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Research Article

The role of temperature in shaping the nervous system phenotype in the epaulette shark (Hemiscyllium ocellatum)

Emily E. Peele[a], Carolyn R. Wheeler[b,c], Jennifer T. Wyffels[d,e], Jodie L. Rummer[b,f], John W. Mandelman[g], Kara E. Yopak[a]

[a] Department of Biology and Marine Biology, University of North Carolina at Wilmington, Wilmington, NC 28403, USA
[b] ARC Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, QLD 4814, Australia
[c] School for the Environment, The University of Massachusetts Boston, Boston, MA 02125, USA
[d] Marine Science Research Center, Ripley's Aquariums, Myrtle Beach, SC 29577, USA
[e] Center for Bioinformatics & Computational Biology, University of Delaware, Newark, DE 19713, USA
[f] Marine Biology and Aquaculture, College of Science and Engineering, James Cook University, Townsville, QLD 4811, Australia
[g] Anderson Cabot Center for Ocean Life, New England Aquarium, Boston, MA 02110, USA

Corresponding Author:
Emily Peele
E-mail address: peelee@uncw.edu

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Abstract

Introduction

Over the last century, sea surface temperatures have increased by more than 0.5 ˚C, with predictions suggesting an increase of 1-4 ˚C by 2100. Oceanic warming poses significant challenges to marine species, particularly those with physiological and developmental processes that are tightly linked to environmental conditions. In cartilaginous fishes, including sharks, the brain grows continually throughout life, supported by the capacity for lifelong neurogenesis. This feature suggests that the nervous system – both peripheral (sensory) and central (brain) – of sharks may be highly plastic and able to adapt dynamically to a changing environment.

Methods

We investigated the effects of elevated rearing temperature on brain development in the epaulette shark (*Hemiscyllium ocellatum*), a species known for its tolerance of environmental fluctuations in intertidal habitats. Eggs (n = 12) were sourced from a breeding stock at the New England Aquarium and reared in either ambient (27°C) or elevated (31°C, 4°C above ambient) seawater temperatures until two months post-hatch. Using histological analyses, we compared the relative volume of the nose (olfactory rosette), total brain, and major brain regions between treatment groups.

Results

Despite this species' natural exposure to temperature variability, Generalized Linear Models revealed that elevated temperature significantly altered the volume of the olfactory sensory epithelium, olfactory bulbs, and medulla oblongata after accounting for overall brain size. Analyses of proportional brain region volumes also showed that elevated temperature was associated with reduced olfactory bulb size and increased subpallial volume relative to total brain size. These changes may suggest potential changes in cognitive capacity related to olfactory processing as well as sensory and / or motor functions at elevated temperatures.

Conclusions

While short-term studies, such as this one, cannot capture long-term adaptive potential, understanding the impacts of elevated temperature on brain phenotypes provides critical insights into how elasmobranchs may cope with changing ocean conditions. Such knowledge will be vital for predicting the resilience of these ecologically important species to future environmental stressors.

Introduction

Environmental stressors driven by climate change can significantly affect the survival of marine organisms; however, the physiological impacts of anthropogenic change on many species remain largely unexplored [1]. Over the past century, sea surface temperatures have risen by more than 0.5°C and are expected to increase by 1–4°C by the year 2100 [2-4]. A temperature increase of 1°C or more is anticipated to have widespread, irreversible effects on species composition and, consequently, ocean ecosystem services [5-8]. Although previous studies have examined the combined effects of various environmental stressors on marine organisms [see 2 for review], it is essential to isolate the effects of temperature on anatomy, physiology, behavior, and survival of marine species, as temperature is a key determinant of biological function.

The thermal physiology of an organism is largely influenced by its geographic distribution and the environmental conditions under which it has evolved. The thermal window, which varies among species, defines the limits and capacity of an organism to acclimatize within a certain temperature range and is thought to be constrained by the effects of temperature on metabolic demands [2, 9]. Within this range, key functions such as growth and reproduction operate optimally. Deviations from these optimal temperatures may restrict performance, and further temperature shifts toward either extreme potentially inhibit various biological processes, including somatic growth and reproduction [10]. As a result, a larger share of available energy must be used to maintain basic physiological functions [11].

It has been proposed that the high energetic cost of brain tissue maintenance may have constrained brain size evolution [12]. While maintaining and developing neural tissue is metabolically expensive [12], larger brains likely provide certain evolutionary advantages [13, 14], though these benefits remain speculative [15]. As a result, trends in encephalization (having a larger brain relative to body size) may be positively associated with aerobic capacity, due to the high energy demands of brain tissue [16-19]. However, within these evolutionary constraints, there is still considerable interspecific variation in brain size and organization, or the relative size of major brain regions (e.g., the olfactory bulbs, telencephalon, diencephalon, mesencephalon, cerebellum, and medulla oblongata) across cartilaginous fishes [20-26]. Much of this variation has been linked to a species' ecology or life history patterns [26-29], a pattern also documented in various other vertebrate groups [30-33], potentially conferring variation in sensory specialization and/or behavior. Despite significant interspecific variation in brain size and organization, much less is known about the extent of intraspecific phenotypic plasticity within the cartilaginous fishes [34]. Understanding this plasticity is crucial for identifying how environmental factors correlate with ontogenetic brain allometries [35], as environmental enrichment has been shown to correlate positively with neural growth across numerous taxonomic groups, including rodents [36-38], birds [39], reptiles [40], and fishes [41]. Similar to how fish experience seasonal shifts in thermal thresholds [2], brain size and organization may also fluctuate seasonally or in response to changing environmental conditions in fishes, birds, and mammals [42-46].

Fishes, along with amphibians and some reptiles, may exhibit indeterminate growth, meaning their bodies continue to grow throughout life [47]. As such, the brains of fishes also grow throughout their lifespan [48-52], often experiencing a period of rapid growth early in life that tapers off after sexual maturity [53-57]. Adult neurogenesis in fishes has been described along the entire rostrocaudal brain axis [49, 51, 58-70]. This contrasts with mammals, where adult neurogenesis is limited to two main proliferative brain regions in the forebrain [71-74]. Thus, lifelong neurogenesis in species with indeterminate growth creates a system with a large potential for plasticity, but at the potential cost of continued energetic demands. Given that the metabolic rate of ectotherms is largely influenced by environmental temperature [75], elevated temperatures beyond an organism's thermal optima are likely to impact normal patterns of brain

growth in fishes. Indeed, in one species of shark (*Heterodontus portusjacksoni*), animals reared at elevated temperatures exhibited significant differences in size of the olfactory bulbs and tegmentum, suggesting normal brain development can be affected by environmental stressors [34].

Elasmobranch fishes (sharks, skates, and rays) play essential roles in ocean ecosystems, and understanding how they adapt physiologically to a changing climate is crucial – not only for assessing direct effects on these species, but also for evaluating potential cascading effects throughout the food web [76]. Since the first global assessment in 2014, elasmobranch populations have significantly declined, with approximately 31% of sharks now listed as threatened (Critically Endangered, Endangered, or Vulnerable) on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species [77]. In addition, their adaptive capabilities may be limited due to life history characteristics, such as late maturity, relatively long lifespans, and low fecundity for many species [78]. This combination of factors may impair their ability to cope with increasing environmental stressors due to climate change [79], with warming potentially impacting embryonic development time, aerobic metabolism, and thermal tolerance [80].

Research on the effects of climate change on elasmobranchs indicates variable and species-specific impacts across different environmental scales [81-87]. The epaulette shark (*Hemiscyllium ocellatum)* in particular has been used to study the effects of environmental stressors on their growth and physiology [88-93]. *H. ocellatum* is a small, oviparous species that inhabits tropical reefs and reef flats on the Great Barrier Reef in Australia [94]. These reef flats are highly variable environments, where temperatures can fluctuate as much as 12°C during a 24-hour period [95]. Due to these natural fluctuations in temperature, this species has potentially adapted to thermally, and therefore physiologically, challenging environments [96]. For example, epaulette sharks are highly tolerant to hypoxia [97-100] and hypercapnia [88, 89, 92], possibly confirming some adaptive abilities to environmental stressors. However, little is known about how environmental perturbations, such as elevated temperatures, impact brain growth and maintenance in *H. ocellatum*.

The brain is central to sensory, motor, and behavioral processes, making it crucial to understand how environmental perturbations influence the neural phenotype for predicting future impacts of anthropogenic stressors. It is predicted that changes in an animal's metabolic rate or physiological processes can influence the development of metabolically costly brain tissue. This study aimed to quantify the effects of elevated temperature on the peripheral olfactory system (olfactory rosette), brain volume, and brain organization (volume of major brain regions) in juvenile epaulette sharks. Despite previous research indicating temperature can affect brain development in sharks [34], it was hypothesized that epaulette sharks reared under elevated temperatures would exhibit no significant change in volume of nervous system structures due to this species' evolutionary history of adaptation to highly variable environmental regimes.

Methods

Embryonic growth and development

In collaboration with the University of Massachusetts Boston and James Cook University, eggs from *Hemiscyllium ocellatum* were collected from a singular breeding pair at the New England Aquarium under the ethical guidelines of the New England Aquarium Animal Care and Use Committee (protocol # 2017-05). Egg collection and husbandry details of the maintenance systems have been previously described in detail [90]. Briefly, eggs were placed in either a tank with present day (termed ambient) (27°C; n=6) or elevated (31°C; n=6) seawater temperatures from 10 to 14 days post egg deposition until hatching; eggs were also assessed 2-3 times per week for viability. After hatching, neonates were measured for total length (cm) and mass (g) (Table 1) before being transferred to nursery tanks maintained at the same temperature as their

incubation conditions. Sharks were offered a mix of minced shrimp and clam daily. Two months after hatching, hatchlings were euthanized in 0.4 g/l seawater of MS-222 (m- aminobenzoic acid ethyl ester, methansulfate salt) according to the Institutional Animal Care and Use Committee (IACUC) guidelines of the University of North Carolina Wilmington (IACUC # A2021-007) and transcardially perfused with 0.1M phosphate buffer (PB) followed by 4% paraformaldehyde (PFA) in 0.1 M PB. Heads were preserved in 4% PFA in 0.1 M PB.

Tissue Processing

After fixation, brains were excised, detached from the spinal cord, and weighed to the nearest 0.01g. Brains were then cryoprotected in 30% sucrose in 0.1 M phosphate buffer and subsequently embedded in OCT compound, rapidly frozen in isopentane, and stored at -80°C. Tissue was sectioned on a cryostat with a tape system (CryoJane, Leica Biosystems). Coronal sections were cut at 30 µm and every third section was collected. Sections were dehydrated in a graded ethanol series, stained with 0.1% cresyl violet acetate, and coverslipped with Permount mounting media (Fisher Scientific). Each section was photographed at 10x magnification on a Leica MC170 HD with a Leica MC190 HD camera (Leica Biosystems), an Aperio AT2 DX Slide Scanner (Leica Biosystems), or an Olympus VS200 Slide Scanner (Olympus LS).

In each histological section, the sensory epithelium of the olfactory rosettes was isolated from the rosette epithelium surface area by thresholding, aided by the high cell density of the sensory region. Preprocessing of images involved applying a Gaussian filter with a smoothing sigma of 5 to reduce noise and enhance signal. Major brain areas were delineated digitally following criteria of previous studies [20, 27], and boundaries within the brain were noted histologically by various *sulci limitans* (distinct sulci within the tissue) and *zona limitans* (areas devoid of cells). Briefly, the olfactory bulbs were digitally separated from the rest of the forebrain by including all tissue anterior to the proximal olfactory peduncle. The telencephalon was subdivided into the pallium and subpallium. The *sulcus limitans lateralis*, *sulcus limitans medialis*, and *zona limitans medialis* were used to mark the boundary between the pallium and subpallium. The caudal boundary of the telencephalon (and rostral boundary of the diencephalon) was delineated at the rostral edge of the optic chiasm. The caudal boundary of the diencephalon was set at the *commissura posterior*. For this study, the diencephalon was separated into the epithalamus, thalamus, and hypothalamus. The epithalamus included the habenula, *habenular commissure*, and *stria medullaris*. The thalamus included both the dorsal and ventral thalamus, and the hypothalamus included the preoptic area, inferior lobe, suprachiasmatic nucleus, nucleus medius hypothalamus, nucleus lateralis tuberis, and hypophysis. The midbrain was separated into the optic tectum (including the superficial tectal zone, central tectal zone, and periventricular tectal zone) and tegmentum, which was separated ventrally below the *sulcus tectotegmentalis*. The caudal boundary of both the optic tectum and tegmentum was set at the caudal end of the *velum medullare anterius*. The cerebellum included both the corpus cerebelli and the vestibulolateral cerebellum. The medulla was subdivided into the two acousticolateralis nuclei, the dorsal octavolateralis nucleus (DON) and medial octavolateralis nucleus (MON). The DON included tissue within the dorsal granular ridge and its *crista cerebellaris,* and the MON included tissue within the lateral granule area and its *crista cerebellaris*. The caudal boundary of the acousticolateralis nuclei was set at terminus of both *crista cerebellaris*. All tissue not included in either the DON or MON was designated as medulla. The caudal boundary of the medulla was marked by the first complete cervical spinal nerve.

All histological images were analyzed using QuPath [101]. Once the area of each structure within each section was determined, the area was multiplied by section thickness (30 µm) and distance between sections (number of skipped sections), to calculate individual volumes of the sensory epithelium and major brain nuclei (Tables 1, 2). Brain region volume was not corrected for tissue shrinkage due to staining, and universal shrinkage across all regions was assumed.

Statistical analyses

Incubation duration (time *in ovo*), log_{10}- transformed neonate mass, and log_{10}- transformed hatchling total length were collected, and a Welch's two sample t-test was used to test for developmental differences between treatment groups. A Welch's two sample t-test was also used to test for differences in age, log_{10}-transformed total length, and log_{10}-transformed body mass 2-months post-hatch between the ambient and elevated treatment groups. A Shapiro–Wilk test was used to test the normality of biometric measurements, and a Levene's test was used to evaluate homogeneity of variances across groups. To account for variation in body mass, total brain mass was 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 (AICc). This method corrects for bias from small sample sizes [102] and was designed to minimize Kullback-Leibler information between the model generating the data and a fitted candidate model [103]. The model with the lowest AIC_c score was selected as the best fit model. Two candidate models for total brain volume were tested, including raw brain volume ~ raw body mass (model 1) and log_{10} brain (region) volume ~ log_{10} body mass (model 2). Modeling of total brain mass against body mass indicated that raw volume was a significantly better fit (model 1; Table S1). Therefore, raw total brain volume was then regressed against body mass, and the standardized residuals were calculated to obtain a measure of relative volume. To assess olfactory rosette sensory epithelium volume, two candidate models were tested, including raw olfactory rosette volume ~ raw body mass (model 1) and log_{10} olfactory rosette sensory epithelium volume ~ log_{10} body mass (model 2). Modeling of region mass against body mass indicated that raw volume was a significantly better fit (model 1; Table S1). Therefore, raw region volume was then regressed against body mass, and the standardized residuals were calculated to obtain a measure of relative volume. Standardized residuals for epithelial volume and total brain volume between the ambient and elevated temperature groups were then compared using the Welch's two sample t-test.

A Generalized Linear Model (GLM) with a Gaussian error distribution and identity link function was used to test whether treatment group (ambient vs. elevated temperature) explained variance in olfactory rosette volume after controlling for total body size. In this model, olfactory rosette volume and total body mass were both covariates, and treatment group was included as a fixed factor. In addition, a GLM was used to test whether treatment group explained variance in brain region volume after controlling for total brain size. In these models, brain region volume and total brain volume were both covariates, and treatment group was included as a fixed factor. This method has been widely used for brain size allometry adjustments [104, 105]. Finally, to account for differences in total brain volume between treatment groups (see results), brain region volume was corrected using the proportion approach, where region volume (numerator) is expressed as a percentage of the total brain volume (denominator). This method has been commonly used to normalize for differences in total brain size in volumetric brain studies [104-106]. Proportional brain region volumes were then compared between the ambient and elevated temperature groups using the Welch's two sample t-test. An alpha value of $p < 0.05$ was considered significant.

Results

Body size

Incubation duration for eggs maintained at elevated temperature was shorter than eggs maintained at ambient temperatures ($t(9.46) = 6.90$, $p < 0.001$). A comparison of biometric data at hatching indicated that sharks reared under elevated temperatures weighed significantly less ($t(9.38) = 4.24$, $p < 0.01$), but were not significantly smaller in total body length ($t(9.70) = -0.04$, p

= 0.97) when compared to sharks reared at ambient temperatures (Fig 1). Two months post-hatch (63 ± 4 days), sharks reared at elevated temperatures were no longer significantly smaller in body mass (t(9.82) = 1.42, p = 0.18) or total length (t(10.00) = 1.49, p = 0.17) compared to sharks reared at ambient temperatures (Fig 2).

The nervous system

Gross images of peripheral and central nervous system morphology of *H. ocellatum* are shown in Figure 3. Representative histological sections of the nervous system, including regional boundaries within the brain, are shown in Figure 4. There was no significant difference in olfactory rosette volume residuals between treatment groups (t(7.86) = 0.68, p = 0.52) (Fig. 5). There was, however, a significant difference in relative total brain volume between ambient and elevated treatment groups (t(9.68) = 2.40, p = 0.04; Fig. 6).

Figures 7–9 present the observed variation in proportional brain region volumes between treatment groups. Across these plots, the olfactory bulbs and subpallium trended larger in the control treatment group, whereas the subpallium and the medulla oblongata suggested an increase in volume in the elevated temperature treatment group. Trends in the data motivated the statistical analyses, which formally tested the significance of these patterns using parametric and scaling-based methods.

GLM analysis indicated a significant effect of treatment on volume of the olfactory epithelium (Treatment: β = 0.00 ± 0.00, t(8) = –2.73, p = 0.03) after correcting for brain size. In addition, GLM results revealed a significant treatment effect in both olfactory bulb (Treatment: β = -0.00 ± 0.00, t(9) = -3.65, p = 0.01) and medulla oblongata volume (Treatment: β = 0.00 ± 0.00, t(9) = 2.32, p = 0.05) after correcting for total brain size. Treatment effect was not significant in any additional brain region [pallium (Treatment: β = -0.00 ± 0.00, t(9) = -1.62, p = 0.14), subpallium (Treatment: β = 0.00 ± 0.00, t(9) = 0.94, p = 0.37), epithalamus (Treatment: β = 0.00 ± 0.00, t(9) = 1.84, p = 0.10), thalamus (Treatment: β = 0.00 ± 0.00, t(9) = 1.10, p = 0.30), hypothalamus (Treatment: β = -0.00 ± 0.00, t(9) = -0.54, p = 0.60), optic tectum (Treatment: β = 0.00 ± 0.00, t(9) = 0.70, p = 0.50), tegmentum (Treatment: β = 0.00 ± 0.00, t(9) = 0.18, p = 0.87), cerebellum (Treatment: β = -0.00 ± 0.00, t(9) = -0.72, p = 0.49), DON (Treatment: β = -0.00 ± 0.00, t(9) = 1.13, p = 0.29), or MON (Treatment: β = 0.00 ± 0.00, t(9) = 0.84, p = 0.42)].

Comparing proportional differences in brain region volume also indicated significant differences in size between the treatment groups. In the forebrain, the olfactory bulbs (t(8.23) = 3.74, p = 0.01) were significantly smaller in sharks that developed and were reared in elevated temperatures. However, the subpallium was significantly larger in sharks reared in the elevated treatment group. (t(9.99) = -3.04, p = 0.01). There was no significant difference in relative volume of the pallium (t(9.59) = 2.04, p = 0.07), epithalamus (t(9.74) = -1.39, p = 0.20), thalamus (t(9.63) = 0.67, p = 0.52), or hypothalamus (t(9.67) = 0.30, p = 0.77) between treatment groups (Fig. 7). In the midbrain, there was no significant difference in relative volume of the optic tectum (t(9.82) = -0.39, p = 0.71) or tegmentum (t(6.36) = 0.57, p = 0.59) between treatment groups (Fig. 8). Finally, in the hindbrain, there was no significant difference in relative volume of the cerebellum (t(7.09) = 1.32, p = 0.23), DON (t(9.44) = -0.73, p = 0.48), MON (t(9.09) = -0.31, p = 0.77), or medulla oblongata (t(8.43) = -2.05, p = 0.07) between treatment groups (Fig. 9).

Discussion

This study evaluated how elevated incubation and rearing temperatures, simulating ocean warming, impact the development of both the peripheral (olfactory rosette) and central (total brain and its organization) nervous system in *H. ocellatum*. It was hypothesized that there would be no difference in rosette volume, brain volume, volume of major brain regions in sharks incubated and reared under elevated temperatures, attributing to this species' highly variable natural environment. However, sharks incubated under elevated temperatures (+4°C above

ambient) hatched sooner and had a smaller body mass at hatching. They also exhibited smaller total brain volume, with reductions that were not uniform across the brain, but localized to the olfactory bulbs. However, sharks reared at higher temperatures also exhibited a significantly larger subpallium when proportional volume was compared, and GLM results indicated a significant treatment effect on the olfactory rosette and medulla. Though this study was not a functional analysis, it is hypothesized that the extent of information processing of any given brain region is proportional to the amount of neural tissue allocated to that specific function [107]. Therefore, a change in volume may confer a difference in processing power or cognitive capacity of the given region. Alterations in the size of the brain and its major regions have significant implications for functional specialization across all vertebrate groups [39, 43, 108-112]. Consequently, modifications in the nervous system due to elevated incubation and rearing temperatures may lead to variations in neural function, potentially affecting laterality, cognition, sensory capabilities, or behavioral patterns in sharks [113].

Adult ectotherms display various physiological and behavioral strategies to mitigate the effects of temperature fluctuations, such as altering their activity patterns to exploit microhabitats with more favorable thermal conditions [114]. However, developing embryos are especially vulnerable to temperature changes because of their limited thermal window and minimal capacity to regulate their body temperature [1]. This is especially true of oviparous species, where the embryos are unable to seek thermal refuge from high-temperature zones [2]. In this study, time to hatching for *H. ocellatum* was significantly reduced when eggs were subjected to a 4°C increase in temperature (Fig. 1) [90]. Faster development times in elevated temperatures have been observed across a diversity of vertebrate and invertebrate species, and in both endotherms and ectotherms, due to the temperature dependence of development [75, 115, 116]. Embryos from this study incubated at elevated temperatures completed embryonic development and consumed yolk significantly faster compared to sharks incubated in present day conditions [90]. In Wheeler et al. [90], resting metabolic rate (RMR) estimates measured throughout incubation were higher in embryos reared at 29°C compared to 27°C, indicating the influence of temperature on metabolic rate regulation in this species. Embryonic oxygen uptake rates were significantly higher at 29°C compared to 27°C but were significantly reduced at 31°C. Sharks reared at 27°C and 31°C, as reported in Wheeler et al. [90] were used in this study, suggesting that the observed effects on the nervous system may stem from the metabolic demands associated with elevated temperatures. The combined change in RMR and decreased oxygen consumption rates suggest a compensatory behavioral strategy to save energy by reducing ventilation, or a reduction in metabolic performance at 31°C. The *pejus* temperature has therefore been estimated for *H. ocellatum* at just below 31°C, where physiological performance of a given trait begins to decline [90].

While sharks from the present study weighed significantly less at hatching after incubation at elevated temperatures, the difference was not significant 63 (± 4) days after hatching (Fig. 2). This compensation in body mass may be due to these sharks feeding much earlier compared to those from ambient temperatures [90]. However, after hatching, *H. ocellatum* reared at high temperatures had reduced aerobic scope and maximum oxygen uptake rates, further supporting a reduction of aerobic performance at 31°C [90]. In addition, muscle tissue from the same neonate sharks sampled for this study also exhibited a lower muscle fiber density and an increase in oxidative damage to muscle proteins at elevated temperatures [91]. This increase in oxidative damage could additionally lead to higher tissue maintenance costs [91, 117]. However, other developmental and structural properties of the muscle were consistent across treatment groups, suggesting a potential absence of muscle plasticity or limited capacity to acclimate to elevated temperatures [91]. Epaulette sharks incubated in the elevated treatment group also exhibited incomplete coloration patterns after hatching, which could affect their ability to camouflage, find mates, or hide from predators [118]. In Gervais et al. [93], *H. ocellatum* showed

no difference in food consumption rate when incubated and reared at high temperatures, but those individuals in higher temperatures also had a significant decrease in body mass at hatching and higher mortality. These varied responses to elevated temperature could be attributed to the fact that the sharks in Gervais et al. [93] were incubated and reared at 32°C, potentially exceeding the *pejus* temperature for this species [90, 93].

Interspecific differences in both anatomy and detection thresholds of sensory systems have been extensively studied in elasmobranchs [119], and these differences are thought to confer variation in sensory capacity across modalities [120-125]. These variations are linked to ecological niche, indicating that sensory systems face selection pressures based on primary habitat [126]. In *H. ocellatum*, there was no difference in olfactory rosette epithelial proportional volume between the temperature treatments (Fig. 5). However, GLM revealed a significant treatment effect in olfactory rosette epithelial volume after accounting for total body size. These results suggest that a 4°C increase may impact the gross development of the neuroepithelial tissue in this species. Considering the continuous proliferation of ORNs in the olfactory epithelium throughout adulthood in cartilaginous fishes [129], there could be variations in peripheral receptors when reared in warmer temperatures. In addition, given significant variation in the size of the olfactory bulbs (Fig. 7), finer resolution data specifically related to odor sampling should be collected in future work. These studies should incorporate measurements of olfactory receptor neuron (ORN) number and density, axon counts of the olfactory nerve and tract [127, 128], and behavioral analysis of odor detection to assess the functional implications for sensory detection and processing.

Despite compensation of body size 63 ± 4 days after hatching (Fig. 2), total brain volume and proportional volume of the olfactory bulbs were significantly smaller in individuals incubated and reared at 31°C. According to metabolic theory and the evolution of encephalization, environmental temperature likely plays a significant role in determining the brain size of ectothermic vertebrates. Although the maintenance of brain tissue incurs similar costs in both ectotherms and homeotherms [130, 131], it is proportionally more expensive for ectotherms, given that their whole-body metabolic rate is ten times lower than that of homeotherms [131]. As such, these metabolic constraints may also limit the energy available for brain growth and maintenance [132]. Since *H. ocellatum* has a reduced aerobic scope at 31°C, a change in metabolic efficiency may have affected normal patterns of brain development and rates of neurogenesis after birth in this species.

Within the brain, proportional volume of the olfactory bulbs were significantly smaller compared to control conditions, and GLM results confirmed that treatment explained the variation beyond the differences in total brain size. Taken together, these results indicated that rearing sharks in elevated temperatures did not decrease total brain volume uniformly, but rather disproportionately impacted the development of the olfactory bulbs. The metabolic efficiency of physiological functions, such as feeding, digestion, and growth, is dependent on a species' optimum temperature [133]. When exposed to elevated temperatures, embryonic yolk stores are not efficiently utilized in birds [134, 135] or fishes [136]. Changes in oxygen uptake rates at elevated temperatures may have resulted in embryos depleting their yolk stores more rapidly [90] or inefficiently. As *H. ocellatum* is oviparous [137, 138] and depends on a finite yolk supply during development, a faster or inefficient use of these resources could adversely affect forebrain development, which takes place later in embryogenesis, potentially resulting in smaller olfactory bulbs. In elasmobranchs, the olfactory bulbs also exhibit a high degree of allometric independence from the rest of the brain, meaning they do not scale as tightly with brain size compared to other brain regions [139, 140]. This decoupling may allow for greater phenotypic plasticity in the olfactory bulbs relative to other parts of the brain and may explain why they were significantly affected by development temperature. The olfactory bulbs were also the only brain region significantly smaller in the Port Jackson sharks (*Heterodontus*

portusjacksoni) when reared in elevated temperatures [34]. Hyperallometry of the olfactory bulbs throughout ontogeny has been observed in the few cartilaginous fishes studied to date; this may be attributed to increased olfactory capacity necessary for long-distance navigation, prey capture, or in finding mates later in life [56, 57]. Since olfaction is essential for key behaviors, such as predator avoidance, prey detection, communication with conspecifics, mate location, and navigation [119, 141, 142], any disruption in olfactory signaling, processing in the brain, or interference with signal transmission to secondary and tertiary targets could significantly impair prey-tracking abilities in this species. Notably, sharks from the current study took a longer time to track food in the elevated temperature treatment compared to sharks reared in present-day temperatures (Wheeler, personal observation).

Within the telencephalon, the ventrally positioned subpallium was significantly larger in sharks incubated and reared at elevated temperatures, when compared using proportional region volume. However, treatment did not explain additional variation in brain region size once total brain size was accounted for using GLM. In elasmobranchs, the telencephalon plays a significant role in processing and regulating various sensory modalities, as well as higher cognitive functions [143-145]. In particular, the subpallium receives input from the pallium and olfactory projections from the olfactory bulbs [146, 147], as well as input from the diencephalon and tegmentum [148]. This region is therefore involved in multimodal sensory integration and likely controls complex behaviors [149, 150]. In the Port Jackson shark (*H. portusjacksoni*), animals reared in higher temperatures exhibited stronger laterality in behavioral trials, which was hypothesized to be adaptive to better cope with the detrimental effects of climate change [113]. Increases in temperature within a narrow range may accelerate nervous system development in specific brain nuclei, as suggested by the enlargement of the telencephalic subpallium, which could in turn have a range of effects on sensory processing in these sharks. As surpassing thermal limitations likely reduced metabolic rate in this study, this may have contributed to changes in nervous system development.

The hindbrain did not show a significant change to the proportional volume of any region; however, GLMs revealed a significant treatment effect in the medulla after accounting for total brain size. The medulla is the site for central termination of multiple sensory-motor nuclei and projections from the octavolateralis senses [151]. A larger medulla has been reported in teleosts under elevated temperatures, where the ocellaris clownfish (*Amphiprion ocellaris*) exhibited significant enlargement of this region when reared in warmer conditions [152]. Common minnows (*Phoxinus phoxinus*) acclimated to a 6°C above ambient conditions also had both larger total brains and a larger medulla than cool- acclimated fish. However, these fishes made more errors during maze exploration, suggesting the increase in brain volume did not confer a behavioral advantage [153]. In addition, as the medulla is one of the first brain regions to differentiate embryologically [154], a change in embryonic metabolic rate at higher temperatures may have affected normal development of this region. In addition, the two analytical approaches used in this study (GLM and proportional comparisons) differ in their sensitivity to variance, such that proportional analyses are more influenced by heterogeneous variability among brain regions, whereas GLMs provide a more conservative test by explicitly modeling variance while controlling for total brain size. As a result, apparent differences (or lack thereof) in significance among regions may reflect variance structure rather than differences in effect magnitude, given the small sample size of this study.

Studies on the effects of environmental stressors on the physiology of aquatic organisms must also consider the inherent variability of aquatic environments. Specifically, physiological and behavioral responses to a stable, average condition are likely different than responses to fluctuating conditions. Therefore, species that have thermally acclimated to a stable environment may not translate to their physiological performance in their natural, variable environment [155]. Previous studies have indicated animals can tolerate greater maximum and

minimum temperatures when conditions are rapidly changed compared to more gradually [156, 157], but a more gradual change in temperature allows for greater adjustments to thermal plasticity [158]. In addition, laboratory studies on adult epaulette sharks reveal that their metabolic rates are more strongly influenced by diel patterns than by temperature, underscoring the importance of considering daily activity cycles when evaluating physiological responses [159]. Since epaulette sharks have evolved in highly variable environmental conditions, the effects of stable, elevated temperatures on brain development observed in this study may not directly reflect the outcomes that would occur in the wild. Therefore, future research should account for diverse responses species may exhibit when scaling up any observed effect to broader, environment-level stressors. Finally, the small sample size in the current study may have limited statistical power, potentially masking differences among brain regions and therefore preventing detection of all treatment-related effects.

Several factors can influence brain size and neural phenotype, such as the number and size of neuronal and non-neuronal (glial) cells, cell size, the density and number of connections between cells, and the timing of developmental events that shape the nervous system [160-162]. Therefore, the effects of temperature on the nervous system may extend beyond differences in brain size or organization to potentially affect the rate of neuro- or gliogenesis. In lizards, elevated rearing temperatures increased neuron density in the telencephalon, even though the relative size of the telencephalon and total neuron count decreased in the high-temperature treatment group [163]. Given the capability for lifelong neurogenesis throughout the brain in fishes [51], future studies should explore how environmental changes influence neurogenesis rates in both the brain and peripheral nervous system from development throughout ontogeny in sharks. Temperature can also affect neurotransmitter function in the brains of developing tilapia (*Oreochromis mossambicus*), and brain regions differentially respond to changes in temperature at different developmental stages, with varying effects in males and females [164]. Since central neurotransmitters are involved in early brain differentiation [165, 166], changes in neurotransmitter levels may affect the development of the central nervous system in these fishes in response to temperature [167].

This study focused on the effects of elevated temperature; however, changes in pH and the expansion of hypoxic zones are also predicted over the next century. The combined effects of these anthropogenic stressors on the physiology and survival of marine organisms are of growing concern [79, 86, 168-171]. Environmental stressors such as temperature, ocean acidification, and hypoxia can have complex interactions, influencing a broad range of physiological processes [168]. Future CO_2 conditions have been linked to significant changes in gene expression involved in the signaling of neurotransmitters in the European sea bass, *Dicentrarchus labrax*, and coho salmon, *Oncorhynchus kisutch* [172, 173]. Additionally, in elasmobranchs, acetylcholinesterase activity decreases in the telencephalon and optic tectum of juvenile white-spotted bamboo sharks (*Chiloscyllium plagiosum*) when exposed to elevated CO_2 conditions [83]. This decrease in acetylcholinesterase is hypothesized to relate to decreased swimming behavior in high CO_2 conditions, as acetylcholinesterase activity has been associated with motivation [174], learning, and memory [83, 175], and muscle responsiveness [176]. Environmental exposure to hypoxia in elasmobranchs is hypothesized to impair development and increase neonate mortality [177]. However, the epaulette shark has demonstrated a buffering capacity to reduced oxygen. During exposure to anoxia, sharks enter a state of neuronal hypometabolism and ventilatory suppression thought to be related to the maintenance of brain energy [97, 98]. This potential ability to alter brain metabolism *via* adenosine receptor activation and reduction in oxidative metabolism during periods of environmental stress may have evolved due to the species' exposure to low oxygen while hunting in intertidal pools [98]. They have shown neuroprotective mechanisms of increased nitric oxide synthase (NOS) activity following exposure to hypoxia, possibly causing vasodilation, therefore

maintaining blood flow withing the central nervous system. They also display no apoptotic figures after hypoxia exposure, thought to be related to delayed neuronal death under hypoxic conditions [178]. However, results from the current study indicate these neuroprotective mechanisms may not extend to all future anthropogenic stressors. Research on individual anthropogenic stressors therefore reveals varying, species-specific effects across different environmental contexts. Understanding the neural mechanisms behind these changes, studied as both singular stressors and combined, is crucial for predicting the impacts of future ocean conditions.

Conclusions

Though the brain is linked to critical biological processes, few studies have aimed to determine the effects temperature as a climate stressor has on phenotypic plasticity in the elasmobranch brain. While studies on acute responses to environmental conditions do not provide insights into true adaptation over broad timescales, quantifying the effects of temperature on nervous system organization may help predict the neural outcomes of environmental perturbations. As *H. ocellatum* is regularly exposed to fluctuating environmental conditions [95], we predicted sharks to display some buffering capacity in response to temperature. However, due to a likely reduction in metabolic efficiency at their *pejus* temperature, which is required for brain growth and maintenance, sharks exposed to elevated temperatures exhibited a smaller total brain and proportional olfactory bulb volume compared to sharks at ambient temperatures, and significant treatment effects after controlling for total body size in the olfactory rosette. In addition, sharks reared in higher temperatures also exhibited an increase in proportional volume of the subpallium and a significant treatment effect in the medulla from GLM analysis, after controlling for total brain size. The waters around the Great Barrier Reef – this species' native range – are experiencing high sea surface temperatures and annual heat waves resulting in mass coral bleaching events [179-181]. As epaulette sharks hunt for prey in shallow intertidal flats [96], they may be particularly susceptible to these heatwaves. In addition, these warm-adaptive species may already live close to their thermal tolerance limits, and therefore have limited capacity to adapt to further increases in temperature [182]. Understanding the effects of elevated temperature on neural phenotypes, especially within the regions responsible for processing sensory and/or motor information, may provide insight in potential cognitive changes or physiological consequences of these species within the next 100 years in response to climate change.

Statements

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Statement of Ethics
This study protocol was reviewed and approved by the New England Aquarium Animal Care and Use Committee (protocol # 2017-05) and the Institutional Animal Care and Use Committee (IACUC) guidelines of the University of North Carolina Wilmington (IACUC # A2021-007).

Conflict of Interest Statement
K. Yopak is an Editor-in-Chief of *Brain, Behavior, and Evolution*. Otherwise, the authors have no conflicts of interest to declare.

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Conceptualization: EEP, KEY; Methodology: EEP, JTW, KEY; Data Collection: EEP, CRW, JTW, JLR, JWM, KEY; Formal Analysis: EEP; Visualization: EEP; Supervision: KEY; Writing—original draft: EEP; Writing—review & editing: EEP, CRW, JTW, JLR, JWM, KEY

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. Further enquiries can be directed to the corresponding author.

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Figure Legends

Fig. 1. Differences in epaulette shark (**a**) incubation time, (**b**) $\log_{10}$ body mass at birth, and (**c**) $\log_{10}$ total length at hatching between the ambient and elevated treatment groups. Asterisks indicate a significance difference.

Fig. 2. Differences in epaulette shark (**a**) $\log_{10}$ body mass, and (**b**) $\log_{10}$ total length 2-months post-hatch between the ambient and elevated treatment groups.

Fig. 3. Representative brain of *H. ocellatum* from the ambient treatment group in (a) dorsal and (b) lateral view. The major brain regions are labeled for comparison. Abbreviations: Cer, cerebellum; Hyp, hypothalamus; Med, medulla oblongata; OBs, olfactory bulbs; ORs, olfactory rosette; Tect, optic tectum; Tegm, tegmentum; and Telenc, telencephalon. Key: A, anterior; D, dorsal; P, posterior; V, ventral.

Fig. 4. Representative cresyl violet-stained histological sections of *H. ocellatum* from the ambient treatment group. Lines through the dorsal brain indicate location of coronal sections. (a) olfactory rosette and sensory epithelium, (b) telencephalon and boundary between the pallium and subpallium (c) representative forebrain, midbrain, and hindbrain subdivisions, (d) cerebellum and medulla oblongata subdivisions, and (e) caudal hindbrain subdivisions. Abbreviations: cer, cerebellum; DON, dorsal octavolateralis nucleus; hyp, hypothalamus; med, medulla oblongata; MON, medial octavolateralis nucleus; pal, pallium; PL, primary lamella; SF, secondary fold; spal, subpallium; tect, optic tectum; tegm, tegmentum.

Fig. 5. Relative volume (residuals) of paired olfactory rosettes of *H. ocellatum* incubated and reared at ambient and elevated temperatures.

Fig. 6. Relative volume (residuals) of total brain in *H. ocellatum* incubated and reared at ambient and elevated temperatures.

Fig. 7. Relative volume (percentage) of six major *H. ocellatum* forebrain regions for ambient and elevated treatment groups, showing the (**a**) olfactory bulbs, (**b**) pallium, (**c**) subpallium, (**d**) epithalamus, (**e**) thalamus, and (**f**) hypothalamus. Asterisks indicate a significance difference.

Fig. 8. Relative volume (percentage) of two major *H. ocellatum* midbrain regions for ambient and elevated treatment groups, showing the (**a**) optic tectum and (**b**) tegmentum.

Fig. 9. Relative volume (percentage) of four major *H. ocellatum* hindbrain regions for ambient and elevated treatment groups, showing the (**a**) cerebellum, (**b**) DON, (**c**) MON, and (**d**) medulla oblongata. Abbreviations: DON, dorsal octavolateralis nucleus; MON, medial octavolateralis nucleus.