inset in (a). For annotations, see List of Abbreviations. Scale
15 bars = 100 μ m.

16

17 **Figure 11** *Rhodeus ocellatus*, development of the torus longitudinalis. Virtual dissection, head to the
18 left. (a) stage 1-ovl/pec-bud, 165 hpf. (b) stage pec-bud, 185 hpf. (c) stage high-pec, 210 hpf. (d)
19 stage long-pec, 235 hpf. For annotations, see List of Abbreviations.

20

21 **Figure 12** *Rhodeus ocellatus*, trochlear nerve, stage long-pec, 235 hpf. (a-f) microCT images, virtual
22 sections. (a and b) midsagittal plane, head to the left, dorsal towards the top. (c and d) transverse
23 plane, dorsal towards the top. (e) horizontal plane, head towards the top. (f) parasagittal plane, head
24 to the left, dorsal towards the top. For annotations, see List of Abbreviations.

25

1 **Figure 13** *Rhodeus ocellatus*, hindbrain segmentation, stage long-pec, 235 hpf. (a) microCT images,
2 virtual sections, midsagittal plane, dorsal towards the top, head to the left. The black dash line
3 indicates the rhombomeric boundaries. (b) horizontal plane, head to the left, direction of section plane
4 indicated in inset at the upper right corner. (c-k) transverse plane, dorsal towards the top, sections go
5 from the rostral to caudal, direction of section plane indicated in inset in (b). For annotations, see List
6 of Abbreviations.

8 **Figure 14** *Rhodeus ocellatus*, hindbrain segmentation, 135 hpf. (a and d) microCT images, volume
9 rendering, show boundaries between rhombomeres. (b) virtual section, horizontal plane, head to the
10 left, show the location of the Mauthner cell at the centre of r4. (c) virtual section, transverse plane,
11 dorsal towards the top. For annotations, see List of Abbreviations.

13 **Figure 15** *Rhodeus ocellatus*, development of the brain region and the pectoral fin bud. Dorsal view
14 of the head region microCT images, pseudo-colored volume-renderings, rostral to the left. Lateral
15 view of the embryo, photomicrographs, rostral to the left. (a) 1-ovl/pec-bud, 165 hpf; (b) pec-bud, 185
16 hpf; (c) high-pec, 210 hpf; (d) long-pec 235 hpf. For annotations, see List of Abbreviations.

17 LIST OF ABBREVIATIONS

18 ac, anterior commissure
19 AIS, anterior intraencephalic sulcus
20 Ccer, commissure cerebelli
21 CeP, cerebellar plate
22 Ch, notochord
23 DIV, trochlear decussation
24 DVe, diencephalic ventricle
25 E, epiphysis
26 EmT, eminentia thalami
27 fp, floor plate
28 fr, fasciculus retroflexus

- 1 GM, gray matter
- 2 Ha, habenula
- 3 Hc, caudal hypothalamus
- 4 Hi, intermediate hypothalamus
- 5 Hr, rostral hypothalamus
- 6 Hy, hypophysis
- 7 III, oculomotor nerve
- 8 IL, inferior lobe of hypothalamus
- 9 IV, trochlear nerve
- 10 IX, glossopharyngeal nerve
- 11 le, lens
- 12 lfb, lateral forebrain bundle
- 13 LR, lateral recess of hypothalamic ventricle
- 14 lr, lateral rectus muscle
- 15 M, Mauthner neurons
- 16 M1, pretectal migrated area
- 17 M2, posterior tubercular migrated area
- 18 M3, EmT migrated area
- 19 M4, telencephalic migrated area
- 20 MHB, midbrain-hindbrain boundary
- 21 mlf, medial longitudinal fascicle
- 22 mou, mouth
- 23 MV, median ventricle of the mesencephalic ventricle
- 24 MVS, median ventricular sulcus of the mesencephalic ventricle
- 25 N, region of the nucleus of the medial longitudinal fascicle
- 26 NIII, oculomotor nucleus
- 27 NIn, interpeduncular nucleus
- 28 NIV, trochlear nucleus
- 29 NVa, rostral trigeminal motor neuron
- 30 NVII, facial motor neuron
- 31 NVp, caudal trigeminal motor neuron
- 32 OA, occipital arch
- 33 OB, olfactory bulb
- 34 oc, optic chiasma
- 35 OC, otic capsule

- 1 OE, olfactory epithelium
- 2 of, oval fossae
- 3 OG, octaval ganglion (VIII)
- 4 ON, olfactory nerve
- 5 on, optic nerve
- 6 ot, otolith
- 7 P, pallium
- 8 pc, posterior commissure
- 9 pf, pectoral fin
- 10 Po, preoptic region
- 11 poc, postoptic commissure
- 12 PoR, preoptic recess
- 13 PR, posterior recess of hypothalamic ventricle
- 14 Pr, pretectum
- 15 ptc, posterior tubercular commissure
- 16 PTd, dorsal part of posterior tuberculum
- 17 PTh, prethalamus
- 18 PTM, posterior tectal membrane
- 19 PTv, ventral part of posterior tuberculum
- 20 r, rhombomere
- 21 re, retina
- 22 RL, rhombic lip
- 23 RVe, rhombencephalic ventricle
- 24 S, subpallium
- 25 SC, spinal cord
- 26 Sd, dorsal subpallium
- 27 sr, superior rectus muscle
- 28 Sv, ventral subpallium
- 29 T, midbrain tegmentum
- 30 tc, tela choroidea
- 31 Tel, telencephalon
- 32 TeO, tectum opticum
- 33 TeVe, tectal ventricle
- 34 Th, thalamus
- 35 TL, torus longitudinalis

- 1 tpc, tract of posterior commissure
- 2 TS, torus semicircularis
- 3 TVe, telencephalic ventricle
- 4 V, trigeminal nerve
- 5 Va, valvula cerebeli
- 6 VI, abducens nerve
- 7 VII, facial nerve
- 8 WM, white matter
- 9 X, vagus nerve
- 10 Zli, zona limitans intrathalamica
- 11

NIII NIV

235 hpf

r0

r1

r8

r7

r6

r5

r4

r3

r2

NIn

(a)

r1

r2

r3

r4

r5

r6

r7

r8

(b)

NIII

m

(c)

NIV

NIn

r0

(d)

NVa

V

r2

(e)

NVp

r3

(f)

M

VII

r4

(g)

VI

r5

(h)

NVII

r6

(i)

IX

r7

(j)

X

r8

(k)

135 hpf

r1 r2 r3 r4 r5 r6 r7 r8

(a)

r3 r4 r5
M

(b)

M

r4

(c)

(d)

100 μ m

165 hpf

pf —

100 μ m

(a)

185 hpf

pf —

100 μ m

(b)

210 hpf

pf —

100 μ m

(c)

235 hpf

pf —

100 μ m

(d)