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Effects of Increased Temperature on Brain and Sensory Development in the Port Jackson Shark (*Heterodontus portusjacksoni*)

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Abstract: Morphological differences in the peripheral (sensory) and central (brain) nervous system may confer sensory and/or behavioral variation in elasmobranchs, both across taxa and throughout ontogeny. Over the last century, sea surface temperatures have increased over 0.5 °C and are predicted to rise 1–4 °C by the year 2100, potentially affecting species' physiological performance negatively. As the nervous system of fishes grows continually throughout their lives, it may be highly plastic in response to environmental changes. This study examined the effects of increased rearing temperature on nervous system development in Port Jackson sharks (*Heterodontus portusjacksoni*). Egg cases (n = 21) were collected from Gulf St. Vincent (Adelaide, SA) and placed into either ambient (17.6 °C) or 3 °C above ambient seawater conditions through hatching and reared for up to five months post-hatch. Relative volumes of the eyes and nose (olfactory rosette) were quantified using magnetic resonance imaging, and relative brain size and size of major brain regions were compared between the two treatment groups. The size of the olfactory bulbs and tegmentum varied significantly between the treatment groups, which suggest differences in primary, secondary, or tertiary sensory processing and/or motor functions at elevated temperatures. While studies on acute responses to environmental conditions cannot inform true adaptation across broad timescales, understanding the effects of increased temperature on the brain phenotype can aid in predicting how elasmobranchs may fare in response to changing ocean conditions.

Keywords: comparative neuroanatomy; climate change; thermal physiology; elasmobranchs

Key Contribution: When reared under increased temperatures, Port Jackson sharks have significantly smaller olfactory bulbs and larger tegmentum. This phenotypic plasticity in the brain may indicate a change in sensory processing in this species when exposed to environmental changes.

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1. Introduction

Environmental stressors caused by climate change can impact the survivability of marine organisms; yet, the effects of anthropogenic changes on the physiology of many species are largely unknown. Over the last century, sea surface temperatures have increased over 0.5 °C, and are predicted to rise 1–4 °C by the year 2100 [1–3]. Temperature increases of 1 °C or more will likely have broad, irreversible effects on species composition and ultimately on ocean ecosystem services [4–7]. Previous studies have examined the combined effects of various environmental stressors on marine organisms (see [1] for a review); but, as temperature dictates the performance of any given biological function, parsing out

the specific effects of temperature on anatomy, physiology, behavior, and survivability of marine species is critical.

An organism's thermal physiology is largely driven by its geographic distribution and the conditions in which it has evolved to thrive under. Changes in physiological performance across temperature ranges and in response to climate change impact numerous biological processes, including body size and growth rate, metabolism, behavior, reproductive success, feeding, and species distribution [4–6,8–13]. In addition, thermal responses are highly plastic and variable, both within and among species. For example, species adapted to a narrow, stable environment, like the tropics, may be pushed beyond their physiological limits with small changes in temperature, compared to species residing under more variable conditions [14]. As individuals experience temperatures either warmer or cooler than a trait's optimal, their performance will decline. Similarly, large shifts in temperature towards either thermal range edge will ultimately inhibit that trait [15]. Processes that are non-essential to survivability, including somatic growth and reproduction, are often the first traits to be impacted, as basic maintenance costs begin to require greater proportions of available energy [16].

While it is metabolically costly to develop and maintain neural tissue [17], a larger brain has been hypothesized to confer some evolutionary benefits [18,19], though these benefits remain speculative [20]. In addition, high energetic costs of brain tissue maintenance is thought to have, in part, constrained brain size throughout evolution [17]. Therefore, evolutionary trends in encephalization (or a larger brain than expected for any given body size) may be positively associated with aerobic capacity due to the high energetic costs of brain tissue [21–24]. Within these evolutionary constraints, there is a large degree of interspecific variation in brain size and brain organization, or the relative size of major brain regions (e.g., the olfactory bulbs, telencephalon, diencephalon, mesencephalon, cerebellum, and medulla oblongata) across cartilaginous fishes [25–31]. A large degree of this variation has been correlated with a species ecology or life history patterns (e.g., [31–34]), a trait also seen in a range of other vertebrate groups (e.g., [35–38]), which may be associated with sensory specialization and/or behavior.

Although variation in brain size and organization between species is high, far less is known about the degree of intraspecific phenotypic plasticity in this group. Understanding this plasticity can be crucial for determining how environmental factors correlate with ontogenetic brain allometries [39]. For example, environmental enrichment has been shown to positively correlate with neural growth across taxonomic groups, including rodents [40–42], birds [43], reptiles [44], and fishes [45]. Similar to seasonal shifts in thermal thresholds of fishes [1], brain size and architecture may also change seasonally or shift with changing environmental conditions in fishes, birds, and mammals [46–50]. Changes in brain size may also be correlated with environmental factors, such as water turbidity, depth, and habitat complexity [51–54]. However, very little is known regarding intraspecific variability in the brain of cartilaginous fishes, (e.g., [55,56]), and even less about variation of the brain in individuals reared under different environmental conditions.

Many species of fish, along with a number of reptiles and amphibians, experience indeterminate growth, whereby their body grows continuously throughout their life [57]. Correspondingly, fish brains also grow throughout ontogeny [58–62], often with a period of rapid growth early in life, tapering off after sexual maturity [55,56,63–65]. This contrasts with mammals, where adult neurogenesis is restricted to two main proliferative brain regions in the forebrain [66–69]. Therefore, lifelong neurogenesis in species with indeterminate growth creates a system with the potential for a high degree of plasticity, but at the potential cost of continued energetic demands [56]. Given that the metabolic rate of ectotherms is largely determined by environmental temperature [70], temperatures elevated above an organism's thermal window may impact normal patterns of brain growth in fish.

Elasmobranch fishes (sharks, skates, and rays) serve many ecological functions and ecosystem services; therefore, assessing their adaptive physiological capabilities in a chang-

ing climate not only helps in understanding environmental effects on these species but also possible effects further down the food web [71]. Elasmobranch populations have been drastically declining over the last few decades, with an estimated 31% of sharks listed as threatened (Critically Endangered, Endangered, or Vulnerable) on the IUCN Red List of Threatened Species™ [72]. In addition, their adaptive capabilities are believed to be limited, owning to the late age at maturity, relatively long lifespan, and low fecundity of many species [73]. This life history strategy may also impair the ability for these species to cope with increased environmental stressors due to climate change [74], with warming critically impacting development time, aerobic metabolism, and thermal tolerance [75]. Studies on the effects of climate change on elasmobranchs indicate variable and species-specific effects across different environmental scales. Combined effects of increased temperature and decreased pH exhibit significant effects on development time in embryos and physiology and behavior in juvenile sharks [76]. In particular, these combined environmental stressors significantly impact metabolism, survival, and body condition during the early life stages of the brownbanded bamboo shark (*Chiloscyllium punctatum*) and little skate (*Leucoraja erinacea*) [77,78]. The sandbar shark (*Carcharhinus plumbeus*) also exhibits a significant reduction in metabolic performance under high temperatures (32 °C) [79]. Although this temperature is outside of its current thermal distribution, the likelihood of encountering higher temperatures will continue to increase with climate change [79].

The Port Jackson shark (*Heterodontus portusjacksoni*) is a temperate, benthic shark species found along the continental shelf in Southern Australia [80]. It is a slow-growing, long-lived species with a low fecundity [81]. This species is also prone to high rates of pre-hatch mortality, reaching over 89% [82]. As the waters of southeast Australia are experiencing sea surface warming faster than the global average [83], this species may be particularly at risk under future ocean conditions. Previous studies on *H. portusjacksoni* have shown that the combined effects of ocean acidification and warming reduces metabolic efficiency [84]. Sharks maintained under increased water temperatures also exhibit atypical swimming patterns, reduced feeding responses, and increased frequency of body injuries [85]. While this species exhibits highly repeatable, individual differences in behavioral trials [86,87], sharks take less time to discriminate quantity when reared under high temperatures [88,89]. The change in laterality and cognition induced by exposure to high temperature was hypothesized to aid in mitigating the deleterious effects of environmental stressors [88,89]. Additional studies on this species have shown the effects of environmental temperature on mortality rates [89], growth rates [90], maximum oxygen consumption and critical thermal limits [85], and muscle capillary density [91]. However, no study has yet investigated whether changes to environmental temperature correlate with morphological changes in the nervous system in the Port Jackson shark.

The brain plays a critical role in controlling physiological and behavioral processes. Thus, understanding the influence and degree of environmental effects on the neural phenotype when exposed to environmental perturbations is crucial for predicting future impacts of anthropogenic stressors. Therefore, this study quantified the effects of increased temperature on the peripheral nervous system (i.e., eyes and olfactory rosette), brain size, and brain organization in juvenile *H. portusjacksoni*. It was hypothesized that sharks reared under increased temperatures would exhibit a reduction in relative brain size, size of major brain regions, and size of peripheral nervous system structures due to the increased oxygen demand and metabolic costs when exposed to higher temperatures.

2. Materials and Methods

2.1. Embryonic Growth and Development

Heterodontus portusjacksoni shark eggs were collected (n = 21) by hand from Christies Beach's Horseshoe Reef in Adelaide, South Australia (35.141° S; 138.465° E), on the same day in November 2016, following the ethical guidelines of the Macquarie University Animal Ethics Committee (ARA # 2016-027; [85]). Egg color and pliability were examined to collect eggs younger than six weeks post-oviposition [92], ensuring that 85% of development

occurred under treatment conditions [85]. Transportation and rearing conditions have been described in detail by Gervais et al. [85]. In brief, eggs were transported and maintained at Macquarie University, New South Wales, at their collection temperature (17.6 °C) for one week; then, temperatures were increased (0.5 °C day^{-1}) until experimental temperatures were reached. Egg cases were then maintained at either ambient (17.6 °C; $n = 11$) or elevated (20.6 °C; $n = 10$) seawater conditions until hatching. The ambient temperature represented the current average sea temperature of Adelaide, SA [93], whereas the elevated temperature represented the end-of-century (EOC) conditions under the RCP 8.5 climate model [2]. After hatching, neonates were placed into a nursery tank under the same temperature their egg cases were maintained in and fed a mixture of prawns, fish, and squid (ad libitum) every other day. Following experimental analyses by Gervais et al. [85] for up to 5 months post-hatch, animals were then euthanized in 0.4 g/L of seawater from MS222 (m-aminobenzoic acid ethyl ester, methanesulfonate salt) if they had not died naturally. Age at death, total length (TL (cm)), and body mass (g) were immediately measured after death to the nearest 0.1 g or 0.1 cm (Table 1). The heads were excised and immersion-fixed in 10% formalin in 0.1 M phosphate buffer and then shipped to the University of North Carolina Wilmington for additional analysis, including collecting white skeletal muscle for Thomas et al. [91] and nervous system tissue for the present study.

Table 1. Brain mass, brain structure mass, and morphometric data collected from the 21 specimens of *H. portusjacksoni* examined in this study. OT mass was estimated using the ellipsoid method, following Wagner [94]. TEG mass was estimated by subtracting the OT mass from the mesencephalon mass. Abbreviations: CER, cerebellum; DI, diencephalon; MED, medulla oblongata; OB, olfactory bulb; OT, optic tectum; TE, telencephalon; and TEG, tegmentum.

ID	Treatment	Age, Days	Sex	Body Mass, g	TL, cm	Brain Mass, g	OB Mass, g	TE Mass, g	DI Mass, g	OT Mass, g	TEG Mass, g	CER Mass, g	MED Mass, g
01A	Ambient	125	M	50.0	23.0	0.688	0.053	0.376	0.061	0.034	0.032	0.078	0.125
02A	Ambient	136	M	81.5	27.0	0.913	0.080	0.441	0.080	0.041	0.036	0.097	0.158
03A	Ambient	126	M	90.0	26.5	0.843	0.080	0.418	0.076	0.043	0.034	0.083	0.130
04A	Ambient	116	M	101.5	26.5	0.909	0.102	0.458	0.078	0.041	0.037	0.094	0.144
05A	Ambient	125	M	77.0	25.2	0.821	0.071	0.408	0.063	0.035	0.037	0.091	0.138
06A	Ambient	112	M	54.5	22.3	0.666	0.056	0.335	0.059	0.041	0.025	0.068	0.117
07A	Ambient	139	F	68.0	24.0	0.857	0.074	0.436	0.069	0.047	0.020	0.085	0.132
08A	Ambient	134	F	63.0	24.5	0.738	0.050	0.398	0.067	0.025	0.041	0.075	0.135
09A	Ambient	116	M	102.5	26.5	0.816	0.091	0.422	0.064	0.037	0.042	0.088	0.132
10A	Ambient	138	M	77.0	26.0	0.827	0.067	0.414	0.066	0.042	0.036	0.091	0.137
11A	Ambient	92	F	75.0	24.1	0.787	0.063	0.393	0.065	0.041	0.032	0.085	0.125
01E	Elevated	177	F	87.5	25.7	0.928	0.082	0.461	0.082	0.036	0.054	0.109	0.163
02E	Elevated	171	M	53.0	22.9	0.771	0.052	0.386	0.078	0.032	0.045	0.084	0.129
03E	Elevated	150	M	45.0	22.5	0.749	0.056	0.358	0.062	0.037	0.037	0.082	0.136
04E	Elevated	177	F	62.0	24.5	0.914	0.061	0.464	0.083	0.036	0.048	0.099	0.145
05E	Elevated	177	M	65.5	23.1	0.825	0.067	0.415	0.074	0.037	0.047	0.091	0.150
06E	Elevated	139	F	33.0	20.0	0.658	0.028	0.357	0.057	0.032	0.031	0.072	0.120
07E	Elevated	145	F	63.8	25.5	0.839	0.062	0.422	0.074	0.046	0.038	0.101	0.160
08E	Elevated	199	M	58.0	25.0	0.996	0.074	0.506	0.083	0.042	0.053	0.115	0.175
09E	Elevated	136	F	41.0	23.5	0.866	0.071	0.419	0.066	0.039	0.039	0.092	0.142
10E	Elevated	206	F	48.0	21.0	0.895	0.047	0.434	0.078	0.047	0.044	0.095	0.138

2.2. Magnetic Resonance Imaging (MRI)

2.2.1. Anatomical Imaging

Following a 16-month minimum period of post-fixation, a subset of specimens ($n = 3$ per treatment) was processed for magnetic resonance imaging (MRI). Prior to imaging, the specimens were placed in a solution of PBS + 0.01% sodium azide for a minimum of one week to remove fixatives and then transferred to a fresh solution of PBS + 0.01% sodium azide + 5 mM of the contrast agent Prohance (Bracco Diagnostics Inc., Monroe Township,

NJ, USA) for a minimum of one week. The addition of a contrast agent reduces the longitudinal relaxation time (T1) and increases the signal-to-noise ratio efficiency [95,96]. The heads were then suspended in a chamber filled with an inert MRI-invisible solution (Fomblin; KJ Lesker). All specimens were scanned on a Bruker 9.4 Tesla small animal scanner (Billerica, MA, USA) with a 20 cm bore and either a 72 mm or 86 mm rat coil, located at the University of North Carolina Chapel Hill's Biomedical Research Imaging Center (BRIC). High-resolution (70–100 µm), T1-weighted anatomical scans, using a 3D FLASH sequence, were acquired from contrast-enhanced tissue. Previous research has demonstrated that these scan parameters produce sufficient contrast between grey/white matter in elasmobranch central and peripheral nervous system tissue [95,97]. The pulse sequence parameters used for this study are shown in Table S1.

2.2.2. Segmentation of MR Images

Peripheral sensory structures (e.g., olfactory rosettes and eyes) from both treatment groups were digitally segmented from the 3D data using ITK-SNAP software (Version 3.8.0), an open-source application that allows for both manual delineation and user-guided automatic segmentation using an active contour (level set) algorithm [98]. The paired olfactory rosettes and eyes from each sample were manually delineated and segmented to calculate the volume of each structure in situ. The volumes of both olfactory rosettes and eyes (left and right) from the MRI analysis were summed before the analyses and recorded to the nearest 0.01 mm^3 (Table 2).

Table 2. Eye and olfactory rosette volume (mm^3) from MRI analysis collected from a subset of 6 specimens of *H. portusjacksoni* imaged in this study.

ID	Treatment	Eye Volume, mm^3	Olfactory Rosette Volume, mm^3
02A	Ambient	716.3	435.4
07A	Ambient	769.6	407.7
10A	Ambient	684.2	405.3
03E	Elevated	591.0	300.9
07E	Elevated	688.7	273.4
09E	Elevated	569.2	254.2

2.3. Tissue Processing

After imaging, brains were removed from the chondrocranium, and the spinal cord was removed at the region of the first complete cervical spinal nerve. The brains were then photographed and weighed to the nearest 0.001 g (Table 1). The brains were then dissected into six major brain regions (the olfactory bulbs, telencephalon, diencephalon, mesencephalon, cerebellum, and medulla) using planar divisions based on external anatomical markers, following the criteria of Yopak et al. [99] and Northcutt [25], and weighed to the nearest 0.001 g (Table 1). Briefly, the olfactory bulbs were separated from the rest of the forebrain within 0.1 mm of the beginning of the olfactory peduncles. The caudal boundary of the telencephalon was delineated at the rostral edge of the optic chiasm, and the remainder of the brain was sagitally bisected to differentiate the four remaining regions. The caudal boundary of the diencephalon was set at a diagonal plane extending from the rostral edge of the optic tectum to the caudal edge of the infundibulum. The midbrain was separated from the hindbrain using a planar vertical division at the caudal edge of both tectal hemispheres. The cerebellum was defined as all of the tissue lying above and including the upper leaf of the cerebellar auricles. The medulla was separated and included tissue from the lower leaf of the auricles and the dorsal and medial octavolateralis nuclei. The caudal boundary of the medulla was marked by the first complete cervical spinal nerve.

The optic tectum volume (a subregion of the mesencephalon) was estimated using the idealized ellipsoid method [100]. The ellipsoid method is a technique to estimate brain region volume, calculated by measuring the length, width, and height of a specified brain region, assuming it is shaped like an idealized ellipsoid or half-ellipsoid [100]. The length

(l), width (w), and height (h) of the left lobe of the optic tectum was measured using digital calipers. The volume (V) of a single hemisphere of the optic tectum was then estimated using the following formula:

$$V = \frac{1}{6}\pi\ (l \cdot w \cdot h) \quad (1)$$

Assuming symmetry of the two tectal lobes, this value was then doubled to calculate the total volume of the optic tectum. The optic tectum volume was then converted into mass using the estimated specific gravity of brain tissue (1.036 mg/mm^3) [94]. Once calculated, the mass of the optic tectum was subtracted from the mass of the mesencephalon to estimate the mass of the tegmentum (Table 1).

2.4. Statistics

The Welch's two sample *t*-test was used to test for differences in age, $\log_{10}$-transformed total length, and $\log_{10}$-transformed body weight between the ambient and elevated treatment groups. A Shapiro–Wilk test was used to test the normality of biometric measurements. Body size varied between treatment groups (see results), and the volume of nervous system (both peripheral and central) structures is known to vary with body size. Therefore, nervous system (peripheral and central) structure volume and brain mass were corrected for body mass using residuals calculated from linear models. To determine whether $\log_{10}$ transformations were necessary prior to the analyses, the best linear model for predicting scaling relationships was determined using the corrected Akaike information criterion (AIC_c). This method corrects for biases from small sample sizes [101], and was designed to minimize Kullback–Leibeler information between the model generating the data and a fitted candidate model [102]. The model with the lowest AIC_c score was therefore selected as the best fit model [103]. Two candidate models of peripheral nervous system (PNS) structure volume and total brain volume were tested, including raw structure size ~ raw body mass (model 1) and $\log_{10}$ structure size ~ $\log_{10}$ body mass (model 2). Modeling of the PNS region and total brain mass against body mass indicated that $\log_{10}$ transformations were a significantly better fit (model 2; Tables S2 and S3). Therefore, $\log_{10}$ PNS structure volume and total brain mass were then regressed against $\log_{10}$ body weight, and the standardized residuals were calculated to obtain a measure of relative size.

For the major brain regions, residuals were calculated from linear models of brain region mass against total brain mass. Two candidate models for each brain region were tested, including brain region mass~total brain mass (model 1) and $\log_{10}$ brain region mass~$\log_{10}$total brain mass (model 2). AIC_c scores indicated that raw brain region mass against brain mass was a significantly better fit (model 1; Table S4). Brain region mass was then regressed against brain mass, and the standardized residuals were calculated to obtain a measure of relative brain region size. Standardized residuals for eye volume, rosette volume, brain mass, and brain region mass between the ambient and elevated temperature groups were then compared using the Welch's two sample *t*-test. Finally, a *post-hoc* Holm–Bonferroni pair-wise correction was used to account for multiple comparisons [104]. An alpha value of $p < 0.05$ was considered significant.

These data were also analyzed using a multivariate approach to visualize the clustering of samples and characterize patterns of brain organization between treatment groups. Principal component analysis (PCA) was used to reduce the number of variables to graphically illustrate the greatest source of variation between individual brain regions. The relative volume of each brain region was first calculated as a fraction of the total brain mass; then, structure proportions were normalized with the arcsine square root transformation before applying the PCA on the correlation matrix. This technique has been widely used in assessing multidimensional datasets in comparative neuroanatomy (i.e., [64,100,105–107]). Finally, PC1 and PC2 scores were compared using the Welch's two sample *t*-test. All data were analyzed using RStudio version 4.0.3 [108] with packages ggplot2 version 3.3.2 [109], FSA version 0.9.3 [110], and MuMIn version 1.43.17 [111].

3. Results

3.1. Body Size

A comparison of biometric data indicated sharks reared under elevated temperatures were significantly older at the time of euthanasia ($t(14.06) = -5.01$, $p < 0.001$) but were significantly smaller in both body weight ($t(17.67) = 2.91$, $p = 0.02$) and total length ($t(17.71) = 2.21$, $p = 0.04$) compared to sharks at ambient temperatures (Figure 1).

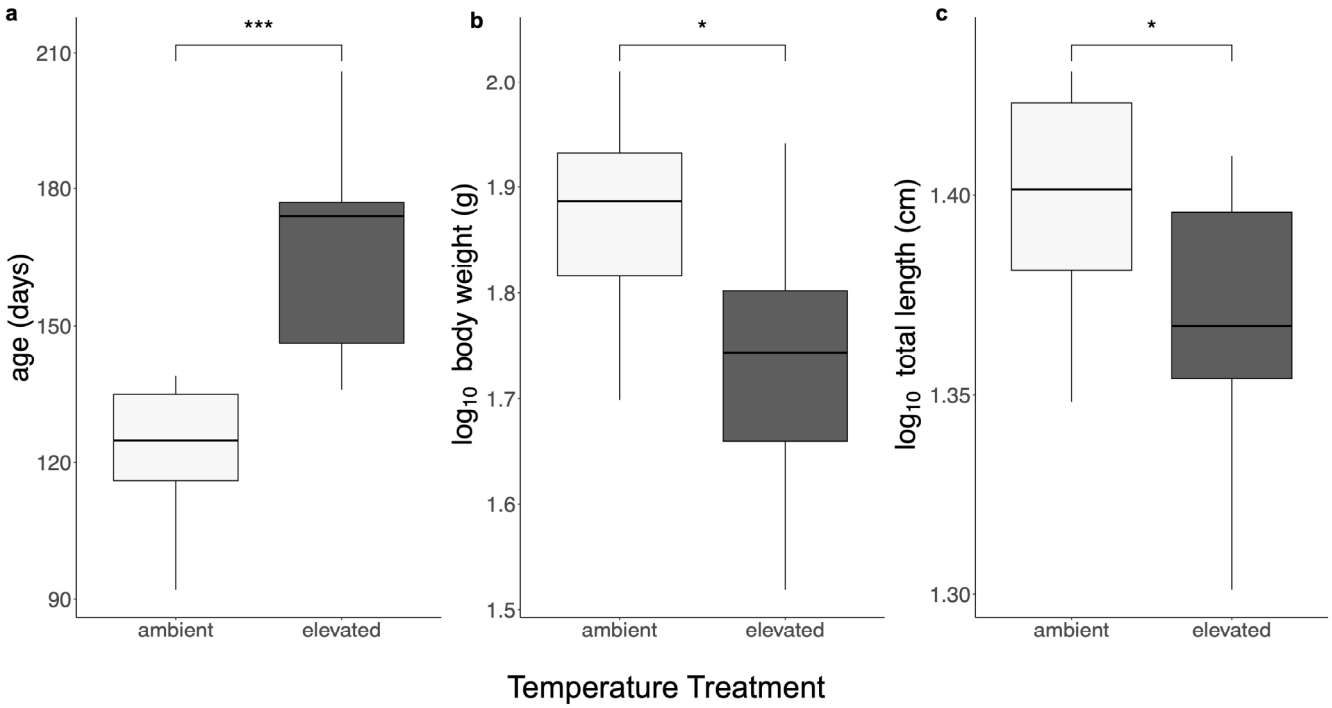

Figure 1. Differences in (**a**) age, (**b**) $\log_{10}$ body weight, and (**c**) $\log_{10}$ total length between the ambient and elevated treatment groups. Asterisks indicate a significance difference.

3.2. The Nervous System

The peripheral and central nervous system morphology of *H. portusjacksoni* is shown in Figure 2. There was no significant difference in eye ($t(2.20) = 0.18$, $p = 0.88$) or olfactory rosette ($t(2.26) = 1.13$, $p = 0.72$) residuals between the ambient and elevated treatment groups (Figure 3). There was also no significant difference in the relative brain mass between the ambient and elevated treatment groups ($t(16.54) = -2.62$, $p = 0.11$; Figure 4). However, the olfactory bulbs were significantly smaller in sharks reared under elevated temperatures ($t(18.81) = 3.71$, $p = 0.01$), while the tegmentum was significantly larger ($t(16.64) = -3.10$, $p = 0.05$). There was no significant difference in the relative size of the telencephalon ($t(18.11) = 0.49$, $p = 0.95$), diencephalon ($t(16.84) = -1.49$, $p = 0.64$), optic tectum ($t(18.82) = 0.95$, $p = 1.00$), cerebellum ($t(18.83) = -2.54$, $p = 0.11$), or medulla oblongata ($t(16.59) = -1.86$, $p = 0.32$) between the treatment groups (Figure 4).

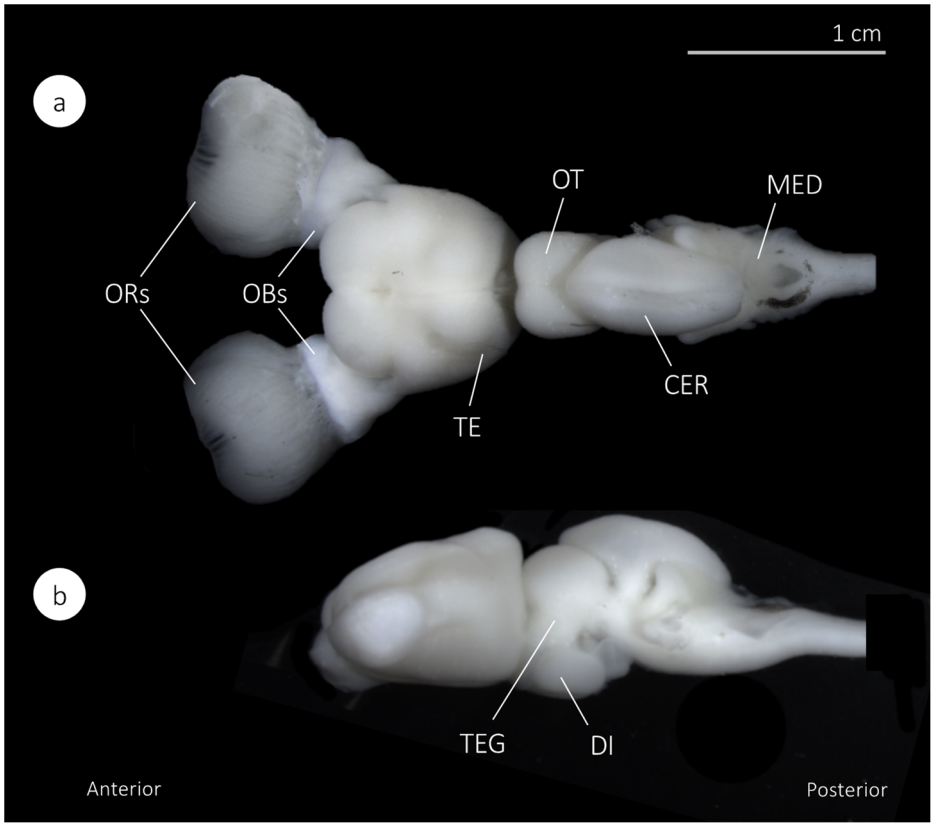

Figure 2. Representative brain of *H. portusjacksoni* from the ambient treatment group in (**a**) dorsal and (**b**) lateral view. The major brain regions are labeled for comparison. Abbreviations: CER, cerebellum; DI, diencephalon; MED, medulla oblongata; OBs, olfactory bulbs; ORs, olfactory rosette; OT, optic tectum; TE, telencephalon; and TEG, tegmentum.

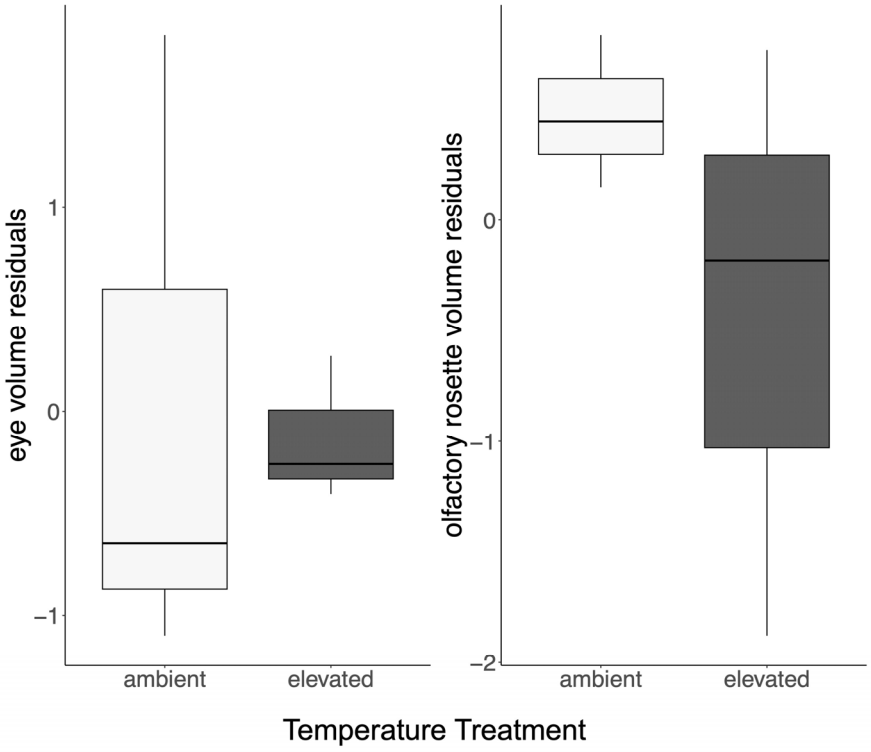

Figure 3. Changes in the relative size (residuals) of paired (**a**) eyes and (**b**) olfactory rosettes of *H. portusjacksoni* reared under the ambient and elevated treatment groups.

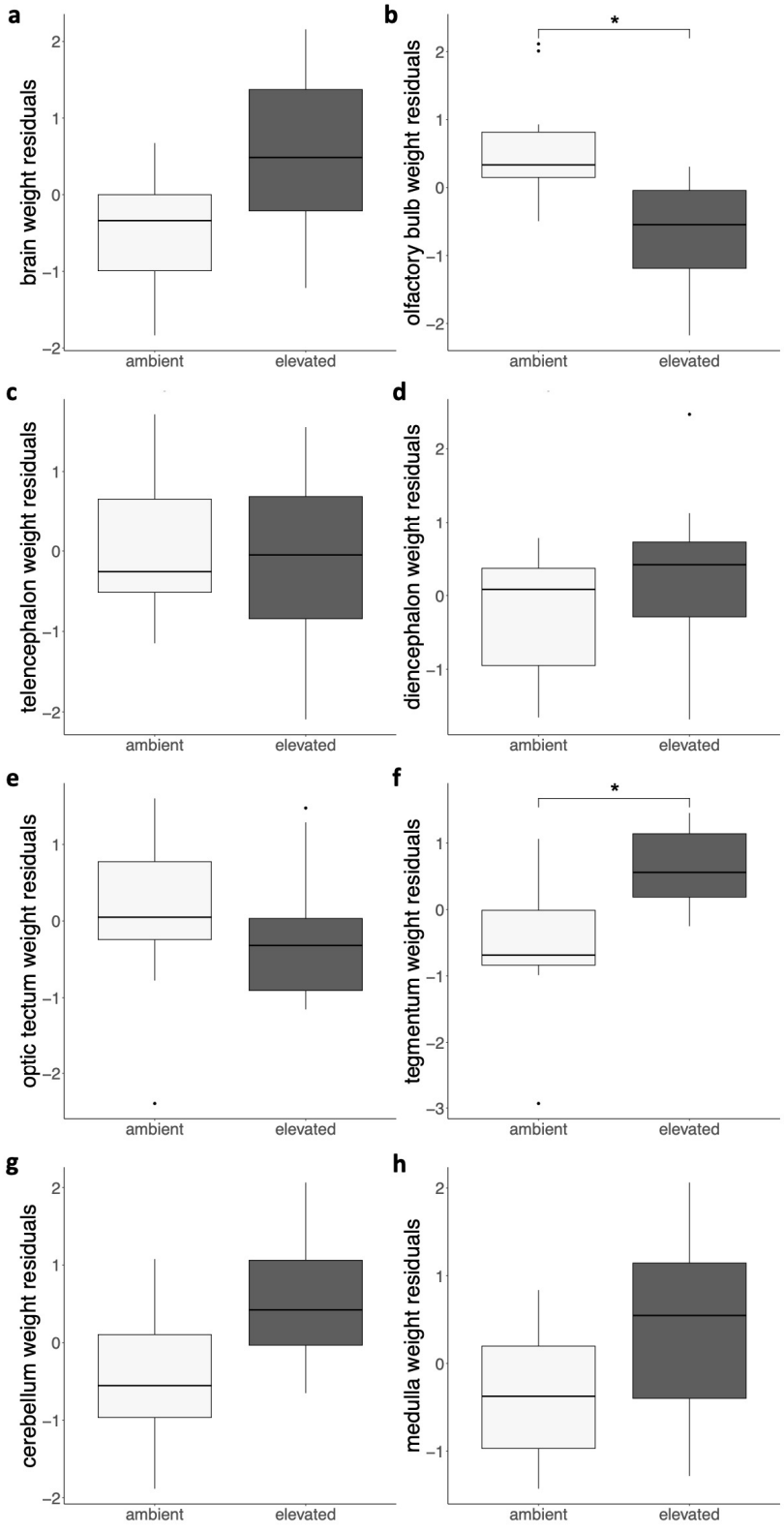

Figure 4. Changes in the relative size (residuals) of the brain and seven major brain regions of *H. portusjacksoni* between the ambient and elevated treatment groups, showing the (**a**) total brain, (**b**) olfactory bulbs, (**c**) telencephalon, (**d**) diencephalon, (**e**) optic tectum, (**f**) tegmentum, (**g**) cerebellum, and (**h**) medulla oblongata. Asterisks indicate a significance difference.

3.3. Principal Component Analysis

Principal component analysis (PCA) performed on the correlation matrix of the relative size of the seven brain regions assessed in this study yielded seven principal components (Table 3). The first axis explained over 53% of the variance, and was primarily related to olfactory bulb size. The second axis was related to tegmentum size, and to a lesser extent optic tectum size, and explained over 78% of the cumulative observed variation. Inclusion of the third component explained 89% of the total cumulative variation, and reflected the loadings for the telencephalon and optic tectum. In the plots of PC1 and PC2 (Figure 5), most individuals from the ambient group clustered on the right, while individuals from the elevated treatment group clustered on the left. There was a significant difference in the PC1 scores between the ambient and elevated treatment groups ($t(18.98) = 3.47$, $p < 0.005$), but not between the scores for PC2 ($t(19) = -1.34$, $p = 0.20$). The clustering was reflective of the ambient treatment group having significantly smaller olfactory bulbs, while the elevated group had a significantly larger tegmentum.

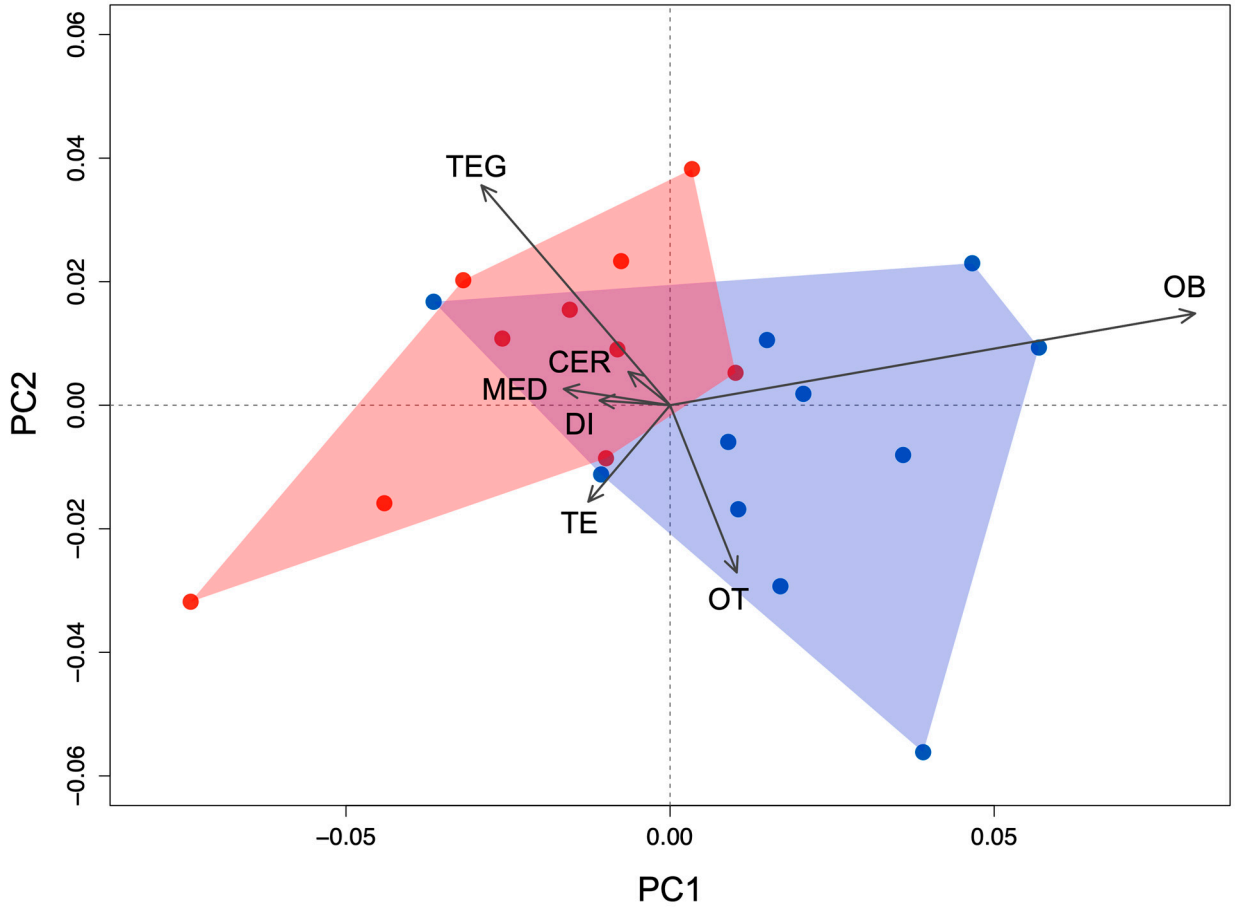

Figure 5. Scatterplot of the principal components PC1 and PC2, representing differences in the relative size of seven major brain regions between the ambient (blue circles) and elevated (red circles) treatment groups. Abbreviations: CER, cerebellum; DI, diencephalon; MED, medulla oblongata; OBs, olfactory bulbs; OT, optic tectum; TE, telencephalon; and TEG, tegmentum.

Table 3. Loadings and cumulative proportion of variation explained by each of the seven principal components.

Brain Region	Principal Component Loadings						
	PC1	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**	**PC7**
Eigenvalue	1.024×10^{-3}	4.874×10^{-4}	2.042×10^{-4}	1.077×10^{-4}	6.900×10^{-5}	3.032×10^{-5}	3.528×10^{-7}
Olfactory bulbs	0.900	0.297	0.043	−0.010	0.036	0.059	0.308
Telencephalon	−0.140	−0.312	0.660	−0.164	−0.180	0.093	0.615
Diencephalon	−0.121	0.015	0.036	0.689	0.585	−0.237	0.332
Optic tectum	0.114	−0.541	−0.513	0.356	−0.343	0.357	0.243
Tegmentum	−0.323	0.712	−0.040	0.242	−0.341	0.396	0.236
Cerebellum	−0.071	0.108	−0.303	−0.089	−0.403	−0.779	0.339
Medulla	−0.182	0.053	−0.452	−0.552	0.477	0.203	0.432
Cumulative proportion of variation	0.532	0.786	0.892	0.948	0.984	0.999	1.000

4. Discussion

Our study assessed the effects of increased rearing temperatures, as a proxy for ocean warming, on peripheral (eyes and olfactory rosettes) and central (total brain and its organization, including the olfactory bulbs, telencephalon, diencephalon, optic tectum, tegmentum, cerebellum, and medulla oblongata) nervous system development in *Heterodontus portusjacksoni*. It was hypothesized that sharks reared under increased temperatures would exhibit a decrease in relative brain size, the size of major brain regions, and the size of peripheral nervous system structures. While the sharks that were reared under increased temperatures exhibited a smaller body size, only the olfactory bulbs were significantly smaller in this group. In addition, another region of the brain (tegmentum) was significantly larger in the elevated treatment group. Though this study was not a functional analysis, Jerison's "Principle of Proper Mass" [112] predicts that an increased size of any specified brain region will, to some degree, reflect a specialized function of that brain region. As a change in the size of either the brain or any major brain regions has implications for functional specialization in all vertebrate groups [43,47,54,113–116], changes to the nervous system at increased rearing temperatures may confer variation in neural function, such as laterality and cognition, sensory impairment, or behavioral anomalies, in these fish [88].

The adult stages of many ectotherms have physiological and behavioral strategies to buffer potential changes in temperature. However, developing embryos are particularly at risk to changes in temperature due to the combined effects of a constrained thermal window, an inability to move away from regions of high temperature [1], and little potential to modify their body temperature [117]. In the present study, embryonic development time was significantly reduced in *H. portusjacksoni* when exposed to a temperature increase of 3 °C (Gervais, unpublished). While the sharks were significantly older in the elevated treatment group, it is due to the fact that embryonic development was shorter and, thus, they hatched earlier than ambient sharks. Despite hatching earlier, they were significantly smaller in body mass and total length, which may be related to differences in oxygen consumption and the increased energetic demand of living under elevated temperatures [85]. Individuals from the current study also exhibited increased capillary density in the elevated treatment group, indicating that they may be able to metabolically restructure their muscle tissue to maintain oxygen supply at higher temperatures [91]. This morphological change in muscle vascularization may indicate a degree of thermal plasticity through acclimatization in this species based on evolutionary adaptations to temperature fluctuations [85,91]. In addition, sharks in the current study were exposed to a constant temperature increase (3 °C), although temperature variability or duration of exposure may also affect the fitness of species facing climate change [118].

Variation in the anatomy and detection thresholds of individual sensory modalities has been well studied across elasmobranchs [119], and are thought to confer variation in sensory capacity to varying degrees [120–125]. These differences correlate with ecological niches, suggesting that these systems are under differential selection pressures associated

with primary habitats [126]. Given these specializations, sensory systems may be limited in their capacity to tolerate changes in environmental parameters in response to anthropogenic disturbance [127]. However, within the peripheral nervous system of *H. portusjacksoni*, there were no differences in the eye volume nor olfactory rosette volume between the two rearing temperatures (Figure 3), suggesting that a 3 °C increase in temperature in this species does not affect gross development of the visual and olfactory system. Given the continued retinogenesis (e.g., [128,129]) and proliferation of olfactory receptor neurons in the olfactory epithelium (e.g., [130]) documented through adulthood in cartilaginous fishes, differences may exist in the peripheral receptors that were not captured by volumetric assessment.

Animals reared under higher temperatures exhibit a significantly larger tegmentum, but no differences in the size of the medulla, cerebellum, optic tectum, diencephalon, or telencephalon. A proposed scaling law for vertebrate brains indicates "late equals large", where brain regions that develop later (e.g., the olfactory bulbs and telencephalon) exhibit steeper allometric relationships compared to brain regions which cease neuronal proliferation earlier [131]. As the brain develops under a hindbrain-to-forebrain gradient [132], a larger hindbrain at higher temperatures may be attributed to the increase in oxygen uptake in embryos. The increase in the size of the tegmentum may also be related to the increase in oxygen uptake rates under higher temperatures [85]. For example, when exposed to increased temperatures, common minnows (*Phoxinus phoxinus*) reared under warmer temperatures have larger brains, specifically larger medullas, a greater metabolic rate, and an overall higher aerobic scope when compared to cool-acclimated fish [133]. The same *H. portusjacksoni* sharks collected from Adelaide double their resting oxygen uptake rates in response to higher rearing temperatures [85]. Taken together, increases in temperature over a narrow range may confer an increase in the rate of nervous system development up to a point. However, surpassing thermal limitations may eventually lead to a reduction in metabolic rate or compromised nervous system development [117,134]. The tegmentum is implicated in a variety of motor functions, including eye movement, and receives secondary fibers from most major sensory, electrosensory, mechanosensory, and auditory fibers [135,136]. Enlargement of this structure may therefore have implications for sensory and motor processing in these sharks reared at higher temperatures.

In contrast to the tegmentum, Port Jackson sharks reared at elevated temperatures exhibit significantly smaller olfactory bulbs, one of the last brain regions to develop embryologically in elasmobranch fishes [137]. The metabolic efficiency of physiological functions, such as feeding, digestion, and growth, is dependent on a species optimum temperature [138]. When reared at high temperatures, embryonic yolk stores are not efficiently used in avians [139, 140] or fish [141]. If oxygen uptake rates are higher under increased temperatures, the internal yolk stores available to embryos may be used up more quickly or inefficiently. As *H. portusjacksoni* is oviparous [142] and therefore relies on a limited yolk supply during development, a faster or inefficient use of available resources may negatively impact affect forebrain development, which occurs during late embryogenesis, leading to smaller olfactory bulbs. The olfactory bulbs also maintain a degree of allometric independence from the rest of the brain in elasmobranchs, whereby they do not scale as tightly with brain size, compared to other brain regions [143,144]. This decoupling may permit a higher degree of phenotypic plasticity in the olfactory bulbs compared to the rest of the brain, and may explain why it is one of the few brain regions that is significantly smaller at higher temperatures. As olfaction is critical for an animal's ability to avoid predators, find prey, identify and communicate with conspecifics, locate potential mates, and navigate [119,145,146], any disruption in olfactory signaling to the brain has the potential to significantly affect prey tracking in *H. portusjacksoni*. In addition, Port Jackson sharks are largely nocturnal and predominantly feed on benthic organisms [147,148], and likely rely heavily on olfactory cues for hunting. This species also has relatively enlarged olfactory bulbs compared to many other benthic coastal species [144], and both juveniles and embryos have been shown to alter their oxygen uptake rates in response to predator-associated odorant cues [149,150]. Therefore, this species may be particularly susceptible to changes in the functionality (and size) of the olfactory bulbs.

Changes in brain size and neural phenotype may be affected by several variables, including, but not limited to, the number and size of neuronal and non-neuronal (glial) cells, the size of these cells, the number and density of connections between cells, and the timing of developmental events forming the nervous system [151–153]. Therefore, the effects of temperature on the nervous system may not be limited to changes in brain size or organization, but likely also affect rate of brain development and/or neuro- and gliogenesis. In lizards, an increased rearing temperature led to an increase in neuron density in the telencephalon, although relative telencephalon size and total neuron number were reduced in the high-temperature treatment group [154]. Due to the potential for lifelong neurogenesis throughout the brain in these species, future research on the effects of environmental perturbations should also focus on the rates of neurogenesis in the brain and peripheral nervous system from development through ontogeny in sharks.

Gross dissection, as a method of brain weight calculation, has been used in many previous studies on fishes (e.g., [25,29,64,155]). Sampling errors from this method in chondrichthyan fishes from additional studies have been reported to be less than 1.3% [25,32,99]. In addition, the ellipsoid method has been widely used in comparative brain studies (e.g., [156–160]), as it is a rapid technique that does not require destructive sampling. While this method is commonly used for its simplicity and speed of data collection [64,100], it can also provide an over- or under-estimation of brain region volume. For example, it supposes that a region perfectly approximates an idealized half-ellipsoid, and often includes ventricles and/or additional brain regions in its volume calculation [31,56,161,162]. This method has therefore been shown to overestimate brain volume, in part, due to the convoluted shapes of the brain [160,161]. Given that the relative optic tectum size in this study was estimated using the ellipsoid method (and the tegmentum size was calculated by subtracting the optic tectum from the rest of the midbrain), these data should be interpreted with caution. Finally, magnetic resonance imaging (MRI) is a technique that has been used for brain volume assessment and anatomical descriptions in fishes [96,97,163,164]. This method has been compared to the ellipsoid method to determine variability in volume assessment in the Australian barramundi, *Lates calcarifer*. Brain regions calculated from MRI were significantly smaller in the telencephalon and optic tectum compared to the ellipsoid method, but there was no difference between MRI and the ellipsoid method in the olfactory bulbs [96]. Therefore, comparing the volume of peripheral nervous system structures (eyes and olfactory rosette) in this study to previous work is potentially limited to similar methodologies.

Although the current study focused on the effects of increased temperature, pH and hypoxic zones have also been predicted to change over the next 100 years, and the consequences of their interactions on the physiology and survival of marine organisms are a topic of increased concern [74,78,165–168]. The combined effects of environmental stressors, including temperature, ocean acidification, and hypoxia, can interact to affect a broad range of physiological processes [165]. While elevated temperatures may not have deleterious effects on sensory structures, other aspects of climate change (such as increasing CO_2) may more greatly affect perceptions. For example, the black sea bream, *Acanthopagrus schlegelii,* exhibited a significantly negative impact on foraging behavior, with longer responses and latency times and reduced swimming velocity, when reared under elevated pCO_2 conditions [169]. Related to the visual system, the phototactic responses of two-spotted goby larvae, *Gobiusculus flavescens*, were significantly affected under elevated CO_2 levels [170]. Future research should investigate the impacts of elevated CO_2 on retinal topography, olfactory receptor neuron density in the olfactory rosette, and neurogenesis in the brain, and how these changes ultimately impact behavioral responses to elucidate how these sensory structures are affected by environmental change [127].

5. Conclusions

Our study is one of the first to quantify the effects of increased temperature on brain development in an elasmobranch species, the Port Jackson shark, *H. portusjacksoni*. While

studies on acute responses to environmental conditions cannot inform true adaptation across broad timescales, quantifying the effects of temperature on the brain phenotype can aid in predicting the consequences of environmental perturbations. These results may also indicate how elasmobranchs will fare in response to changing ocean conditions, particularly when combined with behavioral and cognition studies. The waters of southeast Australia are experiencing sea surface warming faster than the global average [83]. Although many marine animals have the capacity to alter their distribution, a shift in geographic range may be limited for *H. portusjacksoni*, as they exhibit high fidelity to breeding sites [171]. Moreover, as an oviparous species, embryos are incapable of behaviorally thermoregulating during development. Due to the life history strategy of this species, *H. portusjacksoni* may possess limited adaptive capabilities in the face of warming ocean temperatures. This study showed that sharks reared under higher temperatures were significantly smaller in body mass, despite being significantly older at time of euthanasia. The sharks exposed to increased temperatures also displayed a larger tegmentum and smaller olfactory bulbs than sharks at ambient temperatures. Understanding the effects of increased temperature on neural phenotypes, especially within the regions responsible for processing sensory and/or motor information, may aid in determining the consequences of ocean warming in this species and indicate how they will respond to environmental perturbations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes8120611/s1, Table S1: Scan parameters for T1-weighted images of *H. portusjacksoni*; Table S2: Results of the best-fit model for each PNS region; Table S3: Results of the best-fit model for the total brain; Table S4: Results of the best-fit model for each brain region.

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Data Availability Statement: All data needed to evaluate the conclusions in the paper are present in the main text and/or the Supplementary Materials. Detailed numerical data will be made available to individuals upon request.

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