The central nervous system of agnathans

An ontogenetic and phylogenetic study of encephalization and brain organization in lampreys and hagfishes

by

Carlos Andrés Salas López

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Author's Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution. This Thesis contains published work and/or prepared for publication in international journals, some of which has been co-authored. The bibliographical details of the work and where it appears in the Thesis are outline below.

Carlos A. Salas

Statement of Contributors

Chapter 2

Salas CA, Yopak KE, Warrington RE, Hart NS, Potter IC and Collin SP (2015) Ontogenetic shifts in brain scaling reflect behavioral changes in the life cycle of the pouched lamprey *Geotria australis*. Frontiers in Neuroscience 9:251. doi: 10.3389/fnins.2015.00251

Shaun Collin, Nathan Hart, Ian Potter and Carlos Salas contributed to the conception, Rachael Warrington and Carlos Salas acquired the data, and Kara Yopak and Carlos Salas designed the analyses and interpreted the data. Carlos Salas crafted the article, and all authors collaborated in its revision.

Chapter 3

Salas CA, Yopak KE, Lisney TJ, Hart NS, Potter IC and Collin SP (in preparation) The central nervous system of agnathans: encephalization in lampreys and hagfishes.

Shaun Collin, Kara Yopak, Nathan Hart, Ian Potter and Carlos Salas contributed to the conception, Carlos Salas acquired the data, and Kara Yopak, Thomas Lisney and Carlos Salas designed the analyses and interpreted the data. Carlos Salas crafted the article, and all authors collaborated in its revision.

Chapter 4

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Carlos A. Salas **Shaun P. Collin**

Abstract

Brain size relative to body size (encephalization) and the scaling of major brain subdivisions (telencephalon, diencephalon, mesencephalon and rhombencephalon) with brain size (brain organization), have been investigated across nearly all vertebrate clades. It has been shown that encephalization and patterns of brain organization have evolved in relation to a range of phylogenetic, developmental, ecological, physiological and behavioral variables, such as habitat, metabolic constraints, and predatory mode. Although data do not exist across a fully comprehensive dataset, equivalent relationships between encephalization, brain organization and diverse life history traits have been proposed in several vertebrate species during their ontogeny. Despite the cross-vertebrate generalities documented in brain evolution, current knowledge of the encephalization of extant relatives to jawless vertebrate forms (lampreys and hagfishes), at both ontogenetic and phylogenetic levels, is deficient. It is still unknown whether conserved patterns of brain scaling originated in the earliest vertebrates, or at the onset of gnathostomes. This will have important implications for our understanding of the developmental and phylogenetic processes acting on encephalization, filling a critical gap in our knowledge of the evolution of the brain from the origin of vertebrates. Therefore, in order to better understand the evolution of the central nervous system of vertebrates, I aim to: 1. Describe the variation in encephalization and brain organization throughout ontogeny of a species of anadromous parasitic lamprey (*Geotria australis*) 2. Study the predictors of relative brain size in extant species of cyclostomes to reconstruct the history of encephalization in agnathans, and 3. Estimate the effect of diverse life history traits on interspecific patterns of brain organization in lampreys. In the following chapters, I will present several methods that have been implemented to improve previous estimates of encephalization and brain organization, such as the use of phylogenetically-informed statistics and multimodal inference modeling methods. The results obtained in this Thesis will be used to argue that both ontogenetic and interspecific comparisons can be employed to unveil patterns of brain evolution, and that the rules governing brain scaling in extant jawless vertebrates are, in general terms, similar to those that apply to jawed vertebrates, e.g. in all vertebrates, the medulla oblongata scales hypoallometrically with the rest of the brain. However, differences in the scaling relationships between a number of brain structures, e.g. the olfactory bulbs and telencephalon, as well as the absence of a true cerebellum, suggest substantially different patterns of brain organization between jawless and jawed vertebrates. I predict this work will provide a crucial perspective on the evolution of the vertebrate central nervous system.

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List of Abbreviations

Life cycle stages

am	ammocoete or larva
amII	second year class ammocoete
amIII	third year class ammocoete
amIV	fourth year class ammocoete
ds	downstream migrant
us	upstream migrant
sa	spawning
	adult

Life history traits

Parasitism

NP	non-parasitic
P	parasitic

Habitat

A	anadromous
AFW	anadromous and freshwater
FW	freshwater

Feeding ecology

B	blood
PG	parasitic generalist
F	flesh

Brain structures

OB	olfactory bulbs
Te	telencephalic hemispheres
PO	pineal organ
OT	optic tectum
MOR	rostral medulla oblongata
MOC	caudal medulla oblongata

Techniques and statistics

AICc	Akaike information criterion (second order)
Δ AICc	difference between AICcs
ANCOVA	analysis of covariance
ANOVA	analysis of variance
GLS	generalised least squares
OLS	ordinary least squares
pGLS	phylogenetic generalised least squares
PCA	principal components analysis
pPCA	phylogenetic principal components analysis
PC	principal component
d.f.	degrees of freedom

Chapter 1 General Introduction

The comparison of the nervous system at inter- (species) and intra- (ontogenetic) specific levels has been critical for our understanding of the evolution of the brain of vertebrates (Striedter, 2005). In recent years, new comparative methods have been developed, incorporating the phylogenetic relationships between species in the analyses. This approach has greatly improved the estimation of diverse proxies for brain evolution among vertebrates, such as the relationship between brain size and body size (encephalization), as well as the drivers of variation in brain organization (reviewed in Symonds and Blomberg, 2014). Moreover, these techniques use algorithms that can allow for a direct estimation of the ancestral states of these characters (Revell, 2012), providing more effective methods to predict the evolutionary pathways that have contributed to the extant vertebrate brain. Despite the critical position that agnathans occupy in vertebrate evolution, little is known about the diversity of their central nervous system, highlighting the need for a complete analysis of the extant members of this important group.

In this thesis, comparative phylogenetic methods, as well as comparisons of the brain across stages of the life cycle, will be performed on a dataset of 18 extant agnathans to evaluate the variation in complexity of their central nervous system and the ontogenetic trajectory of brain size and structure scaling, in order to increase our understanding of the early evolution of the brain of vertebrates. In the following sections, relevant background of what is known of the early evolution of the vertebrate brain will be presented in addition to the evolutionary history of lampreys. The Introduction will end with a summary of the main aims of the Thesis.

1.1 Evolution of the nervous system

A common characteristic of all organisms that exist within the biosphere is the capacity to both perceive and modify the environment. Therefore, it is expected that each organism will possess a specific set of sensory and motor structures or pathways, which will consequently allow for a particular interaction with the environment (**Figure 1.1**). It has been proposed that these perceptual and motor fields can be understood as a unity, i.e. the *Umwelt*, which is unique for each species (von Uexküll, 1957 [1934]). The operation of this functional cycle thus defines a domain of existence of the species within the environment, which corresponds to their niche, whereas the observation of the interaction between species and niche can be interpreted as their behaviour (Maturana and Varela, 1973; Maturana and Mpodozis, 2000).

During evolutionary history, a large number of both perceptual and motor systems have arisen, with diverse levels of organization and complexity (Arendt, 2008; Jékely, 2011). For example, in unicellular organisms, various molecular networks provide the structural basis of their respective *Umwelten* (reviewed in Bassler, 2002; Krell et al., 2011; Brunet and Arendt, 2016).

The rise of multicellular organisms brought an increase in biodiversity and trophic levels (Stanley, 1973; Huntley and Kowalewski, 2007), which correlates with the development of specialized cells for sensory and motor functions. Indeed, it has been argued that the appearance of predation has been critical in the development of the nervous system (Northcutt, 2002; Monk and Paulin, 2014). In these early nervous systems, sensorial components may have accomplished sensory-motor integration through paracrine stimulation of neighbour effectors, which then evolved to form more complex nets for sensory-motor integration (Mackie, 1990; Jékely et al., 2015; Katz, 2016). There is a general agreement that these "diffuse nerve nets" have served as the basis for the independent evolution of centralized nervous systems in a number of animal taxa (**Figure 1.2**), although different scenarios have been proposed for the evolution of the nervous system of vertebrates (Northcutt, 2012).

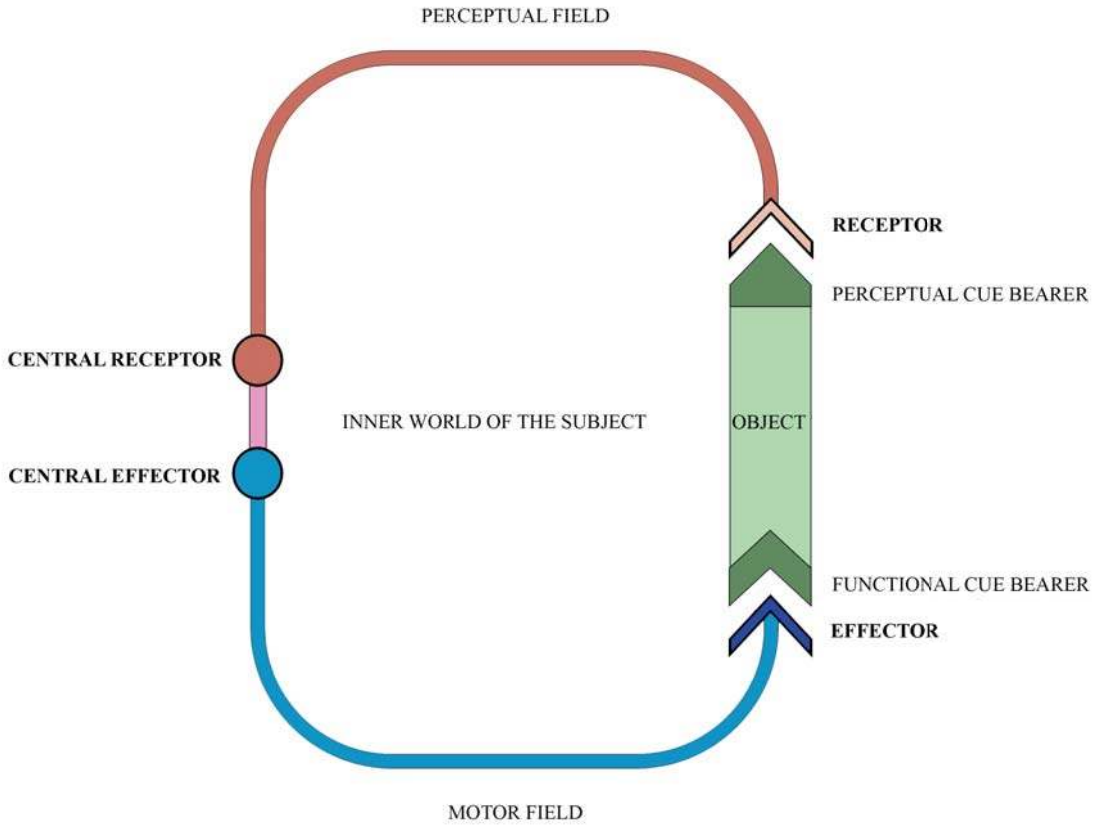

Figure 1.1 | Functional cycle of perception. The diagram illustrates how the relationship between object (environment) and subject (organism) constitutes a systematic whole, determining a unique inner world or *Umwelt*. Adapted from von Uexküll (1957 [1934]).

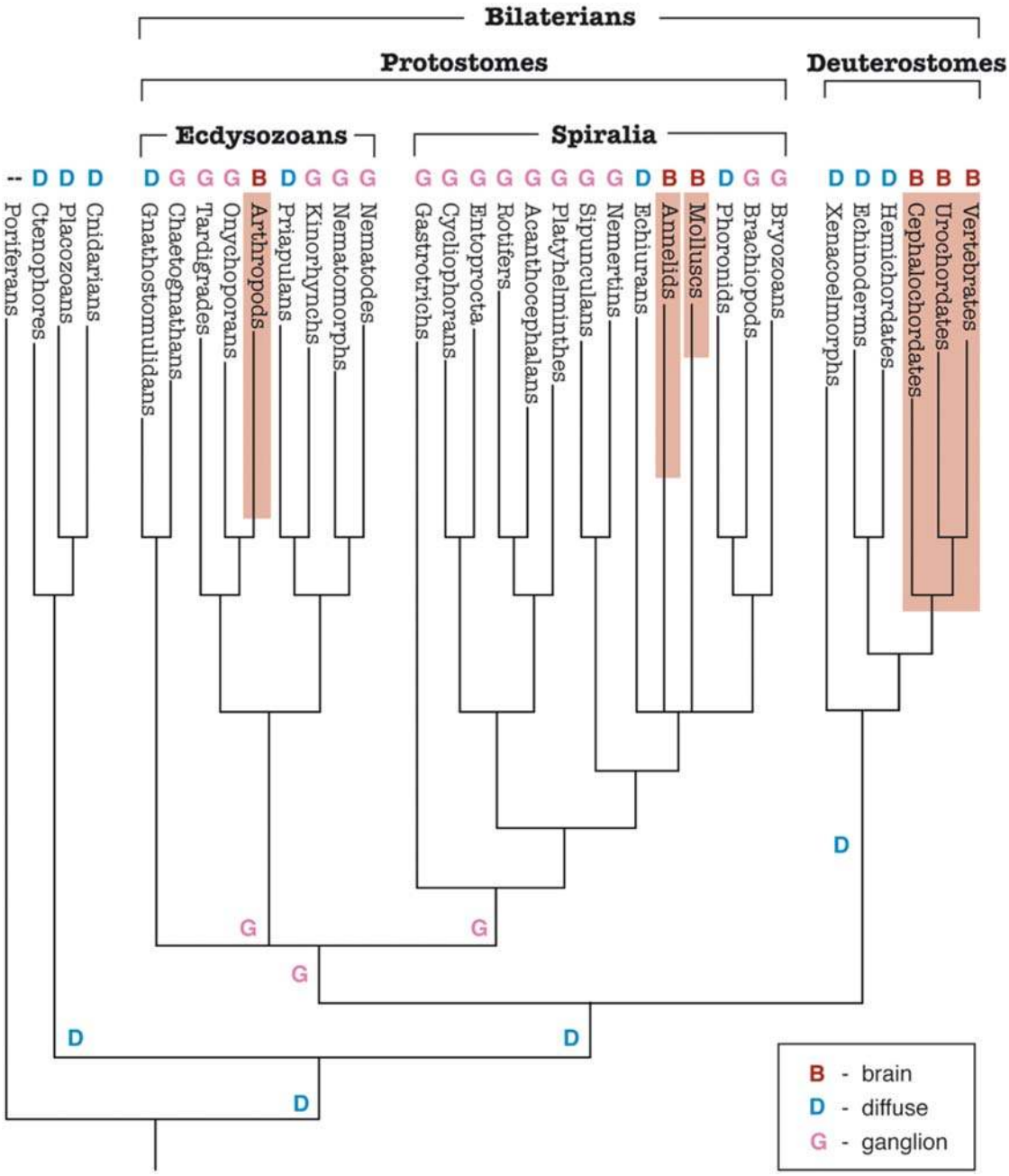

Figure 1.2 | Parallel evolution of the brain-like central nervous systems (CNSs). More complex CNSs have independently evolved in arthropods, molluscs, annelids and chordates (cephalochordates + urochordates + vertebrates). Reproduced from Northcutt (2012).

1.1.1 Evolution of the chordate brain

It has been suggested that complex brains have evolved independently at least four times in eumetazoans (Moroz, 2009; Northcutt, 2010; Northcutt, 2012), with one of them occurring in the last common ancestor of the Chordata (**Figure 1.2**). All chordates possess a number of anatomical features that distinguish them from protochordates and other invertebrates, including a dorsal hollow nerve cord, a notochord, segmented trunk muscles, and a pharynx that is perforated by a variable number of pharyngeal slits, which have been related to developmental

innovations of vertebrates, such as the neural crest and placodes (Gans and Northcutt, 1983; Northcutt and Gans, 1983). Cladistic studies of the brain of chordates have suggested that the condition observed in the brain of extant cephalochordates (i.e. lancelets, **Figure 1.3 A**) may represent the ancestral condition of the brain in this group (Northcutt, 1996; Butler, 2000a; Khonsari et al., 2009). Lancelets are filter-feeding, sedentary chordates that have a relatively simple brain, which consists of a neural tube with only a caudal and a rostral division (Lacalli, 1996; Ekhart et al., 2003). In contrast, it is generally accepted that adults of nearly all vertebrate species possess a common "brain archetype", whereby their brain is composed of the same subdivisions, i.e. from rostral to caudal: telencephalon (including the olfactory bulbs), diencephalon, mesencephalon, and rhombencephalon (reviewed in Striedter, 2005). It was postulated that there was no transitional forms between the organization of the brain found in lancelets and that of vertebrates, and this sudden change in brain organization was attributed to a redundancy of homeobox genes as a consequence of a genomic expansion at the origin of this group (Northcutt, 1996), in line with the hypothesis of "punctuated equilibria," as proposed by Eldredge and Gould (1972). Alternatively, it has been hypothesized that this process occurred gradually, as a serial transformation of the brain of chordates, with a transitional form (**Figure 1.3 B**) between lancelets and vertebrates thought to exist (Butler, 2000a; b; 2006). The first stage in this transition is thought to have occurred with the appearance of paired eyes and a more developed diencephalon, in conjunction with an overall increase in the size of the brain relative to body size. The second transitional stage (**Figure 1.3 C**) was thought to be a more developed telencephalon (pallium + striatum), and enhanced connectivity between brain subdivisions (Butler, 2000a; b; 2006). This hypothesis has received support from the fossil record (Mallatt and Chen, 2003), although there is not yet agreement that this fossil (*Haikouella*) is an actual chordate (reviewed in Janvier, 2015). In all of these cases, it has been considered that a vertebrate-like brain was developed during the time of the shift from filter-feeding to more active modes of predation, with the development of major sense organs, and musculature that allowed for active swimming (Gans, 1989; Northcutt, 1996).

1.1.2 Conservation and divergence of the vertebrate brain

Evidence of the first vertebrates in the fossil record suggests that this group of animals appeared approximately 500 million years ago, during the Palaeozoic era (Shu, 2008). These Cambrian vertebrates were jawless fish-like animals with a pair of large, antero-dorsally facing camera-like eyes, a small median olfactory organ, and 5–7 pairs of gill arches, amongst other traits (Janvier, 2015). Vertebrates are divided into two main groups, agnathan (jawless) and gnathostome (jawed) vertebrates. It is thought that the lineage originating from cyclostomes (extant jawless vertebrates, i.e. lampreys and hagfishes) split from other jawless stem gnathostomes ("ostracoderms") at the beginning of the radiation of jawless vertebrates (**Figure**

1.4). In spite of this early divergence, it can be inferred that at least some of these jawless vertebrate species may have shared common brain characteristics with modern jawless vertebrates, such as a relatively well developed pineal organ and hindbrain (Gai et al., 2011). In fact, it has been recently shown that both lampreys and hagfishes possess a common pattern of gene expression with gnathostomes during brain development, suggesting that these molecular markers may constitute primitive shared characters for all vertebrates, which further supports a generalised plan of brain organization for all vertebrates (Sugahara et al., 2016). However, the comparison of adult brain organization between cyclostomes and gnathostomes (**Figure 1.5)** and even within cyclostomes, indicates important morphological and functional differences between these groups, such as the absence of a developed true cerebellum (Kishida et al., 1987; Weigle and Northcutt, 1998; Montgomery et al., 2012) or myelin in nerve fibres in cyclostomes (Bullock et al., 1984), which are thought to reflect their long history of evolutionary divergence (Braun, 1996; Butler and Hodos, 1996; Wicht, 1996; Khonsari et al., 2009).

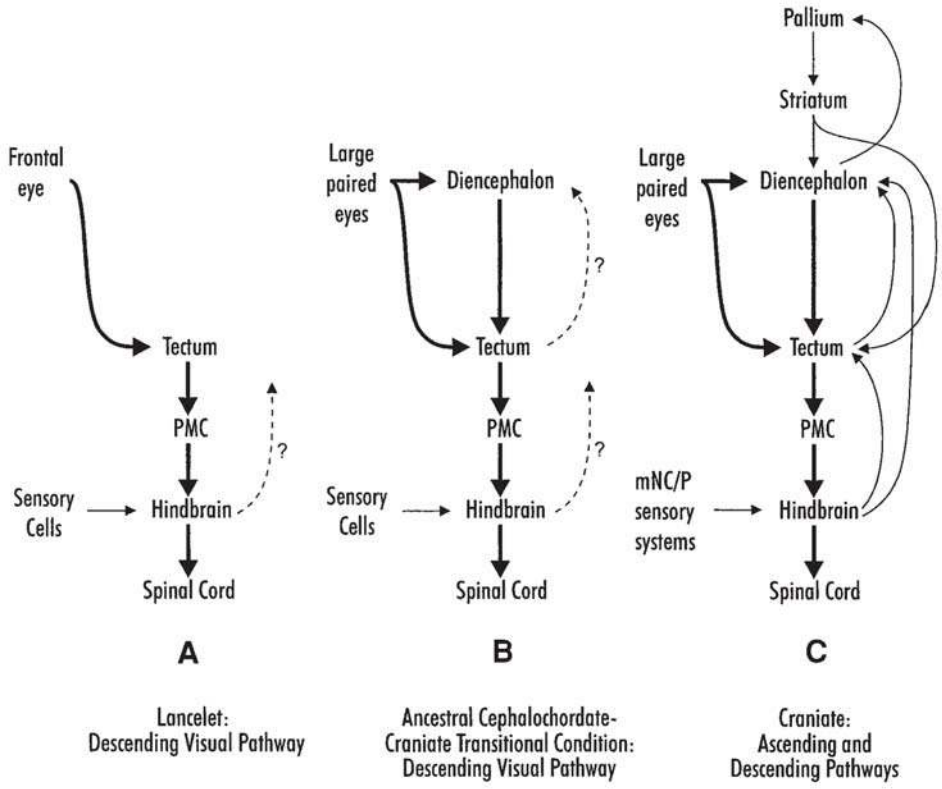

Figure 1.3 | Serial transformation hypothesis of the origin of the craniate (vertebrate) brain. mNC/P, migratory neural crest and placodes; PMC, primary motor centre. Reproduced from Butler (2000a).

Comparisons of the vertebrate brain have received much attention, especially with respect to comparisons of brain size across species (reviewed in Striedter, 2005). These studies show that brain size increases predictably with body size. When brain size is plotted as a response of body size, log-transformed data points usually fall along a predictable slope, where smaller animals have relatively larger brains, i.e. brain size increases allometrically (slope < 1) with body size.

This universal scaling rule for vertebrates is, however, more consistent within taxonomic groups with smaller number of species, where a higher level of variation between the slopes and intercepts can be typically found (Jerison, 1973; van Dongen, 1998). For example, cyclostomes possess, on average, a relatively smaller brain and a shallower slope than gnathostomes (Ebinger et al., 1983; Platel and Vesselkin, 1989). Moreover, within gnathostome vertebrate classes, it has been shown that individual species can deviate significantly from the expected brain size for their body size. It has been suggested that this difference in relative brain size, or encephalization, is related to a number of life history traits in these groups (e.g. Gittleman, 1986; Bauchot et al., 1989; Fish and Lockwood, 2003; Iwaniuk and Nelson, 2003; Yopak, 2012). Nonetheless, no such study has been performed in agnathan vertebrates, so it is still unknown whether these principles apply to this ancient group of vertebrates.

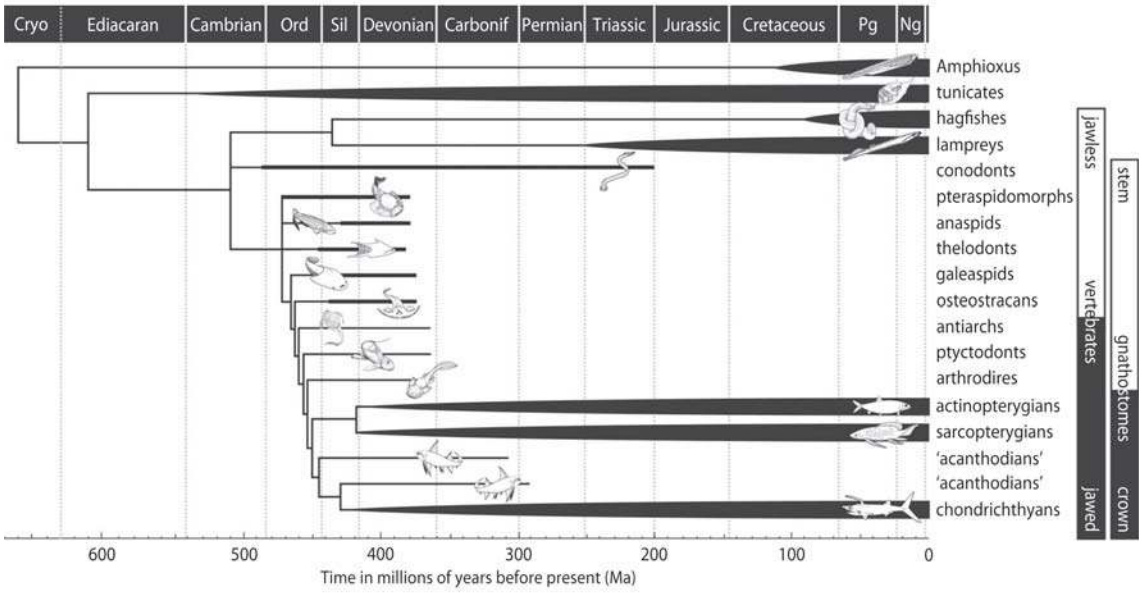

Figure 1.4 | Phylogenetic tree of chordates and a representative selection of extinct jawless vertebrates. Reproduced from Donoghue and Keating (2014).

Interspecific differences in the size of brain structures relative to total brain size (brain organization) have also shown a high degree of variability within and across major vertebrate groups, which has been linked to a combination of diverse factors, such as the lifestyle of each species, as well as to conserved scaling rules that apply to each of their brain structures (reviewed in Striedter, 2005). Proponents of the mosaic mode of evolution argue that brain organization may follow the principle of proper mass (Jerison, 1973), which directly relates the relative size of a given brain structure to the function that it subserves (Barton and Harvey, 2000; Iwaniuk et al., 2004). This principle may be applied to whole subsystems within the brain, such as the visual system, where changes occur in both the cytoarchitecture of neural structures, as well as in the relative size of individual nuclei. This principle may also reflect "grade shifts" or changes in intercept (relative size) that have evolved independent of phylogenetic relationships and are related to life style (e.g. in birds: Gutierrez-Ibanez et al., 2014; Wylie et

al., 2015), which may have a developmental origin (Charvet and Striedter, 2011; Charvet et al., 2011). In fact, it has been proposed that the conservation of a number of mechanisms in early development, in particular the order of developmental events that give rise to each brain structure, may be relevant to their relative size in the adult brain. Some authors have proposed that the later a brain structure develops, the longer the period of neurogenesis, and thus the larger its size will be in the adult brain, i.e. the "late equals large" principle (Finlay and Darlington, 1995; Finlay et al., 2001). One of the expected consequences of such a model is that these late-developing structures, such as the telencephalon and cerebellum, will scale with a hyperallometric slope with brain size and, subsequently, will be extremely large in species with larger brains. This model has been fitted to data on brain structure size for a diversity of vertebrate groups, such as mammals and cartilaginous fishes, and is therefore thought to be a highly conserved strategy for all vertebrates (Yopak et al., 2010; Charvet and Striedter, 2011). Nonetheless, although currently available data for cyclostomes support the applicability of a few of these principles to jawless vertebrates, e.g. the telencephalon of hagfishes increases in size with increasing brain size (Ebinger et al., 1983), very few species of cyclostomes have been studied. This thesis will provide further evidence that a number of these mechanisms are also observed in extant agnathans.

1.2 Diversity of lampreys and their *Umwelten*

Previous studies of the nervous system of cyclostomes have overlooked possible intra- and interspecific differences in the central nervous system of lampreys, e.g. Nieuwenhuys and Nicholson (1998) have not taken into account life history traits or variation throughout ontogeny. In this section, the main characteristics of lampreys and their lifestyles will be used to argue against a homogeneous perspective of these animals. Therefore, a brief review of the characteristics of the peripheral visual system of lampreys will be presented, as it is one of the few aspects of the neurobiology of lampreys that has been extensively studied across all major taxonomic groups and life history traits, revealing significant differences that could be extended to other parts of their nervous system. In addition, a summary of the main sensory systems and functional subdivisions of the brain of lampreys will introduce the brain structures studied in this Thesis.

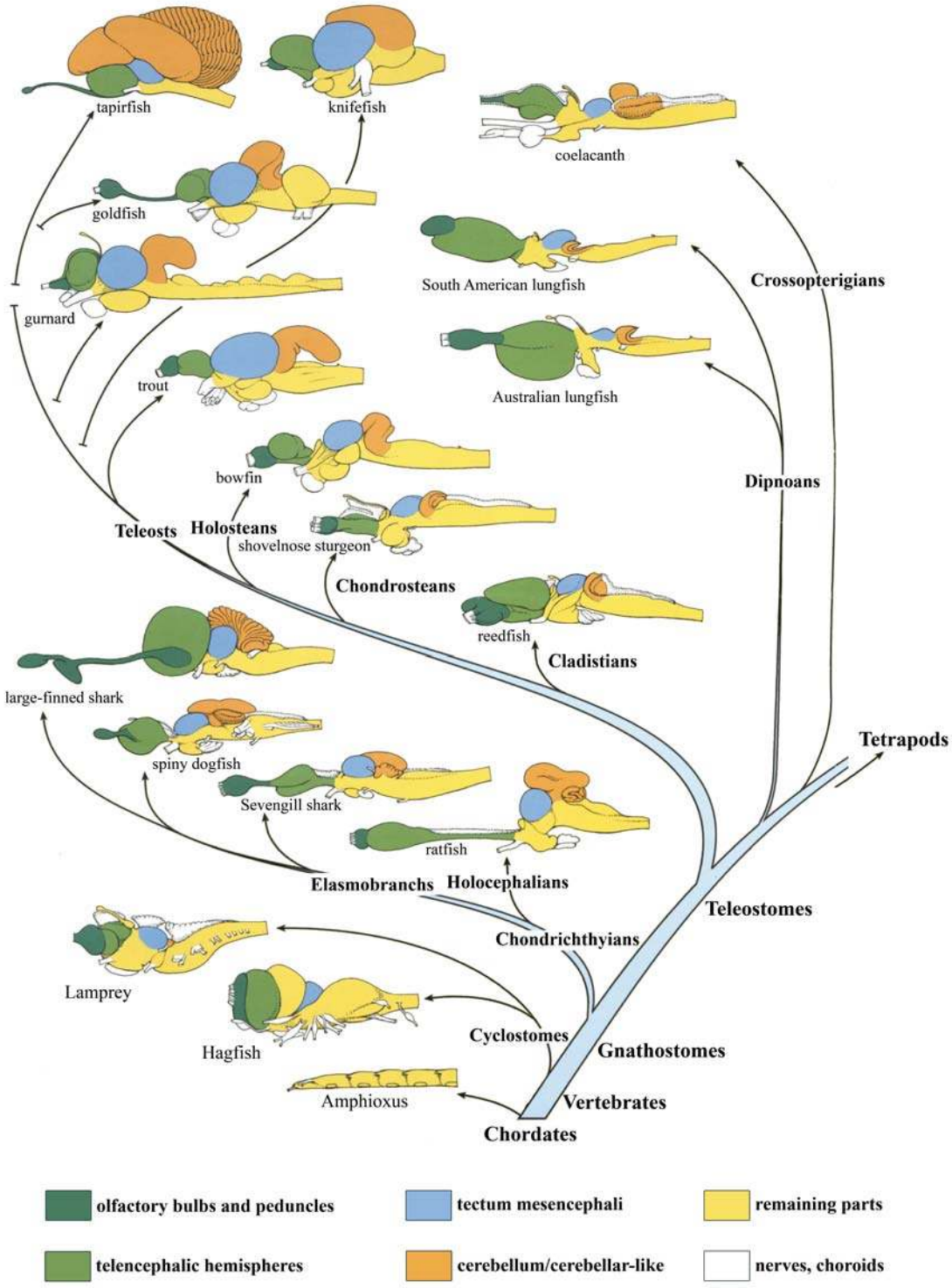

Figure 1.5 | The brain of vertebrates. Comparison of the brain of a cephalochordate (*Amphioxus*), jawless vertebrates (lamprey and hagfish), and a selection of brains of aquatic gnathostomes from a lateral view. A number of brain structures are colour-coded for a comparison across species. Adapted from Nieuwenhuys et al. (1998).

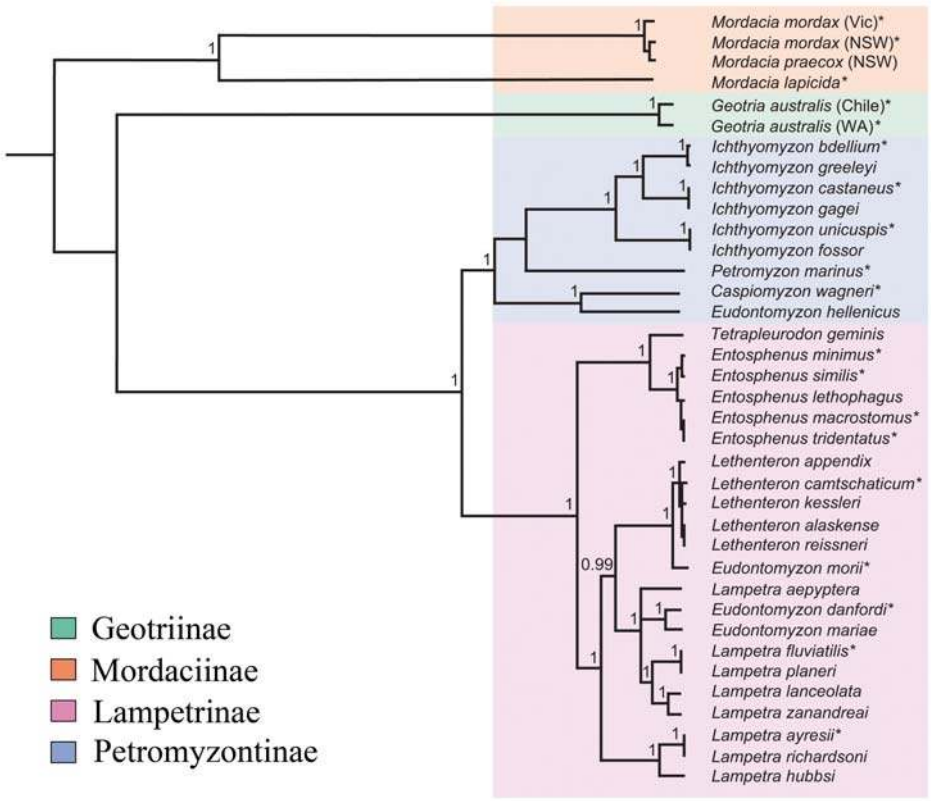

Figure 1.6 | Phylogenetic relationships of 35 extant species of lampreys. The tree is derived from cytochrome *b* sequence data using Bayesian analyses. Subfamilies of lampreys are highlighted with different colours. Species with an asterisk have a parasitic lifestyle as adults. Bayesian posterior probabilities are given for those nodes where values are greater than 0.95. NSW, New South Wales (Australia); Vic, Victoria (Australia); WA, Western Australia (Australia). Adapted from Potter et al. (2015).

1.2.1 Diversity of lampreys

Currently, there are 41 recognized species of lampreys (**Figure 1.6**), which are mostly distributed in temperate regions of both the northern and southern hemispheres. Lampreys are divided into three families, Mordaciidae, Geotriidae, and Petromyzontidae. The Mordaciidae (with only one genus and three described species) and the monotypic Geotriidae constitute the Southern Hemisphere species, which represent early branches of the phylogenetic tree of lampreys (Lang et al., 2009; Potter et al., 2015). In contrast, all Northern Hemisphere species are grouped in the Petromyzontidae, the most diverse family of lampreys, with eight different genera and 37 species. Morphologically, extant lampreys possess several traits that are found uniquely in this group of animals and dictate their taxonomic position within the jawless fishes or agnathans. Agnathans are most notably distinguished from the rest of vertebrates by the absence of jaws and paired fins (**Figure 1.7 A**), but there are other characters that differentiate them, such as the number of semicircular canals in the labyrinth (two in lampreys), a single median dorsal nostril, the structure of the branchial skeleton, and the presence of a mesencephalic plexus, among others (Hardisty and Potter, 1971a; Rovainen, 1979; Nieuwenhuys and Nicholson, 1998; Osorio and Retaux, 2008). However, one of the most

striking characteristics of adult lampreys is their buccal apparatus (**Figure 1.7 B**). It consists of a highly developed oral disc, possessing multiple series of teeth arranged in different rows and locations within the mouth, with a number of teeth and laminae present also on the tongue-like piston, which, together, form a specialised suctioning apparatus (Potter and Strahan, 1968; Hardisty and Potter, 1971a; Renaud et al., 2009). These morphological characters are species-specific, so the number, arrangement, and form of both the lingual and oral teeth series are diagnostic and can be used to differentiate lamprey species (Hardisty and Potter, 1971a; Potter, 1980b; Potter and Hilliard, 1987).

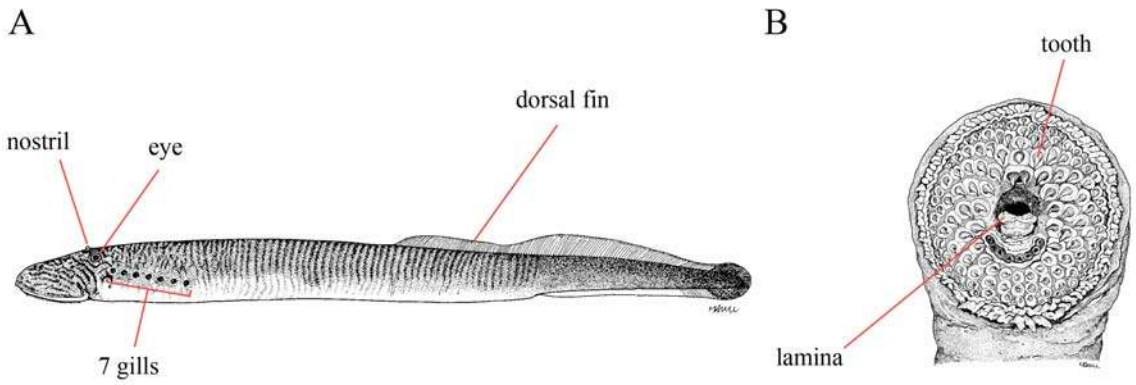

Figure 1.7 | Main anatomical features of lampreys. (A) Parts of the body. (B) Mouthparts of the silver lamprey *Ichthyomyzon unicuspis*. Adapted from Renaud (2011).

Another common characteristic for all lampreys is their unique life cycle, which consists of two phases: a larval phase and an adult phase. The larval stage, which is common for all lampreys, is independent of their ecological specificities. Larvae are called ammocoetes, which have poorly developed eyes and live burrowed in sediments in the bottom of shallow, slow moving riverine waters, where specialized mouthparts filter out microorganisms and detritus (Hardisty and Potter, 1971a). Depending on the species, this period can last from 3 to 7 years (Hardisty and Potter, 1971a). Larval phase individuals then metamorphose into an adult form (Hardisty and Potter, 1971a; Kennedy and Rubinson, 1977), that exhibit a range of life history traits that are likely to impact their nervous system: (1) some species become parasitic, typically feeding on teleost fishes (Hardisty and Potter, 1971a; Wilkie et al., 2004; Kuraku et al., 2012), whereas others attain sexual maturation shortly after metamorphosis without experiencing a feeding phase as adults (non-parasitic species). (2) Some parasitic species remain attached to their hosts for substantial periods of time while feeding, from which they extract mainly blood (passive predation). Other parasitic species remain attached to typically smaller hosts for relatively shorter periods of time, from which they remove ‘chunks’ of mainly muscle tissue. Feeding in these muscle feeders often leads to the death of their hosts. This type of feeder represents a more active mode of predation (Potter and Hilliard, 1987; Renaud et al., 2009). Lastly, a few species feed on both blood and muscle tissue, and a single species, *Caspiomyzon wagneri,* may

feed on carrion and benthic invertebrates (Renaud et al., 2009; Renaud, 2011). (3) A third life history trait can be distinguished based on the habitat in which the adult lampreys are found. Some parasitic species remain for the whole of their life cycle in fresh water, whereas other parasitic species migrate into marine environments. Some species of lampreys even form more complex population structures, where both freshwater and anadromous populations coexist (Potter et al., 2015).

1.2.2 Variation in the visual system

One of the biggest changes experienced by lampreys during metamorphosis is the development of their visual system, where the non-image forming "eye" of the ammocoete transforms into a camera-like, image-forming structure in the adult. This change is so profound in lampreys that it was once thought that the ammocoete was a completely different species (Potter et al., 1980). Even though all species of lampreys share many morphological and physiological characteristics, there are significant differences in the structure and function of their visual system (Collin et al., 1999; Collin and Potter, 2000; Collin et al., 2003a; Collin et al., 2004; Gustafsson et al., 2008; Davies et al., 2009), which can be correlated with differences in the development, behaviour, ecology and phylogeny of these animals.

During ontogeny, a complete differentiation of the retinal photoreceptors is not observed until the lampreys are young adults, i.e. after the ammocoetes undergo metamorphosis (de Miguel and Anadon, 1987; Rubinson, 1990). During this period, the differentiation and growth of the main visual structure within the brain of lampreys, the optic tectum, also occurs (de Miguel and Anadon, 1987), which finally leads to the acquisition of an image-forming eye in the young adults (Kennedy and Rubinson, 1984; de Miguel and Anadon, 1987; Rubinson, 1990). The complement of photoreceptor types present in the retina varies in each of the three families of lampreys: the northern hemisphere lampreys *Petromyzon marinus* and *Lampetra fluviatilis* possess two different photoreceptor types, one large and the other small (Govardovskii and Lychakov, 1984; Harosi and Kleinschmidt, 1993), which are thought to represent a cone and a rod, i.e. specialized photoreceptors for bright and dim light environments, respectively (Govardovskii and Lychakov, 1984; Collin and Trezise, 2006). Moreover, two different visual opsins have been characterised in these species, providing them with the potential for dichromatic vision (Govardovskii and Lychakov, 1984; Davies et al., 2009). On the other hand, in the southern hemisphere lampreys *Geotria australis* and *Mordacia mordax*, a different complement of retinal photoreceptors has been found. Based on morphological criteria, five different photoreceptor subtypes are present in the retina of *G. australis* (Collin et al., 1999; Collin and Trezise, 2004), each containing a different opsin gene (Collin et al., 2003b), with peak spectral sensitivities from the ultraviolet to the long wavelength parts of the spectrum. Peak absorbance of the five visual pigments has been established using microspectrophotometry

and conventional spectrophotometric analysis of recombinant opsins expressed in mammalian cells (Davies et al., 2007; Collin et al., 2009). The presence of five photoreceptor types suggests the potential for pentachromatic vision (Davies et al., 2007). In contrast, only a single type of photoreceptor has been identified in *M. mordax*, which possess morphological characteristics of both a rod and a cone photoreceptor (Collin and Potter, 2000; Collin et al., 2004). The differences found in the complement of photoreceptor types (opsins) and their spectral sensitivities reveal that different species of lampreys have, as for many other species of vertebrates, heterogeneous levels of visual perception that reflect their specific lifestyle (**Figure 1.8**).

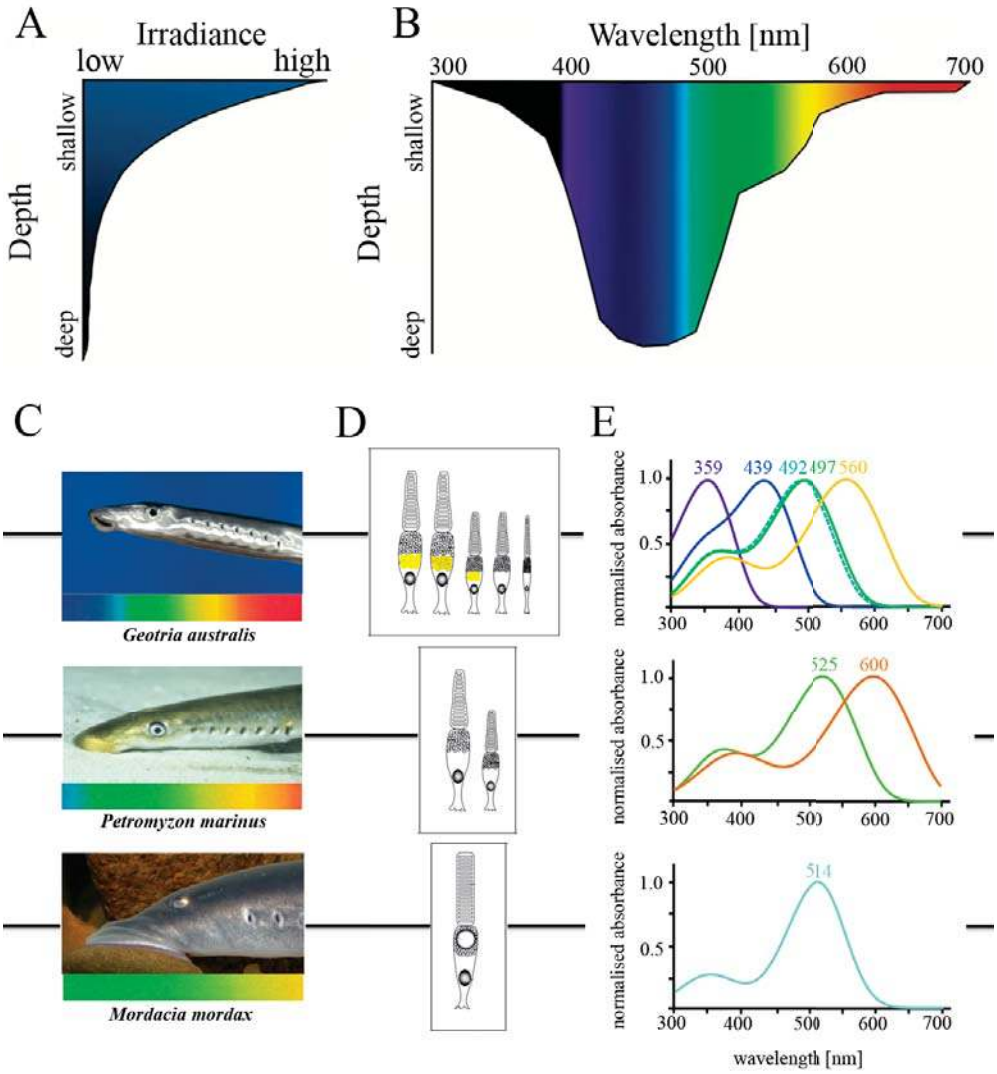

Figure 1.8 | Spectral sensitivity of lamprey species. The relationship between light irradiance (A) and spectral attenuation (B) is shown for different depths in the open ocean. The retinal photoreceptors within the eyes of the three families of lampreys are able to perceive different regions of the light spectrum (C), which have been inferred from the number and type of photoreceptors (D) and peak spectral sensitivities of the visual pigments housed within different photoreceptor types (E). These patterns of spectral sensitivity can be potentially related to the different light environments occupied by each species. Adapted from Collin et al. (2003a); Collin et al. (2004); Davies et al. (2007); Davies et al. (2009); Roth (2014).

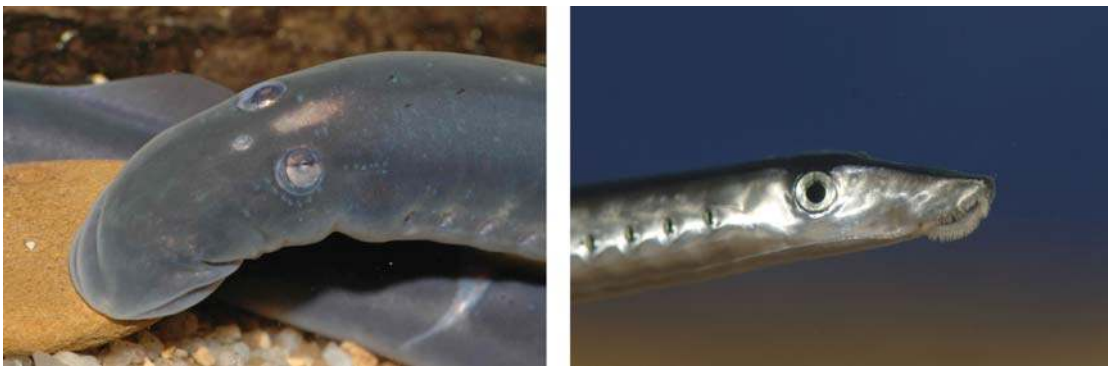

Figure 1.9 | Southern hemisphere lampreys, upstream migrant of *Mordacia mordax* (left) and downstream migrant of *Geotria australis* (right). Compare the different position and orientation of the eyes in the head between these species. Photo credits: T Raadik (left) and G. Westhoff and S.P. Collin (right).

Other visual adaptations in lampreys are related to the spatial distribution of light in natural habitats. For example, in different vertebrates it has been shown that the spectral tuning of photoreceptor types can vary across the retina (Hart, 2004; Temple et al., 2010). Similarly, it has been shown in all vertebrate groups, including lampreys, that each type of photoreceptor is distributed in a specific spatial arrangement across the retina, forming regions of high photoreceptor density (Mammalia: Müller and Peichl, 1989; Reptilia: Wong, 1989; Chondrichthyes: Hueter, 1991; Actinopterygii: Zaunreiter et al., 1991; Amphibia: Zhang and Straznicky, 1991; Ahnelt and Kolb, 2000; Aves: Hart, 2001; Cephalaspidomorphi: Collin et al., 2004; Sarcopterygii: Bailes et al., 2006; For more examples go to the database on the topography of different retinal cell types, see: Collin, 2008. Database at URL http://www.retinalmaps.com.au). In lampreys and other vertebrates, this topographical variation of the distribution of retinal cells is not only true for photoreceptors, but also for other retinal cell types, such as the retinal ganglion cells, which give rise to the visual afferents projecting to the brain via the optic nerve (Fritzsch and Collin, 1990; Wallace, 2001; Schulte and Bumsted-O'Brien, 2008; Fletcher, 2010). The resulting regions of high cell density are called retinal specializations, and are often correlated with regions of the visual field that are behaviourally relevant to the animal (Collin and Pettigrew, 1988a; b; Collin et al., 1998; Bozzano and Collin, 2000; Hart, 2001; Coimbra et al., 2009). The silver lamprey *Ichthyomyzon unicuspis* has a central-dorsal specialization of high retinal ganglion cell density in post-metamorphic juveniles, which migrates to the retinal periphery in adults (Fritzsch and Collin, 1990), while the sea lamprey *P. marinus* has a higher density area of retinal ganglion cells in both the ventro-temporal and dorso-temporal regions of the retina (Jones et al., 2009). In southern hemisphere lampreys, a different pattern has been found. The pouched lamprey *G. australis* has three areas with higher convergence ratios between photoreceptors and retinal ganglion cells, which are located in the dorsal and temporal regions of the retina (Wallace, 2001), including a population of giant retinal ganglion cells (Fletcher et al., 2014). In contrast, *M. mordax* has a specialized area of photoreceptors in the ventro-temporal retina, in concordance with the position and

orientation of their dorsal eyes in the head (Wallace, 2001; Collin et al., 2004, see **Figure 1.9**). These differences are likely reflected in the retinotopic organization and physiology of neurons in the visual areas and other brain structures of lampreys (Jones et al., 2009; Cornide-Petronio et al., 2011).

Various types of photoreceptors and opsins had already evolved before the separation of invertebrates and vertebrates (reviewed in Lamb et al., 2007). In fact, it has been argued that the five opsin types present in the pouched lamprey *G. australis* may represent the ancestral condition of lampreys and all vertebrates (Collin et al., 2003b). In this case, both Mordaciidae and Petromyzontidae could be considered as derived forms, in which the number of photoreceptors and opsins were reduced, correlating with their modes of life and the life conditions under which they live (Collin and Potter, 2000; Davies et al., 2009). If the visual system of *G. australis* indeed approximates that of early vertebrates, a camera-like eye with diverse retinal specializations may have been the ancestral condition to all vertebrates, and the visual system of hagfishes should then be considered as a derived feature. Recent findings suggest that the 'Tully monster' (*Tullimonstrum gregarium*) was an early vertebrate related to basal forms of lampreys, which possessed laterally-displaced eyes mounted on a rigid bar (McCoy et al., 2016). This configuration enlarges the visual field (McComb et al., 2009), supporting the idea that early lampreys may have had a well-developed visual system.

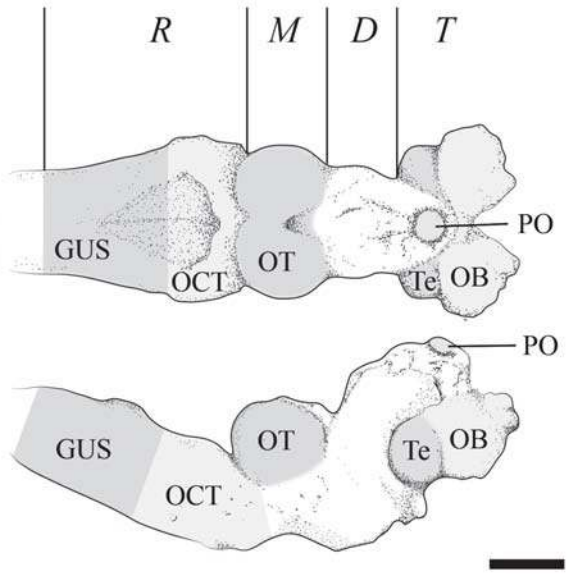

Figure 1.10 | The brain of lampreys. Scheme shows the brain of an upstream migrant of *Geotria australis* from a dorsal (top) and lateral view (bottom). The main subdivisions of the brain (italicized) are *T*, telencephalon; *D*, diencephalon; *M*, mesencephalon; *R*, rhombencephalon. The six brain structures studied in this Thesis are OB, olfactory bulbs; Te, telencephalic hemispheres; PO, pineal organ; OT, optic tectum; MOR, rostral medulla oblongata and MOC, caudal medulla oblongata. Scale bar = 1mm.

1.2.3 Other sensory systems

Lampreys possess a variety of sensory systems, many of which have not yet been the subject of comparative studies as in the case of the visual system. These sensory systems include chemoreception (olfactory, gustatory), visual and non-visual photoreception, various trigeminal-mediated (e.g. pain, temperature and tactile) and octavolateralis-mediated (e.g. electroreception, lateral line) sensory systems, amongst others (reviewed in Braun, 1996). The diverse sensory organs of lampreys form afferents to specific brain regions (Nieuwenhuys and Nicholson, 1998). The following brain structures in lampreys will be examined in this Thesis (**Figure 1.10**). From rostral to caudal:

(1) The olfactory bulbs are well-developed brain structures in both lampreys and hagfishes (Braun, 1996; Wicht, 1996). In lampreys, the olfactory bulbs process olfactory signals such as pheromones and other naturally-occurring odours (Buchinger et al., 2015), which have been linked to diverse behaviours such as mating and migration (Vrieze et al., 2010; Johnson et al., 2015). The olfactory bulbs receive primary sensory fibres from both the main and the accessory olfactory epithelia (Chang et al., 2013). Their main secondary targets are the telencephalic hemispheres (Northcutt and Puzdrowski, 1988), but some fibres bypass these brain structures to reach other motor areas of the brain (Derjean et al., 2010; Green et al., 2013).

(2) The cerebral or telencephalic hemispheres are the evaginated portions of the telencephalon of lampreys. They receive extensive secondary olfactory projections from the olfactory bulbs (Northcutt and Wicht, 1997), as well as other secondary sensory input of various modalities via relays in the thalamus (Polenova and Vesselkin, 1993). These telencephalic brain structures are connected to various other areas of the telencephalon, forming part of an important circuit for sensory-motor integration that has been conserved during the evolution of vertebrates (Khonsari et al., 2009; Grillner and Robertson, 2015).

(3) The pineal organ is a relatively small brain structure that contains photoreceptors involved in non-visual photoreception. It participates in the neuroendocrine control of the circadian rhythms (Freamat and Sower, 2013), including the regulation of the onset of metamorphosis, gonad maturation and spawning (Eddy and Strahan, 1968; Eddy, 1971; Cole and Youson, 1981).

(4) The optic tectum is the roof (most dorsal part) of the mesencephalon. It receives sensory input from multiple brain regions, such as the retina and octavolateralis systems (Bodznick and Northcutt, 1981; Jones et al., 2009). This brain structure participates in

various motor responses involving spatial orientation in lampreys and other groups of vertebrates (Gruberg et al., 2006; Saitoh et al., 2007; Kardamakis et al., 2015).

(5) The rostral end of the medulla oblongata, which is associated with various cranial nerves (V-VIII), possesses both sensory and motor components. The sense organs providing input to this region include neuromasts of the lateral line system, electroreceptors, photoreceptors, and other types of skin receptors that are distributed in many parts of the body (Butler and Hodos, 1996). Motor systems arising from this area innervate muscles of the head, and are also involved in the coordination of feeding and orientation movements (Rovainen, 1996).

(6) The caudal end of the medulla oblongata is associated with cranial nerves IX-XII, which also have sensory and motor components. The sensory component consists of various types of chemoreceptors that are located in the feeding canal and over the gills (Barreiro-Iglesias et al., 2010), whereas the motor components control breathing and other autonomic functions (Rovainen, 1996).

1.3 Aims of this Thesis

The main aim of this study is to gain a better understanding of the evolution of the vertebrate brain. Using a wide range of techniques, the central nervous system of agnathans is investigated at ontogenetic and phylogenetic levels. The work is presented as a series of papers, each examining a different perspective of the evolution of the brain in agnathans. The Chapter titles are as follows:

- Chapter 2. Ontogenetic shifts in brain scaling in lampreys
- Chapter 3. Encephalization of lampreys and hagfishes
- Chapter 4. Patterns of brain organization in lampreys
- Chapter 5. General discussion

Chapter 2 Ontogenetic shifts in brain scaling in lampreys

2.1 Abstract

Very few studies have described brain scaling in vertebrates throughout ontogeny and none in lampreys, one of the two surviving groups of the early agnathan (jawless) stage in vertebrate evolution. The life cycle of anadromous parasitic lampreys comprises two divergent trophic phases, firstly filter-feeding as larvae in fresh water and secondly parasitism as adults in the sea, with the transition marked by a radical metamorphosis. We characterized the growth of the brain during the life cycle of the pouched lamprey *Geotria australis*, an anadromous parasitic lamprey, focusing on the scaling relationship between brain and body size during ontogeny and testing the hypothesis that the vast transitions in behaviour and environment are reflected in differences in the scaling and relative size of the major brain subdivisions throughout life. Body and brain mass and the volume of six major brain structures of *G. australis*, representing six points of the life cycle, were recorded, ranging from the early larval stage to the final stage of spawning and death. Brain mass does not increase linearly with body mass during the ontogeny of *G. australis*. During metamorphosis, brain mass increases markedly, even though body mass does not increase, reflecting an overall growth of the brain, with particularly large increases in the volume of the optic tectum and other visual areas of the brain and, to a lesser extent, the olfactory bulbs. These results are consistent with the conclusions that ammocoetes rely predominantly on non-visual and chemosensory signals, while adults rely on both visual and olfactory cues.

2.2 Introduction

Lampreys are extant relatives of an early and diverse group of jawless vertebrates (Kumar and Hedges, 1998; Heimberg et al., 2008; Janvier, 2008; Smith et al., 2013). The results of early studies on the agnathan nervous system (Johnston, 1902; Heier, 1948; Nieuwenhuys, 1977) have thus been used as an indicator of the ancestral condition of the vertebrate brain (Fritzsch and Northcutt, 1993a; Butler and Hodos, 1996; Northcutt, 2002; Gilland and Baker, 2005; Khonsari et al., 2009; Suárez et al., 2014). The design or bauplan of the vertebrate brain and the developmental mechanisms that underlie their subdivisions are considered to be highly conserved (Striedter, 2005; Ota and Kuratani, 2007; Guérin et al., 2009; Charvet et al., 2011). However, it is expected that the various sensory modalities and other neural specializations will evolve, to a degree, in association with ecological niche, and that this relationship will be reflected in adapted behaviours and/or enhanced cognitive capabilities (Barton et al., 1995;

Barton and Harvey, 2000; de Winter and Oxnard, 2001). Indeed, brain size and the relative development of major brain subdivisions vary at intraspecific, interspecific, and ontogenetic levels across a range of vertebrates (e.g. Kruska, 2005; Gonda et al., 2013) in relation to factors such as lifestyle, habitat, and behaviour (e.g. Pollen et al., 2007; Yopak and Montgomery, 2008; Barton and Capellini, 2011), as well as phylogenetic and developmental constraints (e.g. Finlay and Darlington, 1995; Yopak et al., 2010).

The size of the brain relative to the body (scaling) has long since been used in studies of brain development and evolution (Ariëns Kapper, 1936; Gould, 1975; Deacon, 1990; Aboitiz, 1996), in which brain mass (E) is characterized as a function of body mass (S) with Snell's formula: $E = k * S^{\propto}$ or $\log E = \propto \log S + k$, where $\propto =$ allometric slope or scaling power. It is a common assumption that encephalization (a larger than expected brain size for a given body size) reflects enhanced cognitive capabilities (Jerison, 1977; Ebbesson, 1980; Lefebvre et al., 2004), although this is still the subject of debate (Healy and Rowe, 2007; Herculano-Houzel, 2012). Previous studies have examined encephalization of the brain of jawless fishes (Platel and Delfini, 1981; Ebinger et al., 1983; Platel and Vesselkin, 1989; Wicht, 1996) and have shown that agnathans, particularly lampreys, possess a relatively small brain and some of the highest degrees of intraspecific variation in brain and body mass when compared to any other vertebrate group (Ebinger et al., 1983; Platel and Delfini, 1986). However, these data have been collected from very few species and no consideration has yet been given to changes in encephalization and brain organization that may occur throughout their life cycle. Indeed, ontogenetic studies of diverse groups of vertebrates have shown that the brain grows at different rates during their lifespan, with the rates being greatest in the embryonic and early postnatal phases (Bauchot et al., 1979; Gille and Salomon, 2000; Fu et al., 2013; Ngwenya et al., 2013). Although some studies have shown shifts in ecology and corresponding shifts in brain development occur in fishes (e.g. Brandstätter and Kotrschal, 1990; Wagner, 2003; Lisney et al., 2007; Iribarne and Castelló, 2014), there are no data on the pattern of encephalization or brain subdivision scaling during the ontogeny of lampreys.

The life cycle of lampreys is very conserved (Chang et al., 2014; Potter et al., 2015), consisting of a prolonged and sedentary larval phase, followed by metamorphosis into the free-swimming adult phase (Manzon et al., 2015), as illustrated in **Figure 2.1**. In the pouched lamprey *Geotria australis*, which is widely distributed in temperate regions of the southern hemisphere (Renaud, 2011), the life cycle has an approximate duration of eight years (Potter et al., 1980; Potter et al., 1983; Potter and Hilliard, 1986).

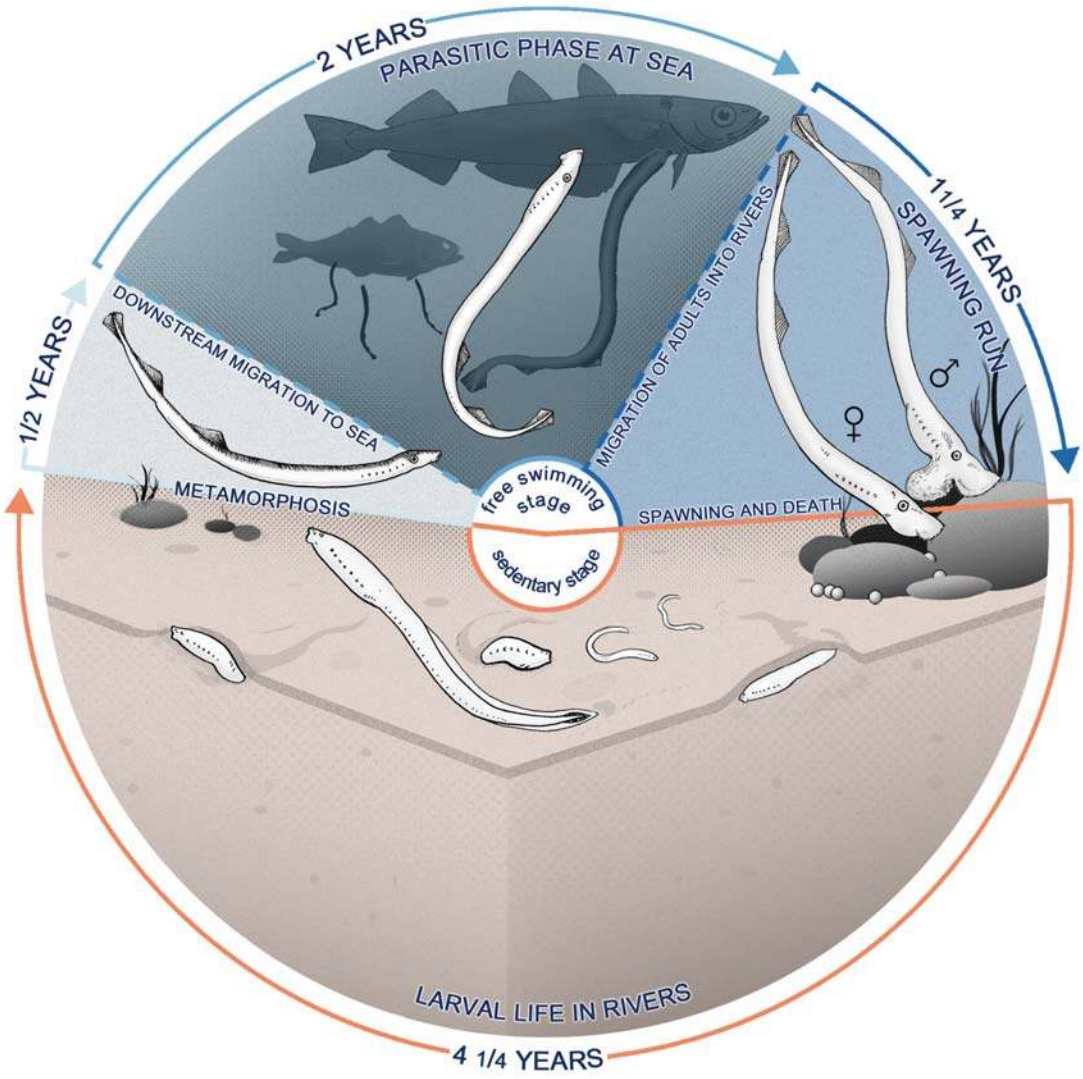

Figure 2.1 | Life cycle of *Geotria australis* presents anadromous reproductive and feeding migrations. After hatching (bottom), the larvae – also called ammocoetes – burrow in the sediments of rivers, becoming a microphagous filter-feeder for approximately four years. The larval phase is followed by a metamorphosis, a non-feeding transition to adult stage that lasts for approximately 6 months (left), where there is a marked transformation in most of the body systems. Animals at this stage start migrating downstream and enter the sea, where they locate a teleost host and feed on its flesh using a specialized buccal apparatus (top). G. australis return years later to the rivers, where they start a long upstream migration, subsisting only on body reserves, which are expended in developing secondary sexual characteristics and reproductive behaviour. Upstream migrants finally spawn and die (right).

After hatching, the larvae (ammocoetes) burrow in the soft sediments of streams and rivers, filtering detritus, algae and other organisms from the overlying water (Piavis, 1971; Moore and Mallatt, 1980; Richardson et al., 2010; Dawson et al., 2015). Ammocoetes have rudimentary eyes, with a largely undifferentiated retina (Meyer-Rochow and Stewart, 1996; Villar-Cheda et al., 2008), and also a well-developed non-visual photoreceptive system, e.g. the pineal organ (García-Fernández and Foster, 1994; Deliagina et al., 1995; Melendez-Ferro et al., 2002; Vigh et al., 2002). In fact, they exhibit nocturnal habits with synchronized, seasonal downstream

movements (Gritzenko, 1968; Potter, 1980a), which may be controlled by circadian rhythms. An octavolateralis system provides additional mechano-, electro-, and photo-perception, with photoreception being mediated by dermal non-visual photoreceptors located in the tail (Ronan, 1988; Ronan and Bodznick, 1991; Deliagina et al., 1995; Gelman et al., 2007). Ammocoetes also have well developed gustatory (Baatrup, 1985; Barreiro-Iglesias et al., 2010) and olfactory (VanDenbossche et al., 1995; Zielinski et al., 2005) systems, and behavioural evidence has revealed that rotting potato haulms attracted ammocoetes when placed on the bed of freshwater streams (Enequist, 1937; Hardisty and Potter, 1971a), indicating that they may actively search for food using chemosensory cues. Therefore, taste and olfaction are likely important drivers of their behaviour.

The metamorphosis of anadromous parasitic species of lampreys, such as *G. australis,* involves major morphological and physiological changes and the development of new sensory and motor capabilities. These include the development of image-forming eyes with the potential for pentachromacy in *G. australis* (Meyer-Rochow and Stewart, 1996; Collin et al., 1999; Collin et al., 2003a; Davies et al., 2007), a reduction of lateral line-mediated negative phototaxis that marks a switch from non-visual to visual perception (Binder et al., 2013), the rearrangement of the gustatory and lateral line systems (Currie and Carlsen, 1988; Jørgensen, 2005; Gelman et al., 2008; Barreiro-Iglesias et al., 2010), and the development of a tooth-bearing suctorial disc and "tongue-like" piston with the associated musculature and trigeminal motor innervation (Homma, 1978; Lethbridge and Potter, 1981). Metamorphosis also involves fundamental changes in a number of internal organs, including the intestine and gills, which enable the lamprey to osmoregulate in the sea (Youson et al., 1977; Hilliard et al., 1983; Bartels and Potter, 2004; Reis-Santos et al., 2008).

During the marine parasitic phase, *G. australis* swims towards and attaches to a host, often a teleost fish, and feeds from its flesh (Hilliard et al., 1985; Renaud et al., 2009), thereby increasing in body size from approximately 100 mm and 0.75 g to 620 mm and 220 g (Potter et al., 1980; Potter et al., 1983). There is strong evidence that during its marine parasitic phase, *G. australis* occupies an epipelagic niche in the sea and exhibits diurnal habits (Potter et al., 1979; Cobley, 1996; Collin et al., 1999; Davies et al., 2007). Following the completion of the parasitic phase, the adult lamprey re-enters rivers cued mainly by pheromones that are released by the ammocoetes (Vrieze and Sorensen, 2001; Sorensen et al., 2005; Vrieze et al., 2010; Vrieze et al., 2011), where they migrate upstream at night (Jellyman et al., 2002; Binder and McDonald, 2007; Vrieze et al., 2011). *Geotria australis* does not feed during its exceptionally long spawning run, using body reserves accumulated during the marine phase to develop secondary sexual characters and mature gonads (Potter et al., 1983; Paton et al., 2011). The life cycle culminates in spawning and subsequent death.

During its life cycle, *G. australis* occupies different ecological niches and encounters diverse environmental conditions, yet there have been no comprehensive studies that have quantified changes in brain organization, corresponding to these marked changes in ecology and behaviour, occur in this species. In this study, we assess changes in relative brain size (encephalization) and in the volume of six major brain structures (brain organization) at different phases of the life cycle in *G. australis*. We hypothesize that differences in brain size and organization will reflect the pronounced environmental and physiological changes that lampreys experience during ontogeny.

2.3 Methods

All the procedures described below were performed in accordance with the ethical guidelines of the University of Western Australia Animal Ethics Committee - Research Project RA/3/100/917.

2.3.1 Data collection

Forty specimens of *Geotria australis* were analysed in this study, representing six different points in their life cycle (ammocoetes of second, third and fourth age class, downstream migrants, upstream migrants, and spawning adults). Specimens within a stage had the same fixation and preservation methods, as shown in Supplementary **Table 2.1**, and were captured in the same year (ammocoetes and downstream migrants) or in different years (upstream migrants and spawning adults). Morphometrics (body mass, body length, sex) were collected for each individual when possible. After a period of fixation, the brain was removed from the chondrocranium. The meninges were removed and the cranial nerves were cut to within 0.5 mm of the base. The brains were blotted and weighed to the nearest 0.1 mg (ammocoetes and downstream migrants) or 1 mg (upstream migrants and spawning adults). Neither brain nor body mass were corrected for shrinkage due to fixation.

Photographs of the lateral and dorsal views of each brain were taken using a Leica EC3 camera attached to a Nikon SMZ-745T dissecting microscope. Brains were submerged in a solution of 0.1 M phosphate buffer while photographed to prevent volume distortions caused by dehydration of the tissue. Measurements of length were taken for each of the six brain structures as shown in **Figure 2.2**. Brain structures were determined from previously published descriptions of the brain and the cranial nerve distribution in lampreys (Nieuwenhuys and Nicholson, 1998). The length (l), height (h), and width (w) of the olfactory bulbs (OB), telencephalic hemispheres (Te), the pineal organ (PO), the optic tectum (OT), the rostral end of the medulla oblongata (MOR; defined as the anterior region of the rhombencephalon comprising the V-VIII nerves), and the caudal medulla oblongata (MOC; defined as the

posterior region of the rhombencephalon comprising the IX-XII nerves) were measured using ImageJ (Rasband, 1997) as described previously (Huber et al., 1997; Wagner, 2001; Yopak and Lisney, 2012). The PO was dissected out of the brain and photographed separately, see **Figure 2.2 B**.

Volumes of each major brain structure were estimated using the ellipsoid method, which approximates the volume of a structure by assuming it takes the shape of an idealized ellipsoid, or a fraction of it as shown below (Huber et al., 1997; Wagner, 2001). The general formula of an ellipsoid is:

$$V = \frac{4}{3}\pi\ a\ b\ c$$

where *a, b, c* are the radii of the ellipsoid. Using the measurements of length (*l*), height (*h*) and width (*w*) shown for each structure in Figure 2, the volumes were defined as:

$$V = \frac{1}{6}\pi\ l\ h\ w$$

for the OB, Te, PO, and the OT, which were all modelled as half ellipsoids,

$$V = \frac{1}{3}\pi\ l\ h\ w$$

while the volume of the MOR and MOC were modelled as a quarter of an ellipsoid. In the case of bilateral structures (i.e. OB, Te, and OT), the values of the volumes were doubled. Volume estimates were neither corrected for ventricular volume nor structure thickness in the case of the OT (see Ullmann et al., 2010). Total brain volume was calculated from total brain mass using the estimated density of the brain tissue, $d = 1.036\ mg/mm^3$(Stephan, 1960).

2.3.2 Age determination

The approximate age of the ammocoete samples was estimated from length-frequency histograms for larval and metamorphosing representatives of *G. australis* (Potter et al., 1980; Potter and Hilliard, 1986). Age of adult stages was inferred from the timing of the upstream migration and sexual maturation (Potter et al., 1983).

2.3.3 Data analysis

All analyses were performed using the open source software R (R Core Team, 2013b). The complete dataset was divided into two subsets, one containing body and brain mass (n=32) and the other containing total brain and brain structure volume estimates (n=39).

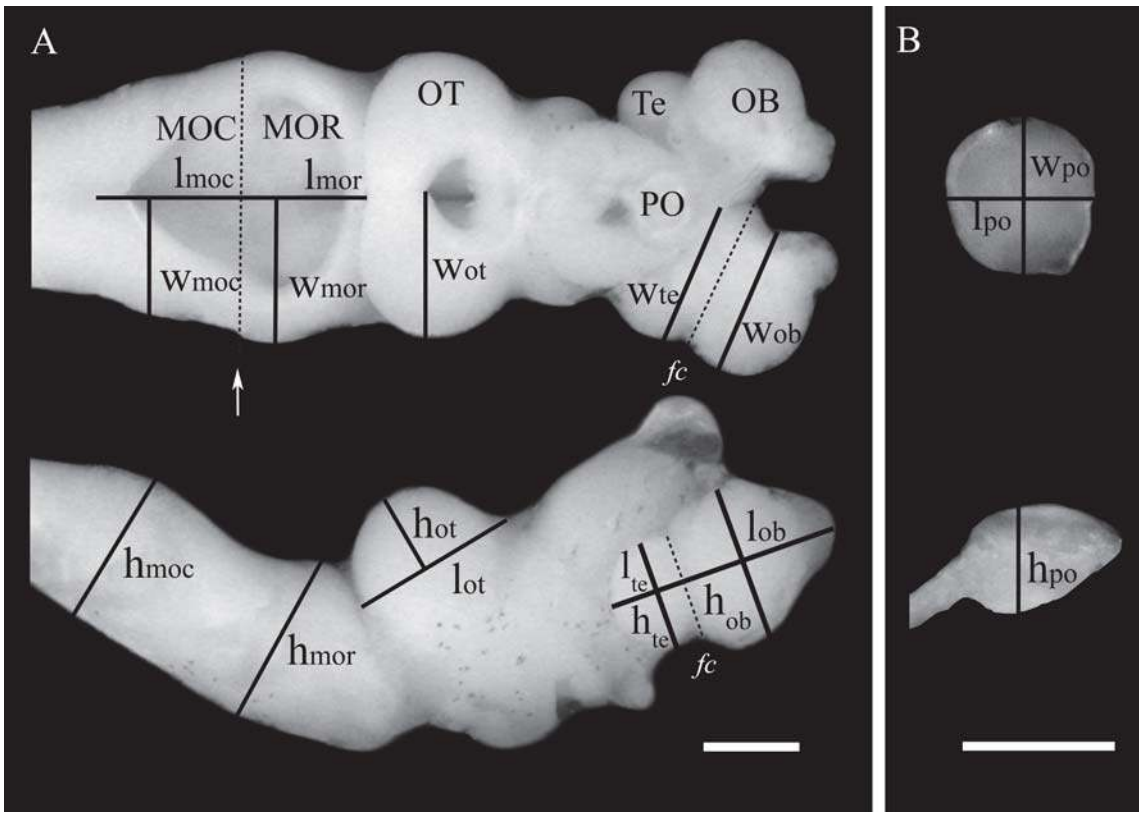

Figure 2.2 | Estimation of the volume of brain structures using the ellipsoid method. Measurements of length (l), width (w) and height (h) of six brain structures taken from a dorsal view (A, top) or lateral view (A, bottom) of the brain of an upstream migrating *G. australis*. In the case of the OB and the Te, these were defined as parallel or perpendicular lines to the *fissura circularis* (fc), which is highlighted with a discontinuous line in the telencephalon. The limit of MOR and MOC was defined by a line running parallel to the posterior end of the head of the eighth nerve (white arrow). (B). The same measurements were performed in the PO after it was dissected and separated from the remainder of the brain. Scale bars = 1mm.

stage abbrev	n	fixation/preservation methods	body mass [g]	brain mass [mg]	OB [µL]	Te [µL]	PO [µL]	OT [µL]	MOR [µL]	MOC [µL]
amII	6	Bouins / ethanol	0.32	0.62	0.03	0.03	4.43E-03	0.04	0.10	0.21
amIII	5	Bouins / ethanol	1.03	1.00	0.05	0.05	9.80E-03	0.05	0.21	0.26
amIV	3 *	Bouins / ethanol	1.92	1.45	0.07	0.07	1.63E-02	0.04	0.28	0.36
ds	6	PFA / ethanol	1.33	5.69	0.40	0.48	1.48E-02	1.21	0.61	0.69
us	11 *	PFA / PBS	180.28	27.60	2.78	1.22	5.48E-02	2.93	4.12	4.49
sa	9 **	PFA / ethanol	133.36	20.00	2.12	1.26	6.74E-02	2.01	3.57	2.80

Table 2.1 | Average of the parameters measured for each stage. (*) Body mass averages from 2 specimens of fourth age class ammocoetes (amIV) and 5 specimens of upstream migrants (us). (**) Brain subdivisions averages calculated from 8 specimens. PFA: paraformaldehyde; PBS: phosphate buffered saline.

2.3.3.1 Linear models

For brain and brain structure scaling analyses, each data point was $\log_{10}$ transformed to improve normality prior to analysis, after being multiplied by an arbitrary factor (10 and 1000, respectively), in order to obtain positive values of the variables following $\log_{10}$ transformation. We conducted similar analyses on both datasets: we fitted least squares regressions within and between stages, and performed analyses of covariance (ANCOVA), with brain mass as the response variable, body mass as the covariate, and stage as a factor for the brain and body mass comparisons. In the case of the brain structures, total brain structure volume was compared to total brain volume minus total structure volume as a covariate. This was done to account for the bias that exists when a brain subdivision is scaled against total brain mass (which includes the subdivision of interest) (Deacon, 1990; Iwaniuk et al., 2010). To control for similarity within the larval or adult phases of the life cycle, stages were combined in "model 1" (no combination), "model 2" (all ammocoetes grouped together), "model 3" (all adults grouped together), "model 4" (all ammocoetes grouped together, downstream and upstream migrants grouped together), "model 5" (all ammocoetes grouped together, upstream migrants and spawning adults grouped together), and "model 6" (all ammocoetes grouped together, all adults grouped together) (see Table 2.2). Linear models were fitted to each of these factors and the linear assumptions for each were checked using the R package gvlma (Pena and Slate, 2014); valid linear models were then compared and selected using the second-order Akaike Information Criterion (AICc); If the best model had a AICc value indistinguishable from the following model(s), they were averaged using multi-model inference methods contained in the R package MuMIn (Barton, 2014), and the relative importance of the factor in the resulting model was used as a criterion for selection. Tukey Post-Hoc tests were used to detect differences between groups in the selected models.

	Stages						
Factor	**amII**	**amIII**	**amIV**	**ds**	**us**	**sa**	**n**
model 1	amII	amIII	amIV	ds	us	sa	6
model 2	ammocoetes			ds	us	sa	4
model 3	amII	amIII	amIV	all adults			4
model 4	ammocoetes			ds + us		sa	3
model 5	ammocoetes			ds	us + sa		3
model 6	ammocoetes			all adults			2

Table 2.2 | Grouping of stages for each of the factors modelled in the ANCOVA analyses. See text for more details. amII: second year class ammocoetes, amIII: third year class ammocoetes, amIV: fourth year class ammocoetes, ds: downstream migrants, us: upstream migrants, sa: spawning adults.

2.3.3.2 Principal component analysis

We also used a multivariate approach to determine the clustering of the samples in multidimensional space and characterize the patterns of brain organization of *Geotria australis* at each point of the life cycle. Principal component analysis (PCA) was performed using the

relative volume of each structure, calculated as a fraction of the sum of the volume of all six brain structures measured within a specimen (Wagner, 2001; Lisney et al., 2007). Structure proportions were normalized using the arcsine square root transformation previous to analysis. PCA was run using the autocovariance matrix and the singular value decomposition method for better numerical accuracy (R Core Team, 2013a).

2.4 Results

2.4.1 Brain scaling

The brain of *Geotria australis* shared similar characteristics with those of other species of lampreys (**Figure 2.3**) (Wicht, 1996; Nieuwenhuys and Nicholson, 1998). Our analysis of the scaling of brain and body mass in *G. australis* at successive stages of development revealed that the brain and body have different scaling patterns during ontogeny **(Figure 2.4 A**). Body mass grows at a higher rate than brain mass in both the adult phase and the analysed period of the larval phase, a trend that is interrupted during metamorphosis **(Figure 2.4 A**, arrows), where body mass was similar between downstream migrants and the latest ammocoete stage (Two-tailed Welch t-test, T=1.98, p=0.201); however, brain mass was significantly higher in downstream migrants as compared to ammocoetes IV (One-tailed Welch t-test, T=7.8, p =0.037).

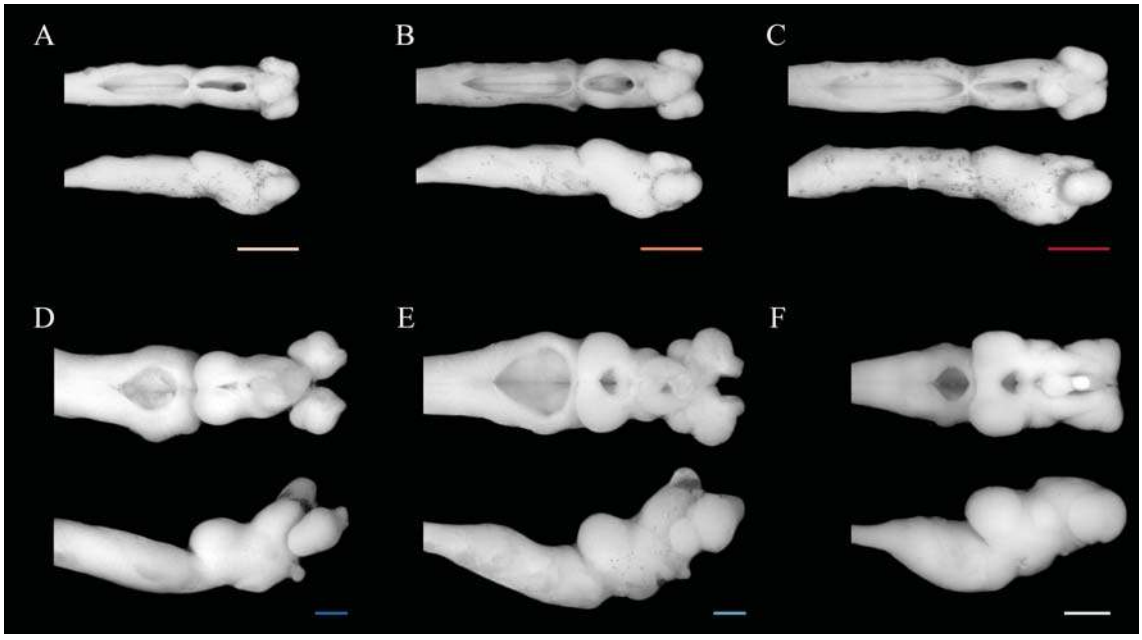

Figure 2.3 | Brain of *Geotria australis* during ontogeny. A representative brain of each stage studied is shown in a dorsal (top) and lateral view (bottom): (A) second age class ammocoete, (B) third age class ammocoete, (C) fourth age class ammocoete, (D) spawning adult, (E) upstream migrant, and (F) downstream migrant. Note the marked difference between the brain of a late ammocoete and a downstream migrant (C and F). Scale bars = 1 mm.

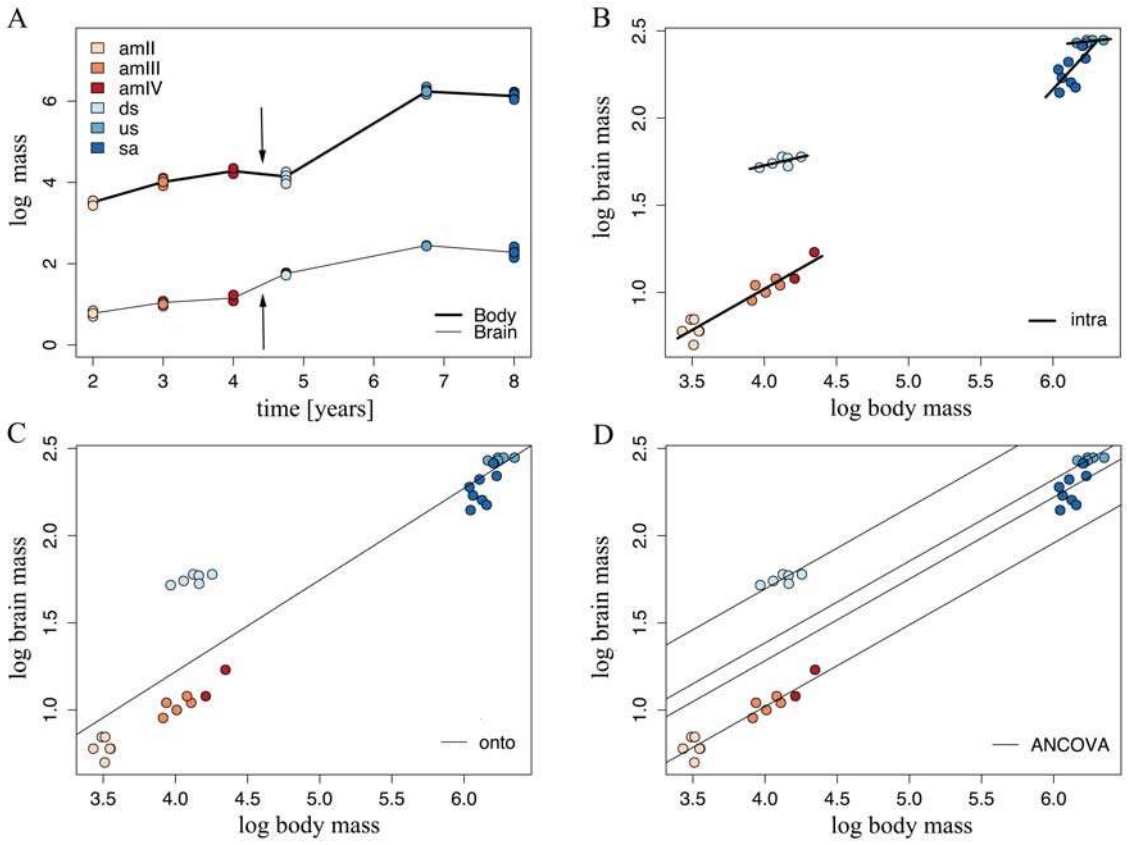

Figure 2.4 | Brain and body growth vary during the ontogeny of *Geotria australis*. (A) Brain and body mass growth traced over time. Arrows mark the period of metamorphosis. (B) Intraspecific linear regressions, (C) Ontogenetic regressions, and (D) Linear regressions fitted for each stage after an ANCOVA analysis. For the values of the parameters of these regressions, refer to Table 2.3. For abbreviations, see List of Abbreviations.

According to the second-order Akaike information criterion, the best model of brain mass as a function of body mass occurred when model 2 was used as a factor, grouping all ammocoetes together (**Table 2.3**). We fitted stage-specific (intraspecific) regressions to each of these groups, whose slopes varied across ontogeny (**Figure 2.4 B**); all groups showed intraspecific negative allometry of brain mass with body mass. The highest rate of brain growth was reached at the larval phase ($\alpha = 0.47$), followed by downstream and upstream migrants (**Table 2.4**), while the period of regression of body mass in the course of maturation was accompanied by a steep reduction of brain mass ($\alpha = 0.90$). We also defined an ontogenetic linear regression as the line of best fit between all specimens, where most of the groups had large deviations from the predicted values of brain mass (**Figure 2.4 C**), indicating that brain mass does not scale linearly with body mass at all stages in the life cycle of *G. australis*. These two sets of regressions were combined in an analysis of covariance (ANCOVA), the results of which are illustrated in **Figure 2.4 D**. These data show that both model 2 and body mass are significant when explaining the observed variance of brain mass (ANCOVA, $p<0.001$), and no significant interaction between factor (model 2) and covariate (body mass) is found, indicating no significant differences in the slopes calculated for each group in the stage-specific regressions.

The ANCOVA calculated a common slope, with a similar value to the slope obtained in the intraspecific regression of ammocoetes, and different intercepts for each group (see **Table 2.4**), which represent differences in relative brain mass between groups. The Tukey Post-Hoc test showed significant differences between all groups of model 2 ($p<0.001$); downstream migrants had the highest intercept, demonstrating an increase in relative brain mass at this stage.

Factor	Linear models													
	brain to body mass		brain subdivision to total brain minus brain subdivision volume											
			OB		Te***		PO		OT		MOR		MOC	
	AICc	ΔAICc	AICc	ΔAICc	AICc	ΔAICc	AICc	ΔAICc	AICc	ΔAICc	AICc	ΔAICc	AICc	ΔAICc
no factor	**	**	-59.79	0*	**	**	-16.48	8.44	**	**	-41.79	34.24	**	**
model 1	-79.84	6.69	-55.32	4.47	-76.13	6.76	-22.22	2.70	-53.28	2.38	-76.03	0	-73.20	2.54
model 2	-86.53	0	-56.91	2.88	-82.19	0.71*	-24.92	0*	-54.85	0.81*	-64.05	11.98	-75.74	0*
model 3	**	**	-52.54	7.25	-79.85	3.04	-17.66	7.26	-51.88	3.78	-51.77	24.26	-62.16	13.58
model 4	-81.12	5.41	-56.88	2.91	-80.64	2.25	-24.76	0.16*	-55.66	0*	-62.80	13.23	**	**
model 5	-81.24	5.29	-59.69	0.10*	-82.61	0.28*	-16.32	8.60	-49.66	6.00	**	**	-73.76	1.98*
model 6	**	**	-57.95	1.83*	-82.89	0*	-18.92	6.00	-52.29	3.37	-54.67	21.36	**	**

Table 2.3 | Summary of model selection. Values of the second-order Akaike information criterion (AICc) and the difference of this value with the selected model (ΔAICc) are given below. (*) ΔAICc < 2, models were selected using model average (see methods), (**) linear model assumptions were violated, (***) the volume of the telencephalic vesicles is compared to the volume of the OB (see results). For abbreviations, see List of Abbreviations.

linear model	factor	n	stage	intercept	slope	r^2	p-value	global stats
ontogenetic regression	none	32	all stages	-0.8855	0.52619	0.8568	-	NO
stage-specific regressions	none	13	am	-0.8656 (***)	0.47135 (***)	0.9031	4.025e-07	OK
		6	ds	0.9631 (+)	0.1912	0.3260	0.1382	OK
		5	us	1.8826 (*)	0.08929	0.3169	0.1896	OK
		8	sa	-3.2174	0.8971 (+)	0.3455	0.0562	OK
ANCOVA	model 2	13	am	-0.8572 (***)	0.46915 (***)	0.9927	< 2.2e-16	OK
		6	ds	-0.1823 (***)				
		5	us	-0.4921(**)				
		8	sa	-0.5945 (*)				

Table 2.4 | Summary of the parameters of the linear models of brain mass as a function of body mass. Plots of these equations are shown in Figure 2.4. (***) p-value < 0.001, (**) 0.001 < p-value < 0.01, (*) 0.01 < p-value < 0.05, (+) 0.05 < p-value < 0.1, ( ) p-value > 0.1. For abbreviations, see List of Abbreviations.

linear model	structure	factor	n	stage	intercept	slope	r^2	p-value	global stats
Ontogenetic regression	OB	none	39	all stages	-2.12 (***)	1.27 (***)	0.984	< 2.2e-16	OK
	Te				-1.27	1.01	0.963	-	NO
	PO				-1.04 (***)	0.63 (***)	0.838	< 2.2e-16	OK
	OT				-1.88	1.24	0.905	-	NO
	MOR				-0.53 (***)	0.95 (***)	0.958	< 2.2e-16	OK
	MOC				0.35	0.73	0.947	-	NO
Best model	OB	none	39	all stages	-2.12 (***)	1.27 (***)	0.984	< 2.2e-16	OK
	Te+	model 6	14	am	-0.32 (***)	0.64 (***)	0.987	< 2.2e-16	OK
			25	adults	0.62 (***)				
	PO	model 2	14	am	-2.54 (***)	1.16 (***)	0.884	2.877e-16	OK
			6	ds	-3.13 (**)				
			11	us	-3.41 (*)				
			8	sa	-3.14 (+)				
	OT	model 4	14	am	2.23(***)	0.47 (***)	0.983	< 2.2e-16	OK
			17	ds+us	3.40 (***)				
			8	sa	3.28 (*)				
	MOR	model 1	6	amII	0.88 (+)	0.41 (*)	0.986	< 2.2e-16	OK
			5	amIII	1.15 (***)				
			3	amIV	1.23 (***)				
			6	ds	1.27 (*)				
			11	us	1.84 (**)				
			8	sa	1.83 (**)				
	MOC	model 2	14	am	1.20 (***)	0.43 (***)	0.977	< 2.2e-16	OK
			6	ds	1.25				
			11	us	1.70 (**)				
			8	sa	1.62 (**)				

Table 2.5 | Summary of the parameters of the linear models of brain subdivisions volumes as a function of total brain volume minus brain subdivision volume. Plots of the best model are shown in Figure 5. (+) Te as a function of total volume of the OB. (***) p-value < 0.001, (**) 0.001 < p-value < 0.01, (*) 0.01 < p-value < 0.05, (+) 0.05 < p-value < 0.1, ( ) p-value > 0.1. For abbreviations, see List of Abbreviations.

2.4.2 Scaling of brain structures

The analysed brain structures showed different patterns of growth during the life cycle of *G. australis*. Ontogenetic regressions of total structure volume against total brain volume minus structure volume (hereafter referred to as brain volume) were fitted to each of the structures analysed and their parameters are tabulated in **Table 2.5**. A general trend between these regressions was the large deviations from the expected values shown by the downstream migrants, which were positive for the Te and the OT, but negative in the case of the PO, the MOR and the MOC.

The olfactory bulb was the only structure where the observed values fitted the expected values closely in all the stages, supporting a linear scaling of this structure with total brain throughout ontogeny (**Figure 2.5 A**). Remarkably, the OB showed the steepest hyperallometric growth reported in this study (α=1.27), generating highly developed OB in upstream migrants and spawning adults. The PO and the MOR also showed a significant linear fit with total brain volume, as shown in **Table 2.5**, although this was not the best model for these structures (see below).

Similar to the OB, the Te showed a close fit to brain volume in most stages, but because of the high heteroscedasticity in the values of spawning adults, the linear assumptions were violated in this case and in other tested linear models of the Te (results not shown). Nevertheless, we found that these assumptions were valid when fitting the telencephalic volume with the volume of the OB, and thus in this case total OB volume was used as covariate in the ANCOVA analysis. The best model for the Te included model 6 as a factor (**Figure 2.5 B**). This structure showed linear growth with the OB along the larval phase and an increase in size after metamorphosis, which is maintained throughout the adult phase of the life cycle. However, only a marginal difference was detected between ammocoetes and adults (Tukey Post Hoc test, p=0.091).

The best models for the PO and the MOC had model 2 as factor, whereas for the MOR it was model 1 and for the OT it was model 4 (**Table 2.5**). The calculated slope for the PO in the ANCOVA was higher than in the ontogenetic regression, and ammocoetes had the highest intercept (**Figure 2.5 C**). We found no significant differences between ammocoetes, downstream and upstream migrants, but the PO in spawning adults was significantly different from that of downstream migrants, although only marginally different from upstream migrants (Tukey Post Hoc test, $p = 0.017$ and $p = 0.053$, respectively). The corrected slope for the OT showed two markedly slow phases of growth, larval and adult, with a significant difference in size between them (Tukey Post Hoc test, $p < 0.05$; **Figure 2.5 D**); the OT of spawning adults was significantly reduced compared to downstream and upstream migrants (Tukey Post Hoc test, $p < 0.05$), and not statistically different from the OT of ammocoetes (Tukey Post Hoc test, $p = 0.45$).

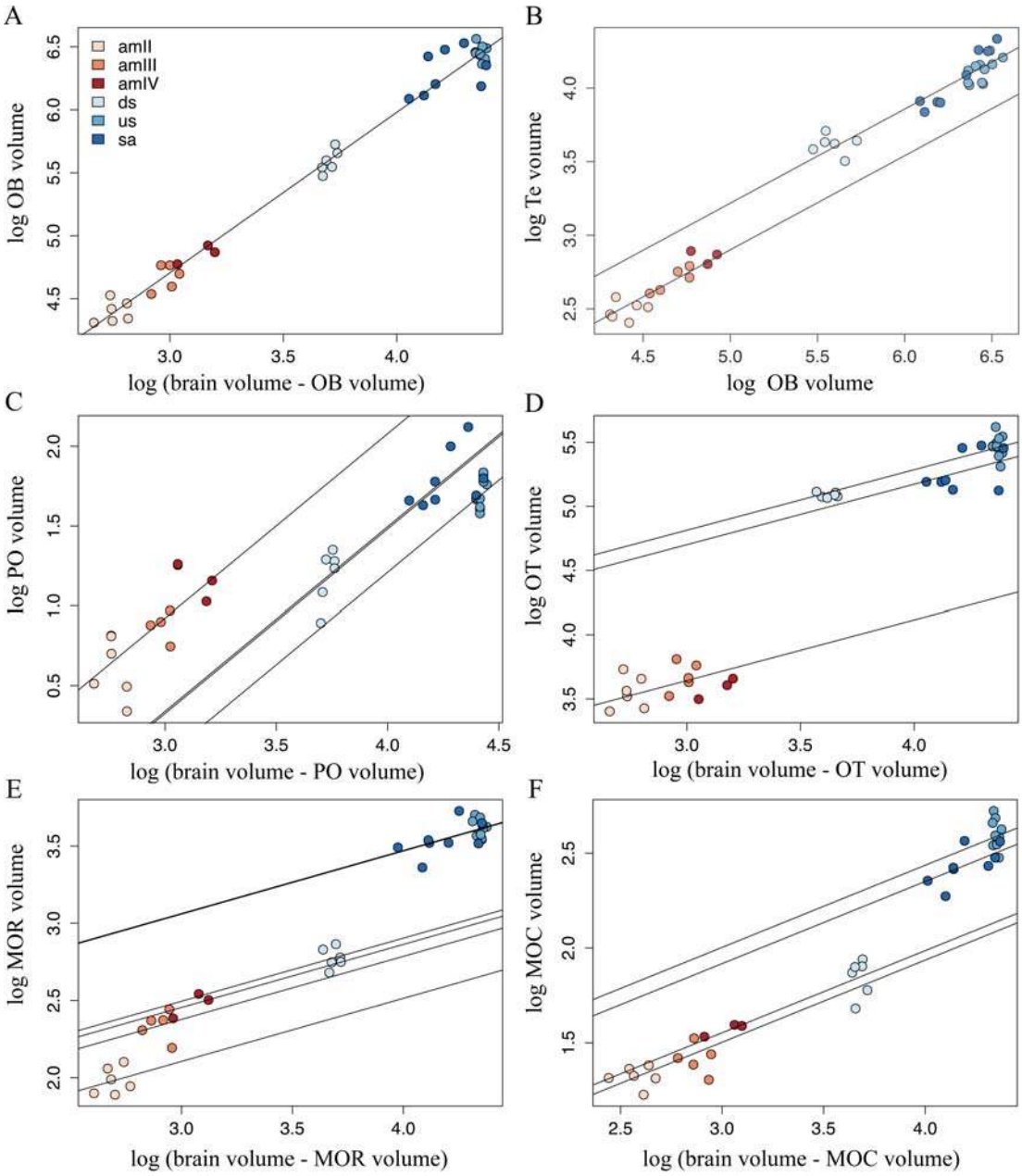

Figure 2.5 | Calculated regression lines after ANCOVA. Best linear models are plotted for each structure, showing the differences in scaling of each structure to the rest of the brain: (A) OB, (B) Te, (C) PO, (D) OT, (E) MOR, and (F) MOC. For the values of the parameters of these models, refer to Table 2.5. For abbreviations, see List of Abbreviations.

The volume of the MOC in the downstream migrants was significantly different to the other stages (Tukey Post Hoc test, $p < 0.05$), with a shallow slope ($\alpha = 0.43$). However, considering the value of the calculated intercepts in the ANCOVA of the MOC, the downstream migrants clustered with ammocoetes, whereas upstream migrants and spawning adults had higher intercepts (**Figure 2.5 E**). This was also the case for the MOR, where the volume in downstream migrants was different from all the other stages (Tukey Post Hoc test, $p < 0.05$) and their volume was closer to ammocoetes than to adults although, in contrast to all other structures, we found that in this area the ammocoetes were best fitted as separate groups, where

the second age class ammocoetes had a smaller intercept than other larval stages (Tukey Post Hoc tests: amIII, p=0.020; amIV, p=0.083; Figure 2.5 E). Some spawning adults possessed a relatively higher MOR than upstream migrants, consistent with the modifications of the oral disc and the appearance of the gular sac in this period (Potter et al., 1983; Neira, 1984). However, we did not observe significant differences between these groups. Our results also showed no statistically significant differences between male and female lampreys in any structure (results not shown).

2.4.3 Multivariate analysis and stage clustering

The principal component analysis performed on the autocovariance matrix of the relative size of the six brain structures measured in this study provided a clear separation in the multidimensional space of the two phases of the life cycle of *G. australis*. The relative loadings of the first four components and their relative importance are given in **Table 2.6**. The first two components explained 93.3% of the overall variance and their scores are plotted in **Figure 2.6**. The first component (PC1) reflects the high loadings for the OT and MOC, and secondarily in the OB, separating larvae, which had a relatively large MOC and PO, from adults, which had relatively larger OT, OB and Te, although the loadings of both PO and Te were small. Similarly, the second component (PC2) separated younger and older individuals within a phase, where older individuals had relatively large OB and MOR than younger individuals in both phases (larval and adult) of the life cycle.

Importance of components	**PC1**	**PC2**	**PC3**	**PC4**
standard deviation	0.189	0.093	0.046	0.031
proportion of the variance	0.749	0.183	0.045	0.021
cumulative proportion	0.749	0.933	0.978	0.998
relative loadings				
OB	0.313	0.523	-0.710	0.150
Te	0.125	-0.044	0.007	-0.868
PO	-0.094	-0.089	0.084	-0.101
OT	0.666	-0.472	0.193	0.345
MOR	-0.186	0.576	0.584	0.217
MOC	-0.633	-0.403	-0.332	0.218

Table 2.6 | Results of the principal component analysis for the first four components

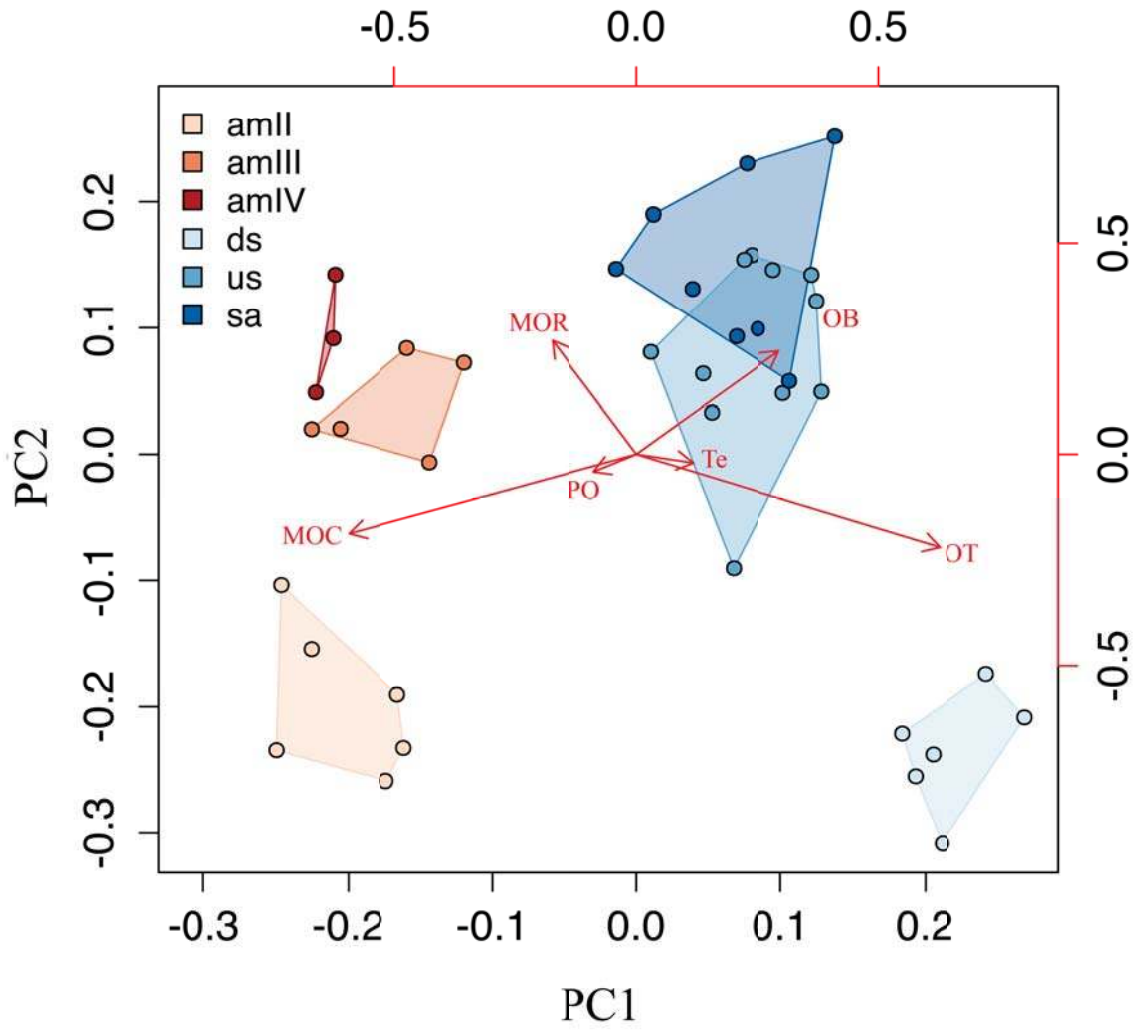

Figure 2.6 | A scatterplot of principal components PC1 and PC2. PC1 represents the major proportion of the variance in the composition of the brain during the life cycle (74.9%), while PC2 represents 18.3% of the variance. For abbreviations, see List of Abbreviations.

2.5 Discussion

Lampreys experience very different behavioural phases during the life cycle, from a microphagous sedentary mode to an active parasitic mode. This study characterized the growth of the brain (encephalization) during the life cycle of *Geotria australis*, focusing on the scaling between brain and body throughout ontogeny and testing the hypotheses that the vast transitions in behaviour and environment are reflected in differences in both encephalization and the relative development of major brain subdivisions.

The changes occurring in the nervous system of lampreys during ontogeny have attracted the attention of many comparative neurobiologists, who have shown extensive morphological and physiological modifications of the peripheral and central nervous system, such as the

development of the visual system (Kennedy and Rubinson, 1977; Kosareva, 1980; de Miguel and Anadon, 1987; Rubinson, 1990; Fritzsch and Northcutt, 1993b; Pombal et al., 1994; Davies et al., 2007; Villar-Cheda et al., 2008). However, in spite of the multiple studies quantifying these changes in the peripheral and/or central nervous system throughout the life cycle (Tamotsu and Morita, 1986; de Miguel and Anadon, 1987; Currie and Carlsen, 1988; Melendez-Ferro et al., 2003; Vidal Pizarro et al., 2004; Antri et al., 2006), an overall view of the pattern of development of the brain and its organization, including larval and adult phases, has been absent until now.

2.5.1 Brain scaling

A description of the changes in encephalization during the life cycle of jawless fishes will improve our current understanding brain development at multiple levels. Previous interspecific studies in agnathans have differed on the scaling relationship between brain size and body size of lampreys, ranging from 0.23 (Ebinger et al., 1983) to 0.56 (Platel and Vesselkin, 1988). In addition to discrepancies in the value of the scaling exponent, both studies suffered from low sample sizes, with data on only three (Ebinger et al., 1983) and two (Platel and Vesselkin, 1988) species, out of 41 currently recognized species of lampreys (Potter et al., 2015). This discrepancy in the scaling exponent requires improved resolution, as one value classifies lampreys as being far less encephalized than other gnathostomes, with a slow rate of growth of the brain in relation to the body ($\alpha = 0.23$), while the other places this group within the known range of the interspecific variation in the scaling exponent between most vertebrate groups ($\alpha = 0.56$), which usually falls between 0.5 and 0.6 (Striedter, 2005). Similarly, no consensus has been reached with regards to the intraspecific scaling exponent in the sea lamprey *Petromyzon marinus*, which ranges from -0.04 (Ebinger et al., 1983) to 0.56 (Platel and Delfini, 1986). However, given the dramatic shifts that occur throughout the life history of lampreys, these published values for brain scaling are likely to be highly dependent on when in the life cycle the brains were sampled. In fact, this study shows that, as lampreys advance in their upstream migration, they lose both body and brain mass at different rates, which is reflected in a higher intraspecific scaling factor in spawning adults (**Figure 2.4**). This variation between upstream migrants and spawning adults may explain previously reported discrepancies in the intraspecific allometric slope in *P. marinus*. Nonetheless, it is possible that the observed differences in relative brain mass may also be related to intraspecific variation between separate populations (Gonda et al., 2011) or according to mating strategies (Kolm et al., 2009), which have also been described in lampreys (Hume et al., 2013b).

The ontogenetic scaling of brain and body mass in other vertebrate groups, such as teleost fishes, has shown that the larvae of both metamorphic (Bauchot et al., 1979; Tomoda and Uematsu, 1996; Wagner, 2003; Sala et al., 2005) and non-metamorphic fishes (Iribarne and

Castelló, 2014) exhibit allometric scaling between brain and body size in the early post-hatching development phase, which may be equivalent to the linear phase of growth reported for ammocoetes in this study. However, in the case of metamorphic fishes, such as the rainbow trout *Oncorhyncus mykiss* or the Japanese eel *Anguilla japonica,* there is no clear evidence of an increase of encephalization associated with metamorphosis (Bauchot et al., 1979; Tomoda and Uematsu, 1996), as our results suggest for lampreys, but constitutes an interesting point that warrants further investigation and should be an area of future study.

Teleost fishes possess continuous growth of both the body and the nervous system throughout life (Bauchot et al., 1979; Leyhausen et al., 1987). This is in contrast to the pattern found in amniotes, where brain growth plateaus before the animal reaches its final body size (reviewed in Striedter, 2005), although there are some exceptions (Ngwenya et al., 2013). In lampreys, our results and previous records on *P. marinus* (Ebinger et al., 1983) suggest that, in early upstream migrants (end of the parasitic phase), brain growth may reach a plateau, where the rate of neurogenesis may be low and the body may have continued growing. This may explain the low intraspecific scaling factor found at this point of the life cycle in both analysed species (**Figure 2.4 B**: $\alpha = 0.09$ for *G. australis,* $\alpha = -0.04$ for *P. marinus*), although these values were not statistically significant in either study. In addition, we found evidence that a relative reduction in brain mass occurs in parallel with the typical reduction of body mass in spawning lampreys (Potter et al., 1983; Paton et al., 2011), which has not been previously shown in other ontogenetic studies of brain scaling in vertebrates. Even though complex behaviour is generally associated with larger brains relative to body size (reviewed by Striedter, 2005), lampreys still exhibit sophisticated behaviours, such as nest construction, in this period (Hardisty and Potter, 1971a; Sousa et al., 2012; Johnson et al., 2015).

Brain growth in vertebrates has been described as the result of several processes, including cell growth and the addition and elimination of cells (Pirlot and Bernier, 1991; Candal et al., 2005; Bandeira et al., 2009; Fu et al., 2013; Boyd et al., 2015). In lampreys, neuro- and glio-genesis are restricted to ventricular proliferative zones in late embryos and early to mid larval stages (Vidal Pizarro et al., 2004; Villar-Cheda et al., 2006; Guérin et al., 2009) and, although adult neurogenesis is widespread in other vertebrate groups (Kaslin et al., 2008), it is considered mostly absent in lampreys (Villar-Cheda et al., 2006; Kempermann, 2012). Taken together, these results suggest that brain growth from late ammocoetes onwards is mainly due to the addition of glia, cell growth, and the establishment of new synapses that contribute to the formation of plexiform tissue or neuropil, as suggested previously for lampreys (Rovainen, 1979; 1996).

2.5.2 Scaling of brain structures

Transitions in habitat and behaviour are common during the development of aquatic vertebrates, even if they do not undergo a metamorphic stage, such as recruitment of fish larvae (Kingsford et al., 2002; Kotrschal et al., 2012a; McMenamin and Parichy, 2013) and the use of nursery areas in sharks (e.g. Bethea et al., 2004; Heupel and Simpfendorfer, 2011). Usually these transitions are accompanied by ad-hoc sensorimotor specializations (Brandstätter and Kotrschal, 1990; Montgomery and Sutherland, 1997; Lisney et al., 2007; Lecchini et al., 2014). Similarly, adults of both bony and cartilaginous fishes, as well as other vertebrates, possess well developed adaptations to their ecological niche, which are generally reflected in their nervous system as a variation in the relative size of brain subdivisions (Kotrschal and Palzenberger, 1992; Gonzalez-Voyer et al., 2009; Gonzalez-Voyer and Kolm, 2010; Yopak, 2012). Surprisingly, the relative size of these brain subdivisions appear to be constant between species of parasitic lampreys, despite the diverse aquatic niches in which they inhabit (Renaud, 2011; Potter et al., 2015). We found that the OT and OB in adults of *G. australis* comprise similar proportions of the brain to that of *P. marinus* (Platel and Delfini, 1986) and other species of lampreys (Platel and Vesselkin, 1989), concordant with the lack of appreciable neuroanatomical differences in the brain between lamprey species, as reported previously (Platel and Vesselkin, 1989; Nieuwenhuys and Nicholson, 1998). However, we consider that more species of lampreys needs to be examined, including those with alternative life style strategies, such as parasitic and non-parasitic paired species of lampreys, to have a wider perspective of the diversity found in the nervous system of extant agnathans.

2.5.2.1 Olfactory bulbs

It has been suggested that the level of variation in the relative size of the major brain subdivisions may occur in the particular structure in a modular or mosaic fashion (Barton and Harvey, 2000), or with a concerted pattern of allometric scaling (Finlay and Darlington, 1995). It has recently been shown that most major brain areas in cartilaginous fishes scale with a characteristic slope that may be conserved across other vertebrates, including mammals (Yopak et al., 2010). One notable exception is found in the OBs, which maintain a level of statistical independence from total brain size in a range of vertebrate groups (Finlay and Darlington, 1995; Gonzalez-Voyer et al., 2009; Yopak et al., 2010; Yopak et al., 2015). At the ontogenetic level, however, our analysis of the scaling of the OBs shows the opposite pattern, whereby the OB scale very tightly with total brain size, with a highly hyperallometric growth (**Figure 2.5 A**).

Multiple functional hypotheses have been proposed to explain the relative size of the OB (reviewed in Yopak et al., 2015), including the role olfaction may play in navigation, which may play an important role in lampreys while finding a host or on their way back to rivers for the spawning run (Siefkes et al., 2003; Johnson et al., 2005; Sorensen et al., 2005; Johnson et

al., 2009; Wagner et al., 2009). The olfactory spatial hypothesis predicts that the size of the OBs should covary with navigational ability, which is supported by the olfactory input to the hippocampus (Jacobs, 2012). The statistical independence of the OB is then substantiated by the fact that the OB, the hippocampus, and other associated areas of the telencephalon do not scale as tightly with brain size as do other brain subdivisions (Finlay and Darlington, 1995; Finlay et al., 2001; Gonzalez-Voyer et al., 2009; Yopak et al., 2010) and can vary across mammalian taxa depending on the influence of olfactory cues in their behaviour (Reep et al., 2007). If these theories can be applied in the context of the lamprey life cycle, we would therefore expect that, should homologous olfactory areas exist in the telencephalon of *G. australis*, they would also scale isometrically with the rest of the brain in this group during ontogeny. Early descriptions of the telencephalon of the lamprey and later hodological evidence have suggested the presence of a hippocampal primordium or medial pallium (Johnston, 1912; Northcutt and Puzdrowski, 1988; Polenova and Vesselkin, 1993; Northcutt and Wicht, 1997). However, scaling of these telencephalic structures have not been studied in agnathans at any level, and even the existence of a medial pallium is disputed by neuroanatomical descriptions based on molecular markers (Pombal and Puelles, 1999; Weigle and Northcutt, 1999; Pombal et al., 2011). Considering that interspecific scaling of the OB has not yet been described in jawless fishes (see Chapter 5), the available evidence does not permit any definitive conclusions to be made with regard to differences found in the scaling of the OB between lampreys and other vertebrates.

An alternative explanation of the involvement of olfaction in navigation in lampreys is the hypothesis of dual olfaction, which assumes parallel processing of distinct sets of molecules or environmental odours by the main olfactory system and pheromones by the vomeronasal system, following independent pathways in the brain, and acting synergistically in the regulation of olfactory-guided behaviours (reviewed in Suárez et al., 2012). In lampreys, two anatomically distinct sets of olfactory epithelia have been described that show different patterns of central projections, which suggests the existence of a precursor of the vomeronasal system in this group (Ren et al., 2009; Chang et al., 2013). This accessory olfactory system is tightly coupled to motor areas of the brain, constituting an unusual motor system in vertebrates, which is capable of eliciting swimming movements after olfactory stimulation with both naturally occurring odours and pheromones (Derjean et al., 2010). Since lampreys can detect very low (subpicomolar) concentrations of pheromones (Sorensen et al., 2005), this system may be employed in navigation and other behaviours involving pheromone perception, such as searching for a natal river environment to spawn (Siefkes et al., 2003; Johnson et al., 2005; Sorensen et al., 2005; Johnson et al., 2009; Wagner et al., 2009). However, whether these differential central projections vary interspecifically and affect the relative size of the OB and/or a tight coupling between development of the OB and motor areas in the brain is unknown and requires further research.

2.5.2.2 Telencephalic hemispheres

Interspecific studies of the scaling of major brain subdivisions have shown that areas of the brain associated with behavioural and motor complexity, e.g. telencephalon and cerebellum, enlarge disproportionately as brain size increases in a range of vertebrates (Finlay and Darlington, 1995; Finlay et al., 2001; Pollen et al., 2007; Yopak et al., 2010). In lampreys, the evaginated portion of the telencephalon considered in this study (the cerebral hemispheres or Te) can be regarded as the multimodal sensorimotor integration centre of the telencephalon, providing a neural substrate for orientation movements of the eyes, trunk, and oral movements, due to direct efferent projections to brainstem motor centres and the OT, in a similar fashion to motor control systems of amniote vertebrates (Ericsson et al., 2013; Grillner and Robertson, 2015; Ocaña et al., 2015). The Te is also the main target of secondary olfactory projections from the lateral olfactory bulb, which, in turn, receives its primary afferents from the main olfactory epithelium (Northcutt and Puzdrowski, 1988; Northcutt and Wicht, 1997; Ren et al., 2009; Derjean et al., 2010). Therefore it is not surprising to find a tight scaling relationship between this structure and the OB ($r^2 = 0.987$). In addition, Te receives afferent fibres from the dorsal thalamus, possibly relaying visual and other sensory input that converge on this thalamic area (Polenova and Vesselkin, 1993; Northcutt and Wicht, 1997). Although not significant, there is some evidence of differences in the size of the Te between larvae and adults (Tukey Post Hoc test, $p = 0.091$), which may be due to the increase of sensory fibres terminating in this area, as both the primary olfactory system (VanDenbossche et al., 1995; Villar-Cheda et al., 2006) and the primary visual projections to the dorsal thalamus (Kennedy and Rubinson, 1977; Kosareva, 1980) develop during metamorphosis. Despite the various studies on the pallial telencephalon of lampreys, no consensus has been achieved yet in relation to the homology of this area with the pallium of other vertebrates (Northcutt and Puzdrowski, 1988; Nieuwenhuys and Nicholson, 1998; Pombal et al., 2009).

2.5.2.3 The pineal organ

The pineal complex in lampreys is formed by the pineal and the parapineal organs (Eddy and Strahan, 1970; Puzdrowski and Northcutt, 1989; Pombal et al., 1999; Yáñez et al., 1999), which participate in non-visual photo-perception and neuroendocrine control of the circadian rhythms in these animals, as it does in a range of vertebrates (Ekström and Meissl, 1997; Vernadakis et al., 1998; Ekström and Meissl, 2003). The PO has also been documented in extinct agnathans, where it was similar in relative size to that of contemporary ammocoetes (Gai et al., 2011), suggesting that non-visual light perception was also highly developed in these extinct groups. The observed morphological and physiological variability of this organ in tetrapods has been linked to latitudinal distribution of the species (Ralph, 1975), nocturnality (Bhatnagar et al., 1986; Haldar and Bishnupuri, 2001), and habitat depth in demersal fishes (Wagner and

Mattheus, 2002; Bowmaker and Wagner, 2004), although none of these factors fully explained the variability found in the size and morphology of this organ across species.

The best model for the PO described three distinctive periods of growth in the life cycle of *G. australis*. First, there was consistent hyperallometric growth throughout the larval phase; in the second period, during early adult life, including the marine parasitic phase, we observed that the growth of this organ plateaus after metamorphosis, where the size of the PO of ammocoetes was not significantly different to that of downstream or upstream migrants, opposite to what was observed in the other brain structures; and third, we found a relative increase in the size of the PO during sexual maturation. A similar pattern of growth has been documented in the PO of the arctic lamprey *Lethenteron camtschaticum* (Tamotsu and Morita, 1986). The larval phase and sexual maturation periods anticipate important milestones in the ontogeny of lampreys, such as the onset of metamorphosis and spawning, both of which likely depend on the timing of circadian rhythms (Freamat and Sower, 2013). In this regard, it was shown that metamorphosis was prevented with pinealoctomy in *G. australis* and other species (Eddy and Strahan, 1968; Cole and Youson, 1981), and maturation was delayed in adults of the river lamprey *Lampetra fluviatilis* after the same procedure (Eddy, 1971).

2.5.2.4 The optic tectum

In lampreys and other non-mammalian vertebrates, the OT is the main primary visual centre of the brain, receiving extensive topographic retinal (retinotopic) projections to the superficial layers (Butler and Hodos, 1996; Iwahori et al., 1999; de Arriba and Pombal, 2007; Jones et al., 2009). Electroreceptive and other sensory input also converge onto this tectal map (Bodznick and Northcutt, 1981; Ronan and Northcutt, 1990; Robertson et al., 2006), where the relevance of salient stimuli can be assessed, as in other vertebrates (Karamian et al., 1966; Karamian et al., 1984; Pombal et al., 2001; Gruberg et al., 2006; Kardamakis et al., 2015), leading to orienting movements of the eye, head and trunk (Saitoh et al., 2007; Ocaña et al., 2015).

Ontogenetic comparisons of the relative size of the OT have been documented in several species of elasmobranchs (Lisney et al., 2007) and teleost fishes (Brandstätter and Kotrschal, 1990; Kotrschal et al., 1990; Wagner, 2003), and have shown a shift from an initially well-developed visual system to a relative reduction in the size of the OT and a corresponding increase in other sensory brain areas, such as those that process olfactory or lateral line input, as the animal matures. This change in brain organization has been associated with shifts in ecological niche, from a well-lit environment in epipelagic fish larvae or nurseries of juvenile elasmobranchs to a different primary habitat as adults. In contrast to these groups, we report an opposite shift in brain organization. In ammocoetes of *G. australis,* the OT underwent moderate growth with total brain size ($\alpha = 0.47$; **Figure 2.5 D**). In fact, this structure remains mostly undifferentiated and poorly layered during most of the larval phase in lampreys (Kennedy and Rubinson, 1977;

de Miguel and Anadon, 1987; de Miguel et al., 1990) and only the central retina is differentiated (Meyer-Rochow and Stewart, 1996; Villar-Cheda et al., 2008). The major growth of the OT occurs in conjunction with the development of the adult eye, in a rapid process that starts at the end of the larval phase and continues during the initial stages of metamorphosis (Potter et al., 1980; de Miguel and Anadon, 1987). Indeed, it is only at the end of the larval phase that the typical retinotopic projections found in adults reach the OT (Jones et al., 2009; Cornide-Petronio et al., 2011). Soon after metamorphosis (downstream migrants), the relative size of the OT is more similar to that of adults than ammocoetes (**Table 2.5**, **Figure 2.5 D**).

This rapid development of the visual system explains the lack of a linear fit of the OT in the ontogenetic scaling of this structure with the rest of the brain. We expect that this fast switch from non-visual to visual perception will also affect the scaling of other visual areas of the brain receiving primary retinal input, such as the dorsal thalamus, and that it may be less pronounced in non-visual areas receiving retinal projections, such as the hypothalamus and pretectal area, which are already developed in ammocoetes, where they participate, for example, in non-visual reflexes (de Miguel and Anadon, 1987; Ullen et al., 1995; 1997; Jones et al., 2009). Nevertheless, the scaling of these visual and non-visual areas of the brain has yet to be studied.

Our results suggest that vision may be important during the parasitic phase, reflected in the high development of the OT during metamorphosis. However, the significant reduction in the size of the OT in spawning adults, which is corroborated with reports of eye degeneration during the spawning run (Applegate, 1950), supports previous evidence that vision is of lesser importance in lampreys during their upstream migration (Binder and McDonald, 2007; Johnson et al., 2015).

2.5.2.5 Medulla Oblongata

Interspecies comparisons in gnathostomes and agnathans have shown that the size of the rhombencephalon, i.e. the medulla oblongata plus the cerebellum, is well predicted from total brain size in both groups (Ebinger et al., 1983; Yopak et al., 2010), although in lampreys only cerebellum-like structures can be identified (Weigle and Northcutt, 1998; Northcutt, 2002; Montgomery et al., 2012). When comparing brain subdivisions, the medulla oblongata had the lowest scaling factor in cartilaginous fishes (Yopak et al., 2010), whereas it was the highest in agnathans (Ebinger et al., 1983). Indeed, the medulla accounts for approximately half of the total brain size in adult lampreys (this study, Platel and Vesselkin, 1989), and even more in early larvae (Scott, 1887), although this is not as obvious in downstream migrants (see below). The medulla is the first to develop cranial nerves in lampreys (Kuratani et al., 1997; Barreiro-Iglesias et al., 2008) and maintains a relatively stable scaling relationship with total brain size during the later larval phase and even throughout metamorphosis (**Figure 2.5 E-F**). However, there was a significant difference in the size of the MOR between the second-age class

ammocoetes and older stages (see intercepts in **Table 2.5**), which may be related to the development of a number of the diverse sensory and motor systems located in this brain structure, as discussed previously.

The growth of the medulla oblongata during metamorphosis maintains a tight scaling relationship with total brain size in late ammocoetes, which supports previous findings that the motoneurons of the trigeminal nucleus in lampreys are conserved through metamorphosis, in spite of the massive replacement of muscle in the head during this period (Homma, 1978; Rovainen, 1996). This has also been documented in other metamorphic vertebrates, such as frogs (Alley and Omerza, 1998).

However, while several brain structures, e.g. the OB and the OT, exhibit greater rate of growth during metamorphosis, both the MOR and MOC grow with a slower rate during this phase, which is expressed as a lower proportion of this area compared to total brain volume in downstream migrants. Nonetheless, our results show a later growth phase of this subdivision during the parasitic phase, particularly of the MOR, which may be associated with the development of the musculature of the ventilatory branchial basket and the oropharyngeal region, and to the scaling of other somatic and sensory functions as body size enlarges during the marine parasitic phase (Aboitiz, 1996; Rovainen, 1996; de Winter and Oxnard, 2001).

2.5.3 Neuroecology of the life cycle

Growth of the central nervous system in lampreys is a discontinuous process, with a variable rate of growth of both total brain and its subdivisions throughout life, which was expressed in the relative size of diverse brain structures in each phase of the life cycle (**Figure 2.6**). These patterns of brain organization may be interpreted as ‘cerebrotypes’ (Clark et al., 2001; Iwaniuk and Hurd, 2005; Willemet, 2012; 2013), whereby similar patterns of brain organization exist in species that share certain lifestyle characteristics. In this case, different cerebrotypes may in fact exist within a species at different phases of the life cycle.

The ammocoetes of *G. australis* are less encephalized compared to young adults (downstream migrants), with brains that are characterized by a relatively large MOC and a highly developed PO (**Figure 2.3**, **Figure 2.6**). The relative size of the MOR is increased in late ammocoetes (**Figure 2.5 E**), whereas the OB, Te and OT were relatively small during the whole larval phase (this study, Scott, 1887). It is possible that these characteristics are related to the requirements of a sedentary, burrower lifestyle and/or to filter-feeding specializations in this group. Patterns of brain organization of other filter-feeding vertebrates has been described previously, such as the basking shark *Cetorhinus maximus* and the whale shark *Rhincodon typus* (Kruska, 1988; Yopak and Frank, 2009), and mobulid rays (Ari, 2011), which similarly possess a relatively small telencephalon and mesencephalon (Kruska, 1988; Yopak and Frank, 2009). However,

given the drastic differences in the ethology between filter feeding jawless and cartilaginous fishes, it is impossible to draw parallels between patterns of brain organization in these groups. Further research is required to determine the existence of common characteristics in brain organization associated with a filter-feeding lifestyle in lampreys.

In contrast to ammocoetes, adult parasitic lampreys are active swimmers who are highly encephalized and possess a battery of well-developed sensory systems during the adult phase, including vision and olfaction. Correspondingly, they also possess a relatively large telencephalon and OB, structures that may be important in navigation (Derjean et al., 2010; Ocaña et al., 2015), and a relatively large OT, which participates in orientation movements and plays a role in visual processing (Saitoh et al., 2007; Kardamakis et al., 2015). Interestingly, some of these features, such high levels of encephalization and a well-developed OT, have also been observed in many coastal-oceanic and pelagic species of both cartilaginous and bony fishes (Lisney and Collin, 2006; Yopak, 2012; Yopak et al., 2015), which may be related to the sensory requirements of the open water habitat across both jawed and jawless fishes.

2.5.4 Conclusions

We have employed a widely-used volumetric approach (Huber et al., 1997; Wagner, 2001; Gonzalez-Voyer et al., 2009; Yopak and Lisney, 2012; Lecchini et al., 2014) to quantify differences in the relative size of major brain structures during the ontogeny of the pouched lamprey. Our results demonstrate shifts in encephalization between larvae and adults, as well as considerable differences in the relative size of brain subdivisions. Taken together, these shifts in brain organization may reflect the sensory requirements of this species at each stage of the life cycle. The inclusion of data of the growth of the brain and its subdivisions in embryonic, prolarva, and early larval stages of ammocoetes, metamorphic, as well as individuals sampled during the parasitic phase, will provide a more comprehensive insight of the growth of the brain and body during the life cycle of lampreys and eventually allow the use of alternative mathematical functions to describe the process of growth in each phase (i.e. Gompertz models, e.g. Calabrese et al., 2013)

It is yet to be determined whether this pattern of brain development is conserved in other species of lampreys, but we anticipate that it is, based on how conserved the life cycle is in this group (Potter et al., 2015), which could explain the reported homogeneity of the central nervous system between species of lampreys. Further studies on the changes in the brain of lampreys throughout ontogeny will contribute to the understanding of the evolution of the brain in agnathans and across vertebrates.

Chapter 3 Encephalization of lampreys and hagfishes

3.1 Abstract

Lampreys and hagfishes are the sole surviving representatives of the early agnathan (jawless) stage in vertebrate evolution, which have previously been regarded as the least encephalized group of all vertebrates. Very little is known, however, about the extent of interspecific variation in relative brain size in these fishes, as previous studies focused on only a few species. Yet, lampreys exhibit a variety of life history traits. Thus, while some species are parasitic as adults, others (non-parasitic species) do not feed after completing their macrophagous freshwater larval phase. In addition some parasitic species remain in freshwater, while others undergo an anadromous migration. On the basis of data for post-metamorphic individuals comprising ~40% of all lamprey species, with representatives from each of the three families, the above differences in life cycle traits are reflected in variations in relative brain size. Across all lampreys, brain mass increases with body mass with a scaling factor or slope (α) of 0.35, which is less than the 0.5 to 0.6 calculated for different groups of gnathostomes (jawed) vertebrates. When parasitic and non-parasitic species are analysed separately, with phylogeny taken into account, the scaling factors of both groups (parasitic $\alpha = 0.43$; non-parasitic $\alpha = 0.45$) approach that of gnathostome vertebrates ($\alpha = 0.43$-0.62). Relative brain size in fully-grown adults of parasitic species is, however, less than that of the adults of non-parasitic species, paralleling the differences between fully-grown adults and recently metamorphosed individuals of anadromous species. Within parasitic lampreys, anadromous species represent the average value of encephalization and thus a condition that might approximate that of the ancestor of extant lampreys. Analyses indicate that the last common ancestor of lampreys and hagfishes was more encephalized than extant lampreys, suggesting that extant lampreys may have evolved a less active mode of feeding behaviour and a corresponding reduction in relative brain size.

3.2 Introduction

Relative brain size is considered a proxy for cognitive capabilities and behavioural complexity in a range of vertebrate species (Jerison, 1973; Gould, 1975; Aboitiz, 1996; Striedter, 2005; Willemet, 2013), and has been widely used in comparative assessment of these traits across diverse taxa (reviewed in van Dongen, 1998; Striedter, 2005). Although brain size generally scales with body size in a predictable manner, the relationship between brain mass and body mass, at interspecific, intraspecific and ontogenetic levels, does vary amongst species with different life history traits, such as primary habitat, pattern of social behaviour, mode of feeding,

and diet (Bauchot et al., 1989; Yopak and Montgomery, 2008; Kolm et al., 2009; Gonda et al., 2011; Kotrschal et al., 2012b; Iglesias et al., 2015b; Salas et al., 2015; Tsuboi et al., 2015). Although there is increasing evidence of a direct link between behavioural repertoire and brain size (Sol, 2009; Kotrschal et al., 2012a; Kotrschal et al., 2014; Tsuboi et al., 2015), the question of whether such a relationship exists has been the subject of debate (Healy and Rowe, 2007; Azevedo et al., 2009; Herculano-Houzel, 2011; Rowe and Healy, 2014; Weisbecker et al., 2015).

Lampreys (Petromyzontiformes) and hagfishes (Myxini or Myxiniformes) are the sole extant representatives of what was once a diverse group of early vertebrates, the agnathans (reviewed in Janvier, 2007; 2015). Both of these groups have an elongated body shape and lack jaws, developing instead a specialized round buccal apparatus from which their collective name of cyclostomes is derived (Hardisty, 1979). In spite of these shared similarities, however, the extent of differences in the organization of the brain (as well as the rest of the body) of lampreys and hagfishes demonstrate a long history of evolutionary divergence (Braun, 1996; Butler and Hodos, 1996; Wicht, 1996; Gill et al., 2003; Khonsari et al., 2009) and the question of whether cyclostomes are monophyletic is still contentious (Stock and Whitt, 1992; Heimberg et al., 2010; Janvier, 2010; Thomson et al., 2014). Fossil records show that cyclostomes have retained a number of primitive characters (Bardack, 1991; Gill et al., 2003; Chang et al., 2014), but it is not clear whether their nervous system represents a derived, specialized form or the ancestral condition of all vertebrates (Northcutt, 1996; 2002; Montgomery et al., 2012). Brain size relative to body size of these two groups has been shown to be reduced as compared to aquatic vertebrate groups, such as bony and cartilaginous fishes (Platel and Delfini, 1981; Ebinger et al., 1983; Nieuwenhuys et al., 1998; Yopak, 2012). Indeed, lampreys are often considered the least encephalized of all vertebrates (Jerison, 1973; Nieuwenhuys and Nicholson, 1998; Striedter, 2005), and even less so than hagfishes (Platel and Delfini, 1981; Ebinger et al., 1983).

Various life history traits are exhibited among the extant species of lamprey (**Figure 3.1**). All lamprey species have, however, a protracted freshwater larval phase, during which the macrophagous larvae (ammocoete) remain burrowed in the sediments of streams and rivers for many years (Hardisty and Potter, 1971a). After a radical metamorphosis, some species become parasitic, typically feeding on teleost fishes (Hardisty and Potter, 1971a; Wilkie et al., 2004; Kuraku et al., 2012), whereas others attain sexual maturation shortly after metamorphosis (non-parasitic species). Furthermore, some parasitic species remain for the whole of their life cycle in fresh water, whereas other parasitic species migrate into marine environments, where they feed parasitically, after which they return to rivers for spawning (Potter et al., 2015).

The adults of parasitic species of lamprey use their suctorial disc for attachment to their hosts, whose body they then penetrate through the action of a multicuspid tongue-like piston (Potter et

al., 2014). Some parasitic species remain attached for substantial periods to their hosts, from which they extract mainly blood, whereas others remain attached for relatively shorter periods to typically smaller hosts, from which they remove 'chunks' of mainly muscle tissue, consequently often leading to the death of those hosts, requiring them to find new hosts more often (Potter and Hilliard, 1987; Renaud et al., 2009). A few species feed on both blood and muscle tissue, and a single species, *Caspiomyzon wagneri,* may feed on carrion and benthic invertebrates (Renaud et al., 2009; Renaud, 2011).

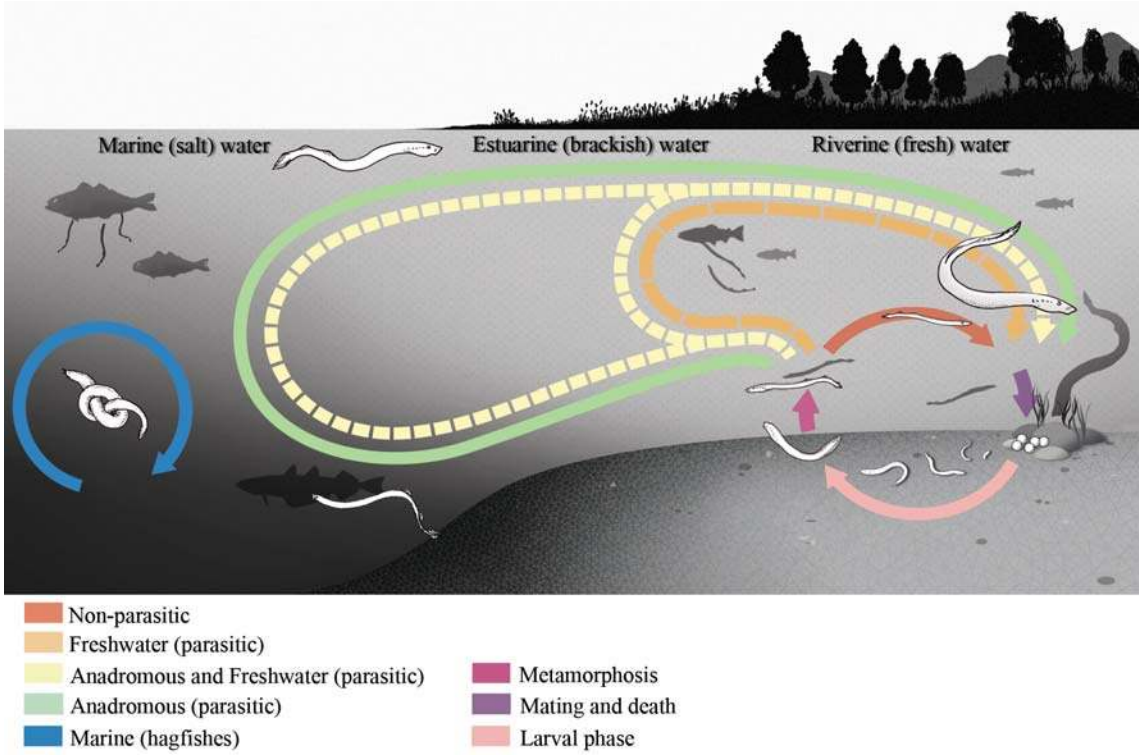

Figure 3.1 | Life cycle types of cyclostomes. The larval phase of all species of lampreys occurs in fresh water, whereas the adult phase differs among species: non-parasitic species are restricted to freshwater environments, as well as freshwater parasitic species. Other species have a strict anadromous adult life, whereas some lampreys present both freshwater and anadromous populations. Hagfishes present a purely marine life cycle.

In contrast to lampreys, hagfishes are found exclusively in marine habitats and undergo direct development (non-metamorphic), with one species at least undertaking seasonal migrations to deeper waters to spawn (Jørgensen et al., 1998). Although hagfishes are often been regarded as scavengers, it is more appropriate to consider them as predators, which feed opportunistically on prey, such as benthic invertebrates and fishes, supplemented, in some species, by scavenging on rare falls of high-level predators such as whales, sharks, and bony fishes (Martini, 1998; Zintzen et al., 2011; Zintzen et al., 2013). The hunting and capture of live prey by hagfishes is a complex and active process, involving the use of sensory barbels and olfactory organ to locate burrows in which prey are present and then employing its dental plates to grasp and begin to swallow that prey. The production of large amounts of mucus may facilitate suffocation of the

prey, while the unique knotting behaviour of hagfishes provides leverage for extracting the prey from they burrow. The more complex and active feeding behaviour of hagfishes than lampreys, including both active predation and scavenging, may help account for these species having a relatively larger brain size (Ebinger et al., 1983; Wicht and Northcutt, 1992; Wicht, 1996). This reiterates the need for a better understanding of the neural basis for the ecological characteristics and particularly the feeding behaviour of hagfishes (Striedter, 2005).

In spite of the critical position of agnathans when discussing the evolutionary history of vertebrates, the central nervous system (CNS) of lampreys and hagfishes has received far less attention than those of gnathostome vertebrates. Thus, while comparative studies on the CNS of other aquatic vertebrates, such as cartilaginous and teleost fishes, have covered a wide phylogenetic and life history range, generalizations on the CNS of lampreys have been based on only a few species and therefore a limited number of life cycle traits. They thus do not take into account the strong possibility that the degree of encephalization or brain organization (see Chapter 4) varies markedly with life cycle characteristics. In the present study, the degree of encephalization has been quantified for adults from each of the three currently described lamprey families, with representatives of both freshwater and anadromous parasitic species, including blood, flesh, and more generalist feeders, and of their non-parasitic derivatives. The resultant data have been used to explore the extent to which variations in brain size reflect differences in the life history traits and phylogenetic relationships. These data on lampreys have been compared to existing data from two species of hagfishes to validate that, irrespective of life cycle traits, the brain size of lampreys is less than that of hagfishes, as this latter group may possesses a more active and complex pattern of feeding behaviour. The data for lampreys and hagfishes are then used to propose the ancestral state of encephalization and life history traits in cyclostomes.

3.3 Methods

All procedures were performed in accordance with the ethical guidelines of The University of Western Australia Animal Ethics Committee (Research Project RA/3/100/917).

3.3.1 Data collection

This study employed data for 201 specimens, representing 16 species of lampreys and two of hagfishes (**Table 3.1**), including those reported in earlier studies on the encephalization (Stähler, 1982; Platel and Delfini, 1986; Platel and Vesselkin, 1988; 1989; Salas et al., 2015). New data were acquired during the present study for 37 adult lamprey specimens, representing 14 species, among which there were 11 species with no previous data on encephalization. The final data set encompassed all three extant families and nine of the 10 genera of lampreys (Potter et al., 2015).

Data for two species of hagfish, derived from 44 specimens and representing the two extant subfamilies of hagfishes (Fernholm, 1998; Nelson, 2006; Fernholm et al., 2013), were taken from Platel and Delfini (1981); Ebinger et al. (1983). When there were data for more than one individual of a species, means were used for the analysis.

Measurements of body mass and brain mass were recorded for each individual. After a variable period of fixation (see below), the brain was removed from the chondrocranium, the choroid plexuses separated from the brain, and the cranial nerves cut off within 0.5 mm of their base. The brain was separated from the spinal cord at the level of the first pair of spinal nerves. Before measuring mass, the brains were blotted dry to remove any liquid remaining in the ventricular system.

The combined data set contained brain and body mass measurements made on both fresh tissue and formalin-fixed samples, using a range of preservation and storage methods (ethanol, formalin or phosphate buffer), some of which had been stored in 70% ethanol for up to sixty-five years. Most studies of the effects of fixation and preservation on aquatic vertebrates have shown that there are significant changes in both body mass and length following fixation and storage in various media (Shields and Carlson, 1996; Kristoffersen and Salvanes, 1998; Buchheister and Wilson, 2005; König and Borcherding, 2012). This applies to both larval and adult lampreys, in which differences of 3 – 6% have been recorded between fixed and fresh tissue (Stähler, 1982; Neave et al., 2006). Mid to long-term tissue preservation studies show that most changes in mass in both formalin- and ethanol-preserved tissue occur within the first few days of storage (Shields and Carlson, 1996; Kristoffersen and Salvanes, 1998; Neave et al., 2006), with additional effects caused by preservation over the long term thus being negligible. However, the combined effects of fixation, the storage media used and the length of preservation on brain and body mass cannot be adequately assessed in our data set. Therefore, previously available data were used as it was published with no further corrections. As it was also shown that there are significant differences between measurements taken of the same object, possibly related to differences in blotting (Shields and Carlson, 1996), one person blotted and weighed all tissues in order to standardize these procedures.

Information on life history traits (diet and habitat) was collected for each species of lamprey from various sources (Renaud et al., 2009; Renaud, 2011; Potter et al., 2014; Potter et al., 2015). First, each species was classed as being either parasitic or non-parasitic. Only the parasitic lampreys were further subdivided according to diet and habitat, since non-parasitic lampreys do not feed as adults and all remain in fresh water during their life cycle (Moser et al., 2015; Potter et al., 2015). According to diet, parasitic lampreys were subdivided into blood-feeders, flesh-feeders, or those with a more generalist diet, which included blood and flesh feeding lampreys and the carrion-feeder *C. wagneri*. Parasitic lampreys were also classed according to habitat, i.e.

anadromous, freshwater or both anadromous and freshwater species. In addition, for comparisons involving both hagfishes and lampreys, four distinct dietary categories in cyclostomes were defined according to diet and the predatory behaviour expected from each diet: blood-feeders are the most passive predators, blood and flesh-feeders and carrion-feeders are semi-passive predators, flesh-feeders are semi-active predators, and non-parasitic, generalist-feeders are the most active predators (i.e. hagfishes). Similarly, in addition to the three categories of habitat for adult parasitic lampreys, we added a fourth category (marine) for hagfishes.

species	species code	family	subfamily	n [#]	body mass [*] [g]	brain mass [mg]	parasitism	habitat	diet	residual [†]
Lampetra planeri	Lpla	P	L	16 [b, f, h]	3.38	12.86	NP	-	-	0.123
Lampetra fluviatilis	Lflu	P	L	56 [b, e, h]	58.35	38.94	P	AFW	F	0.166
Eudontomyzon danfordi	Edan	P	L	2 [e]	6.00	10.30	P	FW	F	-0.061
Lethenteron appendix	Lape	P	L	2 [h]	6.16	12.65	NP	-	-	0.024
Lethenteron camtschaticum	Lcam	P	L	1 [h]	106.75	25.10	P	AFW	F	-0.118
Lampetra richardsoni	Lric	P	L	1 [h]	0.81	4.50	NP	-	-	-0.112
Tetrapleurodon geminis	Tgem	P	L	1 [h]	2.53	9.20	NP	-	-	0.023
Caspiomyzon wagneri	Cwag	P	P	6 [h]	110.52	25.13	P	A	PG	-0.123
Petromyzon marinus	Pmar	P	P	43 [b, d, h]	722.24	57.43	P	AFW	B	-0.054
Ichthyomyzon unicuspis	Iuni	P	P	3 [h]	57.77	9.40	P	FW	B	-0.450
Ichthyomyzon greeleyi	Igre	P	P	1 [h]	4.00	3.70	NP	-	-	-0.443
Ichthyomyzon castaneus	Icas	P	P	4 [h]	9.71	4.38	P	FW	B	-0.507
Geotria australis	Gaus	G	G	14 [g]	152.08	23.07	P	A	F	-0.210
Mordacia lapicida	Mlap	M	M	1 [h]	3.41	8.70	P	A	B	-0.047
Mordacia praecox	Mpra	M	M	4 [h]	2.61	6.05	NP	-	-	-0.164
Mordacia mordax	Mmor	M	M	2 [h]	55.03	17.15	P	A	B	-0.182
Myxine glutinosa	Mglu	My	My	33 [a, c]	32.86	35.61	-	M	G	0.215
Eptatretus burgeri	Ebur	My	Ep	11 [c]	126.18	58.76	-	M	G	0.225

Table 3.1 | Average brain and body mass for each species. Family: P: Petromyzontidae; G: Geotriidae; M: Mordaciidae; My: Myxinidae. Subfamily: L: Lampetrinae P: Petromyzontinae; G: Geotriinae; M: Mordaciinae; My: Myxininae; Ep: Eptatretinae. Parasitism: NP: non-parasitic; P: parasitic. Habitat: A: anadromous; AFW: anadromous and freshwater; FW: freshwater; M: marine. Diet: F: flesh; B: blood; PG: parasitic generalist G: non-parasitic generalist. # Data from: a Platel and Delfini, 1981; b Stähler, 1982; c Ebinger et al., 1983; d Platel and Delfini, 1986; e Platel and Vesselkin, 1988; f Platel and Vesselkin, 1989; g Salas et al., 2015; h this study.* Body size of *L. camtschaticum* extrapolated from (Kucheryavyi et al., 2007). † Residuals obtained from brain scaling in all cyclostomes (n=18).

3.3.2 Data analysis

All the analyses were performed using the R software package (R Core Team, 2013b). We examined brain scaling in agnathans, accounting for phylogenetic relationships and life history traits, with comparative methods available in a number of R packages (Garland et al., 1993; Paradis et al., 2004; Harmon et al., 2008; Revell, 2012), which have been used to test various hypotheses of brain evolution in vertebrates. Prior to analyses, brain and body mass were both $\log_{10}$ transformed to achieve normality.

The phylogenetic relationships between species were established by constructing a bootstrapped neighbour-joining phylogenetic tree with a p-distance model using the software Mega 4 (Tamura et al., 2007), based on sequences of mitochondrial cytochrome B of thirty six species of lampreys and two species of hagfishes (out-group), which were obtained from previous phylogenetic studies (Lang et al., 2009; Potter et al., 2015). The species not available for this study were pruned from the tree.

3.3.2.1 Setup and selection of pGLS models of brain scaling

When scaling parameters are determined with ordinary least squares (OLS) linear models, it is assumed that there is no covariance between the residuals of the model, i.e. the residuals from closely related species are not more similar than those from distantly related species, and all species are independent of each other (reviewed in Symonds and Blomberg, 2014). In contrast, in generalized least squares (GLS) linear models (available in the nlme package for R (Pinheiro et al., 2015)), an additional element is added to the regression, i.e. the variance-covariance matrix, which can account for the covariance of traits in phylogenetic-related species (expressed as residual errors), i.e. phylogenetic generalized least squares (pGLS) models (Symonds and Blomberg, 2014). Each pGLS model of brain scaling was fitted with the correlation structure *corPagel* to estimate the parameter lambda (λ) via maximum likelihood (available in the R package APE, Paradis et al., 2004), which tests the departure from a 'random walk' or Brownian motion (BM) model of evolution (Felsenstein, 1985; Pagel, 1999). In an unconstrained BM model ($\lambda = 1$), it is predicted that trait divergence accumulates over time stochastically on a given phylogeny, such that traits will be more similar between more closely related species. In contrast, a value of $\lambda = 0$ indicates that traits have evolved independently of phylogeny. Intermediate values ($0 < \lambda < 1$) show that the effect of the phylogeny is weaker than in the BM model. Furthermore, under certain circumstances, e.g. in a tree with long terminal edges, λ may be greater than one, which suggests that the residual error in the model is high at the root of the tree and decreases towards the tips, where traits are more similar amongst species than expected from their phylogenetic relationships (Pagel, 1999). In addition, in some scenarios, the equation for λ may become undefined, e.g. if covariance is greater than variance; in such cases, when a maximum likelihood estimation of λ was not possible, a set of eleven

models was constructed, each fitted with fixed values of λ ranging from zero to one, and the model with the largest log likelihood was chosen. Further, since neighbour-joining trees are non-ultrametric, the expected variance of the species as given by the distances from the root to the tips will differ among species; therefore the variance was fixed with weights calculated as the diagonal of the corresponding variance-covariance matrix (Paradis, 2012), using the variance structure *varFixed* (Pinheiro et al., 2015).

We considered a dataset containing all species of lampreys in this study (n=16), in addition to two subsets of parasitic (n=10) and non-parasitic species (n=6). We also constructed a dataset with all species of cyclostomes (n=18). In each of these sets, the pGLS models of evolutionary change were constructed to test for correlates of brain evolution. When considering life history traits, four different candidate models were tested (**Table 3.2**), with body mass alone (model 1, null), and with body mass plus one of three life history categories; parasitism (model 2), habitat (model 3), or diet (model 4). An additional set of analyses was run to test which taxonomic level (order, family or subfamily) best explained the variance in the data (**Table 3.2**). Due to the relatively small sample sizes, only one ecological factor was included in a model each time.

pGLS models	agnathans (n=18)	all lampreys (n=16)	parasitic lampreys (n=10)	non-parasitic lampreys (n=6)
body mass	+	+	+	+
ecological variables lampreys				
+ life history - parasitism		+		
+ life history - habitat			+	
+ diet			+	
taxonomical units				
+ order	+			
+ family	+	+		
+ subfamily		+		

Table 3.2 | Summary of the predictors of brain mass evaluated with pGLS models in each data set.

The most parsimonious pGLS model in each set was selected using the second-order Akaike Information Criterion (AICc) with multi-model inference methods available in the package MuMIn (Barton, 2014), where the best fit model yielded the lowest AICc score. When linear models showed a difference of less than two units ($\Delta AICc < 2$), they were considered to have similar levels of empirical support. Under these conditions, AICc weights were employed instead to define the best model of brain scaling (e.g. Burnham and Anderson, 2002). The significance of the effect of factors in brain scaling was obtained from an ANOVA table of the pGLS model. To show the differences between the levels of a factor graphically, we calculated standardized residuals from model 1. This was achieved by calculating vertical deviations from the predicted slope and dividing each by the square root of the residual variance. We also constructed boxplots with the width of the box adjusted per number of species in each level of the categorical variable.

3.3.2.2 Ancestral state reconstructions

Reconstruction of the ancestral state of the encephalization of cyclostomes, as well as their life history traits (diet and habitat), was performed using several functions implemented in the package phytools (Revell, 2012; Revell, 2013). Character states at internal nodes were unambiguously resolved by assigning them to their most probable state: (1) for continuous characters (encephalization), the function *contMap* was used on unstandardized residuals from the null model of brain scaling (model 1, n=18), where ancestral states were estimated via maximum likelihood. (2) For discrete characters (life history traits), a Bayesian approach was used, where the ancestral state at each internal node of the phylogenetic tree was estimated with stochastic character mapping under an equal rates model (Huelsenbeck et al., 2003; Bollback, 2006; Revell, 2012). This model assumes identical probabilities of each state of the character as a prior, from which posterior probabilities were inferred after 500 simulations. Ancestral states of life history traits were estimated in a tree of 14 species of parasitic lampreys and two species of hagfishes (n=16), which represents approximately 80% of all recognized species of parasitic lampreys (Potter et al., 2015).

3.4 Results

3.4.1 Encephalization of lampreys

3.4.1.1 Effect of parasitism on encephalization and taxonomic predictors of the relative size of lampreys

The impact of a parasitic *vs.* a non-parasitic lifestyle on the scaling of brain mass with body mass was assessed in 16 species of lampreys. Our results show that, across all species examined, brain mass increases with body mass in lampreys with a slope (α) of 0.35, whereas in the pGLS model that included parasitism as a factor (model 2), the slope increased to 0.45, with significantly different intercepts for parasitic and non-parasitic species (**Table 3.3**). Based on AICc scores, pGLS modelling of brain versus body mass showed that parasitism exerted a significant influence on brain size (**Table 3.3**; **Figure 3.2 A**), although the null model also demonstrated substantial support (ΔAICc < 2); In model 2, the factor (parasitism) had significant statistical support (ANOVA, F=5.36, p = 0.038), reflecting the differences observed in the residuals of model 1 (**Figure 3.2 B**). When closely related parasitic and non-parasitic individuals are compared, however, a few exceptions can be observed, e.g. the residuals indicate that the parasitic European river lamprey *Lampetra fluviatilis* possesses a relatively larger brain than the non-parasitic European brook lamprey *Lampetra planeri* (**Figure 3.2 C**). The differences between parasitic and non-parasitic species in all cases are observed on top of greater differences between large taxonomic groups, i.e. between both subfamilies (**Figure 3.2**

C) and families (**Figure 3.2 D**) of lampreys. Therefore, the estimated value of λ in model 2 was 1.58, which indicates that the largest differences between groups may be encountered towards the root of the tree. The members of the family Petromyzontidae accumulated most of the variance observed in the relative size of the brain of all lampreys examined. In fact, the size of the brain in many species was substantially different to that expected for their body size (**Figure 3.2 C**); for example, there was an approximately four fold difference in relative brain size between two species of equivalent body size, the European river lamprey *Lampetra fluviatilis* (residual = 0.166) and the silver lamprey *Ichthyomyzon unicuspis* (residual = -0.450). None of the different taxonomic levels tested in lampreys improved the fit of the null model of brain scaling (ΔAICc > 8); however, subfamily was the best taxonomic predictor of the variance observed in the relative size of the brain, where various members of the subfamily Lampetrinae had residual values above the average (see **Figure 3.2 C**).

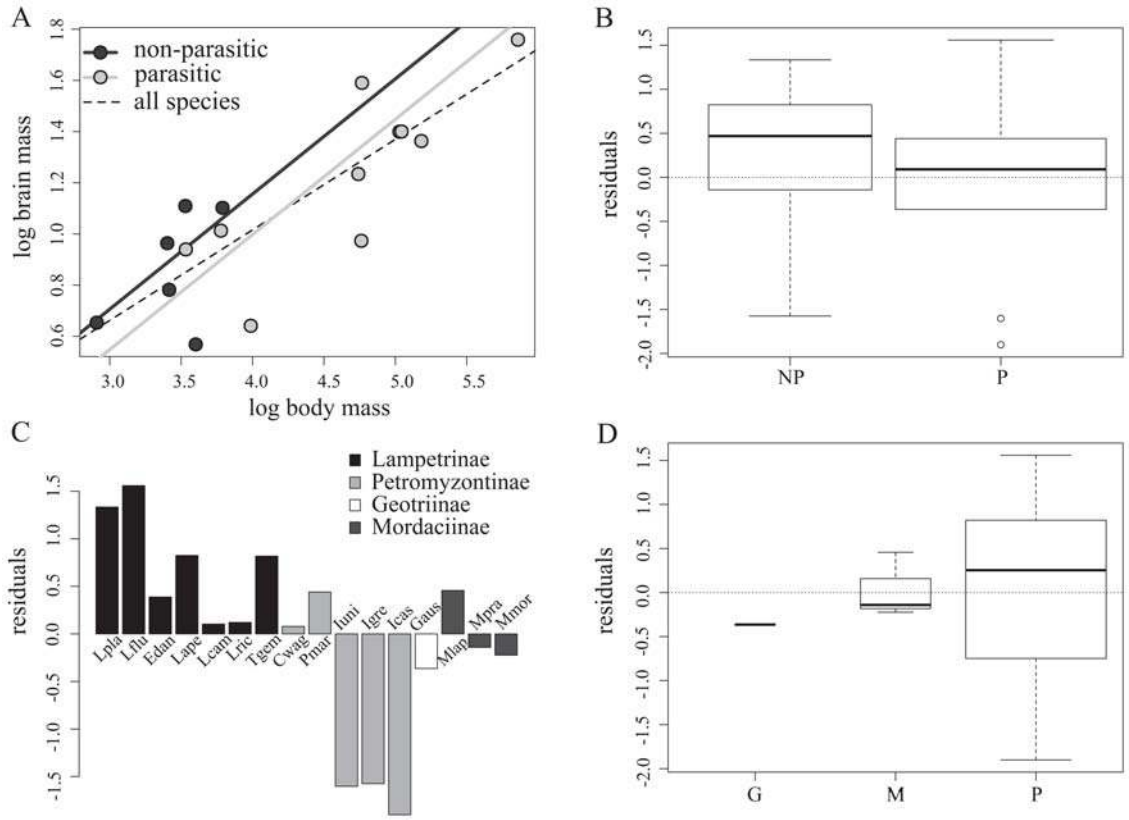

Figure 3.2 | Interspecific scaling of the brain in 16 species of lampreys. (A) Brain mass with respect to body mass. (B) Boxplots of standardized residuals summarizing the differences found between non-parasitic (*NP*) and parasitic (*P*) species. (C) Histogram of the standardized residuals obtained for each species. Species names are abbreviated; a complete list of species names and abbreviations can be found in Table 3.1. (D) Boxplots of standardized residuals summarizing the differences found between families of lampreys, Geotriidae (*G*), Mordaciidae (*M*) and Petromyzontidae (*P*).

3.4.1.2 Effect of life history types on the encephalization of parasitic lampreys

We also studied the effect of the habitat and diet on brain scaling of adult parasitic species (n = 10). This was done by testing if the addition of habitat (**Figure 3.3 A**) or diet (**Figure 3.3 B**) into the model of brain scaling improved the fit when compared to the null model (brain ~ body mass). Between these three models, the scaling of brain mass was best explained by habitat (brain mass ~ body mass + habitat; **Table 3.3**), although the null model also had substantial support (ΔAICc < 2). However, data showed no significant effect of habitat on relative brain mass (ANOVA, F = 2.91, p = 0.131). Possibly due to a low sample size (Freckleton et al., 2002), it was not possible to calculate a maximum likelihood estimation of λ for any of the models fitted with life history characters as categorical variables in parasitic lampreys alone; in both cases, the model with high log likelihood was at a value of λ = 0, suggesting an independence of these traits from phylogeny.

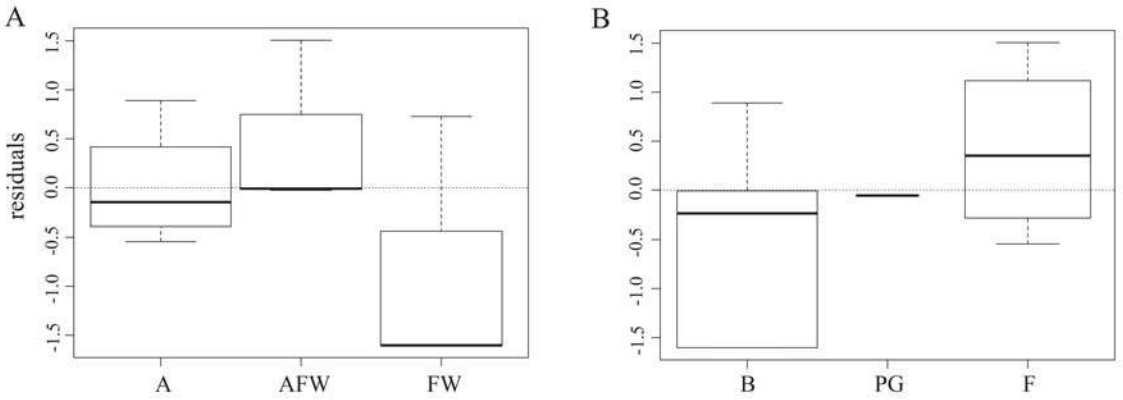

Figure 3.3 | Interspecific scaling of the brain in ten species of parasitic lampreys. Boxplots of standardized residuals summarizing the differences found between (A) anadromous, anadromous-freshwater, and freshwater species, and (B) blood-, generalist-, and flesh-feeding species of parasitic lampreys.

Parameter	all lamprey species (n=16)		parasitic species of lampreys (n=10)		
	body mass (model 1)	+ parasitism (model 2)	body mass (model 1)	+ habitat (model 3)	+ diet (model 4)
slope	0.354***	0.450***	0.429***	0.229*	0.374**
Intercept(s)	-0.399*	-0.644** (NP) -0.802* (P)	-0.751+	0.173 (A) 0.389 (AFW) -0.075+ (FW)	-0.585 (B) -0.486 (PG) -0.408 (F)
Model summary					
λ	0.98	1.58	0.891	0 (fixed)	0 (fixed)
d.f. residuals	14	13	8	6	6
AICc	-7.07	-7.21	9.96	9.63	16.89
ΔAICc	0.136	0.000	0.324	0.000	7.255
AICc weights	0.483	0.517	0.453	0.533	0.014

Table 3.3 | Summary of pGLS model selection in lampreys. For abbreviations, see Abbreviation list. (***) p-value < 0.001, (**) 0.001 < p-value < 0.01, (*) 0.01 < p-value < 0.05, (+) 0.05 < p-value < 0.1, ( ) p-value > 0.1.

3.4.2 Reconstructions of ancestral character states of agnathans

3.4.2.1 Life history traits

The evolution of two life history traits (diet and habitat) in extant agnathans was studied by tracing the history of these discrete traits in the phylogenetic tree of parasitic lampreys and hagfishes (n = 16). Ancestral states of habitat type at deep nodes of the phylogenetic tree of cyclostomes showed low resolution (**Figure 3.4 A**); in the most basal node of cyclostomes, the highest posterior probability in the categories of habitat was anadromy (posterior probability, PP = 32%). With regards to diet, **Figure 3.4 B** shows that highest probability of the ancestral state of this trait was a more passive predation mode (PP = 51%). Unfortunately, the lack of knowledge of the ecology and phylogeny of hagfishes makes it difficult to draw further conclusions in this group.

The probability that basal lampreys were anadromous was 46% and were most likely a passive predator (PP = 81%). It is probable that this condition continued to be present in various ancestral nodes of lampreys, but changed in the last common ancestor of the Petromyzontidae, possibly due to the establishment of freshwater resident populations in addition to anadromous populations (PP = 65%). There were clearer differences in diet between the two subfamilies of the Petromyzontidae: the ancestor of Petromyzontinae was likely a passive predator (PP = 82%), whereas a more active predatory behaviour probably originated in the last common ancestor of Lampetrinae (PP = 63%).

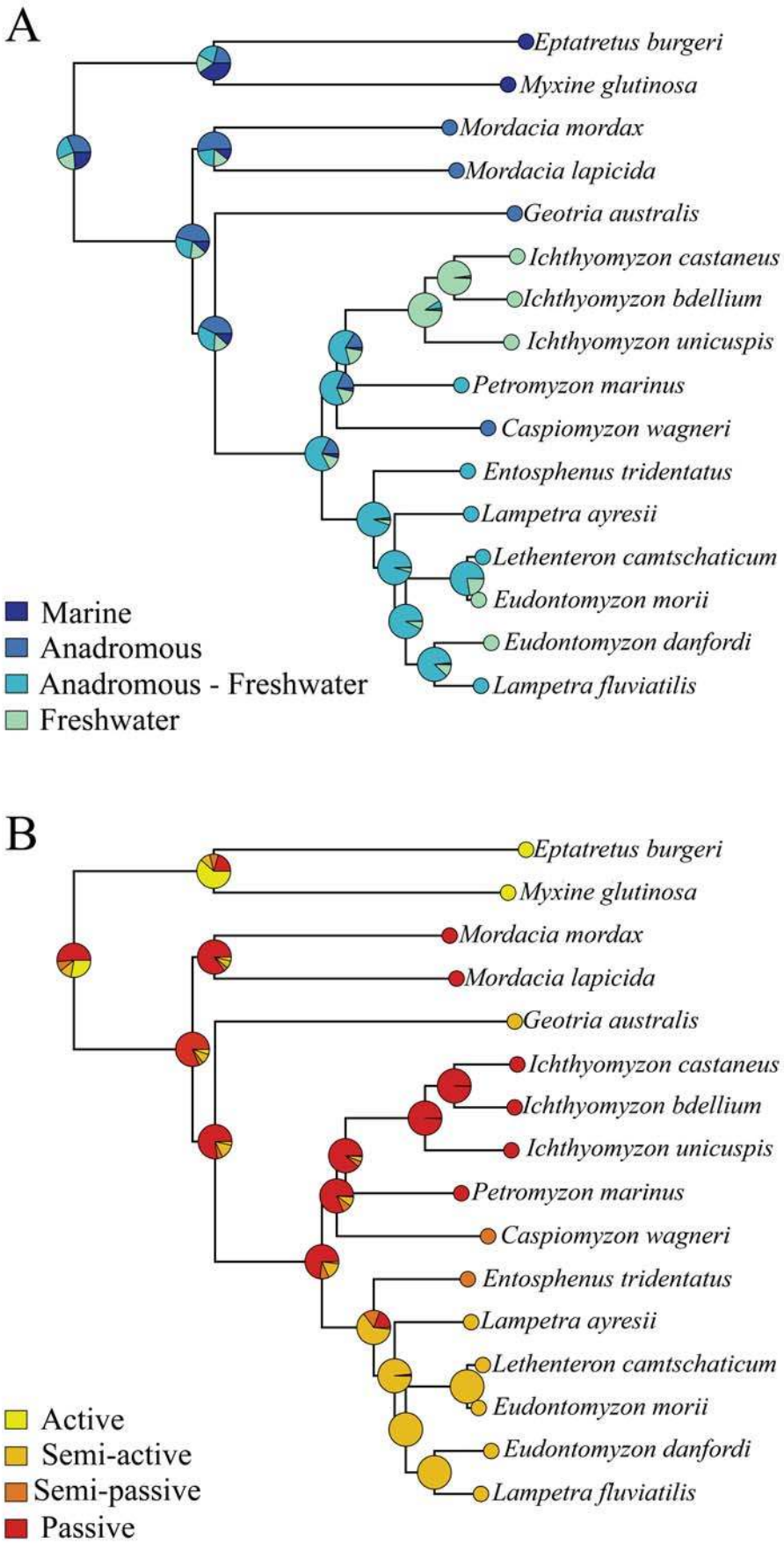

Figure 3.4 | Ancestral state reconstruction of two life history traits of cyclostomes (n=16). (A) habitat of adults. (B) diet. For a definition of each category see Introduction and Methods.

3.4.2.2 Encephalization of cyclostomes

A model of the relationship between brain and body mass was also constructed with data from all available species of cyclostomes (n = 18). The inclusion of the two species of hagfishes had no impact on the slope estimate, but increased the value of the intercept in contrast to the parameters obtained in the analysis of lampreys alone ($y = 0.355x - 0.267$, $\lambda = 0.987$, compare to the values shown in **Table 3.3**). In relation to taxonomic differences, we found equal support of the null model and the one that included Order as a factor ($\Delta AICc=1.02$), where the best model contained Order (AICc weight = 0.619). This model estimated a larger intercept for hagfishes.

We used the residuals obtained from the null model to estimate encephalization in the ancestor of all extant agnathans and in each of the nodes of the phylogenetic tree of this group of vertebrates, whose values are shown color-coded in **Figure 3.5**. According to this estimation, encephalization of the common ancestor of lampreys and hagfishes differed from that observed in most of the extant species of cyclostomes, but was comparable to some of the members of the subfamily Lampetrinae (e.g. the Mexican brook lamprey *Tetrapleurodon geminis* and the American brook lamprey *Lethenteron appendix*). The brain in both subfamilies of hagfishes is relatively larger than those of lampreys, which likely increased before the separation of these two lineages. In contrast, lampreys show a reduction in relative brain size that can be mapped to the ancestor of this group. Data emphasize that the relative size of the brain has remained relatively constant in most species of lampreys, which is especially obvious when comparing anadromous parasitic species. However, two events did not follow this trend: one occurring at the origin of the genus *Ichthyomyzon*, with a pronounced reduction in relative brain size, and another possibly occurring in the ancestor of all species of the subfamily Lampetrinae. In this group, we observed an increase in the relative size of the brain in various species, where in some species (e.g. *L. fluviatilis)*, encephalization approximated the values of relative brain size found in hagfishes (**Table 3.1**).

Taxa	habitat		diet		encephalization	
	type	PP	type	PP	residual value	95% CI
Cyclostomata	anadromous	0.32	passive feeder	0.51	0.000	-0.31 – 0.31
Myxiniformes	marine	0.40	active feeder	0.61	0.106	-0.19 – 0.40
Petromyzontiformes	anadromous	0.46	passive feeder	0.73	-0.090	-0.31 – 0.13
Mordaciidae	anadromous	0.52	passive feeder	0.85	-0.092	-0.32 – 0.13
Geotriidae	anadromous	0.43	passive feeder	0.77	-0.102	-0.32 – 0.11
Petromyzontidae	anadromous-freshwater	0.65	passive feeder	0.73	-0.128	-0.29 – 0.03
Petromyzontinae	anadromous-freshwater	0.63	passive feeder	0.82	-0.150	-0.31 – 0.01
Lampetrinae	anadromous-freshwater	0.93	semi-active feeder	0.63	-0.064	-0.21 – 0.08

Table 3.4 Reconstruction of ancestral character state for various taxa of cyclostomes. For discrete characters (habitat, diet), the value of posterior probability (PP) of the most probable state is given. For the continuous character (encephalization), the estimated value of the residual and 95% confidence intervals (CI) are presented.

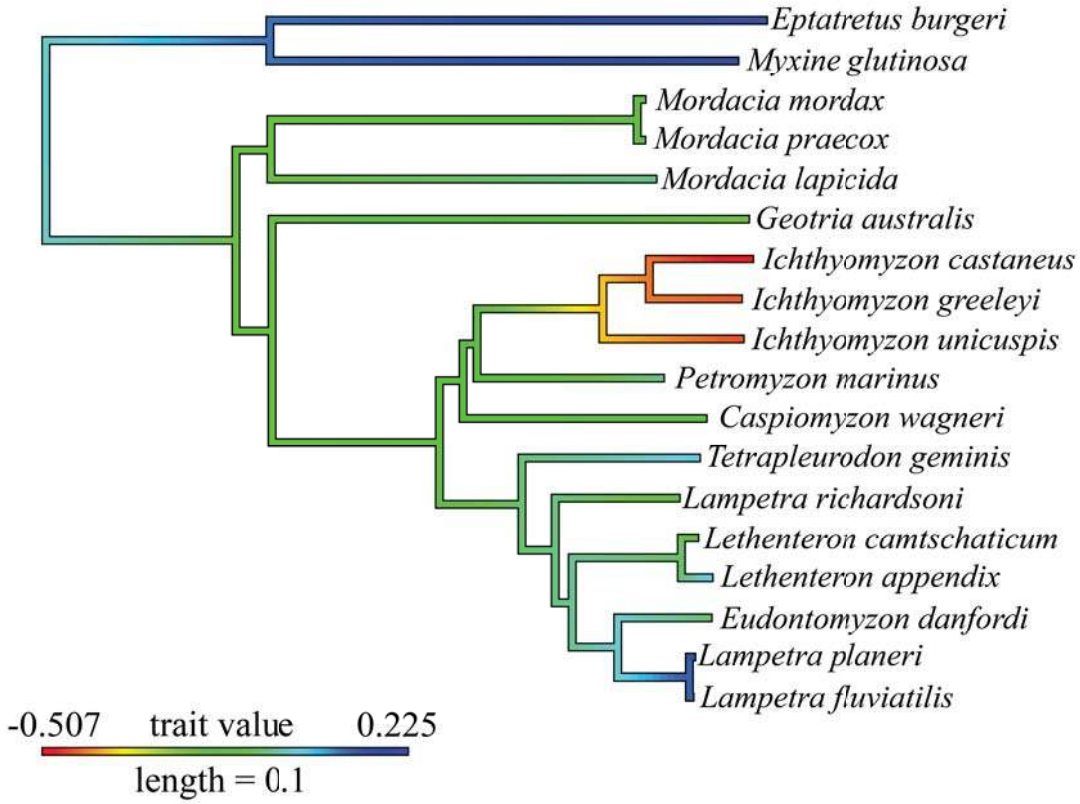

Figure 3.5 | Maximum-likelihood ancestral state reconstruction of encephalization of cyclostomes, estimated from unstandardized residuals from a pGLS model of 18 species of lampreys and hagfishes.

3.5 Discussion

It has been argued that there is a close relationship between brain size and a somatic component at the interspecific level (Deacon, 1990; Aboitiz, 1996; Finlay et al., 2001), where the scaling exponents for brain-body relationships in major vertebrate groups fall between 0.5 and 0.6 (van Dongen, 1998; Striedter, 2005). However, there is also a considerable residual variation in brain size from these allometric lines in vertebrates, implying that brain size also depends on a non-somatic component, which has been associated with a range of life history traits, e.g. parental investment, primary habitat, and feeding mode (Lefebvre et al., 2004; Yopak and Frank, 2009; Mull et al., 2011; Swanson et al., 2012; Corfield et al., 2013; Willemet, 2013; Tsuboi et al., 2015). Previous studies on the allometric relationship between brain and body size have focused primarily on the gnathostomes, with comparatively little known about brain scaling within agnathans. In this study, interspecific variation in encephalization in hagfishes and lampreys has been explored in relation to both phylogeny and ecology. In contrast to previous views that there is very little difference between the brains of different species of lampreys (e.g., Nieuwenhuys and Nicholson, 1998), our results show that there is actually a significant level of variation in relative brain size, which may be indicative of further neuroanatomical differences

related to ecology and specialized behaviours reflecting diverse evolutionary pathways within this group.

3.5.1 Encephalization of lampreys

In lampreys, two previous independent studies reported very different interspecific allometric relationships between brain size and body size, ranging from 0.23 (Ebinger et al., 1983) to 0.56 (Platel and Vesselkin, 1988), although the latter was really an average of the intraspecific scaling rule calculated for each species, instead of an actual interspecific regression (Platel and Vesselkin, 1988). Both studies were biased by a low sample size, with data on only three (the sea lamprey *Petromyzon marinus*, the European river lamprey *Lampetra fluviatilis* and the European brook lamprey *Lampetra planeri*) and two (*P. marinus* and *L. fluviatilis*) species in each study, respectively. It is possible that many other aspects of the biology generalized for lampreys are also biased, as more than 80% of all scientific work on lampreys have been performed on the same three species (Docker et al., 2015). In addition to the deficit of representation, these previous studies on brain-body scaling failed to account for the phylogenetic relationships between species. This is important because closely related species can share many characters through common descent rather than through independent evolution (Felsenstein, 1985; Harvey and Pagel, 1991). In contrast, this current work provides valuable new insights into brain evolution in these ancient lineages of vertebrates for two important reasons: (1) we have included representatives of all major taxonomic subdivisions of lampreys and their life history types, and (2) our analysis uses phylogenetically-informed methods (reviewed in Garamszegi, 2014). The estimated scaling factor of 0.35 for all species of lampreys obtained in this study (both parasitic and non-parasitic) falls between previous estimates for this group (Ebinger et al., 1983; Platel and Vesselkin, 1988), which still falls below the scaling exponent documented for most other vertebrate taxa (Striedter, 2005). However, considering both the current discussion on the concept of "paired species" of lampreys (see below), and the lack of statistical distinction between the null model and the one including life history types (parasitic and non-parasitic species), it may be necessary that these two groups of species be treated separately in future studies of encephalization; in this case, both the model of encephalization controlling for parasitism (slope = 0.45) and the one considering only parasitic species (slope = 0.43) yielded values of slope closer to that reported for gnathostome vertebrates (e.g. Yopak, 2012).

3.5.1.1 Effect of parasitism on encephalization of lampreys and taxonomic predictors

Although an area of controversy (reviewed in Docker, 2009), there may be polymorphic populations within multiple genera of lampreys (Kucheryavyi et al., 2007; Nazarov et al., 2011; Hume et al., 2013b), representing individual epigenetic realizations of the same species

(Makhrov and Popov, 2015). These "paired species" may consist of a parasitic and a non-parasitic form. These paired populations show phenotypic plasticity with regards to the onset of sexual maturation and other morphological characters (Hubbs and Trautman, 1937; Zanandrea, 1959; Hardisty and Potter, 1971b; Vladykov and Kott, 1979; Potter, 1980b). Therefore, while some individuals undergo the typical life cycle of an anadromous parasitic lamprey, reaching large sizes after a period of parasitic feeding, other non-parasitic individuals attain sexual maturity shortly after metamorphosis, remaining close to the size of the ammocoete (**Figure 3.1**). This fact has led various authors to discuss a possible sympatric mechanism of speciation in lampreys resulting from size-assortative mating (i.e. Hardisty and Potter, 1971b; Beamish and Neville, 1992; Hume et al., 2013a; Mateus et al., 2013; Bracken et al., 2015; Rougemont et al., 2015), which was recommended to be evaluated from pair to pair (Docker, 2009). Our data in this sense is not very informative, as it fits both of these alternatives, where differences in the relative size of the brain between the parasitic and non-parasitic pair resemble the differences documented between downstream and upstream migrants in anadromous lampreys (Salas et al., 2015), where non-parasitic species have, on average, a relatively larger brain than parasitic species (**Figure 3.2 B**). In this scenario, it may be important to consider parasitic species separately when evaluating scaling relationships, as non-parasitic species may artificially decrease the value of the slope (Figure 3.2 A; Brandstätter and Kotrschal, 1990). If we accept this, then there is further support that the allometric relationship between brain and body mass may be reasonably conserved across all vertebrates, ranging from 0.43-0.62 (Striedter, 2005; Yopak, 2012; Iglesias et al., 2015b).

Notwithstanding these differences found within paired species, when brain-body data for all species of agnathans were regressed together (model 1, n=18), the values of the residuals were usually most similar within closely related species (**Figure 3.5**), revealing that changes in relative brain size accumulate over time in the different clades of lampreys, including paired species. This is confirmed by the values of lambda obtained in this model ($\lambda = 0.99$), indicating that encephalization may have evolved under a Brownian motion model in this group, where variation in brain size is reflected similarly in paired species of lampreys (or alternative life history forms). Our combined results on encephalization and life history trait reconstructions suggest that these changes can be traced in parallel to changes in the behaviour of these species.

3.5.1.2 Effect of habitat and feeding ecology on the encephalization of parasitic lampreys

Habitat exerted a significant influence on brain size in parasitic adults. In this dataset, freshwater genera (e.g. *Ichthyomyzon* and *Eudontomyzon*) had relatively smaller brains when compared to their closest relatives (**Figure 3.5**). The differences found between parasitic lampreys from different habitats do not corroborate previous results of intraspecific studies in

other species of fishes, where freshwater-resident populations had a relatively larger brain than migratory or marine populations (Kolm et al., 2009; Gonda et al., 2011), as we have shown in the paired species of lampreys (**Figure 3.2 B**). It may be possible that these differences originate earlier than metamorphosis during the ontogeny of these freshwater species, but further research is required. Nonetheless, this result suggests that the differences in brain size in lampreys may be closely related to lifestyle and less to phylogenetic relationships, similar to the results obtained in other groups of vertebrates (Yopak et al., 2007; Yopak and Montgomery, 2008; Eifert et al., 2015; White and Brown, 2015). In any case, these results must be considered with caution, since only a few species per category were included in this comparison.

The model of brain scaling of parasitic lampreys, which included diet as a factor, was the least supported in this analysis. Diet has been thought to influence the size of the brain in at least two ways: (1) acting as a physiological constraint, e.g. the "expensive tissue hypothesis", where various authors have proposed a trade-off between highly energy-demanding tissues such as the brain and gut (Isler and van Schaik, 2009; Navarrete et al., 2011; Tsuboi et al., 2015). Similar arguments relate energetic constraints to brain size, such as basal metabolic rate or body temperature (Gillooly and McCoy, 2014; Yu et al., 2014; Iglesias et al., 2015b). (2) Diet has also been related to the behavioural component of prey capture, whereby active versus passive predation strategies have been correlated with brain size in diverse vertebrate taxa (Gittleman, 1986; Finlay et al., 2001; Hutcheon et al., 2002; Lisney and Collin, 2006; Yopak and Frank, 2009). In the case of lampreys, blood-eaters represent an accentuated parasitism or passive predatory mode, whereby the host usually survives their attack, and the parasite can remain attached to them for a longer period of time, investing less effort in finding a new host. Flesh-eating lampreys behave more like an active predator, perforating the body of their host and consuming internal organs, which usually results in their eventual death, thus necessitating the continual targeting and acquisition of a new host (Beamish, 1980; Potter and Hilliard, 1987; Renaud et al., 2009). Active predation is thought to be related to an increase in the relative size of the brain at the origin of vertebrates (Northcutt and Gans, 1983), which could be similar to the transition from fully parasitic to semi-parasitic represented by flesh-eater species. Our results are consistent with this view, with an increase in relative brain size in some of the members of the family Lampetrinae (flesh-feeding species, **Figure 3.3 B**) in conjunction with more active predatory modes in lampreys (**Figure 3.4**), and a relatively smaller brain in blood-feeding lampreys. However, our analyses found that diet is not a significant driver of brain size in this dataset and requires further investigation in a wider range of species.

3.5.1.3 Encephalization of cyclostomes

Ancestral state reconstruction methods have often been criticised because of their high level of uncertainty, in particular when describing the ancestral state of characters towards the root of

the phylogenetic tree, and because their estimates usually fall within the range of observed values in the data, i.e. they may fail to detect directional trends of trait evolution (Schluter et al., 1997; Pagel, 1999). The reconstruction of the evolutionary history of encephalization presented in this Thesis is not exempt from these caveats (e.g. see confidence intervals in Table 3.4). Therefore, it is recommended that these results should be considered with caution, especially those referring to the most basal nodes of cyclostomes. The reconstruction of the ancestral state of encephalization in agnathans obtained in this analysis suggests that the ancestor of all cyclostomes may have been more highly encephalized than most extant lampreys, and less than extant hagfishes, indicating that none of the extant families of cyclostomes represent the level of encephalization that was present at the origin of this lineage (**Table 3.4**). This supports previous claims based on the organization of the brain that both hagfishes and lampreys constitute derived taxa in relation to their common ancestor, with divergent brain organization, although the main architecture of the brain has remained the same (Northcutt, 1996; 2002). In the case of lampreys, the reconstruction of the ancestral states of encephalization suggests that there was a consistent and progressive reduction of the relative brain size in ancestral lampreys, most of which were apparently anadromous passive predators (**Table 3.4**), which was further incremented in the lineage that gave rise to the genus *Ichthyomyzon*. This substantiates previous views that parasitism may have influenced the relative reduction in brain size (e.g. Striedter, 2005). In contrast, the rise of more active forms of parasitism at the origin of Lampetrinae (flesh-feeding lampreys) may have coincided with an increase in relative brain size in this subfamily. Considering this evidence, and based on the reconstructions of the ancestral life history traits, we hypothesize that the ancestor of extant lampreys may have shared many morphological and behavioural characteristics with extant anadromous, blood-feeding parasitic lampreys, such as members of the Petromyzontidae or Mordaciidae. In contrast, our analysis showed that the encephalization of hagfishes increased in relation to their ancestor. It is possible that this difference may be related to their specialized brain, which possesses a highly-developed olfactory system (reviewed in Ronan and Northcutt, 1998; Collin, 2007), and thus may be reflecting behavioural specializations. It is also relevant that hagfishes are opportunistic feeders and may be considered both scavengers and active predators of invertebrates and even teleost fishes, as described recently by Zintzen et al. (2011; 2013).

In a broader context, large cyclostomes (parasitic lampreys and hagfishes) have a smaller brain relative to their body size than most similarly-sized vertebrates (**Figure 3.6 A**), such as small birds, mammals, cartilaginous fishes and reptiles, although the polygon bounding these data (brain mass to body mass ratio) overlaps other taxa, such as teleost fishes and amphibians. In fact, many species of teleosts fall within the polygon of cyclostomes, such as those from demersal or deep-sea habitats (**Figure 3.6 B**). In addition, it has been shown that any vertebrate taxa containing representatives with an elongated body plan or an “eel-like” shape, such as lampreys, eels, pipefishes, and even salamanders, snakes and slowworms, similarly have a

smaller than expected brain for their body size (Bauchot et al., 1989; van Dongen, 1998; Striedter, 2005), which could explain the relatively reduced brain sizes observed in both cyclostomes and gnathostomes with this body plan. However, a range of species of small-bodied marine fish can be less encephalized than lampreys of similar size, e.g. a few species of pipefishes (Iglesias et al., 2015a; Figure 3.6 A). This suggests that cyclostomes can exhibit relative brain sizes similar to some groups of gnathostomes, and that their primitive condition may not be directly related to the size of their brain.

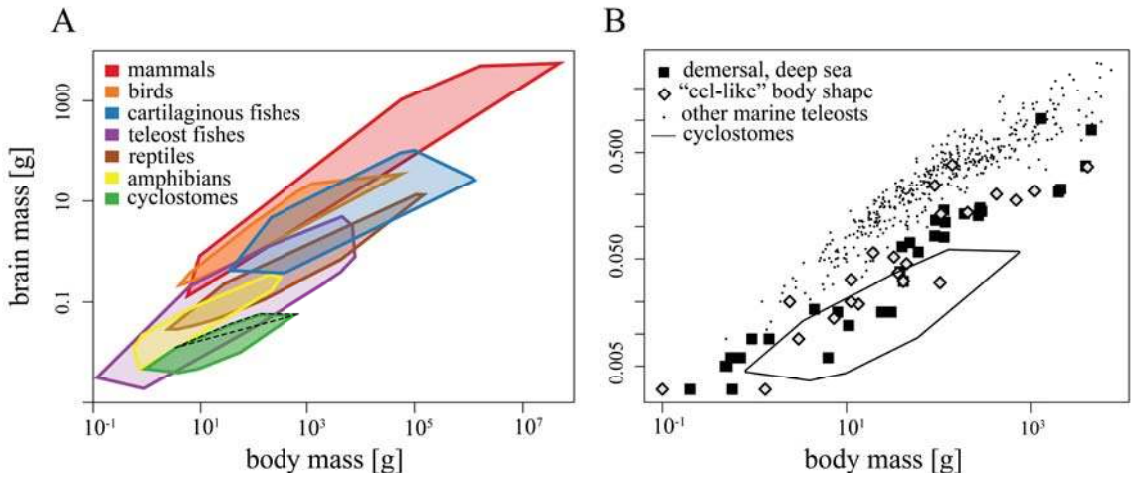

Figure 3.6 | Minimum convex polygons of the relationship between brain mass and body mass in vertebrates. (A) Main taxonomic group trends. Data from Yopak (2012); Iglesias et al., (2015a), after Striedter (2005). The dashed-lined polygon represents cyclostomes as per previous data on three species of lampreys and two species of hagfishes (Stähler, 1982; Platel and Delfini, 1986; Platel and Vesselkin, 1988; 1989). (B) Encephalization in cyclostomes compared to similar-sized marine teleost fishes (data from Iglesias et al., 2015a). Many eel-shaped marine fishes, such as eels, moray-eels, pipefishes and lizardfishes, as well as demersal and deep-sea species, such as scorpionfishes and gobies, shared similar sized brains with cyclostomes (contour).

3.5.2 Conclusions

Body size and parasitism are both significant predictors of brain size in lampreys, but none of the life-history traits analysed in this study (habitat and diet) fully explained the observed residual variance in the relative size of the brain in parasitic lampreys. Nonetheless, the reconstruction of encephalization and the two life history traits of the cyclostomes showed that it is very likely that they have been co-varying during the evolution of this group. Taken together, these results indicate that brain scaling in lampreys, and possibly all cyclostomes more generally, show a similar scaling relationship to those observed in jawed vertebrate groups, suggesting that the evolutionary forces driving brain size may be similar for jawless and jawed vertebrates, and might have been in operation since the advent of vertebrates.

The inclusion of additional species in the study of agnathan brain scaling will allow for the construction of more complex models that incorporate all these life history trait factors

simultaneously and confirm their effect on brain size. Considering that there is a complete genus (*Entosphenus* spp.), and at least eight more species of parasitic lampreys that were not included in this analysis, and an even larger number of non-parasitic species, it is possible that the complete range relative brain sizes in lampreys has not yet been fully realized. In addition, further studies on the largely unexplored hagfishes are much needed to understand the scaling relationships of brain and body size in cyclostomes and how they compare across all vertebrates, to fully reconstruct the evolutionary history of encephalization across vertebrates.

Chapter 4 Patterns of Brain organization in lampreys

4.1 Abstract

A common pattern of brain organization has been described for a range of vertebrate taxa, among which diverse cerebrotypes can be identified and related to a variety of life history characteristics. Patterns of brain organization in agnathans have been studied in only a few species, which does not represent the range of life history types present in this group. This paucity of data about the representatives of the earliest vertebrates severely limits any conclusions that can be drawn about the commonality of brain scaling rules across all vertebrates. This study focused on whether the central nervous system of lampreys (jawless fishes) follows similar scaling rules to jawed vertebrates (with the exception of the cerebellum, which is lacking in agnathans), which are generally considered to be conserved across gnathostomes. Phylogenetic techniques are employed to assess the relative size of six brain structures (olfactory bulbs, telencephalic hemispheres, pineal organ, optic tectum, and the rostral and caudal parts of the medulla oblongata) of post metamorphic adults of 15 species of lampreys. Significant differences in the relative size of specific brain structures, such as the optic tectum and pineal organ, is revealed in addition to differences in the scaling relationships of the olfactory bulbs. The olfactory bulbs show a predictable scaling relationship with the rest of the brain, but show a degree of variation between families of lampreys. In contrast to many groups of gnathostomes, the olfactory bulbs of lampreys also show a steeper scaling rule than that of the telencephalic hemispheres, and are the only brain structures to scale hyperallometrically with the rest of the brain. Despite these notable differences in scaling amongst brain structures, a number of patterns in the relative size of brain structures in lampreys closely parallel the cerebrotypes found in gnathostomes, which suggest convergent patterns of brain organization associated with different aquatic niches. Reconstructions of the ancestral states of brain organization indicate that both *Mordacia* and *Ichthyomyzon* have retained a number of ancestral brain characteristics, such as a relatively larger medulla oblongata and pineal organ, which suggests that the last common ancestor of all lampreys possessed characteristics of an anadromous passive predator.

4.2 Introduction

Various comparative studies of the organization of the vertebrate brain (comprised of the olfactory bulbs, telencephalon, diencephalon, mesencephalon, medulla oblongata and, for gnathostome vertebrates, the cerebellum) have shown that a number of neural specializations

associated with motor and sensory modalities have evolved in relation to their ecological niche and may reflect specific behaviours (Bauchot et al., 1989; Barton and Dean, 1993; de Winter and Oxnard, 2001; Wagner, 2001; Iwaniuk et al., 2004; Yopak et al., 2007; Lisney et al., 2008; Iwaniuk et al., 2010; Liao et al., 2015). These correlations have led to the proposal that the relative size of a brain structure may evolve independently of the rest of the brain in a "mosaic" fashion (e.g. Barton and Harvey, 2000; de Winter and Oxnard, 2001; Iwaniuk et al., 2004). On the contrary, the same neural specializations have also been interpreted as a residual variation from scaling relationships with overall brain size that are widely conserved across gnathostomes (Gonzalez-Voyer et al., 2009; Yopak et al., 2010; Liao et al., 2015), reflecting both phylogenetic and developmental constraints (Finlay and Darlington, 1995; Darlington et al., 1999; Striedter, 2005; Yopak et al., 2010; Charvet et al., 2011). This model predicts that these patterns of brain organization result from conservative developmental events, where the scaling rules for each brain structure are determined by a conserved order of neurogenesis, in which larger brain structures cease neural proliferation later during development, i.e. "late equals large". The observed variability in brain organization is thus explained in terms of the "concerted" scaling of brain structures with changes in absolute brain size during evolution (Finlay and Darlington, 1995; Finlay et al., 2001).

This concerted pattern of brain structure scaling has been described for the majority of gnathostome vertebrates, such as cartilaginous and teleost fishes, amphibians and mammals (Finlay and Darlington, 1995; Gonzalez-Voyer et al., 2009; Yopak et al., 2010; Liao et al., 2015). Many of these studies show that some brain structures, such as the cerebellum and the telencephalon, scale hyperallometrically to the rest of the brain, with a slope exceeding 1.0, whereas others, such as the medulla, show shallower slopes, potentially as a consequence of a larger rate of growth and differentiation earlier in development. However, this model has often been criticized, such that it masks differences in scaling between lower taxonomic levels, does not take into account potential grade shifts (differences in intercept), and overgeneralizes on developmental processes across species (Clark et al., 2001; Weisbecker, 2009; Weisbecker, 2010; Willemet, 2012). Indeed, it is now largely accepted that some brain structures, such as the olfactory bulbs, do not conform to a concerted pattern of evolution in all gnathostomes and maintain a high degree of statistical independence from overall brain size (Finlay and Darlington, 1995; Reep et al., 2007; Gonzalez-Voyer et al., 2009; Yopak et al., 2010; Gutierrez-Ibanez et al., 2014; Liao et al., 2015; Yopak et al., 2015).

Despite these opposing models regarding the constraints and modularity in the evolution of brain organization across vertebrates (Striedter, 2005; Willemet, 2012), it has been suggested that the size of different brain structures may evolve under both concerted and mosaic processes (Gonzalez-Voyer et al., 2009; Gutierrez-Ibanez et al., 2014; Herculano-Houzel et al., 2014), where common scaling rules would apply to specific (smaller) clades, in which specialized

structures may arise as “grade shifts” or changes in the relative size of singular structures or functionally related subsystems with respect to diverse life history traits (Barton and Harvey, 2000; Striedter, 2005; Yopak et al., 2010; Anderson and Finlay, 2014; Smaers and Rohlf, 2016). In this regard, the concept of various cerebrotypes has received support, whereby species from different clades that share certain life history characteristics possess similar patterns of brain organization, demonstrating both concerted tendencies and grade shifts between taxa (Clark et al., 2001; Iwaniuk and Hurd, 2005; Willemet, 2012; 2013).

Variation in both encephalization (relative brain size) and brain organization have been described during ontogeny of a parasitic lamprey species, where “cerebrotypes” were identified within distinct phases of the life cycle of a single species and reflected ecological parameters (Chapter 2, Salas et al., 2015). Similarly, lamprey species have been found to share a common pattern of encephalization, although diverse life history types are correlated with marked differences in relative brain size (Chapter 3). Morphological characteristics of the brain have been extensively described for a few species of lampreys (Johnston, 1902; Heier, 1948; Nieuwenhuys, 1977; Northcutt, 1981; Wicht, 1996; Weigle and Northcutt, 1998; Khonsari et al., 2009; Salas et al., 2015), and revealed interspecific variability of brain composition and brain subdivision scaling (Ebinger et al., 1983; Platel and Vesselkin, 1989). However, interspecific scaling of brain subdivisions in agnathans has only been performed on five species and is therefore not necessarily representative of the taxonomic and ecological diversity within this group. Thus, it is possible that true interspecific variation in brain organization in lampreys has been largely overlooked. Understanding brain structure scaling in agnathans provides a unique opportunity to test whether the patterns of brain-body and brain structure scaling mirror those previously found for gnathostomes, and establish whether these scaling rules are conserved across all vertebrates. In this study, the morphological variation and patterns of brain organization are characterized in 15 species of lampreys to test the hypotheses that common patterns of brain structure scaling are found between agnathan (jawless) and gnathostome (jawed) vertebrates, and whether brain organization of lampreys can be characterized by distinct cerebrotypes.

4.3 Methods

All procedures were performed in accordance with the ethical guidelines of The University of Western Australia Animal Ethics Committee - Research Project RA/3/100/917.

4.3.1 Data collection

We collected volumetric data on the brain from 48 specimens of adult lampreys, representing 15 species, whose relative brain size had been analysed in relation to encephalization among

lampreys (Chapter 2; Chapter 3; Salas et al 2015). Ecological data for each species (parasitism, habitat, diet) were gathered from various sources (Renaud et al., 2009; Renaud, 2011; Potter et al., 2014; Potter et al., 2015). A summary per species can be found in **Table 4.1**.

species	code	family	subfamily	n	Brain [µL]	OB [µL]	Te [µL]	PO [µL]	OT [µL]	MOR [µL]	MOC [µL]	parasitism	habitat	diet
Lampetra planeri	Lpla	P	L	4	7.22	0.98	0.82	0.02	0.85	1.23	1.5	NP	-	-
Lampetra fluviatilis	Lflu	P	L	3	17.86	2.9	1.11	0.07	2.19	3.11	3.52	P	AFW	F
Lethenteron appendix	Lape	P	L	2	12.21	1.58	1.08	0.04	1.88	1.48	3.81	NP	-	-
Lethenteron camtschaticum	Lcam	P	L	1	24.23	4.23	0.93	0.09	2.75	5.31	3.55	P	AFW	F
Lampetra richardsoni	Lric	P	L	1	4.34	0.55	0.26	0.01	0.33	0.47	0.91	NP	-	-
Tetrapleurodon geminis	Tgem	P	L	1	8.88	0.8	0.99	0.03	0.96	1.41	1.96	NP	-	-
Caspiomyzon wagneri	Cwag	P	P	6	24.26	4.51	1.58	0.04	1.35	4.64	4.69	P	A	PG
Petromyzon marinus	Pmar	P	P	4	33.52	5.59	2.99	0.06	3.05	6.29	5.49	P	AFW	B
Ichthyomyzon unicuspis	Iuni	P	P	3	9.07	1.1	0.35	0.06	0.49	1.82	1.99	P	FW	B
Ichthyomyzon greeleyi	Igre	P	P	1	3.57	0.45	0.31	0.03	0.31	0.76	0.92	NP	-	-
Ichthyomyzon castaneus	Icas	P	P	4	4.22	0.52	0.42	0.04	0.34	1.07	1.24	P	FW	B
Geotria australis	Gaus	G	G	11	26.33	2.78	1.33	0.05	3.00	4.13	3.93	P	A	F
Mordacia lapicida	Mlap	M	M	1	8.40	0.51	0.45	0.04	0.97	1.37	1.75	P	A	B
Mordacia praecox	Mpra	M	M	4	5.84	0.22	0.33	0.04	0.45	1.05	1.24	NP	-	-
Mordacia mordax	Mmor	M	M	2	16.55	1.77	1.06	0.11	1.52	3.47	3.57	P	A	B

Table 4.1 | Average brain and brain structure volume for each species. Family: P: Petromyzontidae; G: Geotriidae; M: Mordaciidae. Subfamily: L: Lampetrinae P: Petromyzontinae; G: Geotriinae; M: Mordaciinae. Parasitism: NP: non-parasitic; P: parasitic. Habitat: A: anadromous; AFW: anadromous and freshwater; FW: freshwater. Diet: F: flesh; B: blood; PG: parasitic generalist. For other abbreviations, see List of Abbreviations.

Even though we have previously shown that there are significant differences in brain organization among diverse stages of the adult phase of lampreys (Chapter 2), this Chapter presents data on all available post-metamorphic specimens in order to increase sample size, which is critical to obtain a better estimation of diverse parameters of the linear models (Freckleton et al., 2002).

Measurements of the volumes of six brain structures were taken following a protocol established earlier for lampreys (Salas et al., 2015). Briefly, photographs of the lateral and dorsal views of each brain were taken using a Leica EC3 camera attached to a Nikon SMZ-745T dissecting microscope. The length, height, and width of the olfactory bulbs (OB), telencephalic hemispheres (Te), the pineal organ (PO), the optic tectum (OT), the rostral end of the medulla oblongata (MOR); defined as the anterior region of the rhombencephalon comprising the V–VIII nerves), and the caudal end of the medulla oblongata (MOC); defined as the posterior region of the rhombencephalon comprising the IX–XII nerves) were measured using ImageJ (Rasband, 1997). Volumes were estimated using the ellipsoid method, which approximates the volume of a structure by assuming it takes the shape of an idealized ellipsoid, or a fraction of it, as described previously (Huber et al., 1997; Wagner, 2001; Salas et al., 2015). In the case of measurements taken in one hemisphere of the brain (i.e. OB, Te, and OT), the values of the volumes were doubled. In order to make the results comparable to previous studies of brain scaling of both jawless and jawed vertebrates, the sum of the estimated volumes of the MOR and MOC was considered as the total volume of the medulla oblongata (MO). Total brain volume was calculated from total brain mass using the estimated density of the brain tissue, d = 1.036 mg/mm^3 (Stephan, 1960). Volume estimates were not corrected for preservation media.

The phylogenetic relationships between species were established by constructing a bootstrapped neighbour-joining phylogenetic tree with a p-distance model using the software Mega 4 (Tamura et al., 2007), based on sequences of mitochondrial cytochrome *b* of 35 species of lampreys and two species of hagfishes (out-group), which were obtained from previous phylogenetic studies of lampreys (Lang et al., 2009; Potter et al., 2015). The species that were not available for this study were pruned from the tree (**Figure 4.1**).

4.3.2 Data analyses

Quantitative differences in brain structure scaling were examined using averaged values of volume per species to delineate a number of aspects of brain organization in lampreys (n=15). In addition, the morphological variability of the brain was assessed qualitatively through visual inspection of all specimens (n=48). The patterns of brain structure scaling in lampreys were studied in relation to the rest of the brain, as well as to each other using a multivariate analysis of absolute volumes. In these calculations, the parameters were obtained using species averages when more than one specimen was available per species. To examine the existence of

cerebrotypes in lampreys, we characterized the clustering of lampreys in a multivariate analysis of the relative size of each brain structure, from which an ancestral state of brain organization was estimated. The ancestral states of morphological characters were also estimated.

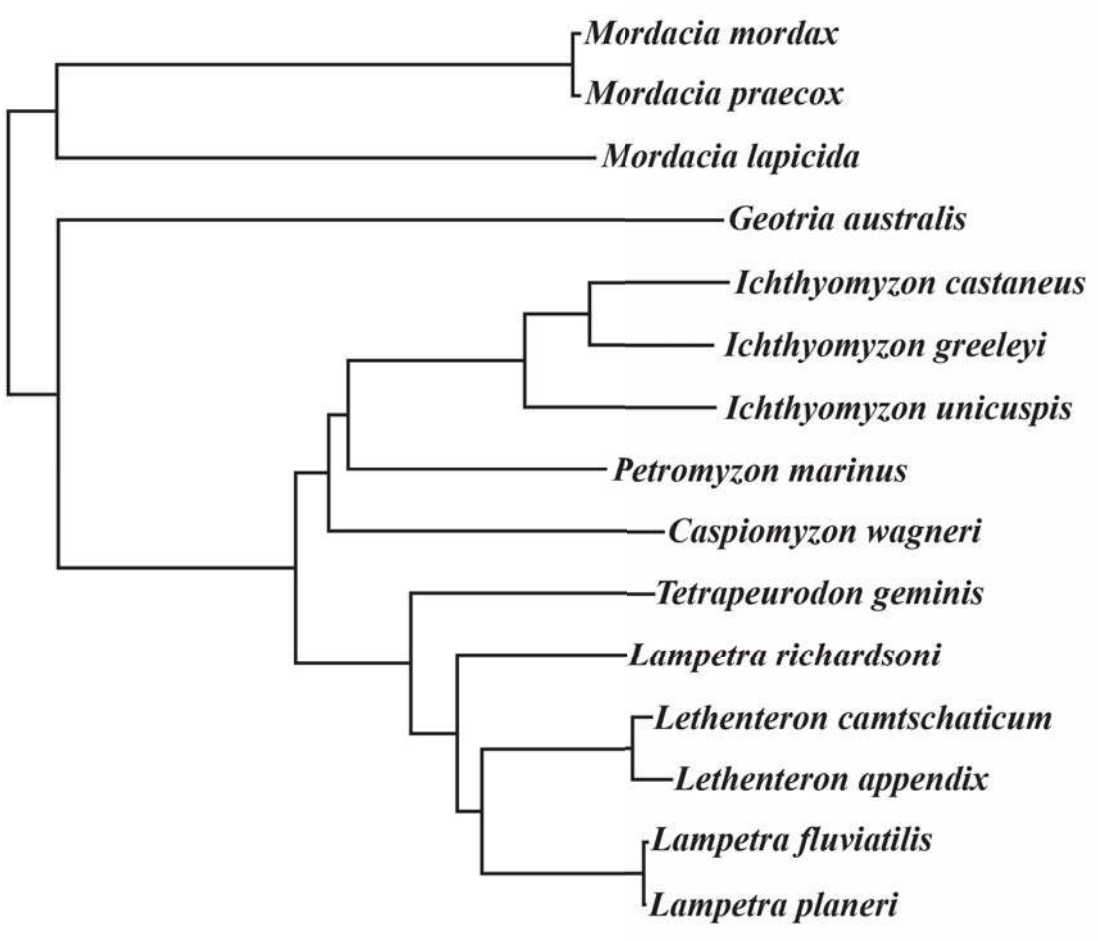

Figure 4.1 | Neighbour-joining phylogenetic tree of the species examined in this study, based on sequences of mitochondrial cytochrome *b* of 35 species of lampreys and two species of hagfishes. Data obtained from Lang et al. (2009) and Potter et al. (2015).

4.3.2.1 Brain structure scaling

All calculations were performed using the R software package (R Core Team, 2013b). In order to examine the scaling of brain subdivisions compared to the rest of the brain in lampreys, data were amplified by a factor of 1000 and $\log_{10}$-transformed to obtain positive values of the transformed variables and improve normality. Linear models were constructed with the volume of each brain structure compared to the rest of the total brain volume minus the volume of the corresponding brain structure (Deacon, 1990; Iwaniuk et al., 2010), hereafter simply referred to as brain size.

Three distinct models of evolutionary change were fitted to describe the scaling of each brain structure with brain size, as described earlier (Chapter 3). Briefly, each evolutionary model was constructed as a phylogenetic generalized least squares (pGLS) model (reviewed in Paradis,

2012; Garamszegi, 2014), which accounts for the covariance of traits in phylogenetically-related species. pGLS models were implemented with the function *gls* (Pinheiro et al., 2015), where each evolutionary model was fitted with a correlation structure, which was available from the package *ape* (Paradis et al., 2004). *corPagel* was used to estimate the parameter lambda (λ) via maximum likelihood (model 1), which tests the departure from a Brownian motion (BM) model of evolution, where trait divergence accumulates stochastically over time. When a maximum likelihood estimation of λ was not possible, a set of 11 models was constructed, each fitted with fixed values of λ ranging from zero to one, where the model with the largest log likelihood was chosen. We also fitted models with fixed values of λ for every structure, at λ=1 (model 2), which represents an unconstrained BM model and at λ=0 (model 3), which simulates the evolution of traits independent of phylogeny. In each of these pGLS models, the variance was fixed with weights calculated as the diagonal of the corresponding variance-covariance matrix of the tree, to control for differing variances among species obtained from non-ultrametric trees, such as neighbour-joining trees (Paradis, 2012). For each of the structures examined, the best pGLS model was determined using the second-order Akaike Information Criterion (AICc), where the best model had the lowest AICc score (Barton, 2014). When linear models showed a difference of less than two units (ΔAICc < 2), AICc weights were employed instead to define the best model of brain scaling (Burnham and Anderson, 2002). In order to graphically represent the differences between categories in each life history trait, we used standardized residuals from the selected model, i.e. orthogonal deviations from the predicted slope that are divided by the square-root of the residual variance.

Distinct scaling relationships for each family were observed from the brain structure – brain size plots, which reflected the marked differences in the morphology of the brain of lampreys between taxa. However, this interaction could not be assessed using a pGLS model due to a low sample size within families (Freckleton et al., 2002). Therefore, in order to evaluate the significance of the observed differences in the scaling of brain structure with brain size between taxa of lampreys, an analysis of covariance (ANCOVA) was used, with family or subfamily used as a factor, and with no phylogenetic correction. All data points (not averages) were used to increase the sample size. Coefficients of determination were calculated with this dataset (see Appendix A).

4.3.2.2 Phylogenetic multivariate analyses

A multivariate approach was employed to analyse the variation in the size of each brain structure in relation to all other structures. We performed a phylogenetic principal component analysis (pPCA) using the function *phyl.pca* (Revell, 2012), which allows for a simultaneous maximum likelihood estimation of the parameter λ to test departure from a BM model of evolution. pPCAs were run using the covariance matrix on two different datasets: (1) $\log_{10}$-

transformed, absolute brain structure volume, where the first principal component (PC1) represents a variable that is isometric with brain size (Finlay and Darlington, 1995). In this case, the ratio between the loadings of any two variables in PC1 correspond to the allometric bivariate coefficient of those variables (Klingenberg, 1996), which has been interpreted as evidence of concerted evolution (allometric bivariate coefficient = 1) or mosaic evolution (allometric bivariate coefficient ≠ 1) between that pair of variables (e.g. Gonzalez-Voyer et al., 2009; Gutierrez-Ibanez et al., 2014). (2) A pPCA was also performed using the relative volume of each structure, calculated as a fraction of the sum of the volume of all six brain structures measured within a species, where structure proportions (relative volumes) were normalized using the arcsine square root transformation prior to analysis (Wagner, 2001; Lisney et al., 2007; Salas et al., 2015). A pPCA on the relative size of each brain structure allows for the clustering of the species in multidimensional space, and therefore the characterization of the patterns of brain organization or cerebrotypes independent of brain size (Clark et al., 2001; Iwaniuk and Hurd, 2005; Lisney et al., 2008). In addition, a phylomorphospace plot, i.e. a projection of the phylogenetic tree into the morphospace, was produced with the values of the first three principal components from the pPCA run on relative brain structure size, using the homonym function available in the R package *phytools* (Revell, 2012) in order to graphically represent the patterns of brain organization in the extant species of lampreys. A cluster analysis based on the Ward method was applied to the values of the first four principal components to describe general cerebrotypes available for lampreys. The Ward method computed clusters in the Euclidean space, which is also the reference space in multivariate ordination methods, such as PCA. Since both PCA and clustering methods such as Ward's are fit to the data using the same mathematical principle (sums of squares, or variance), it is likely that the Ward method will delineate clusters that visually correspond to regions of high density of points in PCA ordination (Murtagh and Legendre, 2014). The significance of each cluster was assessed with approximately unbiased (AU) p-values obtained from multiscale bootstrap resampling after 10000 samples using the package *pvclust* (Suzuki and Shimodaira, 2006).

4.3.2.3 Reconstruction of ancestral states

The ancestral state of brain organization in lampreys was estimated using the function *fastAnc* (Revell, 2012), from the scores per species of the first four principal components calculated with pPCA on relative volumes. Subsequently, we compared the Euclidean distances between the ancestral state of lampreys and each extant species, where the minimum distance corresponded with the species of lamprey with the most similar brain organization.

In order to estimate the ancestral state of morphological characters, the variability observed in the olfactory bulbs and the pineal stalk between families of lampreys (see results) was coded into discrete characters. Since these characters co-varied in all examined specimens, the same

three states were considered for both the olfactory bulbs and the pineal organ, where 1 = no displacement (Mordaciidae); 2 = late displacement (Geotriidae, Salas et al., 2015) and 3 = early displacement (Petromyzontidae). The ancestral state of these discrete characters at each internal node of the phylogenetic tree was estimated with stochastic character mapping under an equal rates model (Huelsenbeck et al., 2003; Bollback, 2006; Revell, 2012). This model assumes identical probabilities of each state of the character as a prior, from which posterior probabilities were inferred after 500 simulations. Since the concept of paired species of lampreys must be considered from pair to pair (Docker, 2009), for this analysis, the ancestral states were obtained exclusively from the phylogenetic relationships between parasitic species, which have been hypothesized to constitute the original lineages of lampreys (Hardisty and Potter, 1971b; Bartels et al., 2015). We estimated the ancestral state of morphological characters in a tree of 14 species of parasitic lampreys, which represents approximately 80% of all recognized species of parasitic lampreys (Potter et al., 2015). We assigned a state of 3 to four species of Petromyzontidae (*Ichthyomyzon bdellium*, *Entosphenus tridentatus*, *Eudontomyzon morii* and *Eudontomyzon danfordi*) based on their closest relatives, given that this was the condition observed in all examined species of this family.

4.4 Results

4.4.1 Gross morphology of the brain

In general terms, the brains of all species examined matched previously described characteristics of the brain of adult lampreys, e.g. the presence of well-developed ventricular and choroidal plexus systems (Johnston, 1902; Wicht, 1996; Nieuwenhuys and Nicholson, 1998). The main subdivisions of the brain, i.e. olfactory bulbs, telencephalon, diencephalon, mesencephalon and rhombencephalon, were readily identified in all species, although an externally identifiable cerebellum (Wicht, 1996; Weigle and Northcutt, 1998; Montgomery et al., 2012) was not found in any of the species examined. Moderate variability was observed in the overall shape of the brain of adult lampreys; most of the brains were characterised by a slender shape, which is particularly evident in the Caspian lamprey *Caspiomyzon wagneri* (**Figure 4.2 I**). However, the brain of other species of lampreys were considered more compressed, showing an expansion in the medio-lateral axis, e.g. the Mexican brook lamprey *Tetrapleurodon geminis* (**Figure 4.2 O**).

There was also morphological variation within the brain structures examined. One of the most striking differences at the gross level was found in the telencephalon, which showed different morphological patterns with brain size across families of lampreys (**Figure 4.3**). In the most basal species of lampreys (Mordaciidae), along the range of total brain sizes examined (brain size = 6.0 – 17.2 mg, body size = 11.9 – 37.3 cm), both the olfactory nerves and the olfactory

bulbs of each hemisphere were adjacent to each other, close to the midline (**Figure 4.2 A-C** and **Figure 4.3 A**). However, in more derived species, such as the members of both the Geotriidae and Petromyzontidae, the olfactory bulbs and olfactory nerves in all adults were displaced laterally (e.g. **Figure 4.2 D-O**, **Figure 4.3 B-C**). Correlates of these morphological differences were observed in other regions, such as the telencephalic or cerebral hemispheres, and the *fissura circularis*, which marks the separation between the olfactory bulbs (Nieuwenhuys and Nicholson, 1998). All the species with laterally displaced telencephalic hemispheres showed a pronounced groove at the level of the *fissura circularis*, whereas in the Mordaciidae, although it was also present, the groove was largely reduced in comparison to both the Geotriidae and Petromyzontidae (**Figure 4.3**).

Another source of morphological variability at the family level was found in the pineal stalk, which connects the pineal complex with the brain (Eddy, 1971; Nieuwenhuys and Nicholson, 1998). Our results are consistent with previous descriptions that reveal the length of this structure increases throughout development, as the pineal complex migrates anteriorly, especially after metamorphosis (Scott, 1887; Eddy and Strahan, 1970). Therefore, larger specimens generally had longer pineal stalks and consequently more displaced pineal organs (e.g. **Figure 4.2 H**, **K**). However, in Mordaciidae, the pineal stalk was shorter at all brain sizes; hence the pineal organ in this group was comparatively closer to the brain than in any other family of lampreys, even in large specimens (**Figure 4.2 A-C**, **Figure 4.3 A**). A similar pattern in the pineal stalk and the telencephalon was previously observed in downstream migrants (early metamorphosed) of the pouched lamprey *Geotria australis* (Salas et al., 2015), but not in the upstream migrants (**Figure 4.3 B**).

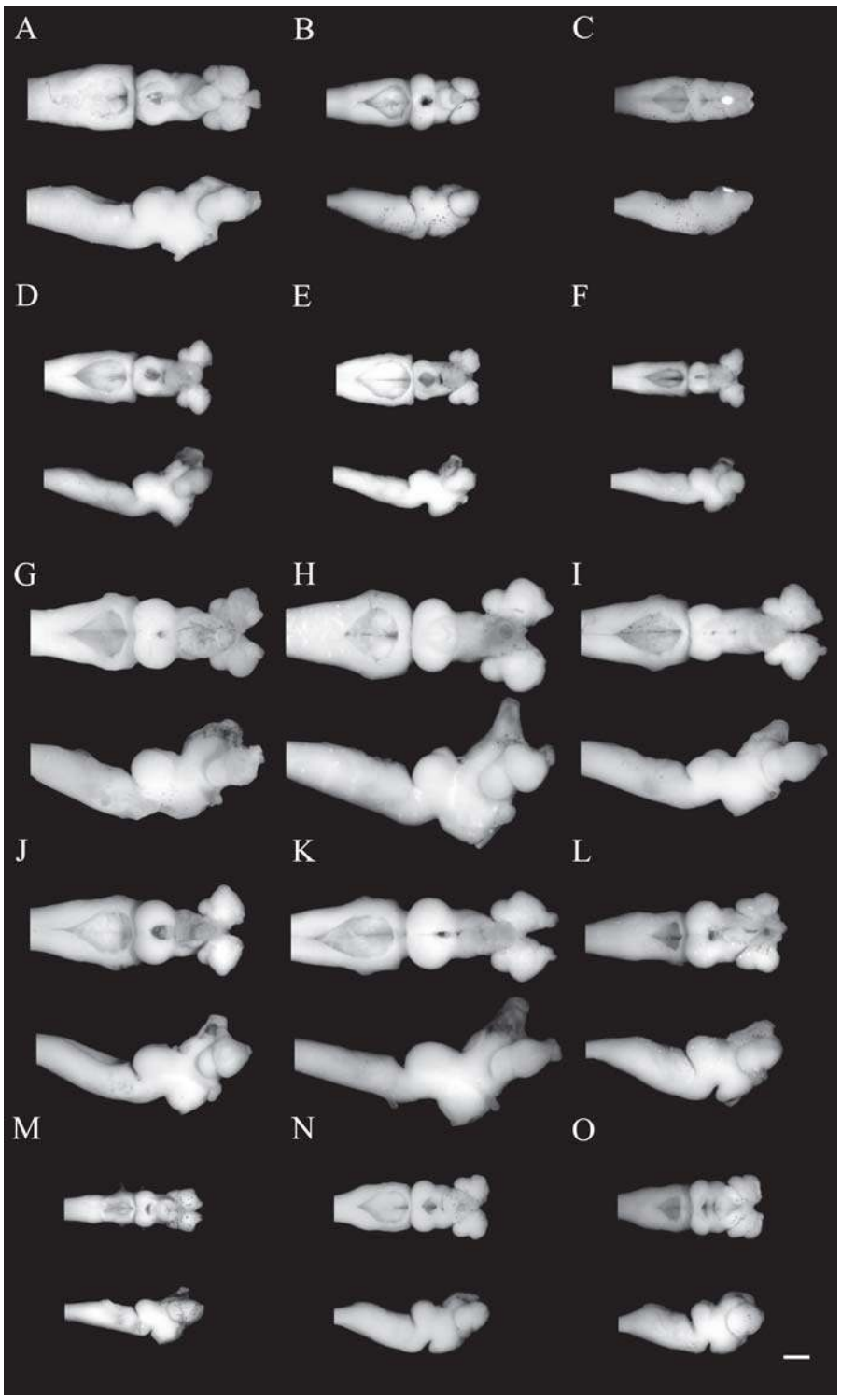

Figure 4.2 | Dorsal and lateral views of representative brains of each species examined in this study. (A) the short-headed lamprey, *Mordacia mordax*; (B) the Chilean lamprey, *Mordacia lapicida*; (C) the precocious lamprey, *Mordacia praecox*; (D) the silver lamprey, *Ichthyomyzon unicuspis*; (E) the chestnut lamprey, *Ichthyomyzon castaneus*; (F) the mountain brook lamprey, *Ichthyomyzon greeleyi*; (G) the pouched lamprey, *Geotria australis*; (H) the sea lamprey, *Petromyzon marinus*; (I) the Caspian lamprey, *Caspiomyzon wagneri*; (J) the European river lamprey, *Lampetra fluviatilis*; (K) the arctic lamprey, *Lethenteron camtschaticum*; (L) the American brook lamprey, *Lethenteron appendix*; (M) the western brook lamprey, *Lampetra richardsoni*; (N) the European brook lamprey, *Lampetra planeri*; (O) the Mexican brook lamprey, *Tetrapleurodon geminis*. Scale bar = 1 mm.

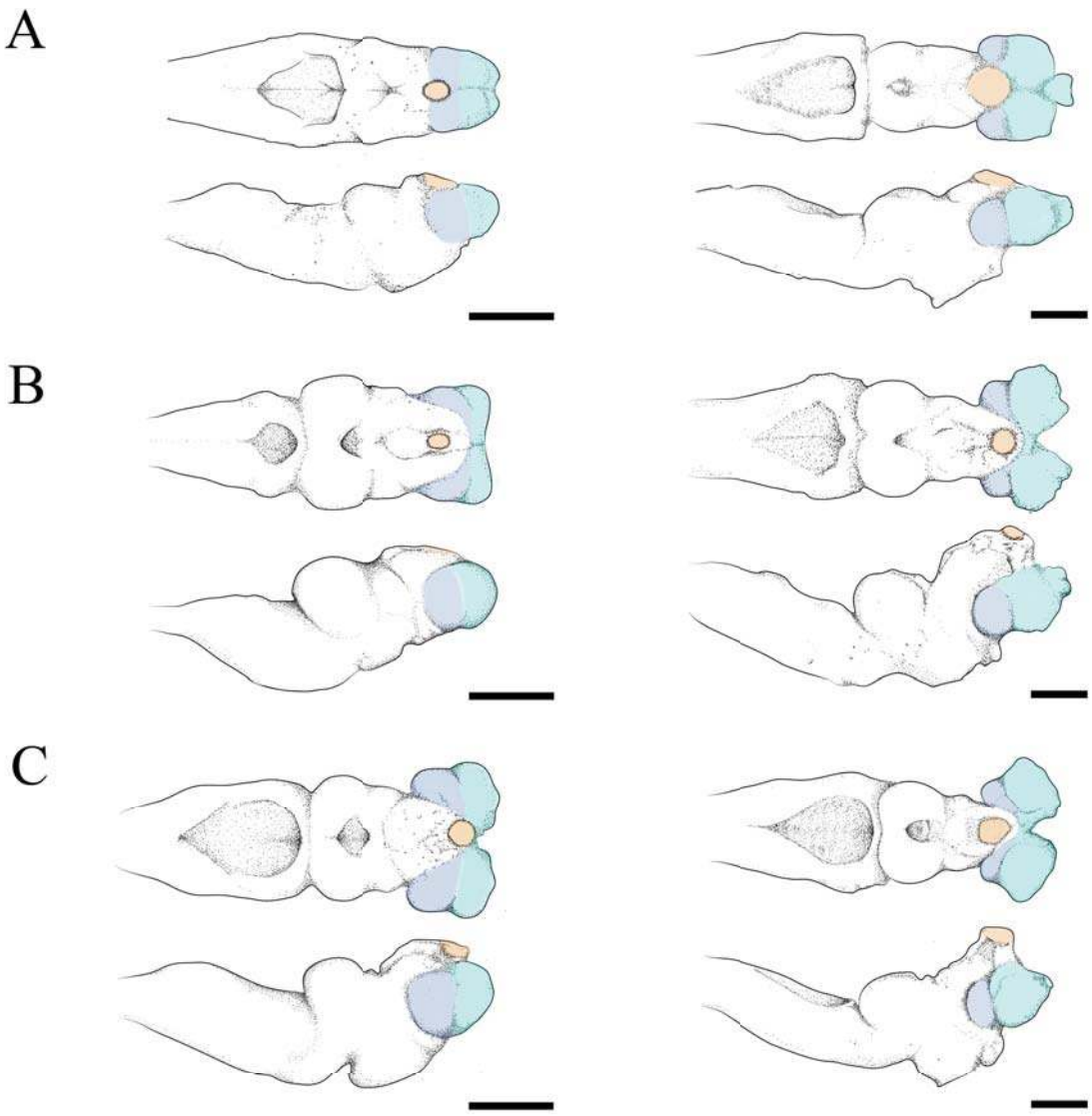

Figure 4.3 | Variation of morphological patterns with increasing brain size. Dorsal and lateral views of the brain of adult (postmetamorphic) lampreys, which suggest specific patterns of growth of the pineal organ (orange), telencephalic hemispheres (purple), and olfactory bulbs (green) in each of the three families of lampreys: (A) Mordaciidae (left: *Mordacia praecox*, right: *Mordacia mordax*); (B) Geotriidae (left: downstream migrant *Geotria australis*, right: upstream migrant *Geotria australis*); (C) Petromyzontidae (left: *Lampetra planeri*, right: *Lampetra fluviatilis*). See Figure 4.2 for more examples. Scale bars = 1mm.

4.4.2 Brain structure scaling

We examined the scaling of each brain structure with the rest of the brain of lampreys using averaged volumes per species and taking into consideration the phylogenetic relationships between species, fitting three different models of evolution. Model selection (Akaike Information Criterion) indicated differing patterns of evolution in the studied structures. Considering the selected models, only the pineal organ (PO) showed a strong association with phylogeny (λ=1), whereas in other structures, such as the telencephalic hemispheres (Te) and

the rostral (MOR) and caudal (MOC) areas of the medulla oblongata, the values of lambda in this dataset (λ=0) suggest evolution independent of the phylogeny. In contrast, the olfactory bulbs (OB) and the optic tecta (OT) exhibited intermediate values of lambda, suggesting a degree of independence from phylogeny (**Table 4.2**).

We found differing relationships between each brain structure and overall brain size. The OB possessed the highest slope of all six examined structures, being the only structure of the brain to show hyperallometric scaling with the rest of the brain (slope = 1.35, CI = 1.08 – 1.61). The PO, OT and MOR all showed nearly isometric scaling with brain size, whereas the value of the slope in the Te, and especially MOC, indicated a hypoallometric scaling of these structures with brain size (**Table 4.2**). When the values for the MOR and MOC were added together (MO), this brain structure scaled hypoallometrically with the rest of the brain (slope = 0.83, CI = 0.69 – 0.97).

The residuals obtained for each selected model were different between the various life history traits of lampreys, which are summarized in **Figure 4.4**. The most remarkable differences between parasitic and non-parasitic species were found in the Te, where non-parasitic species had, on average, a larger relative Te size, and in the MOR, which was more developed in the large parasitic species. Anadromous species had, on average, the smallest OB and Te in comparison to both anadromous-freshwater and purely freshwater species. Blood-feeding lampreys had a larger PO and MOR, whereas flesh-feeding species possessed a relatively larger OT. We found that some of these differences can be attributed to phylogeny; all species of the Petromyzontidae had OB above the average value, but that was not always accompanied of a larger Te. The Geotriinae and Lampetrinae (flesh-feeding lampreys) had a relatively larger OT, whereas the analysis of Mordaciinae and Petromyzontinae revealed relative values of OT on or below the average, while both the Geotriidae and Mordaciidae have on average smaller OB and Te.

The correlations between residuals for selected structures are shown in **Figure 4.5**. Neither Mordaciidae nor Geotriidae possess species with a relatively larger OB or Te, whereas all species of the Petromyzontidae had OB above the average value (Figure 4.4). Nonetheless, the size of the Te was not strongly correlated with the size of the OB (**Figure 4.5 A**), or the size of the OT (**Figure 4.5 B**). A larger PO was common in blood-feeding species (Figure 4.4), which was generally associated with a smaller OT (**Figure 4.5 C**) and a larger MO (**Figure 4.5 D**).

	Structure					
Parameter	OB	Te	PO	OT	MOR	MOC
Slope	1.35***	0.85***	1.08***	1.04***	1.01***	0.76***
CI	1.08 – 1.61	0.59 – 1.11	0.98 – 1.17	0.84 – 1.24	0.82 – 1.21	0.62 – 0.89
Intercept	-2.36***	-0.53	-2.75***	-1.13*	-0.72+	0.39
CI	-3.43 – -1.29	-1.58 – 0.51	-3.23 – -2.27	-1.96 – -0.31	-1.50 – 0.06	-0.15 – 0.92
Model summary						
λ	0.6	0 †	1	0.8 †	0 †	0 †
d.f. residuals	13	13	13	13	13	13
AICc	-1.4	-6.7	-21.3	-10.8	-15.5	-24.9
ΔAICc	1.61	26.67	16.32	0.88	2.68	27.56
AICc weights	0.694	1	1	0.495	0.791	1

Table 4.2 | Parameters of brain structure scaling against the remainder of the brain. For abbreviations, see List of Abbreviations. (***) p-value < 0.001, (**) 0.001 < p-value < 0.01, (*) 0.01 < p-value < 0.05, (+) 0.05 < p-value < 0.1, ( ) p-value > 0.1; † Models chosen based on log likelihood.

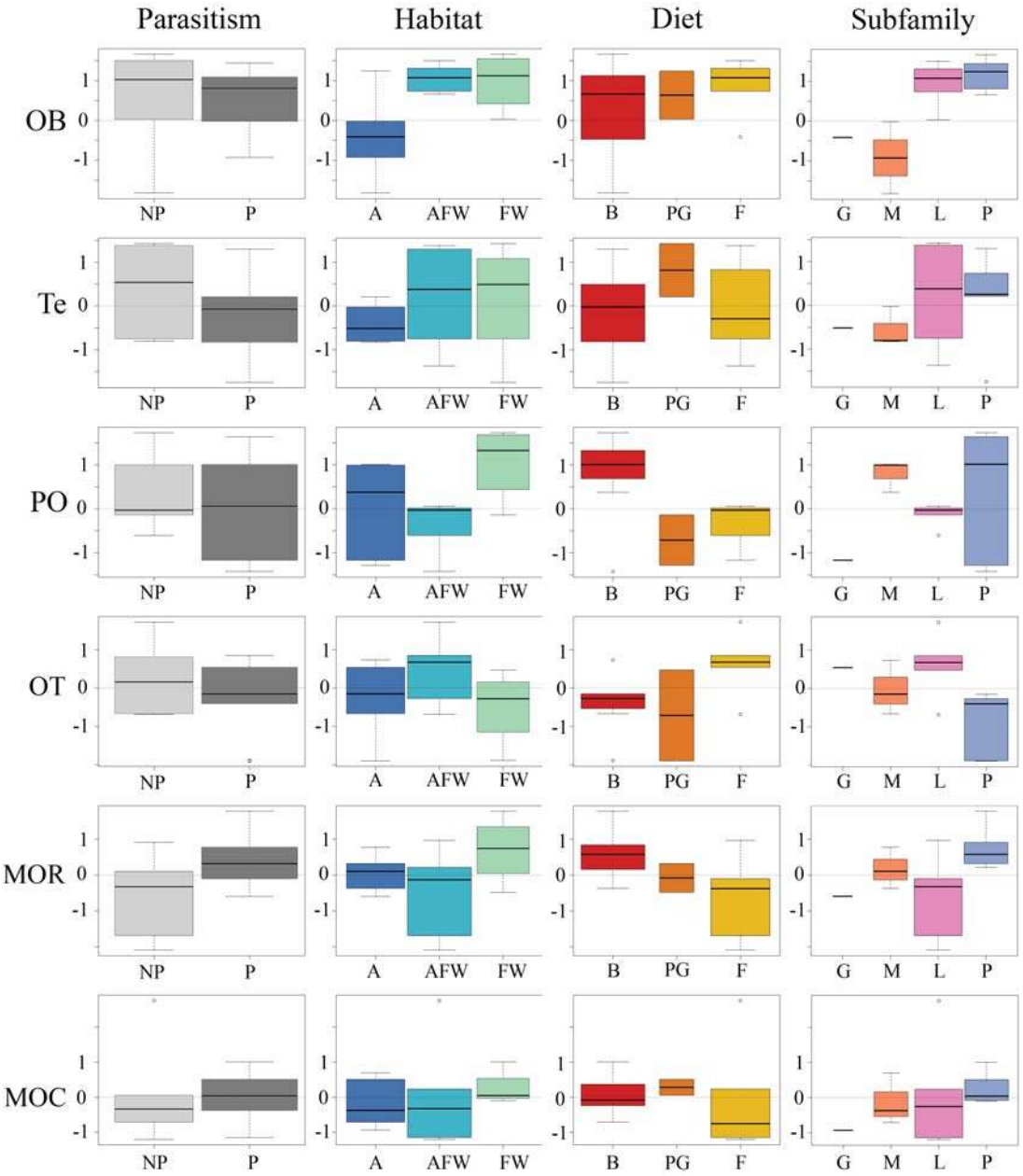

Figure 4.4 | Residuals compared amongst categories per life history trait. For the parameters of these relationships, see Table 4.2. For abbreviations, see List of Abbreviations.

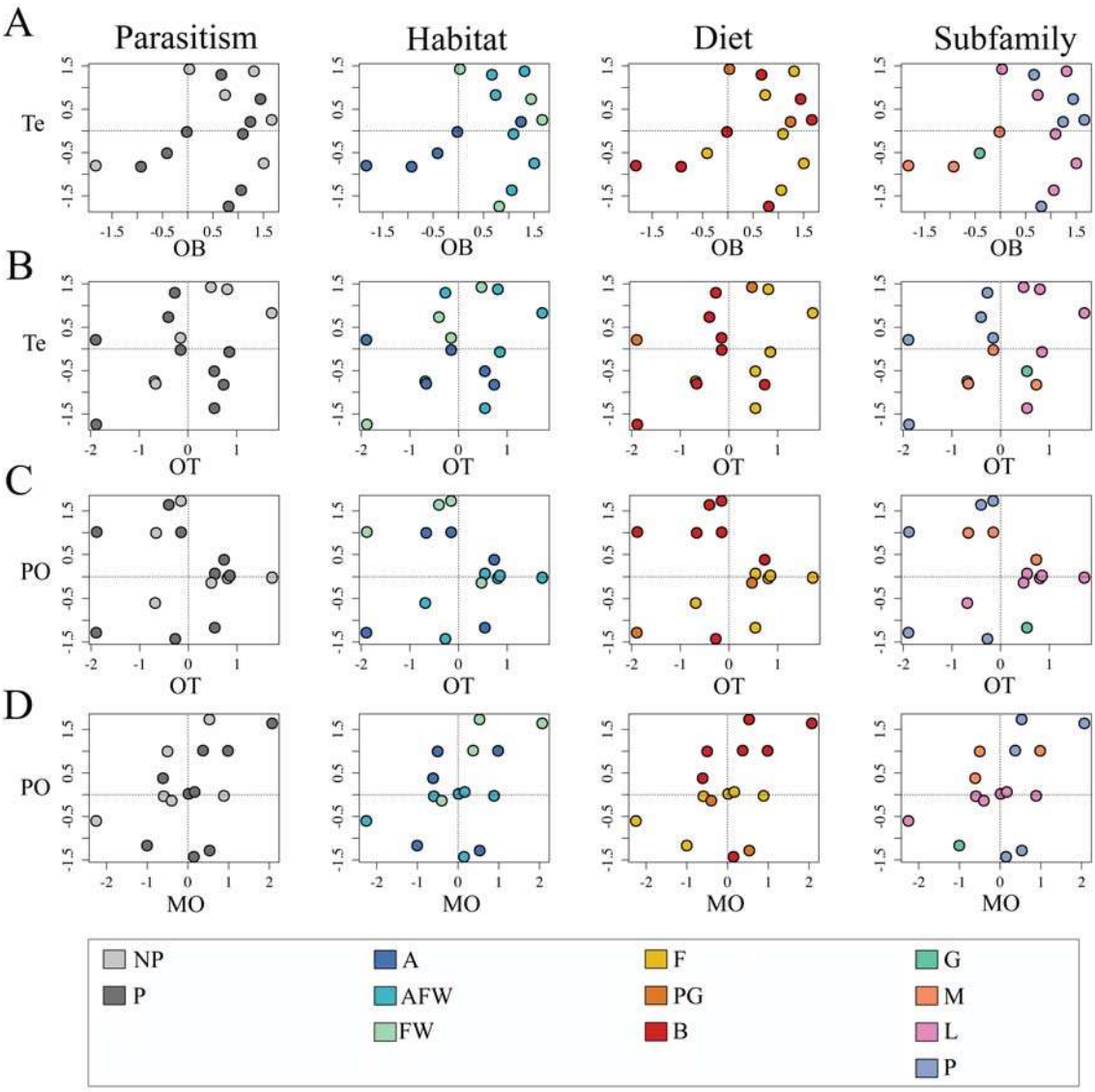

Figure 4.5 | Comparison between residuals for selected structures, according to categories per life history trait. For abbreviations, see List of Abbreviations.

4.4.3 Multivariate analyses in brain structure

4.4.3.1 pPCA in absolute volumes

The first component of the pPCA explained more than 85% of the variance in the absolute volume of the analysed brain structures, most of which loaded strongly and with same direction in PC1. The species scores in PC1 were highly correlated with brain size (r^2 = 0.95, $F_{1,13}$ = 297.7, $p < 0.001$), confirming that changes in brain size can explain up to ~ 87% of the variance in the absolute volume of the examined brain structures. The parameter lambda indicates that these values are likely not related to phylogeny (λ= 6.61e-05). The MOC and MOR had the highest loading in PC1, followed by the OB, in contrast to the PO, and to a lesser extent, the Te, which possessed a greater independence from brain size (**Figure 4.6**). PC2 represented ~ 7% of the variance, which was mainly due to the PO. PC3-4 together represented ~ 5% of the variance,

but none of the structures loaded heavily in these variables. The bivariate allometric coefficients confirmed this trend (**Table 4.3**). These coefficients suggest that most of the structures followed a concerted scaling pattern with each other. The OB varied isometrically with all structures except for the PO. In contrast, Te showed an allometric relationship with the PO, MOR and MOC (PO = 1.26, MOR = 0.96, MOC =0.95), but was close to isometry with the OT (0.98). The PO possessed only allometric relationships with the rest of the examined structures (**Table 4.3**), whereas in the MOR, the relationships were close to isometry with the OT and MOC (OT = 0.98 and MOC = 0.99), and both medullar structures (MOR and MOC) also scaling isometrically to each other (0.99).

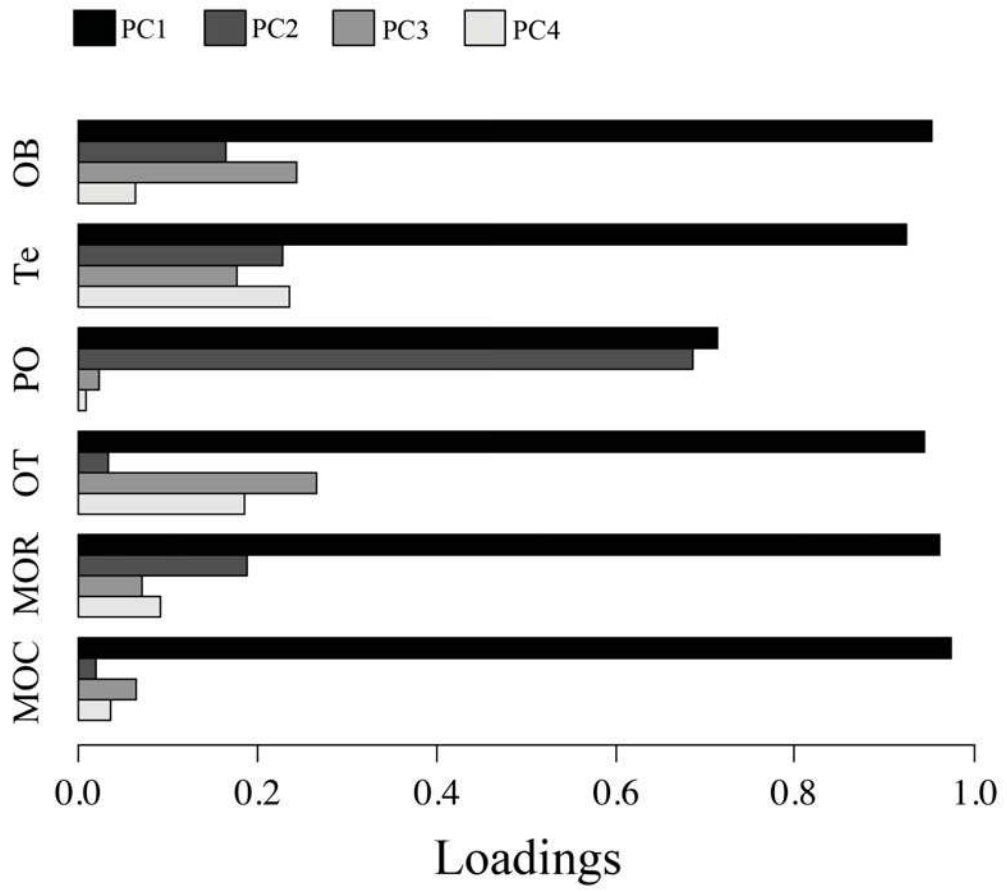

Figure 4.6 | Relative loadings of the first four factors of a phylogenetic principal components analysis (pPCA) of 6 brain structures across 15 species of lampreys. A pPCA was calculated from absolute log-transformed brain structure size. For abbreviations, see List of Abbreviations.

4.4.3.2 pPCA on relative volumes

A pPCA on the relative size of the six brain structures obtained in this study provided a measure independent of brain size and allowed a clear separation of the life history traits of lampreys in multidimensional space. The results of the estimation of the parameter lambda indicated a low association of the clusters with phylogeny (λ= 4.75e-05). The first component (PC1) explained

less variance compared to the PCA on absolute volumes (47.5% vs. 86.5%), which reflected a strong loading for the OB, but also for the MOC and PO, whereas the second component (PC2) loaded heavily for the MOR and PO with the third (PC3) explaining the variance mainly in the OT, and the fourth (PC4) explaining the variance in the Te.

The values of the scores of the extant species of lampreys and their phylogenetic relationships were projected in a tridimensional plot of PC 1 – PC2 – PC3, producing a phylomorphospace plot. **Figure 4.7** shows this graphical representation from three different angles, illustrating the clustering of species of lampreys with similar life histories, according to brain organization patterns or cerebrotypes. Most non-parasitic lampreys possessed low values of PC1, indicating a negative correlation with the OB, whereas parasitic lampreys typically had relatively larger OB and MOR (**Figure 4.7 A**). Freshwater species possessed relatively smaller OB than their closest relatives; most anadromous species were best characterized as having relatively larger MOR, whereas most anadromous-freshwater species had a relatively larger sized OB and OT (**Figure 4.7 B**). Similar to anadromous species, many blood-feeding species had a relatively larger MOR, whereas flesh-feeding lampreys possessed a relatively larger OB and OT than anadromous-freshwater species; parasitic generalists possessed larger values of PC2 than blood feeders and lower PC1 values than flesh feeders (**Figure 4.7 C**).

	Te	PO	OT	MOR	MOC
OB	1.03	1.33	1.01	0.99	0.98
Te	-	1.29	0.98	0.96	0.95
PO		-	0.76	0.74	0.73
OT			-	0.98	0.97
MOR				-	0.99

Table 4.3 | Allometric bivariate coefficients between brain structures. For abbreviations, see List of Abbreviations.

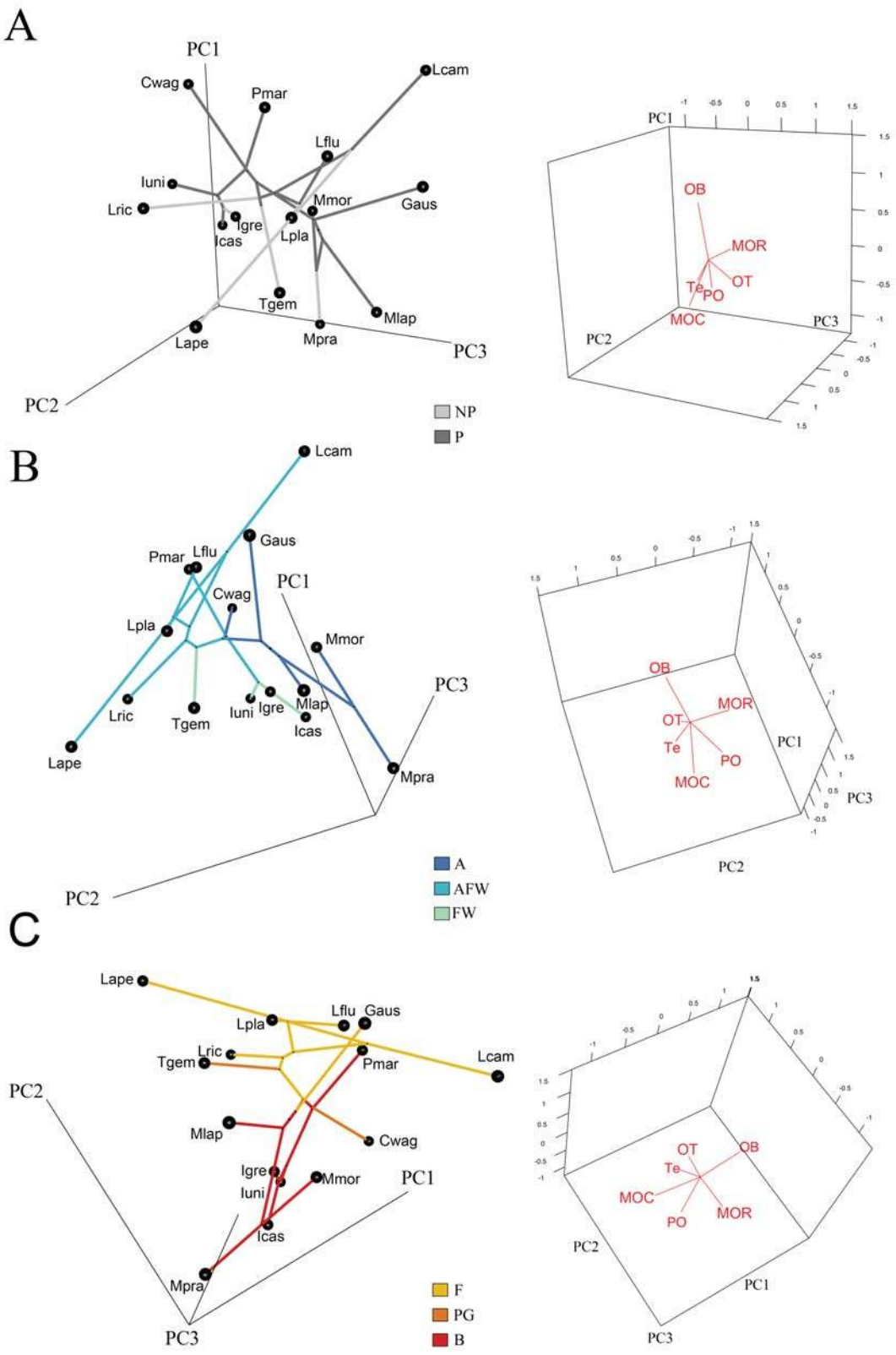

Figure 4.7 | Phylomorphospace plots representing clusters of lampreys according to diverse life history traits. The plots show the distribution of species and ancestral states in the 3D space of the first three principal components calculated from relative brain structure size (left), and eigenvectors of each brain structure viewed from the same perspective (right), for (A) parasitism; (B) primary adult habitat and (C) predatory mode. For abbreviations, see List of Abbreviations.

We used the values of the PC 1-4, which represented the 99.6% of the variance, to determine general groups of lampreys according to the relative size of their brain structures. From the analysis, we identified at least three significant general patterns of brain organization (**Figure 4.8**). The first separation formed two main groups according to the relative size of the OB, one formed by parasitic species with relatively larger OB (cluster 1), and other containing both parasitic and non-parasitic species, with relatively smaller OB (cluster 2). Within cluster 1, two subgroups were then segregated according to the relative size of the OT, clustering species with a similar feeding ecology and from different phylogenetic groups. Cluster 3 consisted of both blood-feeding and more generalist species of parasitic lampreys with relatively smaller OT. Cluster 4 was formed by flesh-feeding parasitic species, all of which have large OT ($p < 0.05$). In both Clusters 3 and 4, there are species from either anadromous or anadromous-freshwater life history types. Cluster 2 is divided into two groups; one comprised of non-parasitic species from the Lampetrinae (Cluster 5), which is closely related to flesh-feeders and exhibited a relatively larger OT and Te, and Cluster 6, which contained two whole genera of lampreys from different families, *Ichthyomyzon* and *Mordacia*, both blood-feeders but from different life history types, i.e. anadromous and freshwater species. Lampreys in Cluster 6 had the largest PO but generally less developed OT, and a relatively large MO. Within Cluster 6, two significant clusters ($p < 0.05$) were found according to the relative size of OB, where species in Cluster 7 had a relatively small OB compared to Cluster 8.

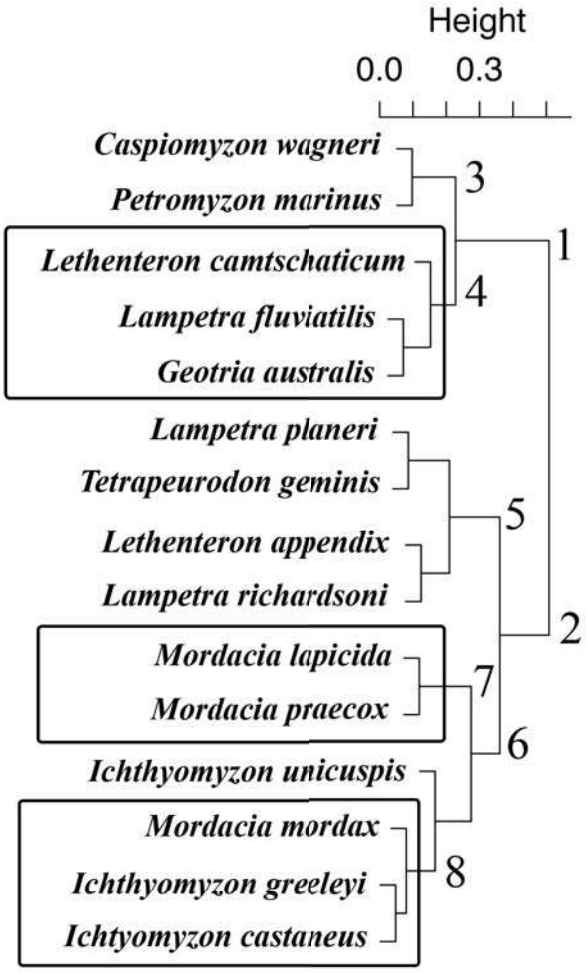

Figure 4.8 | Cluster dendrogram showing grouping of species of lampreys according to cerebrotypes, calculated from PC 1-4 on relative brain structure size. Significant clusters ($p < 0.05$) are marked with a contour.

4.4.4 Reconstruction of ancestral states

The values obtained for the first four principal components of the pPCA on relative brain structure size were also used to estimate the brain organization in the ancestor of a number of clades of lampreys (**Table 4.4**). Overall, the most similar extant species of lamprey to their last common ancestor was the mountain brook lamprey *Ichthyomyzon greeleyi,* based on the Euclidean distance between this pair. The brain organization of *I. greeleyi*, in terms of relative size, was very close to the values of PC 1-2 of the basal node, which represent variation in the OB, PO and MO. However, in PC3 (OT), the closest was the Mexican lamprey *Tetrapleurodon geminis*, whereas in PC4 (Te), the closest was the short-headed lamprey *Mordacia mordax* (**Table 4.4**).

Taxa	PC1 (47.5%) [+OB, -MOC-PO]		PC2 (23.1%) [-MOR, -PO]		PC3 (17.4%) [+OT]		PC4 (11.4%) [-Te]	
ancestral states	residual	CI	residual	CI	residual	CI	residual	CI
Petromyzontiformes	-0.027	-0.39 – 0.34	-0.017	-0.24 – 0.21	0.021	-0.07 – 0.11	0.002	-0.29 – 0.29
Mordaciidae	-0.039	-0.41 – 0.33	-0.021	-0.25 – 0.21	0.022	-0.06 – 0.11	0.004	-0.28 – 0.29
Geotriidae	-0.014	-0.37 – 0.34	-0.013	-0.23 – 0.21	0.019	-0.06 – 0.10	0.001	-0.28 – 0.28
Petromyzontidae	0.031	-0.22 – 0.28	0.005	-0.15 – 0.16	-0.014	-0.07 – 0.05	-0.013	-0.21 – 0.18
Petromyzontinae	0.041	-0.20 – 0.29	0.001	-0.15 – 0.15	-0.022	-0.08 – 0.01	-0.014	-0.21 – 0.18
Lampetrinae	0.017	-0.21 – 0.25	0.032	-0.11 – 0.18	-0.004	-0.05 – 0.06	-0.015	-0.20 – 0.17
extant species								
Ichthyomyzon greeleyi	-0.025		-0.024		-0.045		-0.019	
Tetrapleurodon geminis	-0.069		0.054		0.021		-0.073	
Mordacia mordax	-0.023		-0.060		0.000		0.001	

Table 4.4 | Reconstruction of ancestral state for the first four principal components. For abbreviations, see List of Abbreviations.

We also estimated the most likely morphological pattern of two brain structures (OB and PO) in the last common ancestor of lampreys using stochastic character mapping. These results indicated that the condition in the most basal species of lampreys, Mordaciidae (**Figure 4.3 A**) is the most likely state in the last common ancestor of lampreys (posterior probability (PP) = 61.6%). Some degree of displacement in the OB and PO was most probable in the common ancestor of the Geotriidae and Petromyzontidae (sum PP = 51.6%), whereas fully migrated OB and PO, even at smaller brain sizes, was the most likely state in the ancestor of all the Petromyzontidae (PP = 99.7%), indicating that the morphological characteristics of the OB and PO, with larger overall brain size in these adult lampreys (**Figure 4.3 B-C**), constitutes a derived morphological state.

4.5 Discussion

Due to the limited number of lampreys examined previously, the misconception that most lampreys occupy similar ecological niches and a lack of appreciation of how speciose these jawless fishes are, has led to the assumption that lampreys show little interspecific differences in the morphology of their central nervous system (Nieuwenhuys, 1977; Nieuwenhuys and Nicholson, 1998). However, recent evidence suggests that diverse life history traits, such as habitat, feeding ecology and other behavioural aspects, may significantly influence relative brain size in this group (Chapter 3). In this study, the scaling patterns of six major brain structures were characterized in adult (post-metamorphic) lampreys, covering all three families of extant species and life history traits, testing the hypothesis that the scaling of brain structures in lampreys will follow similar rules to those described for gnathostome (jawed) vertebrates. A number of cerebrotypes were identified and the most likely condition of brain organization in the last common ancestor of lampreys was reconstructed, based on the relative size and the morphological variability found in size different brain structures of extant species.

4.5.1 Brain structure scaling laws in lampreys

Previous studies have proposed that the relationship between the size of brain structures and the rest of the brain has been conserved across all groups of vertebrates, where similar changes in relative size occur in brain structures of species with similar lifestyles, irrespective of their phylogenetic relationships (reviewed in Striedter, 2005). However, although some commonalities in scaling rules can be found in specific brain structures across jawless and jawed vertebrates, our results indicate that some aspects of brain organization in lampreys may radically differ to those of gnathostome vertebrates, suggesting that conserved allometric patterns across gnathostomes may have originated with the earliest jawed vertebrates.

4.5.1.1 Scaling parameters of brain structures with brain size

Across many mammalian clades, brain structures that cease neuronal proliferation late exhibit the steepest slopes and enlarge disproportionately with the rest of the brain, whereas in other structures,'born' earlier, such as in the MO, exhibit shallower slopes (reviewed in Striedter, 2005). Although not empirically shown across all vertebrate groups, patterns of allometric scaling of major brain structures in other groups support this trend (Gonzalez-Voyer et al., 2009; Yopak et al., 2010; Liao et al., 2015). Inconsistent with this conserved pattern, however, is in scaling of the OBs, which often show a high degree of statistical independence from the rest of the brain (Finlay and Darlington, 1995; Yopak et al., 2015). Unlike the patterns documented in many gnathostomes, however, the OB in lampreys showed a relatively higher degree of predictability from overall brain size (r^2 = 0.78), especially within families of lampreys (**Table App 4.2**), and it was the only brain structure to demonstrate hyperallometry, exhibiting a steeper slope than even the Te (**Table 4.2**). Interestingly, a similar interspecific scaling pattern has been found in the olfactory bulbs and telencephalon in previous studies in teleost fishes (i.e. in Tanganyikan cichlids), where the olfactory bulbs scaled with a steeper slope than the telencephalon (OB: α = 1.01; Te: α = 0.76, Pollen et al., 2007), although in lampreys the slopes of the olfactory bulbs are greater (OB: α = 1.35; Te: α = 0.85), and the former analysis did not incorporate the phylogeny in these estimations. Similarly, a recent study on anurans also showed a large slope for the OB (α = 1.37, Liao et al., 2015), although the confidence intervals (0.74 – 1.44) do not confirm this hyperallometry unambiguously. These results suggest that in lampreys, as the brain increases in size, it is becoming disproportionately composed of the olfactory bulbs, as opposed to expansion of the telencephalon and cerebellum, as in other vertebrates (Yopak et al., 2010). Further differences in scaling were revealed for the OT, which mediates sensory-motor responses in many groups of vertebrates (Gruberg et al., 2006). In lampreys, the OT scaled close to isometry with the rest of the brain (α = 1.04), while it has been found to be hypoallometric in both teleosts and cartilaginous fishes, α = 0.80 (Pollen et al., 2007; Yopak and Lisney, 2012). Therefore, lampreys present sleeper slopes in the scaling of the OB and OT than most groups of gnathostomes, which can be expressed as a disproportionate addition of these structures as brains increase in size. These differences may reflect functional and/or developmental specificities of the central nervous system of agnathans that warrants a more detailed study of the brain in this group.

The data obtained for the scaling of other brain structures in lampreys (**Table 4.2**) also suggest a number of similarities with those of other vertebrates. For example, in both agnathan and gnathostome vertebrates, the hindbrain has one of the lowest scaling slopes with brain size, and is well predicted from brain size in various interspecies comparisons (Ebinger et al., 1983; Yopak et al., 2010). According to this dataset, this common pattern was more evident in

lampreys, where both the MOR and MOC areas, i.e. MO, were considered together in the scaling against brain size (α = 0.83, CI: 0.69 - 0.97, λ=0, $p < 0.001$). However, the MOR alone showed isometric scaling with the rest of the brain (**Table 4.2**). These results suggest that the scaling rule of the MO, particularly the MOC, may represent a common pattern of brain scaling across vertebrates, which could be related to an earlier parturition of this structure during development, though this has not yet been empirically shown. The scaling of the homologous regions of the brain of jawed vertebrates to MOR of lampreys, i.e. cerebellar-like structures and trigeminal and octavolateralis nerves, may indeed show a shallower slope than in lampreys, where the scaling of MOR in lampreys is more similar to scaling relationships between the cerebellum and brain of some jawed vertebrates (Montgomery et al., 2012).

The values of lambda obtained in the best model of each brain structure suggest diverse patterns of evolution in the scaling of these brain structures in lampreys. Half of the examined structures indicated that evolution of these structures has been independent of phylogeny, i.e. $\lambda = 0$ (**Table 4.2**). Nonetheless, a maximum likelihood estimation of lambda was not possible for any of these structures, likely due the small sample sizes, where more species are needed to confirm these values (Freckleton et al., 2002; Freckleton, 2009). For other brain structures, such as the OB and the OT, the maximum likelihood estimation of lambda showed intermediate estimates of this parameter, indicating that these structures may be moderately consistent within groups of lampreys. In contrast, the pineal organ showed the strongest correlation with phylogeny ($\lambda = 1$). These diverse values of lambda obtained in each brain structure can be interpreted as evidence that both concerted and mosaic mechanisms may be operating during brain evolution in lampreys, as it has been suggested in other vertebrate clades (Gonzalez-Voyer et al., 2009; Gutierrez-Ibanez et al., 2014; Herculano-Houzel et al., 2014).

4.5.1.2 Allometric independence

Common scaling laws have been found in gnathostomes, where many brain structures, e.g. the telencephalon and cerebellum, are highly correlated with the size of the brain (Finlay and Darlington, 1995; Gonzalez-Voyer et al., 2009; Yopak et al., 2010). The size of the OB, however, is poorly correlated with brain size, and typically has an inconsistent relationship with the size of other brain structures in major vertebrate groups, although these correlations become stronger within more restricted clades and lifestyles (Finlay et al., 2001; Reep et al., 2007; Yopak et al., 2015). Similar to gnathostomes, a higher correlation between the OB and brain size was found within smaller clades of lampreys (see **Appendix A**); however, the results of the principal component analysis on absolute brain structure volume contradicts this, showing a high loading of the OB in PC1 (**Figure 4.6**), which possessed a high correlation with brain size. However, this parameter still indicated some degree of residual variance ($r^2 = 0.95$, $F_{1,13} = 297.7$, $p < 0.001$), which could be correlated with morphological differences of a number of

structures amongst groups of lampreys (**Figures 4.2**, **4.3**). Further, the loading of the OB in PC1 was as high as in other brain structures, such as the OT and the MOR, where the OB had an isometric scaling with most brain structures (**Table 4.3**). Therefore, it can be inferred that, in lampreys, the scaling of many of these structures is largely concerted and primarily determined by overall brain size. However, our estimates indicate that up to 12% of the variance is independent of the size of the brain, similar to what was found in cichlid fishes (Gonzalez-Voyer et al., 2009), which may be related to taxa-specific grade shifts of brain structures such as the Te and PO. For example, the rules of scaling in the PO were less attributable to changes in brain size and may be better explained in terms of a mosaic process of evolution, where an increase in the relative size of this organ was observed only in a few genera, i.e. *Mordacia* and *Ichthyomyzon* (**Figure 4.2 A-F**). Notably, the Te also revealed a degree of independence of brain size ($r^2 = 0.73$), and thus had a greater departure from isometry in its scaling relationships with other brain structures, e.g. the MOR and MOC (**Table 4.3**), in contrast to what has been found in other vertebrates (Yopak et al., 2010).

These differences in allometry warrant further research into the scaling of the OB, Te, and associated brain areas to understand how they could affect olfactory processing in agnathan and gnathostome vertebrates. In addition, a detailed study of neurogenesis and the timing and sequence of other events during the development of the brain across vertebrates, including agnathans, may confirm whether these differences in scaling rules and allometric independence are related to differences in the developmental plan between jawless and jawed vertebrates. According to the results of Chapter 2, it can be hypothesised that lampreys may have a more extended period of neurogenesis in the OB in comparison to gnathostomes, which is expressed as a larger rate of growth of this structure during ontogeny (Table 2.5), i.e. these patterns of growth may be explained in terms of the "late equals large" hypothesis, which needs to be confirmed in future experiments.

4.5.2 Cerebrotypes of lampreys

It has been shown that there is a close relationship between brain organization and ecological factors in many vertebrate taxa, where those species sharing similar lifestyles, independent of phylogeny, possess similar relative brain structure size or grade shifts, whereby these species can be categorized by cerebrotypes (reviewed in Willemet, 2012). The results of the phylogenetic principal component analysis (pPCA) on relative brain structure volumes clustered species of lampreys from different clades, but with similar life history traits, suggesting that different cerebrotypes can be described for lampreys, as has been done for many other vertebrate clades (Clark et al., 2001; Iwaniuk and Hurd, 2005; Yopak et al., 2007; Lisney et al., 2008).

Lampreys exhibit a wide range of life history traits, which have been correlated with significant differences in encephalization and brain organization during the life cycle and across species (Chapters 2-3). In many taxa of lampreys, a parasitic and non-parasitic pair has evolved (Hubbs and Trautman, 1937; Potter, 1980b; Mateus et al., 2013; Mateus et al., 2016). The parasitic species undergo a feeding phase, where they reach large sizes, in contrast to the non-parasitic species, which mature shortly after the metamorphosis, and remain small. These paired species exhibit significant differences in body size, and consequently, relationships between relative and absolute brain size are highly divergent (Chapter 3). It then follows that each of these life history types (parasitic and non-parasitic) will likely possess different patterns of brain organization. In fact, many non-parasitic species possessed relatively larger Te and a relatively smaller MO, and in some cases, a bigger PO or OT. In contrast, parasitic species possessed a more highly developed OB and MOR (**Figure 4.4, 4.7**). A relatively larger OB is likely a consequence of the disproportionate scaling of the OB with brain size (**Table 4.2**). Therefore, we can infer that a number of these differences in brain organization between parasitic and non-parasitic species may be attributed to variation in absolute brain size, which reflect similar scaling rules during ontogeny, and therefore will produce different cerebrotypes as the outcome of concerted scaling of brain subdivisions across these life history types. In addition, other structures such as the MOR may scale in relation to specific somatic parameters, such as the growth of a relatively larger feeding apparatus (Neira, 1984; Murakami and Kuratani, 2008), and/or special sensory capacities that may be more developed in these parasitic species, e.g. electroreception (Chung-Davidson et al., 2004). A comparison of parasitic and non-parasitic species in most cases showed that, independent of the observed differences in brain organization, paired species tend to occupy similar regions of the phylomorphospace (**Figure 4.7**), a result which is in concordance with previous findings that there is a strong phylogenetic signal in the encephalization of lampreys (Chapter 3). Nonetheless, there were significant differences in brain organization in species that have long been geographically separated, e.g. *Lethenteron* (Balakirev et al., 2014; Li, 2014), compared to species that are found to occur sympatrically, e.g. *Mordacia* and *Ichthyomyzon* (see **Figure 4.7**).

Brain organization also varies according to other life history traits, such as primary adult habitat. It has been proposed that the ancestor of all contemporary (non-parasitic) lampreys underwent anadromous migrations, from which have evolved other life history types, such as anadromous-freshwater species with both anadromous and freshwater-resident parasitic populations, and exclusively freshwater parasitic species with shorter migrations (reviewed in Moser et al., 2015; Potter et al., 2015). It has been suggested that the relative size of the OB across vertebrates is related to navigational ability (Reep et al., 2007; Jacobs, 2012; Yopak et al., 2015), and other ecological parameters such as home range, activity pattern, foraging strategy and habitat (Gittleman, 1991; Barton et al., 1995; Huber et al., 1997; Hutcheon et al., 2002; Lisney et al., 2007). When the relative value of brain structures are analysed in a

multivariate analysis (pPCA), our results support this suggestion, whereby most species of lampreys with a parasitic stage (and therefore involving some degree of anadromy) exhibit a relatively enlarged OB, and anadromous-freshwater species have on average, the relative largest OB (**Figure 4.7 A-B**). Nonetheless, the analysis of the residuals per category (Figure 4.4, OB), indicates that a number of exclusively anadromous species (i.e. *Mordacia* spp. and *G. australis*) possess a relatively reduced OB, which could be linked to developmental differences of these species of lampreys (Figure 4.3). Similarly, the values of the residuals do not support a relatively larger OB in migratory species, where freshwater species possessed similar values to anadromous and freshwater species (Figure 4.4, OB). Therefore, it is expected that the relative size of the OB may be the result of various factors and not exclusively related migratory behaviour..

However, the establishment of parasitic, freshwater-resident populations of lampreys in addition to a migratory lifestyle, which probably occurred in the last common ancestor of the Petromyzontidae (Chapter 3), does correlate with a relative increase in the size of the OB in most members of this family compared anadromous families of lampreys that show a morphologically ancestral state of the OB (**Figure 4.3, Figure 4.4, see section 4.4.4**). This transition in life history types involved a shift from a purely anadromous lifestyle, where all recently metamorphosed lampreys migrated to marine habitats, to another type where species developed into a diversity of populations (Abou-Seedo and Potter, 1979; Kucheryavyi et al., 2007; Bracken et al., 2015), including resident parasitic populations that remained in landlocked freshwater systems that were relatively richer in olfactory cues compared to marine habitats; in lampreys, diverse behaviours are driven by pheromonal interaction with conspecifics (Siefkes et al., 2005; Sorensen et al., 2005; Vrieze et al., 2011). It has been estimated that this transition was not accompanied by an increase in encephalization in the ancestor of the Petromyzontidae, or any change in feeding ecology (Chapter 3). Therefore, the relative increase in the size of the OB could be interpreted as a grade shift that was independent of brain size, which became a developmental constraint in this family, where young adults had a relatively larger OB and already possessed typical morphological traits of mature adults, i.e. displaced olfactory bulbs (**Figures 4.2-3**, **Figure App A.1**). This may explain why species such as *Ichthyomyzon* and other freshwater genera possess a relatively larger OB (**Figure 4.4**), despite having a relatively smaller brain size than their closest relatives (Chapter 2). In contrast, in the anadromous species of lampreys from the southern hemisphere, i.e. *Geotria* and *Mordacia*, differences in absolute brain size (Figure 3.2 C) and the morphologically ancestral state of this structure (section 4.4.4) may explain the relatively smaller size of the OB in these groups of lampreys.

The feeding ecology of animals has also been proposed as an important driver of the diversification of the vertebrate nervous system (Gans, 1989; Northcutt, 1996). The diverse levels of predation by which animals source their food, which are usually manifested in

structural specializations to fulfil specific sensory-motor requirements, are reflected in the relative size of the brain (encephalization) and brain organization in a number of vertebrate groups (Striedter, 2005). The results of this Chapter indicate that the predatory modes previously described in lampreys are reflected in a convergence of brain organization patterns amongst phylogenetically distant species. Lampreys with more active predatory modes (i.e. flesh-feeding lampreys) possess relatively enlarged OTs and OBs (**Figure 4.4**, **Figure 4.7 C**), which are typical characteristics of many active predators in a number of other vertebrate groups that live in well-lit habitats (Kotrschal et al., 1998; Lisney and Collin, 2006). Flesh-feeding species of lampreys also possess specialized visual systems (Fritzsch and Collin, 1990; Collin et al., 2003a; Davies et al., 2009), which may be reflected in the relative size of the visual centres of the brain as it has been shown in other vertebrates (Jacobson, 1962; Schwassmann, 1968; Hueter, 1991; Cornide-Petronio et al., 2011; Salas, 2011). Unlike apex predators, however, which generally have a relatively large Te (Lisney and Collin, 2006), the Te is not particularly well developed in flesh-feeding lampreys as compared to lampreys from other predatory modes, such as blood-feeders (**Figure 4.4**). As opposed to flesh-feeders, blood-feeders have a relatively small OT, indicating that more passive modes of predation in adult lampreys may rely less on visual cues (Fritzsch and Collin, 1990; Collin et al., 2004), and possess a relatively larger MOR, suggesting a higher relevance of chemical, electric, and other types of signals (Bodznick and Northcutt, 1981; Chung-Davidson et al., 2004; Chung-Davidson et al., 2008; Buchinger et al., 2015). This correlation appears to conform to the cerebrotype typical of benthic species in a range of other aquatic vertebrates, where species have a relatively small brain, OT and Te, but well-developed medullary systems (Kotrschal and Palzenberger, 1992; Northcutt et al., 2000; Schluessel et al., 2008).

General patterns of brain organization in lampreys were estimated using a cluster analysis of the first four principal components of relative brain structure size, which represented ~ 99.6% of the variance (**Figure 4.8**), where species were grouped according to cerebrotypes. Cluster 3, or the "generalist" cerebrotype, is formed by species of relatively large body size, whose main feature is relatively large OB and MOR, i.e. the sea lamprey *Petromyzon marinus* and the Caspian lamprey *Caspiomyzon wagneri*. The adults of both these species are considered as generalists: *P. marinus* is known to occupy coastal and pelagic habitats across a wide depth (Halliday, 1991) and geographical (Potter et al., 2015) range, feeding on a variety of hosts, including teleost and cartilaginous fishes, and cetaceans (Silva et al., 2014). In contrast, *C. wagneri* inhabits a more restricted environment (Caspian Sea), where it is thought to be associated with the benthos as an opportunistic scavenger, whose diet could be constituted by carrion, fish eggs, and invertebrates (Renaud et al., 2009; Renaud, 2011). These life history traits are consistent with the large reduction of the OT observed in this species (**Figure 4.2**). Cluster 4 or the "active predator" cerebrotype, is represented exclusively by flesh-feeding lampreys from different taxa, whose main characteristic is a well developed OT, although some of these species also possess

relatively large OB and enlarged MOR, similar to the generalist cerebrotype (Figure 4.4). They are generally found in shallow riverine, estuarine, coastal or epipelagic waters (Potter et al., 1979; Beamish, 1980; Orlov et al., 2014). Cluster 5 or the "active non-parasitic" cerebrotype, clustered all non-parasitic species from the Lampetrinae, i.e. species with relatively large OT and Te (but generally not enlarged OB or MO). These species showed a similar cerebrotype to downstream migrants of *G. australis* (Salas et al., 2015). Since non-parasitic species may constitute more than half of all species of lampreys (Potter et al., 2015), it is expected that many species of lampreys fall into this category. The last cluster (Cluster 6), or the "passive predator" cerebrotype, grouped both parasitic and non-parasitic species from two genera of passive predators, *Mordacia* and *Ichthyomyzon*. In this cluster, there are fewer differences in the relative size of each brain structure between parasitic and non-parasitic species with both groups possessing a relatively larger MO and PO (**Figure 4.7**). Surprisingly, this cerebrotype also corresponds to the one observed by Salas et al. (2015) in ammocoetes of *G. australis.* Therefore, it can be inferred that adults may differ less from the ammocoete stage in this cerebrotype than in the active predatory cerebrotype.

The reconstruction of the ancestral cerebrotype of lampreys indicates that most characteristics of the passive predator cerebrotype were also present in the last common ancestor (**Table 4.4**, Chapter 3). Nonetheless, the relative size of the OT in the ancestor was similar to the OT size in the Mexican brook lamprey *Tetrapleurodon geminis,* which is relatively larger than those of species from the passive predator cerebrotype. We have previously estimated that encephalization in lampreys was reduced in comparison with the last common ancestor of hagfishes (Chapter 3). Therefore, it is possible that extant species of lampreys are derived forms in relation to ancestral states of brain organization. Studies of brain organization including evidence from other agnathans and fossils of earlier lamprey species may provide further insights of the evolution of the brain in this group of vertebrates.

4.5.3 Conclusions

Lampreys are extant relatives of the earliest vertebrate lineages, which provide a unique opportunity to study the ancestral condition of the vertebrate brain. This work provides evidence that brain organization in lampreys has been modified during their evolutionary history, in conjunction with diverse ecological variables, in a similar fashion to gnathostomes. Importantly, many cerebrotypes are conserved across agnathan and gnathostome vertebrates. However, jawless and jawed vertebrates showed differing scaling rules, which may be evidencing profound functional and developmental differences between jawless and jawed vertebrates. These results suggest that the plesiomorphic condition of brain organization in vertebrates may be different to the one observed at the onset of gnathostomes. Further studies of brain organization across ontogeny of diverse groups of lampreys, and other agnathans such as

hagfishes and earlier fossilized forms, will provide a better understanding of the early evolution of the vertebrate brain.

Appendix A Supplementary results: Analyses of covariance

A.1 Supplemental methods

Analyses of covariance (ANCOVAs) were fitted by employing generalized least squares (GLS) with the variance function *varIdent* as weights, which allowed different variances for each level of the tested factors to be included (Pinheiro et al., 2015). The monotypic family Geotriidae was excluded from this analysis, as it was not represented along the whole range of brain size, but was included in the plots as a reference. We performed this analysis in two steps: (1) we first tested if there was any significant interaction in the slopes between the families Mordaciidae and Petromyzontidae (model 1) or between the subfamilies Mordaciinae, Petromyzontinae, and Lampetrinae (model 2) in the scaling of each brain structure, or were best fitted as additive (different intercept) models; (2) model inference methods were then applied to the resultant models in the ANCOVAs of family and subfamily, and a model incorporating no factor (model 3). The best model of brain structure scaling was determined using the second-order Akaike Information Criterion (AICc), where the best ANCOVA model had the least AICc score (Barton, 2014). When linear models showed a difference of less than two units (ΔAICc < 2), AICc weights were employed instead to define the best model of brain scaling (Burnham and Anderson, 2002). Ordinary least squares models were fit to each subgroup and all data in the best model for each brain structure to calculate the coefficient of determination in each of these regressions.

A.2 Supplemental results

There were significant differences in the slope and/or the intercept between families of lampreys (**Figure App A.1**), as shown in the selected model for each brain structure (**Table App 4.1**). The coefficient of determination (r^2) for each of these subgroups is shown in **Table App 4.2**. The scaling of the olfactory bulbs showed a strong interaction between family (factor) and brain size (covariate). In this model, different slopes and intercepts were obtained for Mordaciidae and Petromyzontidae (**Figure App A.1 A**), indicating that at smaller brain sizes, the Petromyzontidae possessed significantly larger olfactory bulbs (OB) than members of the Mordaciidae, a difference that was reduced at larger brain sizes. A similar relationship was observed in the telencephalic hemispheres (Te, **Figure App A.1 B),** although the size of this structure was more variable amongst members of the Petromyzontidae, and there was less

correlation within families for the olfactory bulbs. Analyses of both the pineal organ (PO, **Figure App A.1 C**) and the optic tectum (OT, **Figure App. A.1 D**) revealed differences that were better fitted with subfamily as a factor. However, the scaling of the PO with brain size showed a strong interaction between the subfamilies of the Petromyzontidae, where Petromyzontinae had significantly larger PO than members of the Lampetrinae at smaller brain sizes. Mordaciidae had a similar slope to Lampetrinae, but with a larger intercept, indicating a relatively larger PO. In the case of the OT, all subfamilies had a common slope, but different intercepts, where Lampetrinae had the largest relative size of all subfamilies analysed. The pouched lamprey *Geotria australis* had similar values of the OT to members of Lampetrinae (**Figure App A.1 D**). The rostral (MOR) and caudal (MOC) areas of the medulla oblongata also scaled differently between lamprey taxa: MOR showed an interaction between brain size and subfamily (**Figure App A.1 E**), where Petromyzontinae had a lower slope, whereas MOC presented different slopes for each family (**Figure App A.1 F**). A summary of the trends per taxonomical unit, and the line representing the pGLS model selected for that structure, is shown in **Figure App A.2** with data presented as average per species.

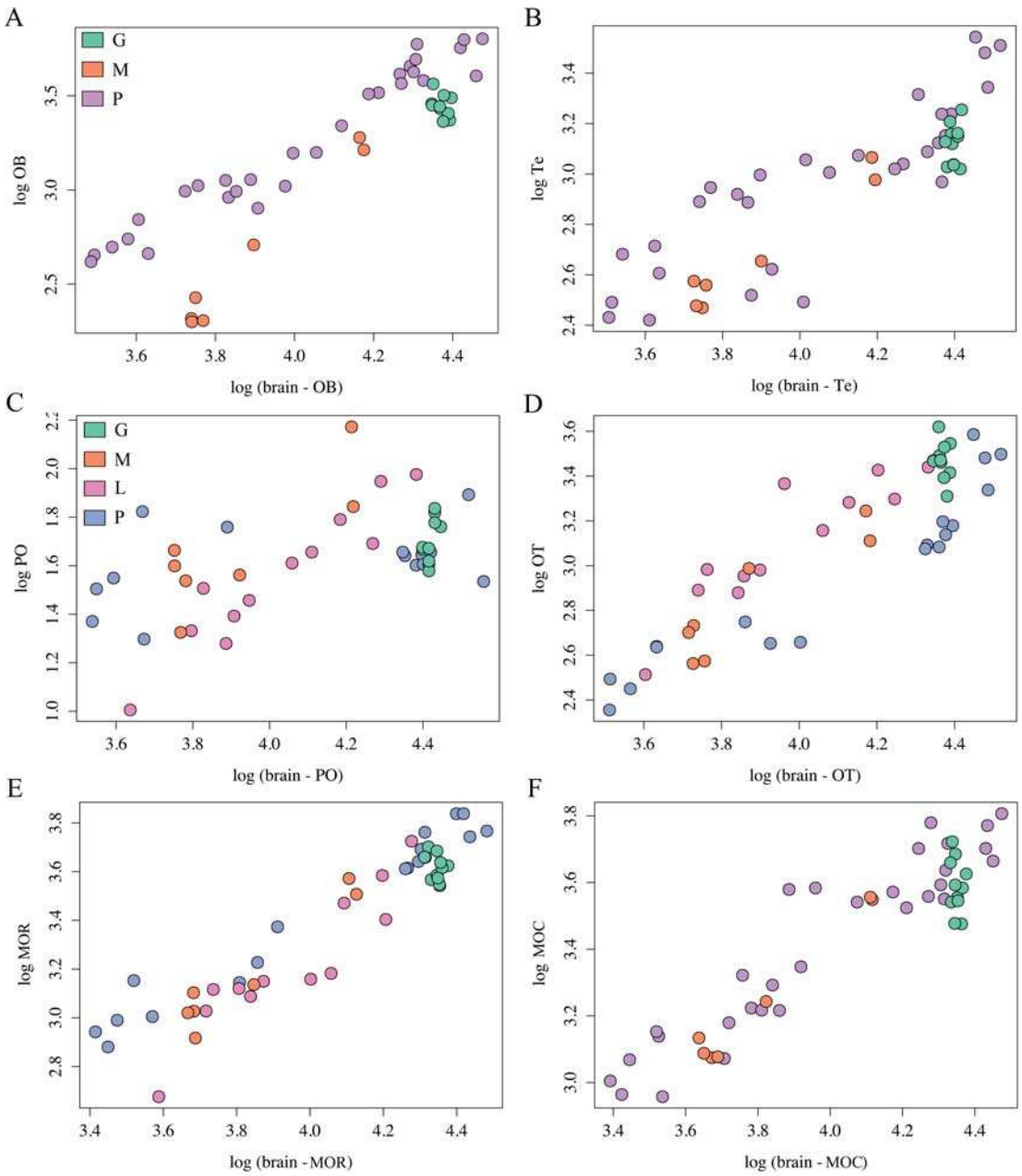

Figure App A.1 | Data points per individuals. Legend in (A) corresponds to families of lampreys as shown in (A), (B) and (F); Legend in (C) corresponds to subfamilies of lampreys as shown in (C), (D) and (E). For abbreviations, see List of Abbreviations.

model	parameter	structure OB	Te	PO	OT	MOR	MOC
model 1	brain size (M)	2.15 ***	1.11 ***				1.00 ***
	brain size (P)	1.22 ***	0.84 +				0.72 **
	Mordaciidae	-5.73 ***	-1.63 ***				-0.59 *
	Petromyzontidae	-1.65 ***	-0.42 *				0.51 **
model 2	brain size (M)			1.03		1.19	
	brain size (P)			0.16 ***	1.01 ***	0.85 +	
	brain size (L)			1.18 ***		1.17 ***	
	Mordaciinae			-2.37	-1.07 **	-1.36	
	Petromyzontinae			0.93 ***	-1.18 ***	0.01*	
	Lampetrinae			-3.19 ***	-0.91 **	-1.39 *	
model 3	brain size intercept						
pGLS	brain size	1.35 ***	0.85 ***	1.08 ***	1.04 ***	1.01 ***	0.76 ***
	intercept	-2.36 ***	-0.53	-2.75 ***	-1.13 *	-0.72+	0.39
	model summary						
	d.f. residuals	33	33	27	33	31	33
	AICc	-67.0	-31.3	-21.8	-35.0	-62.6	-73.1
	ΔAICc	9.41	3.94	12.91	13.41	9.88	3.76
	AICc weights	0.991	0.858	0.997	0.999	0.992	0.804

Table App 4.1 | Parameters of regressions per taxa. Values of the slopes (brain size) and intercepts (lamprey taxa) are given for selected models only; pGLS values are presented as a reference. (***) p-value < 0.001, (**) 0.001 < p-value < 0.01, (*) 0.01 < p-value < 0.05, (+) 0.05 < p-value < 0.1, ( ) p-value > 0.1.

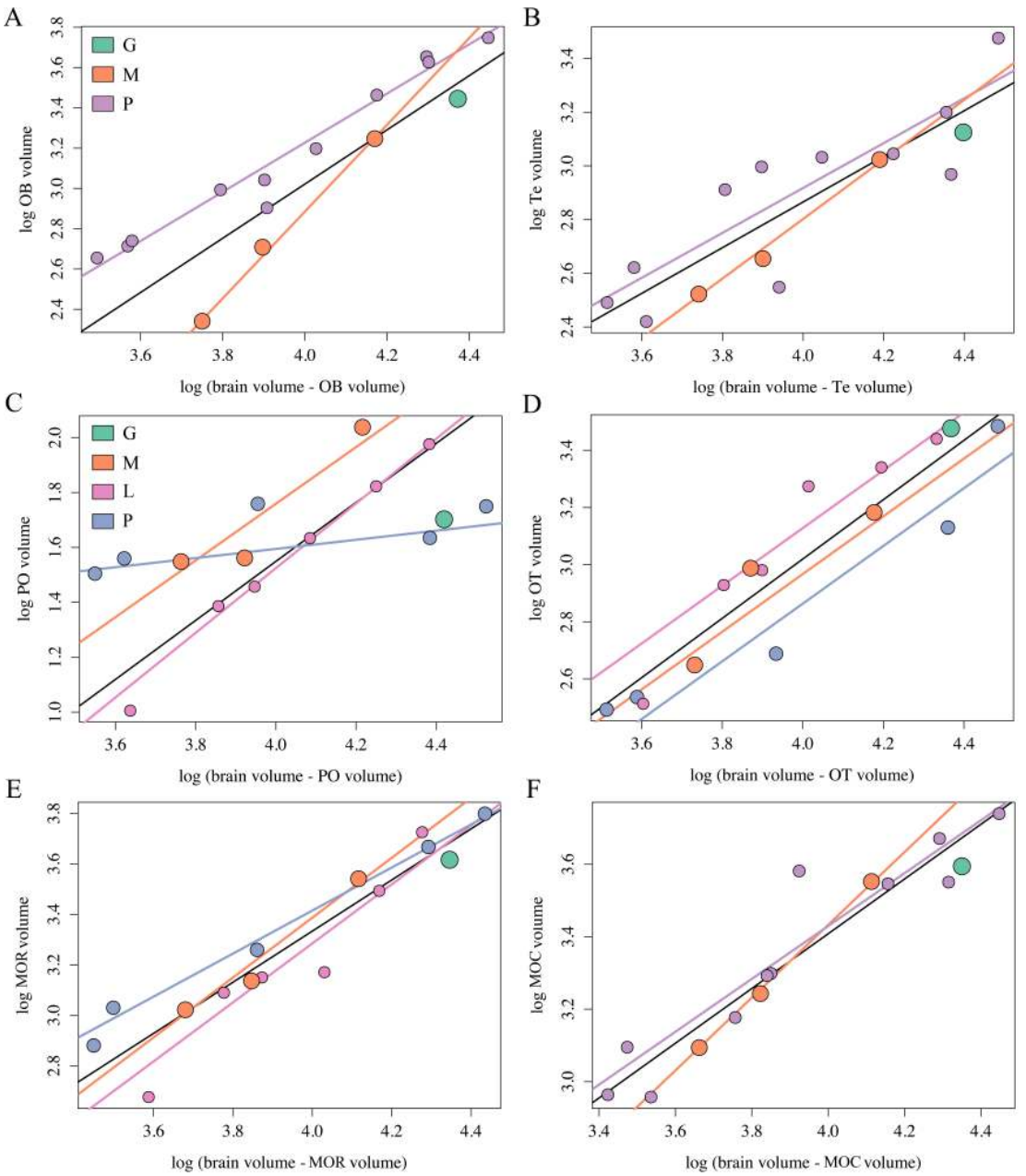

Figure App A.2 | Summary of brain structure scaling with the rest of the brain. Points represent averages per species; black line represents the pGLS model. Legend in (A) corresponds to families of lampreys as shown in (A), (B) and (F); Legend in (C) corresponds to subfamilies of lampreys as shown in (C), (D) and (E). For abbreviations, see List of Abbreviations.

		structure					
taxa	n	OB	Te	PO	OT	MOR	MOC
Mordaciidae	7	0.98***	0.94***				0.97***
Petromyzontidae	30	0.95***	0.73***				0.88***
Mordaciinae	7			0.60*	0.81**	0.91***	
Petromyzontinae	18			0.11	0.88***	0.96***	
Lampetrinae	12			0.89***	0.80***	0.84***	
Petromyzontiformes	48	0.78***	0.74***	0.28***	0.80***	0.89***	0.86***

Table App 4.2 | Coefficient of determination (r-squared) per taxa. Values are given for selected models only as in Table App 4.1. (***) p-value < 0.001, (**) 0.001 < p-value < 0.01 (*) 0.01 < p-value < 0.05, (+) 0.05 < p-value < 0.1, ( ) p-value > 0.1.

Chapter 5 General discussion

The central nervous system of vertebrates shows great variability within and across different taxa; but thus far, there is no definitive explanation for its origin or relevant drivers of variation. In recent years, two schools of thought have dominated our understanding about the evolution of the nervous systems (reviewed in Northcutt, 2012).

(1) The phenetic school of evolutionary thought is represented by those who attempt to resolve evolutionary relationships mainly informed by overall similarity of observable traits, such as patterns of gene expression during early development (e.g. Puelles and Rubenstein, 1993; Lowe et al., 2003). Under this paradigm, it has been suggested that the last common ancestor of protostomes and deuterostomes (i.e. Urbilateria) already possessed a complex tripartite brain (i.e. divided into the forebrain, midbrain and hindbrain), and that enteropneusts and/or annelids, amongst others animal groups, may best represent the transition from invertebrate to vertebrate brains, because of the similarities found in the molecular control of body and brain patterning between these two groups (de Robertis, 1997; Holland et al., 2013; Holland, 2016). However, these molecular networks regulate the development of fundamentally different nervous systems. Therefore, it is considered that the use of molecular markers expressed during early development is an intrinsically misdirected criterion to recognize homology of nervous systems or its subdivisions between species (Faunes et al., 2015), as developmental mechanisms of clearly homologous structures can often differ at early ontogenetic stages, and similar developmental mechanisms can generate different structures in adults (de Beer, 1971; Striedter and Northcutt, 1991; Weiss and Fullerton, 2000; Faunes et al., 2015; Sugahara et al., 2016). Although similar patterns of gene expression may occur during development in a wide range of animals, it cannot be concluded that their presence dictates the organization of brain-like nervous systems or the presence of specific structures in every case.

(2) The cladistic school of evolutionary thought is primarily based on the phylogenetic relationships between species (reviewed in Striedter, 2005), which can be obtained from many different characters, such as behaviour, morphology and DNA sequences. In cladistics, the phylogenetic relationships are defined according to the distribution of these characters; a monophyletic group is considered as a group of species that possess common *shared derived* characteristics, as opposed to *shared primitive*, or *independently evolved* characters, which are phylogenetically non-informative. Using cladistics, it has been inferred that more complex brains have evolved independently multiple times in eumetazoans from a brainless urbilateria (Moroz, 2009; Northcutt, 2010; Northcutt, 2012). Therefore, it is thought that an increase in overall morphological and functional complexity can consistently be correlated with more complex central nervous systems during evolutionary history. It has been hypothesized that one

of these complex nervous systems may have originated in early chordates, but the mechanisms and history of the transition to a more complex brain in vertebrates is still not completely understood. In this regard, it has been argued that these changes occur during ontogeny (Garstang, 1922; Katz et al., 1981; Northcutt, 1990; Maturana and Mpodozis, 2000), where behaviour, environment and developmental systems may all act on the organismal phenotype, which, in successive iterations, can produce evolutionary change. Despite the importance of ontogenetic studies to understand phylogenetic change (Northcutt, 1990), there is a paucity of these studies for a large majority of vertebrate taxa.

In this Thesis, the central nervous system of extant jawless vertebrates has been studied from both a phylogenetic and an ontogenetic point of view, in order to gain a better understanding of the evolutionary history of the vertebrate brain. This work has been presented in three previous chapters:

The shifts in encephalization and brain organization during the life cycle of lampreys are examined during the ontogeny of a representative parasitic species of lamprey, the pouched lamprey *Geotria australis* (Chapter 2).

The encephalization of cyclostomes is then compared amongst various taxa, in addition to predicting the state of this character in the last common ancestor of cyclostomes (Chapter 3).

Finally, the patterns of brain organization are described in lampreys according to a number of life history traits, which allowed for statistical predictions regarding the brain organization of the earliest lampreys to be reconstructed (Chapter 4).

The results of these investigations suggest that both jawed and jawless fishes have common rules governing encephalization and brain organization, which may have originated at the juncture between cephalochordates and vertebrates, although lampreys, and more generally cyclostomes, show some characteristics that may be unique to this group. In the following sections, the results presented will be contrasted to previous knowledge of the evolution of the brain of both jawless and jawed vertebrates to provide a new perspective on the evolution of the brain in early vertebrates.

5.1 Encephalization of vertebrates

The size of the brain relative to body size (encephalization) in vertebrates has long been considered as a proxy for behavioural complexity. In this context, body size has historically been a widely explored predictor of brain size across vertebrate taxa (Striedter, 2005), where both brain size and many of its component parts are thought to have a close relationship with body size (Katz and Lasek, 1978; Deacon, 1990; Aboitiz, 1996; Finlay et al., 2001). In fact, it

was proposed that this close relationship between brain size and body size may be common to a wider range of animals, including invertebrates (Chittka and Niven, 2009). The results presented here indicate that adult lampreys, neither at ontogenetic nor phylogenetic levels, are an exception to this rule: all taxa showed an increase in absolute brain size with larger body sizes (**Figure 2.4, Figure 3.2**). Furthermore, lampreys, like some other groups of vertebrates (Striedter, 2005), also show a large residual variation according to a common scaling rule, which, in the case of lampreys, can be correlated with a number of life history traits, such as the primary adult habitat or feeding behaviour of adult individuals (**Figure 3.3**). The results of Chapter 3 showed that lampreys, as a group, may have a relatively smaller brain than the last common ancestor of cyclostomes (Table 3.4); nonetheless, these results also support previous views that the relative brain size of early jawless vertebrates could have been similar to the level of encephalization of extant lampreys (Northcutt, 1985), where relative brain size of a number of the examined species was similar to the estimated value of encephalization in the ancestor of cyclostomes, e.g. *Tetrapleurodon geminis* (**Figure 3.5**). In contrast, the level of encephalization in some parasitic lampreys, e.g. genus *Ichthyomyzon*, demonstrates a marked reduction in relative brain size, to a level lower than that of the ancestor of cyclostomes (**Figure 3.5**). Therefore, it is hypothesized that not all extant species of lampreys represent the encephalization predicted for early vertebrates.

The rapid growth of the brain during metamorphosis in lampreys (**Figure 2.4**) may correspond to a transition from a passive feeding mode (filter-feeding), in keeping with a sedentary lifestyle, to a more active feeding mode as part of a free-swimming lifestyle that could have its origins at the very beginning of the lineage giving rise to vertebrates (Gans, 1989; Northcutt, 1996). The high rate of growth of the brain of lampreys during metamorphosis may imply an exception to the scaling rules governing brain size with body size observed during other phases of the life cycle in this group of jawless fishes, since body size does not increase during metamorphosis, whereas the brain increases approximately four times over the same period (**Table 2.1**). Interestingly, the growth pattern of a number of brain structures, such as the optic tectum (OT), also undergoes radical increases in growth during metamorphosis (**Figure 2.5**), which resembles the development of major sense organs at the origin of vertebrates (Butler, 2000a; b; 2006). These results support the assertion that the life cycle of lampreys may represent a good model for evolutionary developmental (evo-devo) studies of the nervous system of vertebrates, as proposed previously (Kuratani et al., 2002; Osorio and Retaux, 2008). A similar analysis of additional species of lampreys with a wider range of life history traits may reveal variations in these ontogenetic scaling rules (Chapter 2) and will confirm whether this trend is common to all lampreys, or alternatively, provide different examples of possible scenarios for this transition during the origin of vertebrates.

5.2 Brain organization of vertebrates

The brains of vertebrates are highly diverse, despite a number of commonalities found in both their physiology and anatomy. Comparative studies of the gross anatomy of the vertebrate brain have proposed at least two ways to explain this diversity. (1) Conservative developmental events determine a basic vertebrate brain plan, with common scaling rules of brain structures for all vertebrates, which produce regional variation as a consequence of concerted changes in relative size with changes in absolute brain size (reviewed in Charvet et al., 2011). (2) Grade shifts, or independent increases in the relative size of a given structure, occur due to a degree of plasticity of these developmental mechanisms, allowing change in a modular fashion. These grade shifts can dictate specific requirements with respect to life history traits or behaviours of species, while preserving a basic vertebrate plan (reviewed in Anderson and Finlay, 2014). These mechanisms define common patterns of brain subdivision scaling that have been described for gnathostome vertebrates, as well as equivalences in changes in the relative size of specific structures across species with similar lifestyles (Yopak et al., 2010), where a common denominator of gnathostomes is the pronounced, highly correlated development of higher-order sensory-motor structures such as the telencephalic hemispheres and cerebellum. The results of the present study show that brain structures in lampreys exhibit diverse scaling rules with brain size. For example, similar rules are found across species in the scaling of medullar brain structures, which possess a nearly isometric scaling with most other brain structures examined (**Figure 4.6, Table 4.3**), and a higher correlation across all species (**Table App 4.2**), suggesting a concerted mechanism of brain structure scaling. In contrast, changes in the relative size (grade shifts) of other brain structures may be specific to certain life history traits. For example, although there is a correlation between brain size and the size of the optic tectum, which is higher than that obtained in the telencephalic hemispheres and olfactory bulbs, there is still some degree of statistical independence from the rest of the brain ($r^2 = 0.80$). The optic tectum is relatively larger in species of lampreys with more active modes of predation, which may necessitate higher visual acuity (**Figure 4.4**, **Figure App A.2**). This residual variation with (potential) links to behaviour can be interpreted as a mosaic change in brain structure size (Yopak and Lisney, 2012). Taken together, these results suggest that both concerted and mosaic mechanisms of brain structure scaling may be intrinsic to all vertebrates.

An exception to these common patterns of brain scaling can be found in the olfactory bulbs (reviewed in Striedter, 2005). In many groups of gnathostomes, a number of studies have shown that the size of the olfactory bulbs possess a high degree of residual variance and a lack of predictability from overall brain size (Finlay et al., 2001; Reep et al., 2007; Gonzalez-Voyer et al., 2009; Yopak et al., 2015). These studies indicate a lower correlation between the olfactory bulbs and the rest of the brain compared to other brain structures, which nonetheless becomes

more pronounced in clades with lesser number of species. This pattern of scaling has been addressed by the olfactory spatial hypothesis, which suggests that the primary function of the olfactory bulbs may be to map odorant distributions in time and space; therefore, the relative size of the olfactory bulbs and a few olfactory-recipient structures of the telencephalon should covary with the navigational needs of the organism (Jacobs, 2012). In contrast to gnathostomes, the results of the present study show that there is a tighter relationship between the olfactory bulbs and the remainder of the brain in families of lampreys (**Figure App A.2**, **Table App 4.2**), where all of these groups showed a disproportionate increase in the relative size of the olfactory bulbs when compared to the size of the brain (**Table 4.2**, **Table App 4.1**). Following previous suggestions in gnathostomes (Jacobs, 2012; Bett and Hinch, 2015; Yopak et al., 2015), it can be hypothesized that this hyperallometric law across species of lampreys is related to the anadromous (migratory) behaviour of many of these species of lampreys during their life cycle, where olfactory cues are employed during their migration to find suitable streams to spawn (Johnson et al., 2015). In fact, adult lampreys are extremely sensitive to olfactory stimuli (Sorensen et al., 2005), which may confer greater processing requirements for encoding olfactory information, reflected in highly developed (larger) olfactory bulbs. Interestingly, an enhanced rate of growth of this brain structure has also been reported with increasing brain size during the ontogeny of lampreys (Scott, 1887; Zielinski et al., 2005; Salas et al., 2015), which may be correlated with marked differences in behaviour between the two phases of the life cycle (Hardisty and Potter, 1971a). The large rate of growth of the olfactory bulbs could constitute a developmental constraint for all lamprey species (Chapter 2), which may indicate a pattern of neurogenesis that is unique to lampreys, reflecting a common evolutionary history from an anadromous ancestor (Chapter 3). Given this highly hyperallometric relationship with brain size, it is expected that lamprey species with larger brains, from any life history type, will possess relatively larger olfactory bulbs. A comparison of the relative size of the olfactory bulbs across the three families of lampreys showed that the ancestor of northern hemisphere species (Petromyzontidae) may have undergone a grade shift in the size of the olfactory bulbs, which are relatively large in these species compared to southern hemisphere lampreys (Mordaciidae + Geotriidae), particularly at smaller brain sizes (**Figure 4.4**, **Figure App 4.2**). Since this purported grade shift in the size of the olfactory bulbs appears to coincide with the establishment of freshwater populations predicted for the ancestor of the Petromyzontidae, which originated from species with a more complex population structure, including both non-parasitic and freshwater-resident parasitic populations (Nazarov et al., 2011; Bracken et al., 2015), more sophisticated intraspecific interactions in these species of lampreys needs to be addressed in future research.

The results of interspecific comparisons between the olfactory bulbs, the telencephalic hemispheres and the remainder of the brain in lampreys (Chapter 4) indicate a number of differences and similarities with jawed vertebrates (**Table 5.1**). In both chondrichthyans and

mammals, it has been shown that a hyperallometric scaling relationship exists between the telencephalon and overall brain size, as well as with other brain structures, such as the medulla oblongata and the mesencephalon (Barton and Harvey, 2000; Yopak et al., 2010). This is thought to be linked to developmental constraints, i.e. it is expected that the neurogenic events that take place in the telencephalon occur relatively late during early development (Finlay and Darlington, 1995). Nonetheless, it has been shown in anurans, teleosts fishes and lampreys that the value of the slope in the scaling of the olfactory bulbs and the remainder of the brain is higher than that of the telencephalon and the remainder of the brain (**Table 5.1**). In addition, the olfactory bulbs did not have a significant hyperallometric scaling relationship with the telencephalon in many of species of chondrichthyans (Yopak et al., 2010), whereas in anurans the olfactory bulbs do show a hyperallometric scaling relationship with the telencephalon (Liao et al., 2015); our results suggest that hyperallometry may also be the case in lampreys (**Table 4.2**), but this needs to be tested in further experiments. Considering that many anurans, teleost fishes and lampreys possess a life cycle with a metamorphic stage (Laudet, 2011; McMenamin and Parichy, 2013), which is characterized by heterochronic changes in development (i.e. a change in the timing or rate of a developmental event), the existence of eventual delays in development and/or changes in the rate of growth of the olfactory bulbs in relation to the remainder of the brain in these metamorphic groups of vertebrates may be inevitable. These issues can be tested in further experiments.

Parameter	Brain structure	
Slope (CI)	OB	Te
Chondrichthyans	0.94 (±0.17) (Yopak et al., 2015)	1.05 (Yopak, 2012)
Teleost fishes	1.00 (Pollen et al., 2007)	0.76 (Pollen et al., 2007)
Anurans	1.37 (0.74 – 1.44) (Liao et al., 2015)	0.99 (0.89 – 1.09) (Liao et al., 2015)
Reptiles	*not available*	1.05, p = 0.09 (Powell and Leal, 2012)
Mammals	Carnivores: 0.89 (Gittleman, 1991)	*not available*
	not available	Insectivores: 1.11 (1.03 – 1.20) (Barton and Harvey, 2000)
	not available	Strepsirhines: 1.13 (1.04 – 1.22) (Barton and Harvey, 2000)
	not available	Haplorhines: 1.20 (1.14 – 1.26) (Barton and Harvey, 2000)
Lampreys	1.35 (1.08 – 1.61)	0.85 (0.59 – 1.11)
r-squared		
Chondrichthyans	0.73, $p < 0.001$ (Yopak et al., 2015)	0.95 (Yopak, 2012)
Teleost fishes	0.50, $p < 0.001$ (Pollen et al., 2007)	0.62, $p < 0.001$ (Pollen et al., 2007)
Anurans	0.55, $p > 0.05$ (Liao et al., 2015)	0.90, $p > 0.05$ (Liao et al., 2015)
Reptiles	*not available*	1.00 (Powell and Leal, 2012)
Mammals	Primates: 0.56, $p < 0.0001$ (Barton et al., 1995)	*not available*
	Bats: 0.85, $p < 0.0001$ (Barton et al., 1995)	*not available*
	Insectivores: 0.83, $p < 0.0001$ (Barton et al., 1995)	*not available*
	Various groups: 0.70 (Finlay and Darlington, 1995)	Various groups > 0.96 (Finlay and Darlington, 1995)
Lampreys	Petromyzontiformes: 0.78, $p < 0.001$	Petromyzontiformes: 0.74, $p < 0.001$
	Petromyzontidae: 0.95, $p < 0.001$	Petromyzontidae: 0.73, $p < 0.001$
	Mordaciidae: 0.98, $p < 0.001$	Mordaciidae: 0.94, $p < 0.001$
Relative loading PC1		
Chondrichthyans	0.90 >PC1> 0.80 (Yopak et al., 2010)	1.00 >PC1> 0.90 (Yopak et al., 2010)
Teleost fishes	0.86 (Gonzalez-Voyer et al., 2009)	0.94 (Gonzalez-Voyer et al., 2009)
Anurans	0.02 (Liao et al., 2015)	0.82 (Liao et al., 2015)
Mammals	0.40 > loading> 0.30 (Yopak et al., 2010)	1.00 > loading> 0.80 (Yopak et al., 2010)
Lampreys	0.95	0.92

Table 5.1 | Comparison of diverse scaling parameters of the olfactory bulbs and the telencephalon in jawed and jawless vertebrates.

Importantly, the correlation between the olfactory bulbs and the remainder of the brain of lampreys is more pronounced than most groups of vertebrates that have been examined, i.e. it has a larger r-squared value (**Table 5.1**). Furthermore, in lampreys, the correlation between the olfactory bulbs with brain size is more pronounced than that of the telencephalic hemispheres, whereas all examined gnathostome groups present the opposite pattern (**Table 5.1**). Another difference in scaling between these two telencephalic structures can be found when multiple brain structures are analysed in a phylogenetic principal component analysis (pPCA). In lampreys, the first factor of the pPCA (PC1) represents a variable that is nearly isometric with absolute brain size ($r^2 = 0.95$, $F_{1,13} = 297.7$, $p < 0.001$), similar to other vertebrates (Finlay and Darlington, 1995). The olfactory bulbs possess the highest loading in PC1 when compared to those previously documented for diverse groups of vertebrates (**Table 5.1**), indicating that the variation in the relative size of the olfactory bulbs of lampreys is closely related to changes in absolute brain size. Further, in a PCA, the ratio between the loadings of any two variables in PC1 corresponds to the allometric bivariate coefficient of those variables (Klingenberg, 1996). Many brain structures of lampreys have isometric bivariate coefficients with the olfactory bulbs (**Table 4.3**), which has been interpreted in other groups of vertebrates as evidence of concerted evolution between a pair of brain structures (e.g. Gonzalez-Voyer et al., 2009; Gutierrez-Ibanez et al., 2014). These results suggest that the relationship between the olfactory bulbs and brain size in lampreys represent characteristics that have been previously described in the telencephalon of a number of gnathostome vertebrates i.e. (1) a highly correlated, well-predictable relationship with brain size and other brain structures, and (2) a hyperallometric scaling relationship with many of these structures, which suggest late-occurring events in the olfactory bulbs during development (Finlay and Darlington, 1995). Considering these similarities in the scaling rules of different subdivisions of the telencephalon (i.e. the olfactory bulbs and telencephalic hemispheres) between jawless and jawed vertebrates, it can be hypothesized that there are diverging patterns of brain organization in these groups of vertebrates, ranging from an olfactory dominated agnathan type of brain organization ("swimming noses"), where increases in brain size produce disproportionately larger olfactory bulbs, to another type of pattern of brain organization, which has been described in various groups of gnathostomes, where increases in the size of the brain affects increases in the size of brain structures more correlated with higher cognitive functions, such as the telencephalic hemispheres and cerebellum (e.g. Yopak et al., 2010).

A number of neuroanatomical and physiological studies support this hypothesis. It is known that various aspects of the organization of major circuits for motor control in lampreys are shared with the rest of vertebrates, such as the optic tectum (Zompa and Dubuc, 1998; Gruberg et al., 2006; Saitoh et al., 2007; Jones et al., 2009; Kardamakis et al., 2015) and the connectivity of the telencephalic hemispheres with other brain regions, where olfactory and other sensory modalities converge and are integrated into motor output (Northcutt and Puzdrowski, 1988;

Polenova and Vesselkin, 1993; Northcutt and Wicht, 1997; Ocaña et al., 2015). However, there is also evidence of another neural circuit in lampreys that may provide olfactory-driven motor output that is relayed in the medial olfactory bulb, bypassing the telencephalic hemispheres (Derjean et al., 2010; Ericsson et al., 2013; Green et al., 2013; Pérez-Fernández et al., 2014). Considering that signals from both the main and the accessory olfactory epithelia may convey in this network (Ren et al., 2009; Derjean et al., 2010; Chang et al., 2013; Green et al., 2013), it is possible that many olfactory-guided behaviours of lampreys, such as mating and navigation (reviewed in Buchinger et al., 2015), may be segregated and employ a more direct circuitry to "service" such elaborate behaviours. A similar neural circuit may indeed exist in gnathostomes (Anadon et al., 1995; Huesa et al., 2000; Gayoso et al., 2011; Northcutt, 2011; Gaudin et al., 2013), in which case, both of these alternative circuits for olfactory processing could have been present in the last common ancestor of all vertebrates, retained in the ancestor of both lampreys and gnathostomes, but have since then adopted alternate developmental pathways in each lineage throughout vertebrate evolutionary history. In the case of lampreys, many of these olfactory-driven behaviours may require minimal integration with other senses, and thus be directly transformed into motor output without further elaboration. In contrast, multisensory, integrative motor responses may be common for most behaviours in gnathostome vertebrates, which may explain a hyperallometric scaling of major higher order, integrative brain structures in this group, such as the telencephalic hemispheres and cerebellum.

It has been shown in mammals that in a PCA analysis, the first component, which accounts for approximately 96% of the total variance of related brain parts to total brain size, loads most highly on the telencephalon and cerebellum (Finlay and Darlington, 1995). Additional PCA analyses in other gnathostome vertebrates have shown a similar trend (Gonzalez-Voyer et al., 2009; Yopak et al., 2010; Liao et al., 2015). In all of these groups, the olfactory bulbs load most highly in the second or third component, representing a fraction of the variance not explained by absolute brain size. Therefore, in many of these groups of gnathostomes, an increase in overall brain size represents a disproportionate growth of both the telencephalon and the cerebellum and not necessarily a relative increase in the size of the olfactory bulbs. In contrast, the olfactory bulbs of lampreys load more highly in PC1, i.e. the relative increase in the size of these brain structures is correlated with changes in overall brain size (**Table 5.1**). Based on hodological and physiological criteria, it has been proposed that the telencephalon of lampreys is anatomically and functionally similar to that of gnathostomes (Weigle and Northcutt, 1999; Stephenson-Jones et al., 2011; Ocaña et al., 2015), whereas only cerebellar-like structures are thought to exist in lampreys (Weigle and Northcutt, 1998; Montgomery et al., 2012), which has been recently supported by early developmental data (Sugahara et al., 2013; Sugahara et al., 2016). If the pattern of brain organization observed in lampreys represents the ancestral condition of vertebrates, it is possible that the switch from an agnathan-like to a gnathostome-like pattern of brain organization, in which there is a shift in the relative size of the olfactory bulbs and

telencephalic hemispheres, could be directly linked to the origin or the elaboration of a morphologically distinct cerebellum within the origin of gnathostomes, when vertebrates developed more complex motor patterns of feeding and/or locomotion (Northcutt, 2002) that coincided with the appearance of jaws and paired fins (Montgomery et al., 2012). In that case, the described rules of scaling for the telencephalic hemispheres with the remainder of the brain in a number of jawed vertebrate groups may be restricted to gnathostomes and not to all vertebrates. Further insights on this hypothesis will be obtained after equivalent studies have been performed in hagfishes, although there is evidence that these cyclostomes may possess a relatively larger telencephalon, which scale with a steeper slope than do lampreys (Ebinger et al., 1983). However, a differential analysis of the scaling of the olfactory bulbs and the telencephalic hemispheres is still absent for this group. Only after these studies have been performed, will it be possible to conclude whether these patterns of brain organization are typical for all cyclostomes or constitute a specialization of the lamprey lineage. A more representative sample of hagfish species will allow more definitive conclusions to be made and further improve our understanding of vertebrate brain evolution.

5.3 Conclusions and future directions

Evolutionary studies are frequently presented to explain phenomena from the past, producing hypotheses that cannot be directly tested. In this context, this study has highlighted the importance of using comparative studies as a tool for improving our understanding of the evolutionary processes occurring in the central nervous system since the onset of vertebrates. For many years, the diversity of the central nervous system of cyclostomes has been dismissed, because it was assumed that the relative paucity of change between the few species of lampreys examined was representative of the whole group. This collection of papers has shown that a larger sampling size and the inclusion of species from diverse life history traits and phylogenetic groups supports a greater diversity in the nervous system of lampreys than previously thought. Consequently, using only previous knowledge of the nervous system of lampreys, the evolutionary trends and polarity of change in this group was never recognized as a way to explore alternative patterns of brain organization in early radiations of vertebrates. Using a comparative approach, this study provides evidence for the evolutionary history of encephalization and brain organization of agnathans, which may have important implications towards understanding the state of these traits in the earliest vertebrate. In this regard, it can be concluded that early agnathans may have had a relative brain size that lays in between the estimated values for lampreys and hagfishes as groups, similar to the degree of encephalization observed in a number extant species of lampreys. More importantly, this study is the first to describe the correlation between the degree of encephalization and life history traits in

agnathans, both at interspecific and ontogenetic levels, which proposes that a level of plasticity is present in the nervous system of both jawless and jawed vertebrates.

This study also confirmed that both relative and absolute brain size are important parameters for the understanding of brain evolution. In lampreys, absolute brain size was correlated with different patterns of brain organization as a result of the specific brain structure scaling laws that apply within this group of vertebrates, reflecting diverse sensory specializations, in a similar fashion to changes in brain organization described in gnathostomes. One of the most notable examples in lampreys was found in the olfactory bulbs, which show a hyperallometric scaling relationship with brain size, producing disproportionally larger OB in larger brains. In addition, as in gnathostomes, specific groups of lampreys showed grade shifts in the size of a number of brain structures, which can be correlated to particular life history traits, e.g. more active modes of feeding in this group were correlated with a relatively larger optic tectum. Similar results were obtained when comparing lampreys at different stages of their life cycle, where variation in absolute size of the brain was linked to large differences in brain organization, which, in turn, correlated with divergent behaviour of lampreys in each of these phases of the life cycle. In conclusion, these differences in brain organization can be linked to different cerebrotypes that can be described in lampreys both at ontogenetic and phylogenetic levels.

Future studies in comparative studies of the central nervous system of agnathans will complement these results in at least three major areas: (1) the differences expressed across lamprey species demonstrate how important taxonomical diversity is in order to draw true conclusions about brain organization. A large sample of lamprey species, and most importantly, a study of the variation of brain organization patterns in hagfishes, are critical for a comprehensive understanding of the diversity of the central nervous system of agnathans. (2) Despite of the importance of ontogenetic studies for understanding evolutionary trends, this topic has been mostly neglected in comparative brain research. A comparison of brain scaling rules during the life cycle of species with different life history traits, as well as comparative studies across ontogenetic stages of hagfishes, will provide important evidence for the evolutionary pathways shaping the patterns of encephalization and brain organization in vertebrates, and (3) these studies can be complemented with data on brain size from extinct species of jawless and jawed vertebrates, which may clarify important transitional stages from cephalochordates, to jawless, to jawed vertebrates.

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