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Neuroanatomy of a sex changing fish: the New Zealand spotty wrasse (*Notolabrus celidotus*) brain atlas

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ABSTRACT

For most vertebrates, sexual fate is genetically determined and remains fixed throughout life. However, for some teleost fishes sex is more plastic. Significant progress has been made in characterising the cellular and molecular processes that underpin gonadal sex change. The brain-mediated mechanisms that underlie and initiate this transformation, however, remain poorly understood. One reason for this is the current lack of a neuroanatomical reference work for sex changing fishes. Here, we present a brain atlas for the New Zealand (NZ) spotty wrasse (*Notolabrus celidotus*), developed from 10 wild caught specimens (2 male and 8 female). From each fish, coronal cryosections were collected and stained with cresyl violet solution. Photomicrographs were taken of each section using an inverted bright field microscope and 3D reconstructions were rendered of each brain for annotation and volumetric comparison of specific brain regions. The brain atlas describes the general features of the NZ spotty wrasse brain as well as its specificities. This atlas provides the necessary foundation for further investigation of the brain mechanisms driving protogyny in this species.

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Introduction

For most vertebrates, sexual fate is genetically determined and remains fixed throughout life (Capel 2017). However, for some teleost fishes sex is more plastic. The complete sex change that sequentially hermaphroditic fishes undergo during their reproductive lives is taxonomically widespread across teleosts and considered to be one of the best examples of phenotypic plasticity. Functional sex change has been documented in about 2% of teleost species spanning over 20 families, and generally occurs in three ways: protogynous (female-to-male), protandrous (male-to-female), and sequentially bi-directional (De Mitcheson and Liu 2008; Liu et al. 2017).

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Although it is clear that the brain initiates and underlies sex change (Godwin et al. 1996), the mechanisms through which it does so remain poorly understood. Significant progress has been made in characterising the cellular and molecular processes that underpin gonadal sex change, in particular in the bluehead wrasse (*Thalassoma bifasciatum*) (Todd et al. 2019). These and other studies (Gardner et al. 2005; Diotel et al. 2010) have shown the aromatase gene, encoding the enzyme converting androgens to estrogens, is a key player in gonadal sex change. Sex change in the brain, however, involves a multitude of different brain regions and neurotransmitters. For example, the number of gonadotropin-releasing hormone (GnRH) neurons in the forebrain of the protogynous Ballan wrasse (*Labrus berggylta*) increased in males compared to females (Elofsson et al. 1999). More recently, upregulation of isotocin, the teleost homolog of mammalian oxytocin, was observed in the midbrain early in the protogynous transformation of the bluehead wrasse (*Thalassoma bifasciatum*) (Todd et al. 2019). While in the bidirectional hermaphrodite, *Lythrypnis dalli,* female to male sex change was associated with a reduction in the number of isotocin immunoreactive cells in the forebrain (Black et al. 2004). These neural signals are subtle and highly localised, making it difficult to study them at a coarse anatomical scale. Brain atlases provide the neuroanatomical reference work required to study the brain in more detail and have therefore been developed for all the main neuroscientific animal models (Bradford 1983; Wulliman et al. 2012). To date, however, no such atlas exists for a sex changing fish species. In order to elucidate the brain mechanisms underlying sex change in any particular species of fish, the general neuroanatomy of this species must first be understood. Therefore, a sex changing fish-specific brain atlas is warranted.

The spotty wrasse is an endemic protogynous labrid that is well suited to laboratory studies. Like most wrasses, they have dimorphic initial phase (IP) and terminal phase (TP) colour morphs (Choat 1965). Sexually mature fish will spawn in captivity and sex change is induced in IP fish through the manipulation of social structure (Thomas et al. 2019; Goikoetxea et al. 2021). The ability to complete natural sex change under laboratory conditions is especially important as other model species such as the bluehead wrasse adapt poorly to captivity, thus most sex change experiments have to be done in wild populations (Liu et al. 2015). We have recently identified genes involved in sex differentiation, epigenetic reprogramming, and cell fate in this species (Muncaster et al. 2023). Collectively, these attributes make the spotty wrasse an excellent subject for a brain atlas of a sex changing species.

Materials and methods

Spotty wrasse sampling

Fish were handled in accordance with New Zealand National Animal Ethics Advisory Committee guidelines. Ethics applications were approved by the Animal Ethics Committee of Toi Ohomai Institute of Technology and by the University of Otago Animal Ethics Committee (AUP-22-87). Eight female adult spotty wrasses of size ranging from 12 to 20 cm and two adult terminal phase males of size ranging from 14 to 16 cm were caught by line and hook, deeply anesthetised and euthanized with a 0.06% solution of

ethyl 3-aminobenzoate methane sulfonate (MS 222; A.5040 Sigma). Each brain was removed from the skull and fixed in a solution of 4% paraformaldehyde in 1x phosphate-buffered saline (PBS) for 72 h at 4°C. Brains were subsequently immersed in a 30% sucrose 1xPBS solution until the tissue sank. The brains were then rinsed with 1xPBS, flash-frozen in liquid nitrogen and stored at −80°C. Although terminal phase males are easily distinguishable from females, initial phase males are more difficult to distinguish (Goikoetxea et al. 2021). To confirm sex of the females, a gonadal sample was dissected from each fish, fixed in 4% paraformaldehyde for 24 h, and subsequently stored in 1xPBS. Paraffin-embedded sections of 4 µm were cut on a rotary microtome (Leica RM2125 RTS) and stained with Mayer's haematoxylin and eosin (supplemental Figure 1).

Brain sectioning and histochemical Nissl-Staining

Brain tissue was cut into coronal sections of 30 µm thickness on a Leica CM3050S cryostat. Sections were mounted on SuperFrost plus slides (Thermo Fisher Scientific NZ Ltd, North Shore City, New Zealand), air dried for 1 hr at room temperature, and stored at −20°C till further processing. Prior to staining, a hydrophobic barrier was drawn onto the SuperFrost slide, surrounding the sections. Sections were then washed with 1xPBS and subsequently stained with Cresyl Violet Stain Solution (0.1%) (ab246816) according to the manufacturer's instructions. Finally, sections were rinsed in distilled water, dehydrated with ethanol, cleared with xylene and coverslipped using DPX as mounting medium.

Image processing

A Nikon Ti2-E inverted bright field and fluorescence microscope was used to photograph the sections stained with cresyl violet. Keeping brightness and exposure parameters consistent throughout, photomicrographs were taken with a 4x objective, requiring up to 2 × 3 fields per section for the bigger brain sections. These composite images were taken with

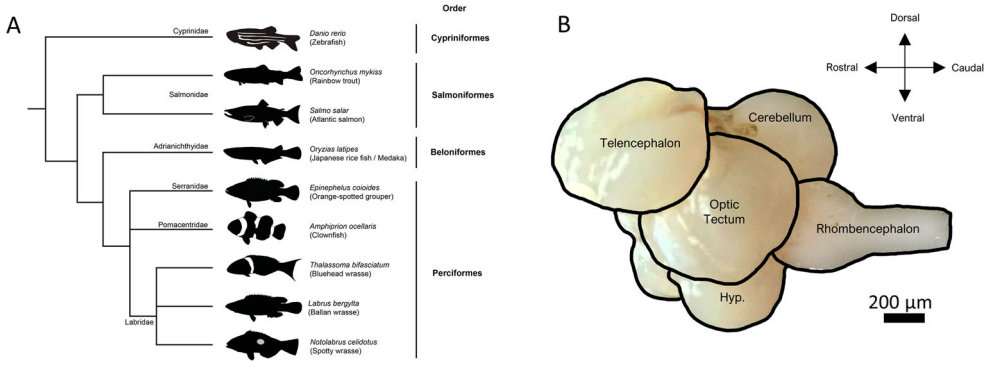

Figure 1. A, Phylogenetic tree showing how the spotty wrasse relates to some of the model fish species (zebrafish, rainbow trout, Atlantic salmon and medaka), and model sex changing species (orange-spotted grouper, clownfish, bluehead wrasse and Ballan wrasse). **B,** Overview of the spotty wrasse brain pictured from the side. Hyp: Hypothalamus.

15% overlap and stitched together using Nikon NIS-Elements software. Raw images were processed in Fiji/ImageJ to correct background noise and minor tissue artefacts. Image segmentation was performed using the Trainable Weka Segmentation plugin (Arganda-Carreras et al. 2017). Nissl-stained cell counts were performed using the Cell Counter plugin.

Neuroanatomical analysis

The nomenclature used in this atlas is based on the one developed for the two most widely used atlases by Wullimann et al. (2012) for the zebrafish (*Danio rerio*), and Bradford and Northcutt (1983) for the goldfish (*Carassius auratus*). When careful comparison with the central nervous system of vertebrates in general was made, the work of Meek and Nieuwenhuys was consulted (Meek and Nieuwenhuys 1998). We identified the different structures mainly by comparison with other teleost fish and placing the spotty wrasse in a more general phylogenetic context (Figure 1A). In addition, because wrasses belong to the order of Perciformes we use the nomenclature of two other Perciformes in a complementary way: the turquoise killifish (*Nothobranchius furzeri*) (D'angelo 2013) and the archerfish (*Toxotes chatareus*) (Karoubi et al. 2016).

Results and discussion

This atlas provides a comprehensive overview of the central nervous system of the NZ spotty wrasse, focusing on both its macrostructure and microstructure. While the basic morphology of the spotty wrasse brain is similar to that of other teleosts (Figure 1A), there are noteworthy differences in the neuroanatomical architecture that are both significant and relevant. The brain of the spotty wrasse is characterised by telencephalic hemispheres with relatively small olfactory areas, a diencephalon with large paired inferior lobes of the hypothalamus bulging out on the ventral surface of the brain, two pronounced lobes of the optic tectum, a prominent cerebellum on the dorsal side, and a large medulla oblongata (Figure 1B). The cranial nerves exhibit a typical pattern that is consistent with previously described teleost brain atlases, with 10 pairs of nerves ranging from the olfactory nerve (I) to the vagal nerve (X) (Wulliman et al. 2012).

Telencephalon

The telencephalon is responsible for higher brain functions, such as memory, emotions, cognition and higher-level multisensory integration and motor control (Savage 1980). The most rostral divisions of this structure are the paired olfactory bulbs. The primary olfactory fibres entering the olfactory bulbs are the axons of the olfactory receptors, which are of placodal origin and not considered part of the central nervous system (Døving 2007). The olfactory system of the spotty wrasse is relatively small, being more integrated in the rostral telencephalon, rather than a separate bulb as found in many other species. Like mammals, fish GnRH neurons originate in the medial olfactory placode during embryonic development and migrate along the olfactory bulb to their final positions within the hypothalamus (Elofsson et al. 1999).

The remainder of the telencephalon is comprised of two subdivisions, area dorsalis and area ventralis telencephali. The telencephalon in the spotty wrasse lacks clearly defined nuclei, similar to the telencephalon of other actinopterygians (Burr 1928). The neuronal groups in the telencephalic hemispheres are divided into two main areas: dorsal and ventral. The dorsal area is characterised by extensive medial (Dm), central (Dc), dorsal (Dd), lateral (Dl), and posterior (Dp) divisions. The dorsal area is defined by its large and highly elaborated lateral part (Dld), which includes the dorsal part (Dld), ventral part (Dlv), and lateral part (Dll). Rostrally, Dld borders Dlv by a tiny cell-free zone. Dlv extends from the most rostral position of the telencephalon to commissural levels. More posteriorly, between Dld and Dlv, there is Dll, whose borders are demarcated by a continuous line of small and tightly packed cells.

In the spotty wrasse, the ventral telencephalon includes four main cell groups, namely, dorsal (Vd), ventral (Vv), similar to other actinopterygians (Anken and Rahmann 1994). The ventral telencephalon is considered homologous to the subpallium of tetrapods (Meek and Nieuwenhuys 1998). Rostral to the anterior commissure (Cant), Vd appears as the olfactory bulbs recede, with Vv ventral to Vd becoming more prominent and Vd taking a progressively more dorsal position. It is important to note that the embryonic development of the teleost telencephalon is significantly different to other vertebrates (Zupanc 2006). While in other vertebrates the telencephalic hemispheres are formed through paired evagination and thickening of the embryonic neural tube, with each hemisphere containing a lateral diverticulum of the ventricle centrally, in teleosts the roof plate of the embryonic telencephalon extends laterally, causing the paired plates forming the hemispheric walls to roll out ventrolaterally, a process called eversion. Consequently, determining the homologous structures of teleostean telencephalic cell masses in other vertebrates solely based on adult topology is challenging.

Diencephalon

The first novel structure that appears when moving towards the caudal end of the telencephalon is the optic tectum. This is noteworthy because the optic tectum is, developmentally, part of the mesencephalon. For the sake of consistency with other brain atlases, we will discuss the diencephalon prior to the mesencephalon. The diencephalon (Figure 2C–N) acts as a primary relay and processing centre for sensory information and autonomic control. In the spotty wrasse, it does not have clear boundaries with the telencephalon and can be subdivided into the preoptic area, epithalamus, thalamus, hypothalamus, tuberal region, pretectum, and optic tracts. The epithalamus, which is the most rostro-dorsal part of the diencephalon, includes the habenula (Ha) and the epiphysis, and nuclei of the dorsal thalamus arise ventrocaudally to Ha. The preoptic area is an area of particular interest in sex changing fish, due to its role in the release of gonadotrophic hormones (Lamm et al. 2022). It is located at the rostral margin, partially covered by the caudal portions of the dorsal telencephalic divisions, while the pretectal area is found at the caudal margin. The anterior preoptic area (PPa) is situated ventro-caudal to the postoptic commissure (cpop). PM is distinguished from the parvocellular preoptic nucleus (PPp), which is bounded dorsally by PM and ventrally by the suprachiasmatic nucleus (SC). The nucleus glomerulus (NG), a prominent visual relay

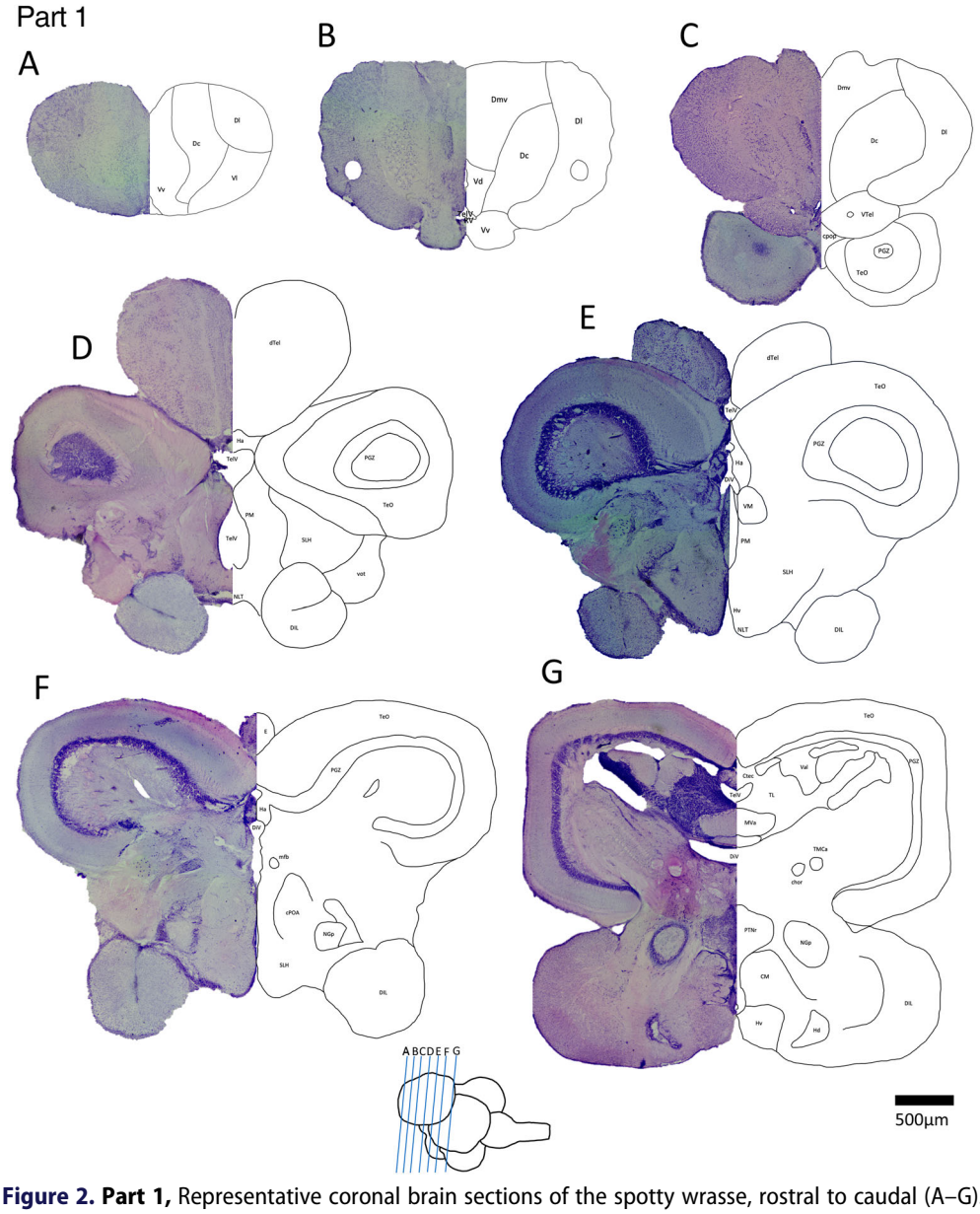

Figure 2. Part 1, Representative coronal brain sections of the spotty wrasse, rostral to caudal (A–G).

nucleus, is remarkably well-defined by a dense border of nuclei and can be found lateral from the tuberal nucleus. The pituitary gland is lacking in this atlas. Because this structure sits in a protective bony enclosure called the sella turnica this structure repeatedly tore off during dissection of the brain. Based on comparison with the archerfish brain atlas (Karoubi et al. 2016), this structure is likely located between the inferior lobes of the hypothalamus, ventral of the diencephalic ventricle (Figure 2H and I).

The hypothalamus emerges ventrally to the caudal pole of the preoptic area with no clear border and includes dorsal (Hd), ventral (Hv), lateral (Hl), and caudal (Hc)

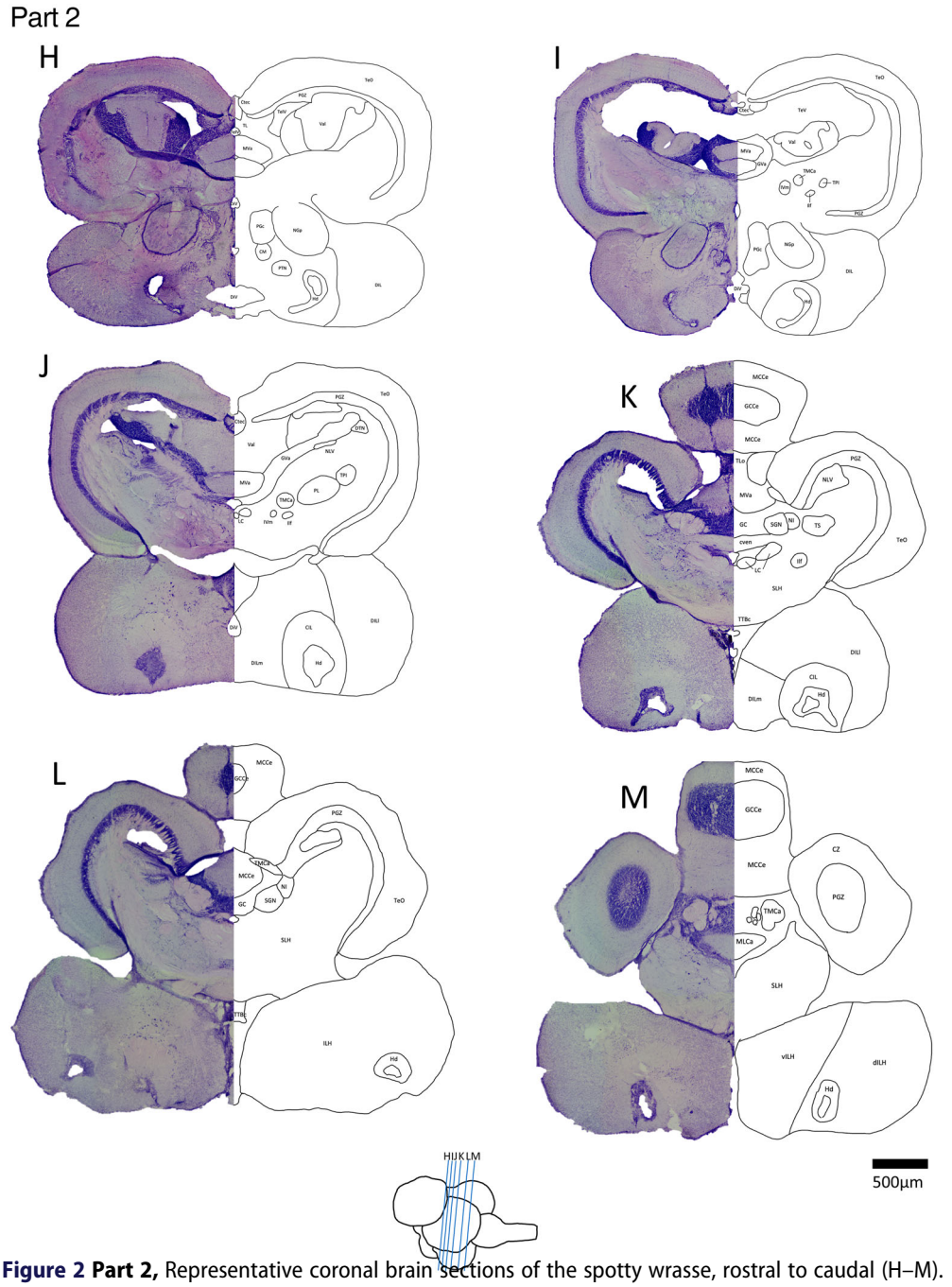

Figure 2 Part 2, Representative coronal brain sections of the spotty wrasse, rostral to caudal (H–M). See Figure 2 Parts 1 and 3 for images A–G and N–U, respectively.

hypothalamic subdivisions, as well as the paired inferior lobe of hypothalamus (DIL). The corpus mammilare are also found in this region, ventral to the tuberal nucleus. Whether this structure is part of the hypothalamus remains a matter of debate among neuroanatomists (Carpenter and Sutin 1983), but in the spotty wrasse this seems to be

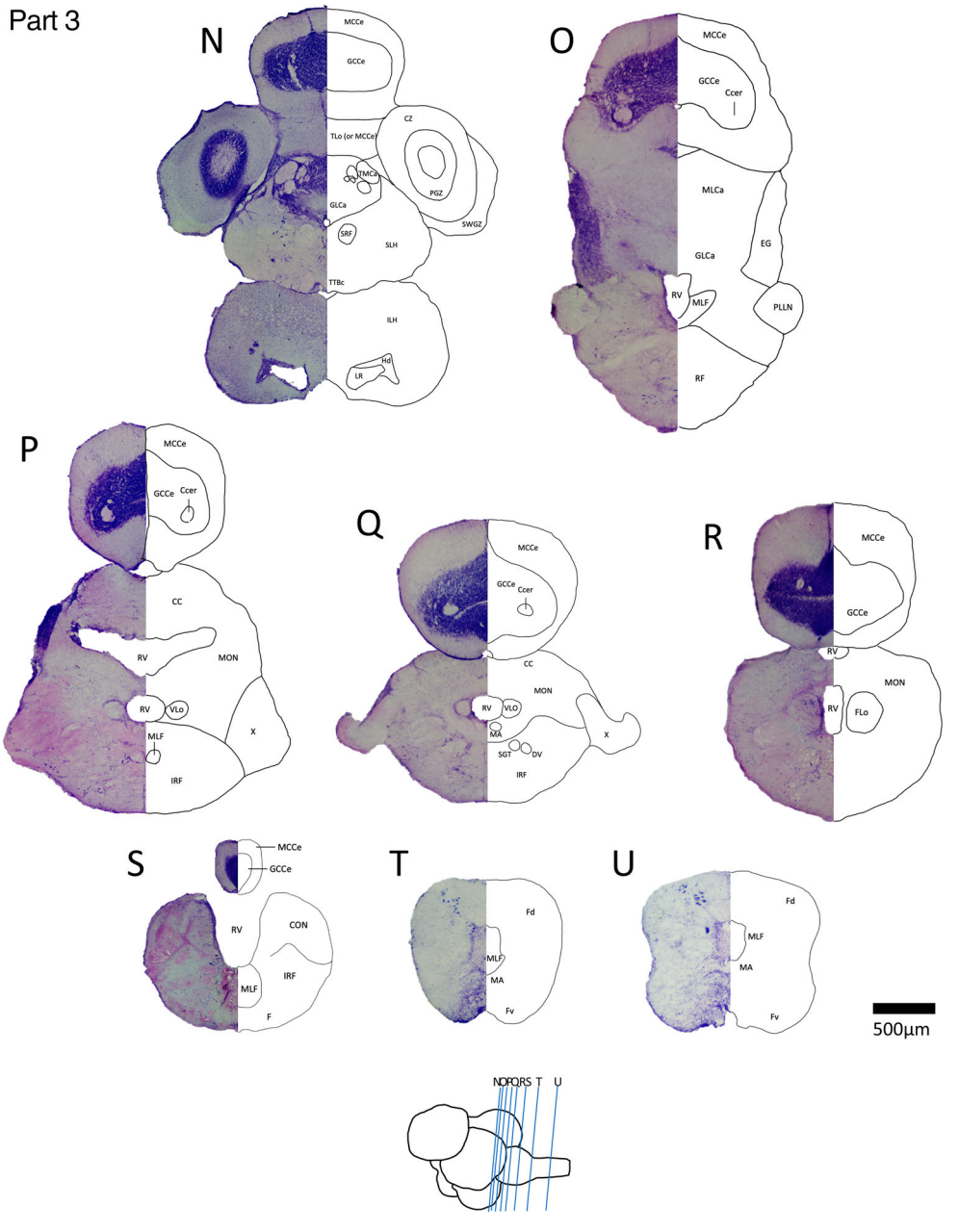

Figure 2 **Part 3,** Representative coronal brain sections of the spotty wrasse, rostral to caudal (N–U). See Figure 2 Parts 1 and 2 for images A–G and H–M, respectively.

the case. The pretectal region is subdivided into a periventricular, central, and superficial part, with the periventricular part including the nucleus of dorsal (PPd) and ventral (PPv) periventricular pretectum, located respectively in a dorsolateral and ventrolateral position. The central pretectal region is located lateral to the periventricular pretectal region and surrounds the superficial pretectal region.

Mesencephalon

The mesencephalon or midbrain is the rostral-most portion of the brainstem and is associated with vision, hearing, motor control, sleep and wakefulness, arousal, and temperature regulation (Nieuwenhuys 1982). Within the spotty wrasse mesencephalon (Figure 2C–O), two distinct regions can be identified: a dominant bulging optic tectum (TeO) and a more centrally located midbrain tegmentum. TeO comprises of two large lobes that border the ventricle, exhibiting a layered structure. From the innermost to the outermost, the layers are the periventricular grey zone (PGZ), the deep white zone, the central zone, and the superficial white and grey zone. The lobes of the optic tectum are remarkably large in the spotty wrasse, overlapping the diencephalon both caudally and rostrally. Functioning as the main visual processor of the brain (Northmore 2011), the relative size of the optic tectum suggests that the spotty wrasse is a highly visually oriented species.

Situated at the caudal pole of the diencephalon, the midbrain tegmentum contains the semicircular torus (TS) and is positioned above the inferior lobe of hypothalamus.

The medial zone of the tegmentum is rostrally bounded by the medial longitudinal fascicle (mlf). The lateral region, on the other hand, accommodates the lateral longitudinal fascicle (llf) and its respective nucleus placed dorsally, along with several fibre tracts, such as the mesencephalo-cerebellar tract (TMCa) and the pretecto-isthmic tract (TPI). The oculomotor and trochlear cranial nerves can be found towards the caudal end of the mesencephalon.

Rhombencephalon

The caudal part of the brain is known as the rhombencephalon (Figure 2P–U). The rhombencephalon assists in the regulation of autonomic functions such as breathing and heart rate, maintaining balance and equilibrium, and movement coordination (Nieuwenhuys 1982). Bounded by the mesencephalic tegmentum rostrally and the spinal cord caudally, it comprises of three main regions: the dorsocranial and ventrocaudal regions, and the medulla. The cerebellum characterises the dorsocranial region, which shows recognisable subdivisions such as the valvula (Va), the corpus of cerebellum (CCe), the granular eminentiae (EG) and cerebellar cristae (CC). The Va occupies most of the mesencephalic ventricle, while the CCe shows a similar layer organisation to Va, with molecular and granular layers and the Purkinje cells layer. To the best of our knowledge, the two paired protuberances EG appear to be continuous with the granular layer of the CCe, and caudally, are placed at the lateral surface of the brain.

The medulla oblongata is organised into four zones: dorsal, intermediodorsal, intermedioventral, and ventral (Meek and Nieuwenhuys 1998). The dorsal zone contains the sensory component of trigeminal nucleus and the nuclei belonging to the lateral area. The inferior reticular formation is also part of the dorsal zone. In the intermediodorsal zone, the vagal lobe (V) appears evident as a small non-lobulated and non-laminated enlargement. The Mauthner axons, unique to fish and amphibians and notable for their unusual use of both chemical and electrical synapses (Eaton et al. 2001), appear ventral to the vagal lobe and adjacent to the rhombencephalic ventricle. The intermedioventral zone contains visceromotor nuclei, such as the medial octavolateralis

nucleus (MON) which appears dorsal to the superior reticular formation and medial to the octaval and lateral area. The nucleus of the motor branch of the vagal nerve is located along the ventro-lateral margin of the rhombencephalic ventricle, and more lateral to the nucleus, the respective nerve branch emerges. Towards the caudal end of the cerebellum, the rhombencephalic ventricle enlarges, with the medulla detached. The medial longitudinal fascicle and the Mauthner axon remain clearly visible all the way through. An extended version of Figure 2 is available as Supplemental Online Material.

Although the brain size of the specimens used for this atlas varied widely in size, no differences in brain size were found when corrected for body size. A very strong ($r^2 = 0.993$) correlation was found between brain size and standard body length as measured from the tip of the snout to the posterior end of the last vertebra (Figure 3). We did not observe any sex differences in gross brain anatomy. However, with the limited number of males used in this study, robust statistical analysis of sex differences was not possible. We hypothesise that significant sex differences can be found in the brain. These differences are likely to be specific to certain cell types and would therefore not be detected with a Nissl-staining. Future studies should therefore compare male and females spotty wrasse brains using a more cell type-specific approach, such as immunohistochemistry for GnRH neurons in the preoptic area (Elofsson et al. 1999).

In conclusion, the present work provides the first brain atlas for a sex changing fish species. It has been well-known that sex change is a brain-mediated transformation. Bluehead wrasses display rapid increases in aggressive and courtship behaviours during sex change, independent of the presence of gonads (Godwin et al. 1996). Although significant progress has been made in understanding the neurobiology of sex change, a detailed mechanistical understanding is yet to be achieved. This brain atlas can be used as a neuroanatomical resource to study the sex changing fish brain in more detail. RNA-sequencing techniques, for example, are powerful tools but need to be applied at a fine anatomical level in order to pick up subtle and highly localised signals. The present work allows for the identification of specific brain structures for future cellular and molecular analyses. We hope this work will assist in advancing our understanding of the mechanisms that underlie the fascinating process of sex change.

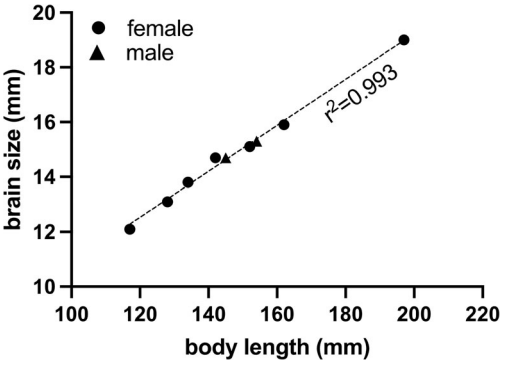

Figure 3. Correlation between captured spotty wrasse brain size and body length.

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Disclosure statement

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