UNIVERSIDADE DE LISBOA
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Fish Brain Development in a Changing Ocean

Francisco José Ferraz de Oliveira Soeiro de Carvalho

Mestrado em Ecologia Marinha

Dissertação orientada por:
Professor Doutor Rui Rosa
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To the fish and the humans of sea and land
Aos peixes e aos humanos do mar e da terra

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my “almost advisor” José Ricardo Paula to the humorous cleaner,
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Abstract

Unravelling how marine species invest in brain tissues (or brain regions) matching the fitness-relevant cognitive demands dictated by a changing environment is a priority in climate change-related (ocean warming and acidification) research. Within this context, this dissertation aimed to assess the combined effects of ocean warming (Δ 4 °C) and acidification (Δ 700 µatm pCO_2 and Δ 0.4 pH) in the brain development (brain/body mass ratio and brain macro-region growth) of several juvenile fish species from different climate regions. Namely: three species adapted to a more stable (tropical) environment (clown anemonefish *Amphiprion ocellaris,* orchid dottyback *Pseudochromis fridmani* and neon goby *Elacatinus oceanops*), and other three adapted to a less stable (more seasonal; temperate) environment (seabream *Diplodus sargus*, flatfish *Solea senegalensis* and meagre *Argyrosomus regius*). The results show that the temperate species used in this study are only affected by ocean acidification in both total brain and specific brain regions, while the used tropical species are affected by ocean acidification, ocean warming and also by the interaction of ocean warming and ocean acidification. In fact, both total brain and every brain-region except for Telencephalon are affected by future conditions of ocean warming and ocean acidification differently according to each species. The lack of responses to ocean warming by the temperate species is here attributed to the widespread latitudinal distribution of those species, and thus the adaptation to a wider temperature range than tropical species. Curiously, all the significant interactions between the two studied stressors are antagonistic interactions with a cross-tolerance mechanism, meaning that under those interactions, the brain weight is closer to control levels than under each of the stressors separately. Possible behavioural and ecological implications of those results are also discussed. Despite the distinct dichotomic pattern between temperate and tropical habitats, the results among fish species and specific brain macro-regions do not exhibit a subjacent pattern. This different results highlight the idea of species-specific phenotypic responses to these climate change-related stressors.

Keywords: Fish brain development, climate change, ocean warming, ocean acidification, temperate and tropical ocean habitats

Resumo

Uma das prioridades da Ecologia Marinha relacionada com as alterações climáticas (aquecimento e acidificação dos oceanos) tem sido perceber como as espécies marinhas investem em tecidos cerebrais (ou regiões cerebrais) de modo a adaptarem-se às necessidades cognitivas relevantes para a aptidão ditada pelo ambiente em mudança. Neste contexto, esta dissertação teve como objetivo avaliar os efeitos combinados do aquecimento (Δ 4° C) e acidificação (Δ 700 µatm pCO_2 e Δ 0,4 pH) oceânicos no desenvolvimento encefálico (relação massa encéfalo/ massa corporal e crescimento das macro-regiões do encéfalo) de várias espécies juvenis de peixes de diferentes regiões climáticas, a saber: três espécies adaptadas a um ambiente mais estável (tropical) (o peixe-palhaço *Amphiprion ocellaris*, o orquídea dottyback *Pseudochromis fridmani* e o neon-goby-azul *Elacatinus oceanops*), e outras três adaptadas a um ambiente menos estável (sazonal, temperado) (o sargo *Diplodus sargus*, o linguado *Solea senegalensis* e a corvina *Argyrosomus regius).* Os resultados mostram que as espécies temperadas usadas neste estudo são afetadas apenas pela acidificação do oceano, tanto ao nível da totalidade do encéfalo, como de cada macro-região específica, enquanto as espécies tropicais usadas são afetadas pela acidificação dos oceanos, aquecimento dos oceanos e também pela interação entre aquecimento dos oceanos e acidificação dos oceanos. De facto, tanto a totalidade do encéfalo como as macro-regiões encefálicas, exceto o telencéfalo, são afetados de maneira diferente pelas condições futuras de aquecimento dos oceanos e acidificação dos oceanos de acordo com cada espécie. A falta de respostas ao aquecimento dos oceanos pelas espécies temperadas é aqui atribuída à ampla distribuição latitudinal dessas espécies e, portanto, à adaptação a uma faixa de temperatura mais ampla que as espécies tropicais. Curiosamente, todas as interações significativas entre os dois fatores estudados são interações antagonísticas com um mecanismo de tolerância cruzada, o que significa que nessas interações, o peso do cérebro está mais próximo dos níveis das condições controlo do que sob cada um dos fatores separadamente. Possíveis implicações comportamentais e ecológicas desses resultados também são discutidas. Apesar do padrão dicotómico bem definido entre habitats temperados e tropicais, os resultados entre espécies de peixes e macro-regiões encefálicas específicas não exibem um padrão subjacente. Estes diferentes resultados destacam a ideia de respostas fenotípicas específicas de cada espécie em resposta às condições de alterações climáticas futuras.

Palavras-chave: Desenvolvimento cerebral dos peixes, alterações climáticas, aquecimento do oceano, acidificação do oceano, habitats marinhos temperados e tropicais

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List of abbreviations

Ach	Acetylcholine
AIC	Akaike Information Criterion
ASAB	Association for the Study of Animal Behaviour
B/Bo	Brain/Body Mass Ratio
BS/B	Brainstem/Brain Mass Ratio
CH_4	Methane
C	Cerebellum
C/B	Cerebellum/Brain Mass Ratio
Cl	Chlorine
CO_2	Carbon dioxide
CO_3^{2-}	Carbonate ion
CMIP5	Coupled Model Intercomparison Project Phase 5
CNS	Central nervous system
°C	Degree Celsius
D	Diencephalon
D/B	Diencephalon/Brain Mass Ratio
DA	Dopamine
DivAV	Divisão de Aquacultura e Valorização
FCUL	Faculdade de Ciências, Universidade de Lisboa
GABA	γ-aminobutyric acid
GABA-A	Binding site for GABA
GLMM	Generalized Linner Mixed Models
Glu	Glutanate
H^+	Hydrogen proton
HCO_3	Bicarbonate
H_2CO_3	Carbonic acid
IEC	International Ethological Conference
IPCC	Intergovernmental Panel on Climate Change
IPMA	Instituto Português do Mar e da Atmosfera
KCC2 (K^+/Cl^-)	potassium chloride cotransporter 2

LMG	Laboratório Marítimo da Guia (Marine and Environmental Sciences Centre, FCUL)
MAGICC6	Model for the Assessment of Greenhouse Gas-Induced Climate Change 6
MS222	Tricaine mesylate
N_2O	Nitrous oxide
NAd	Noradrenaline
NeuroD	transcription factor involved in the differentiation of nervous system
NE	Norepinephrine
NKCC1 ($Na^+/K^+/Cl^-$)	sodium, potassium, and chloride cotransporter 1
nm	nano meters
O/B	Optic Tectum/Brain Mass Ratio
OA	Ocean acidification
OP	Optic Tectum
OW	Ocean warming
pCO_2	Carbon dioxide partial pressure
pH	power of Hydrogen
ppm	parts per million
RAS	Recirculating Aquaculture Systems
RCP	Representative Concentration Pathways
SPE	Sociedade Portuguesa de Etologia
SST	Sea Surface Temperature
T	Telencephalon
T/B	Telencephalon/Brain Mass Ratio
TMC-Iberia	Tropic Marine Centre
UV	Ultraviolet
5-HT	Serotonin
µatm	micro atmosphere
µm	micro meters

1 Introduction

1.1 Climate change

The advent of the industrial era implied that human activities develop cumulative consequences on global ecosystems since the early nineteenth century (Oreskes 2004; IPCC 2013). The emissions of anthropogenic greenhouse gases (carbon dioxide CO_2, methane CH_4, and nitrous oxide N_2O) have increased dramatically, due to the combustion of fossil fuel, industrial processes and changes in the land use as the intensive agriculture and farming improves (IPCC 2013). Those activities are responsible for an annual release of over six billion metric tons of carbon (IPCC 2013), leading to a rising concentration of CO_2 from the pre-industrial 280 µatm (Petit et al. 1999) to the current 390+ µatm over the past 200 years (IPCC 2013). As predicted by the Intergovernmental Panel on Climate Change (IPCC 2013), this concentration will reach 790-1140 µatm, whether the emissions are reduced, kept constant or increased (Figure 1.1). This tremendous growth of emissions enhances the atmospheric greenhouse effect, leading to global warming of 0.3°C (in a most optimistic emission scenario) to 4.8°C (in a most pessimistic emission scenario) by 2100. In Figure 1.1 it is shown the global surface temperature change (in °C) from 1900 to 2100, and CO_2 concentration (ppm) from 1850 to 2100.

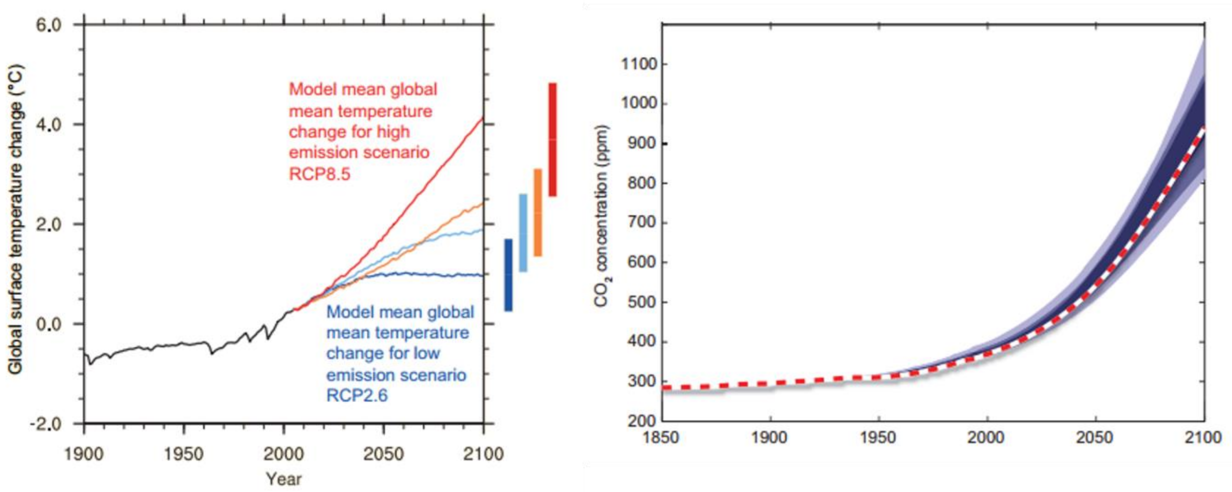


Figure 1.1 – Global surface temperature change and CO_2 concentration (adapted from IPCC (2013)) In the left chart: Global mean surface temperature from 1986 to 2005 and respectively predicted evolution according to the four Representative Concentration Pathways scenarios (RCP2.6 (dark blue), RCP4.5 (light blue), RCP6.0 (orange) and RCP8.5 (red). Likely range for global temperature change by the end of the 21st century is indicated by vertical colored bars. In the right chart: range of CO2 concentrations change simulated by the Model for the Assessment of Greenhouse Gas-Induced Climate Change 6 (MAGICC6) (Adapted from (IPCC 2013)).

Being the climate change the most important anthropogenic stressor on the planet ecosystems, it has received great attention from the scientific community and from most of the world countries' governments. Educational campaigns are developed in many countries in national and in international programs and initiatives. However, human behaviour seems in most cases still individually and collectively unprepared to react by mitigating the causes for such climate changes.

1.2 Marine ecosystems and climate change

Marine ecosystems are very delicately balanced systems, supported by a wide range of complex and intricate biological interaction such as predator-prey relationships, competition, facilitation and mutualism. The success of a single species or of an entire ecosystem rely on such interactions (Doney et al. 2012). Any disruption of this fragile web may have consequences such as the extinction of a species or the fragmentation of an ecosystem. Physical and chemical changes in the habitat as the ones promoted by climate change are expected to differently affect behavioural and demographic traits, leading to shifts in the size, structure, spatial range and seasonal abundance of populations (Brierley and Kingsford 2009). The species-specific impacts of climate change make the holistic prediction of the ecosystem impairment very difficult to perform. Although, it is expected that more stable habitats, such as coral reefs, populated with more specialist species will suffer a greater effect of climate change when compared to more variable habitats with more generalist species as temperate coastal ecosystems (Pörtner and Farrell 2008; Pörtner et al. 2011).

During the past decade, predicted conditions of ocean warming and acidification for the end of the 21st century, as well as their effects on marine biota, have been object of attention from the scientific community and have been increasingly intensively studied (Fabry et al. 2008; Brierley and Kingsford 2009; Doney et al. 2012; Poloczanska et al. 2016). These studies have shown that the combined effect of different stressors may be additive, when the combined effects of two stressors is the result of the sum of each one of the stressors effects ($= a + b$); synergistic, in this case the combined effect of two stressors is greater than their effects sum ($> a + b$) and antagonistic, the effect of each stressor annul the effect of the other, and the combined effect is closer to the control conditions ($< a + b$) (Crain et al. 2008). Several studies were conducted aiming to assess the interactive effect of OW and OA (Crain et al. 2008) and several antagonistic effects were found (Parker et al. 2010; Catarino et al. 2012; Byrne and Przeslawski 2013; Davis et al. 2013).

1.3 Ocean acidification

The global ocean is the most important buffer absorbing an estimated 30% of the emitted CO_2. The atmospheric CO_2 is dissolved in the ocean surface, resulting in a higher CO_2 partial pressure (pCO_2) or a hypercapnic ocean. The hypercapnic water undergoes some adjustments in its chemical equilibrium. In the chain reaction of the CO_2 hydrolysis equation carbon dioxide is hydrolyzed to form carbonic acid (H_2CO_3), which subsequently ionizes to form bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions, releasing a proton (H^+) in each ionization step of the following reaction (England et al. 2011).

$$CO_{2(g)} + H_2O \leftrightarrow CO_{2(aq)}$$

$$CO_{2(aq)} + H_2O \leftrightarrow H_2CO_3 \ (K_{eq} = 2.6 * 10^{-3} \ at \ 25°\mathrm{C})$$

$$H_2CO_3 \leftrightarrow H^+ + HCO_3^- \ (pK_a = 6.35)$$

$$HCO_3^- \leftrightarrow H^+ + CO_3^{2-} \ (pK_a = 10.33)$$

Simultaneously, with the increase of CO_2 concentrations [CO_2(aq)], the carbonate ion concentration decreases.

$$CO_{2(aq)} + CO_3^{2-} + H_2O \leftrightarrow 2HCO_3^-$$

The increase of hydrogen ions by 23% is responsible for a phenomenon called Ocean acidification, and has decreased the pH of the ocean by 0.1 units up until now, and is expected to decrease by 0.3-0.4 units by the end of the century (IPCC 2013). This decrease is equivalent to a 100 to 150% increase in the concentration of H^+ ions (Orr et al. 2005) (Meehl 2007). Different levels of ocean acidification exist in diverse habitats. Coastal areas where acidification is intensified by nutrient runoffs and hypoxia, coastal upwelling areas, shallow coral reefs, tidal marshes, kelp forests, and closer to natural CO_2 sources, are now experiencing higher levels of ocean acidification. Concomitantly, the deep and open ocean zones are experiencing lower levels of ocean acidification (Heuer and Grosell 2014).

Calcifying organisms form calcium carbonate shells, skeletons or internal structures, using the available carbonate in the sea water. More acidic waters have a lower concentration of carbonate ions, since these ions react with CO_2 turning into bicarbonate. This carbonate reduction will promptly impair the calcification ability of those species, or even dissolve their carbonate shells (Hofmann et al. 2010). Ocean acidification is also known to have several neurologic effects in a broad range of species, impairing their learning abilities; a range of sensory systems including olfaction, hearing and vision; behaviours as lateralization, activity, boldness; consequently, affecting ecologic traits as dispersion, recruitment, connectivity, social interactions, predator-prey dynamics, population replenishment, biodiversity, habitat preference, and settlement timing (Heuer and Grosell 2014). Other important biological traits such as growth, development, survival, reproduction, swim performance, metabolism, cardiorespiratory function and osmoregulation are also impaired (Heuer and Grosell 2014). All those aspects could dramatically affect populations and ecosystems (Heuer and Grosell 2014).

1.4 Ocean warming

Although the mean global SST will suffer an increase of 2.7°C, it is predicted to happen in a regional variation range with latitude (Figure 1.2) (IPCC 2013).

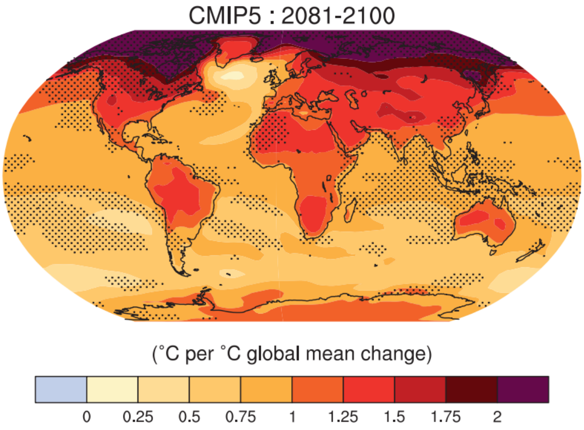


Figure 1.2 – Pattern of global surface temperature regarding CMIP5 model average (adapted from IPCC (2013))

One of the most studied impacts of ocean warming is the distributional shifts of marine species towards the poles and deeper waters, as species adapted to warmer temperatures are finding their optimal temperature migrating towards these locations (Beare et al. 2004; Perry et al. 2005; Dulvy et al. 2008; Cheung et al. 2009). Those horizontal and vertical migrations have several constraints as the possible inexistence of suitable habitats in deeper waters and higher latitude, and the specific dispersal capabilities, which may possibly lead to widespread extinctions (Thomas et al. 2004; Perry et al. 2005; Rutterford et al. 2015). Evidence demonstrates that different species show different variations on distribution ranges responding to the same temperature variation, as the species with faster lifecycles, smaller body sizes and faster maturation respond faster, being the ones with a faster rate of population turnover (Perry et al. 2005). Furthermore, those differential shifts are related to the species specific thermal tolerance. Species that live close to their thermal tolerance limit have the urge to migrate, concomitantly. Species with a wider thermal tolerance, or species that do not live close to their thermal tolerance limits, are more prone to adapt to warmer temperatures (Stillman and Somero 2000; Helmuth et al. 2006; Donelson et al. 2011; Pecorino et al. 2013).

Thermal tolerance curves, or thermal windows can be measured using the aerobic scope, a measure of an organism performance that corresponds to the difference between the maximum and the resting oxygen consumption in specific temperatures, where performance falls below the optimum in higher and lower temperatures (Figure 1.3) (Pörtner and Farrell 2008).

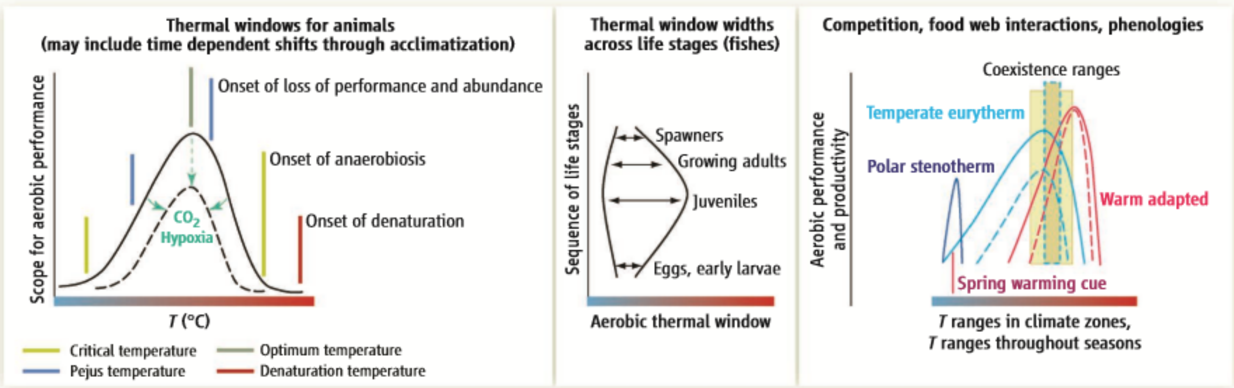


Figure 1.3 – Temperature effects on aquatic animals (adapted from Pörtner and Farrell (2008)).

“The thermal windows of aerobic performance (left) display optima and limitations by pejus (pejus means “turning worse”), critical, and denaturation temperatures, when tolerance becomes increasingly passive and time-limited. Seasonal acclimatization involves a limited shift or reshaping of the window by mechanisms that adjust functional capacity, endurance, or protection. Positions and widths of windows on the temperature scale shift with life stage (middle). Acclimatized windows are narrow in stenothermal species, or wide in eurytherms, reflecting adaptation to climate zones. Windows still differ for species whose biogeographies overlap in the same ecosystem (right, examples arbitrary). Warming cues start seasonal processes earlier (shifting phenology), causing a potential mismatch with processes timed according to constant cues (light). Synergistic stressors like ocean acidification (by CO_2) and hypoxia narrow thermal windows according to species-specific sensitivities (broken lines), modulating biogeographies, coexistence ranges, and other interactions further”(Pörtner and Farrell 2008).

Thermal windows tend to be as narrow as possible to minimize maintenance costs, and are normally narrower in more complex organisms as in fish species. Therefore, organisms adapted to more variable habitats have wider thermal windows (eurytherms) than organisms adapted to more stable habitats (stenotherms). Ocean acidification and hypoxia are known to reduce the aerobic scope and narrow thermal windows, evidencing a synergistic effect of ocean warming and acidification in most fish species (Pörtner and Farrell 2008). All these exposed factors may also contribute to trophic, biogeographic, community composition mismatch and asynchronous phenology events across the global ocean (Figure 1.4) (Edwards and Richardson 2004; Durant et al. 2007).

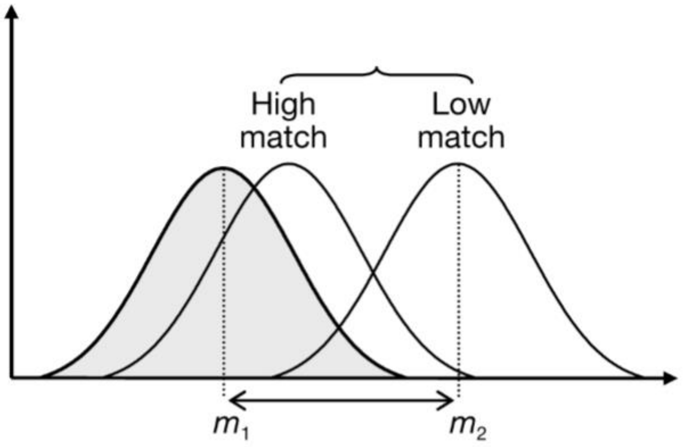


Figure 1.4 – Conceptual model representing ecological mismatches (adapted from Durant et al. (2007))

In the case of Figure 1.4, a trophic mismatch, the “y” axes may plot abundance, while “x” axes may plot time, latitude or depth, and the shadowed curve may represent predator abundance and white curves prey abundance. In the occasion of a community composition mismatch, the “y” axes may plot abundance, while “x” axes may plot time, latitude or depth, and shadowed and white curves may plot mutualistic species abundance (adapted from (Durant et al. 2007)). Ocean warming thus unbalances ecological and behavioural patterns motivating different sorts of adaptations.

1.5 Animal behaviour under climate change

Behavioural changes are the first animal's response to an environmental modification, usually leading to an alteration on species' interactions and ecological processes. As a result, climate change affects, in a persistent way, a large scope of critical behaviours responsible for the prevalence of species and ecological communities (Nagelkerken and Munday 2016).

Ocean warming and acidification affects animal behaviour directly and indirectly through different mechanisms (Figure 1.5). Differences in physiological processes such as higher metabolic rates have direct impacts on fish activity levels and risk-taking behaviour. Another direct impact is related to the deficient calcifying capacity, as the organisms lose their ability to produce their protective structures, and thus, altering their predator-avoidance behaviour. Brain function impairment also affects behavioural traits directly and indirectly. Shifts in physical and biological cues used in the animals' decision-making process will indirectly affect the behaviour. Interspecific behaviours are also affected as the ocean warming and acidification may modify their abundances, distributions and behaviours in different ways (Nagelkerken and Munday 2016).

All the mentioned impaired behaviours will affect a wide range of ecological processes across the ecosystems and specific ontogenesis (Figure 1.6). Larval dispersion, orientation, settlement, habitat selection, ontogenetic migration, feeding migrations, homing, competition for resources, prey detection, foraging success, food selectivity, predator detection, recognition, avoidance, habitat use, spawning migration, timing of spawning and spawning behaviour are the main ecological traits affected by climate change (Nagelkerken and Munday 2016).

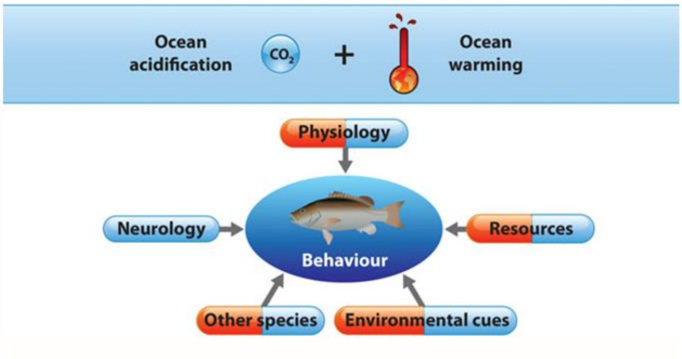


Figure 1.5 – Mechanisms by which ocean warming and acidification may affect fish behaviour (adapted from Nagelkerken and Munday (2016))

The figure above illustrates a representation of the mechanisms by which ocean warming (red) and ocean acidification (blue) may affect, directly or indirectly, fish behaviour, according to Nagelkerken and Munday (2016).

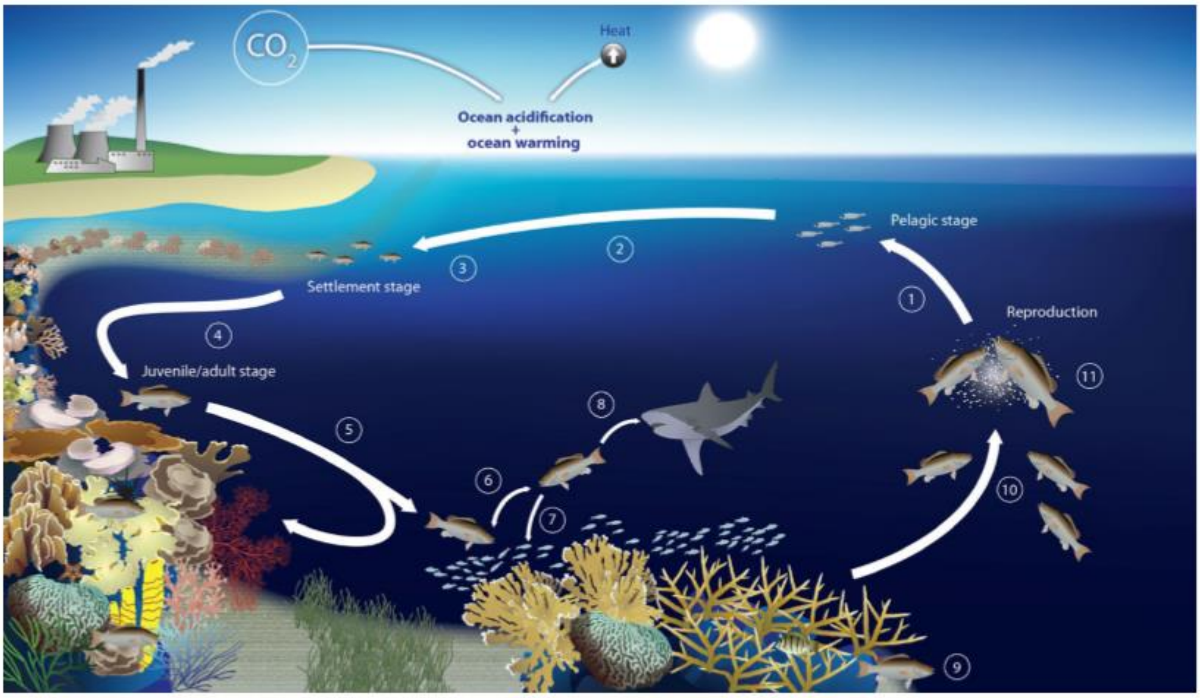


Figure 1.6 – Ecological processes affected by altered behaviour due to acidification or warming. 1 = pelagic dispersal behaviour, 2 = orientation & habitat detection, 3 = settlement, 4 = post settlement habitat selection & ontogenetic migration, 5 = feeding migrations & homing, 6 = competition for resources, 7 = prey detection/foraging success/food selectivity, 8 = predator detection/recognition/avoidance, 9 = habitat use, 10 = spawning migration, 11 = timing of spawning & spawning behaviour"(Nagelkerken and Munday 2016).

Thus, changes in ecological processes do reflect the adaptation induced by altered animal behaviour under climate change.

1.6 Neurobiology and sensory systems under climate change

A significant number of studies shows that exists a consistent impairment over a range of sensory systems (Heuer and Grosell 2014), being the olfaction (Munday et al. 2009b; Dixson et al. 2010; Cripps et al. 2011), hearing (Simpson et al. 2011), and vision (Ferrari et al. 2012; Forsgren et al. 2013) the most studied ones. Moreover, some studies indicate also that ocean acidification does not affect the sensory structures directly, rather impacting the neural processing of their signaling (Heuer and Grosell 2014).

Teleost fish have the capacity of regulating their acid-base balance, by maintaining a relatively constant extracellular alkaline pH (7.7-8.1). To avoid acidosis in hypercapnia, acid-base regulation is achieved with the uptake of HCO_3^-, compensated by the secretion of Cl^-, and secretion of H^+ across the gill epithelium and kidney (Nilsson et al. 2012; Heuer and Grosell 2014). Some also argue that neural processing impairments are due to the ocean acidification impact over the inhibitory neurotransmitter GABA and GABA-A receptor function (Nilsson et al. 2012).

γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the vertebrate brain, being the GABA-A the main receptor for this neurotransmitter. In normal conditions, binding of GABA to GABA-A receptors leads to an inflow of Cl^- and/or HCO_3^- into neurons, leading to a hyperpolarization of the neuron, inhibiting the neural activity. As the neuronal gradients for Cl^- are relatively close to electrochemical equilibrium, the concentration changes of HCO_3^- or Cl^- caused by the acid-base balance lead to an efflux rather than an influx of those ions. This inversion of the GABA-A receptor function origins a membrane depolarization and subsequently an excitatory neuronal transmission, instead of the normal hyperpolarization (Figure 1.7) (Farrant and Kaila 2007; Heuer and Grosell 2014).

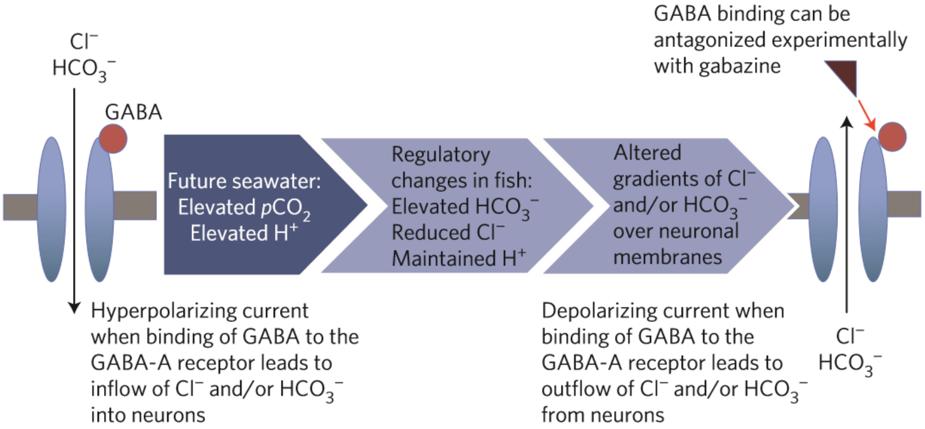


Figure 1.7 – Response of GABA-A receptor function to elevated seawater pCO_2 (adapted from Nilsson et al. (2012)

“This GABA-gated ion channel has conductance for Cl^- and HCO_3^-.Normally, ion gradients are such that channel opening results in Cl^- and HCO_3^- inflow, causing membrane hyperpolarization and inhibited neural activity. Elevated seawater pCO_2 leads to ion-regulatory adjustments in blood and tissues that affect transmembrane gradients for Cl^- and/or HCO_3^- in some neurons. GABA-A receptors thereby become depolarizing and excitatory, resulting in behavioural abnormalities. Support for this hypothesis is provided by showing that the behavioural effects of high CO_2 are reversed by treatment with a GABA-A receptor antagonist (gabazine)” (Nilsson et al. 2012).

In immature neurons, the action of GABA-A receptors is naturally excitatory, due to a higher intracellular concentration of Cl^- (Sernagor et al. 2010). This higher concentration is caused by the superior ion inflow promoted by the augmented expression of the cotransporter NKCC1 ($Na^+/K^+/Cl^-$) (Blaesse et al. 2009), and the reduced ion outflow caused by the diminished expression of the cotransporter KCC2 (K^+/Cl^-) (Rivera et al. 1999), causing a depolarization, and consequently triggering action potentials when GABA activated channels are open (Sernagor et al. 2010). Later in neural maturation, under normal conditions, the expression and functionality of the KCC2 increases, and the action of the GABA-A receptors turns from excitatory to inhibitory (Blaesse et al. 2009). This excitatory action of GABA-A receptors plays an important role during neurogenesis, being active during the earliest stages of network activity, promoting neurite elongation and branching, not only during early development but also during adulthood (Sernagor et al. 2010). Furthermore, the depolarization of the progenitor cells promotes the expression of NeuroD, a transcription factor that positively regulates the neural differentiation (Tozuka et al. 2005).

Lai et al. (2017) found that in the temperate fish, three-spined stickleback (*Gasterosteus aculeatus*), under OA, the expression levels of the transcription factor mentioned is higher than in control conditions. However, in two other tropical fish species, cinnamon anemonefish (*Amphiprion melanopus*) and spiny damselfish (*Acanthochromis polyacanthus*), such transcription factor regulatory increase did not happen.

Not only GABA plays an important role in the immature brains. Other neurotransmitters such as Serotonin (5-HT), dopamine (DA), noradrenaline (NAd) and acetylcholine (Ach) also work as developmental signalers and regulators (Whitaker-Azmitia 1991). Note that in the mature brain they play an entirely different role (Whitaker-Azmitia 1991).

Temperature is also known to quantitatively affect neurotransmitters in the CNS (Tsai and Wang 1997; Wang and Tsai 2000). In Tilapia (*Oreochromis mossambicus*), 5-HT, norepinephrine (NE), GABA, and glutamate (Glu) production was differently affected by different temperatures during the first 25 days post-hatch (Wang and Tsai 2000), while in the adult phase of the same species, 5-HT and NE were affected by warming differently depending on the sex and brain region (Tsai and Wang 1997). Altered neurotransmitter function and quantity not only affects brain development, but also fish behaviour (Winberg and Nilsson 1993; Nilsson et al. 2012).

Mechanisms such as the metabolic suppression under ocean warming and acidification (Pörtner and Farrell 2008; Calosi et al. 2013), and the effect of climate change on specific neurotransmitters are suspected to impact the development of neurons and glia cells, which may lead to an effect on the brain weight.

1.7 Fish brain anatomy and function

The brain is the frontal part of the central nervous system and is located in the cranium of any vertebrate. It commands and manages the adaptation of species within the respective habitats. There is a wide variety of habitat usages, environmental demands and taxonomic differences that promote a range of differential brain functions and morphologies among species (Kotrschal et al. 1998). Despite this fact, the teleost brain follows the same anatomic plan as any other vertebrate with well differentiated regions with the same general functions (Kaas 2016). From the posterior to the anterior part, the brain can be divided into different natural morphogenetic units resulting from the specification of the neural tube, firstly the hindbrain, divided in myelencephalon (brainstem) and metencephalon (cerebellum and pons). The mesencephalon, comprising the optic tectum and smaller parts as the cerebral peduncles (the torus semicircularis and the tegmentum). The forebrain or prosencephalon is formed by the diencephalon and telencephalon (Figure 1.8) (Kaas 2016). Figure 1.8 shows the five brain regions of the teleost fish: the telencephalon, the optic tectum, the diencephalon, the cerebelum and the brainstem and their respective positions.

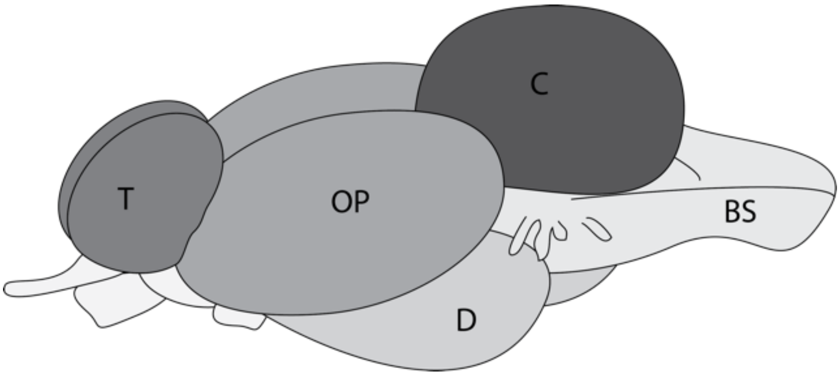


Figure 1.8 – Anatomic model of the teleost brains, comprising the five main macro regions (adapted from Gonçalves et al. (2010))

The brain is constituted by five main macro regions: T - telencephalon, OP - optic tectum, D - diencephalon, C- cerebellum and BS - brainstem. In teleost fish, the main function of the telencephalon is related to the reception and transmission of olfactory stimuli (Helfman et al. 2009). However, it is also responsible for schooling, learning, reproductive and aggressive behaviour (de Bruin 1980; Savage 1980).

The diencephalon comprises four main regions: the thalamus, the subthalamus, the hypothalamus and the epithalamus. These regions are responsible for the control of the autonomic nervous system, the coordination and regulation of homeostasis and the endocrine system, processing environmental changes and regulating circadian and seasonal cycles, changing in coloration (Helfman et al. 2009), relaying motor and sensory signals and regulating consciousness states (Vanderah 2016).

The mesencephalon, mainly the optic tectum, is responsible for the reception and processing of visual stimuli. This part is also important as a coordination center for other sensory systems (audition, lateral line, somatosensory, electrosensory) (Helfman et al. 2009; Northmore 2011). Thus, the topographic perception of the external ambient, such as the location of objects and distance perception, is built in the optic tectum. As a developed neural processor, the optic tectum is highly important for rapid decision-making behaviours as predator avoidance (Northmore 2011).

The cerebellum is responsible not only for the muscle tone (motor control) and equilibrium (Helfman et al. 2009), but also for motor learning, and for having importance in cognitive behaviours, emotions and spatial cognition (Lalonde and Botez 1990; Rodriguez et al. 2005; Sullivan 2010).

Last, the brainstem is the posterior part of the brain and the anterior part of the spinal cord, and the “communication part” between the rest of the brain and the sensory systems, except for smell and vision. It is also known to control some somatic and visceral functions, with respiratory and osmoregulatory centers (Helfman et al. 2009).

Recent literature suggests that the size of the total brain and the relative size of separate brain regions are relatable to specific behavioural responsivity and sensorial acuity, among other capacities in brained species (Corral-López et al. 2017a; Corral-López et al. 2017b; Buechel et al. 2018)

1.8 Brain growth and development

Fish specific brain development and different brain regions' development are mainly affected by natural or artificial selection pressure over generations (Kihslinger and Nevitt 2006) and by means of genetic adaptation (Schlichting and Pigliucci 1998). The size of brain regions responsible for processing sensorial information is positively correlated with the size of their related sensory organs, meaning that a bigger brain region leads, for instance, to a better sensorial reception, also showing the relative importance of the organ or the related brain area in each individual, species or life history stage (Evans 1940; Kotrschal et al. 1998; Shultz and Dunbar 2006; Ito et al. 2007). The same happens with specific behaviours and intelligence, as species with more complex and cognitive demanding social interactions tend to develop larger brains with larger behaviour-related brain areas (Dunbar 1998; Soares et al. 2015; Kotrschal et al. 2017). For example, environmental complexity has an effect on the generality of the fish brain growth, while social complexity shapes the telencephalon and hypothalamus development (Pollen et al. 2007; Shumway 2010), predation pressure is positively related with overall brain, telencephalon and optic tectum development, and negatively correlated with olfactory bulbs and hypothalamus (Kotrschal et al. 2017).

The fact that environmental factors shape the brain evolution was described for mammals too with emphasis on social complexity more than ecological factors (Barton and Dunbar 1997; Dunbar and Bever 1998; Shultz and Dunbar 2006; Byrne and Bates 2007). In fact, ecological and behavioural demands shaped the basic brain conformations alongside evolutionary history (Nieuwenhuys et al. 1998), paving the way for the existence of different morphologic brain types between major taxonomic groups (Kotrschal et al. 1998), with their organization and size varying according to their life history traits (Lecchini et al. 2014).

As fish grow continuously through life, with only a gradual decrease in the growth rate with age, there is a significant influence of environmental pressures experienced by the individuals shaping the brains' development along all the ontogeny (Ekström 1994; Kotrschal et al. 1998; Kihslinger et al. 2006; Kihslinger and Nevitt 2006) through phenotypic plasticity (Schlichting and Pigliucci 1998). For instance, aquaculture salmon alevins (*Oncorhynchus mykiss*) reared in more complex habitats (tanks with stones) developed bigger cerebella than genetically identical individuals reared in simple tanks. Concomitantly, individuals reared in the wild developed larger brains than the ones reared in laboratory conditions (Kihslinger and Nevitt 2006). Other salmon species, *Oncorhynchus tshawytscha*, developed larger olfactory bulbs and telencephalon in the wild than in hatchery conditions (Kihslinger et al. 2006).

Brain development and function are high energetic demanding features. For instance, in humans it consumes about 20% of total energy intake (Aiello and Wheeler 1995) as the neuronal electronic signaling has an energy cost per-neuron as high as ten of other body cells (Yu et al. 2014). As stated before, animals benefit for having larger brains, however, due to such energy costs, larger brains only come with higher ecological and social demands, resulting in a cost-benefit equilibrium (Dunbar 1998).

2 Objectives

Differences in the evolution of the size of different brain regions has aroused two leading hypothesis for their development: the “concerted” one, proposed by Finlay and Darlington (1995) that stated that the evolution of different brain regions is interdependent and that a specific brain region will only evolve if the entire brain responds to the selective pressure; and the “mosaic” one, proposed by Barton and Harvey (2000), that defends that the evolution of each brain region is independent of the rest of the brain.

Unraveling how species invest in brain tissues (or brain regions) matching the fitness-relevant cognitive demands placed by the environment is a priority in ocean warming and acidification research (cf. O’Donnell (2018)). Within this context, this dissertation aimed to assess and compare the phenotypic plasticity responses to both ocean warming and acidification in brain/body mass ratio and brain macro-region growth of juvenile of six different teleost species – three adapted to more stable (tropical) environment (clown anemonefish *Amphiprion ocellaris,* orchid dottyback *Pseudochromis fridmani* and neon goby *Elacatinus oceanops*), and the other three species adapted to a less stable (temperate) environment (seabream *Diplodus sargus*, flatfish *Solea senegalensis* and meagre *Argyrosomus regius*).

3 Materials and methods

3.1 Ethics statement

The experimental procedures implemented for this work were in accordance with the requirements of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. They were also reviewed and approved by the animal ethics committee ORBEA ± Animal Welfare Body of FCUL within the framework of the project PTDC/MAR-EST/5880/2014.

3.2 Specimen's collection and incubation

Forty-eight individuals of each of six different fish species, three tropical species (*P. fridmani* (total length of 1.9-3.9 cm), *A. ocellaris* (total length of 2.0-3.7 cm)*, E. oceanops* (total length of 2.2-3.2 cm)) and three temperate species (*D. sargus* (total length of 3.4-5.2 cm), *S. senegalensis* (total length of 2.5-5.8 cm)*, A. regius* (total length of 3.3-5.7 cm)) were used in this work to fulfil my objective. The three tropical species were reared at the aquaculture facility of Opérculo-LDA (Cascais, Portugal), while the three temperate species were reared at the aquaculture facility of Estação Piloto de Piscicultura em Olhão (IPMA/DivAV). After larval settlement, all the individuals were transported to the aquaculture facilities of Laboratório Marítimo da Guia (LMG-MARE, Faculty of Sciences, University of Lisbon) as recently-staged juveniles.

Each individual was then randomly assigned to 24 independent recirculating aquaculture systems (RAS), in such a way that each RAS contained four elements of each species (separated in tropical and temperate conditions) (see supplemental material). Each one of the RAS was filled with filtered (0.20 µm) natural sea water and equipped with biological (ouriço, Fernando Ribeiro Lda, Lisbon, Portugal), mechanical (100 µm filter, TMC-Iberia, Portugal) and physical (ReefSkimPro 400, TMC-Iberia) filtration, as well as UV sterilization (Vecton 600, TMC-Iberia, Lisbon, Portugal).

Temperature was kept stable by means of a water chiller (FRIMAR, Fernando Ribeiro Lda., Portugal) and submergible water heaters (Eheim, Germany). The pH values were adjusted automatically, via solenoid valves, with a Profilux controlling system (Kaiserslautern, Germany) connected to individual pH probes. PH values were continuosly monitored and regulated by injection of a certified CO_2 gas mixture (Air Liquide, Portugal). Seawater carbonate system speciation was monitored spectrophometrically (595 nm) from total alkalinity according to (Sarazin et al. 1999). Ammonia and nitrite levels were determined daily, using colorimetric test kits (Aquamerk, Merck Millipore, Darmstadt, Germany) and kept below detectable levels. Temperature, pH and salinity were measured daily using a thermometer (TFX 430, EBRO), a pH portable probe (SevenGo Pro, Mettler Toledo) and a refractometer (V2 refractometer, TMC-Iberia, Lisbon, Portugal). A photoperiode of 12h of light and 12 h of darkness was used for every RAS.

On one hand, tropical species were acclimated for 30 days in four different experimental treatments: twelve individuals of each species acclimated to the present conditions of temperature (26°C) and pCO_2 (400 µatm); twelve were acclimated to the future conditions of temperature (30°C, 4°C above the actual mean tropical water temperature) and actual pCO_2 (400 µatm); twelve were acclimated to the present conditions of temperature (26°C) and future pCO_2 (1100 µatm); twelve were acclimated to the future conditions of temperature (30°C, 4°C above the actual mean tropical water temperature) and future pCO_2 (1100 µatm).

On the other hand, temperate species were acclimated for 30 days in four different experimental conditions: twelve individuals of each species were acclimated to the present conditions of temperature (18°C) and pCO_2 (400 µatm); twelve were acclimated to the future conditions of temperature (22°C, 4°C above the actual mean temperate water temperature) and actual pCO_2 (400 µatm); twelve were acclimated to the present conditions of temperature (18°C) and future pCO_2 (1100 µatm); twelve were acclimated to the future conditions of temperature (22°C, 4°C above the actual mean temperate water temperature) and future pCO2 (1100 µatm). We used three replicate tanks to each one of the treatments described.

3.3 Brain sampling

After the acclimation 30 days, all the individuals were euthanized with an overdose of tricaine solution (MS222, Pharmaq; 500–1000 mg/L), to ensure the effectiveness of the euthanasia, the spinal cord was sectioned. The brain was dissected from the cranial cavity into 5 macro-areas: forebrain (olfactory bulbs + telencephalon), diencephalon, optic tectum, cerebellum and brainstem, under a stereoscope (Zeiss; Stemi 2000), using dissection material (Almeida et al. 2012; Teles et al. 2013; Soares et al. 2015). The macro-areas were then weighted using a SARTORIUS CPA225D analytical scale with readability to the nearest 0.01 mg, in a temperature and humidity controlled room.

3.4 Statistical analyses

The analysis were all performed using the statistical software R version 3.3.3 (Another Canoe) (R Core Team 2017). To assess the influence of ocean warming and acidification on the total brain growth I used GLMM with the brain/body mass ratio, to eliminate the effect of different body size, as dependent variable with tank as random effect. To evaluate the effect of the same environmental stressors on the development of each brain macro-region I used GLMM with the total brain macro-region mass/total brain mass ratio as dependent variable, in both cases I used temperature and pCO_2 as covariates. The full models included all 2-way interactions. I simplified the models, removing the factors that did not influenced data variation using the Akaike Information Criterion (AIC), an indicator that balances model complexity with model quality of fitness to select the best model to fit my data (Quinn and Keough 2002). Data was fitted using gaussian family models, and model residuals were then checked for homogeneity of variances. Independence and leverage were used to perform model validation. When assumptions were not met, I turned to gamma family models to fit my data, and model validation was assessed following the same procedure. (For detailed model tables, please refer to supplemental material)

4 Results

4.1 Brain/body mass ratio

After 30 days of acclimation, high pCO_2 significantly increased the brain/body mass ratio (B/Bo) of *A. regius* (Fig. 4.1A, $p<0.001$). In opposition, a significant decrease in B/Bo was observed in *S. senegalensis* (Fig. 4.1C, $p<0.01$). The third temperate species – *D. sargus* - revealed no OA-induced changes in B/Bo (Fig. 4.1B, $p>0.05$). Moreover, warming did no elicit any significant B/Bo change in the three-studied temperate species ($p>0.05$).

Regarding the tropical species, in both *A. ocellaris* and *P. fridmani* there were no significant temperature and pCO_2 effects (Fig. 4.1D and F, $p>0.05$). In contrast, there was a significant positive increase with temperature, and a significant interaction between temperature and pCO_2 in *E. oceanops* (Fig. 4.1E, $p<0.01$).

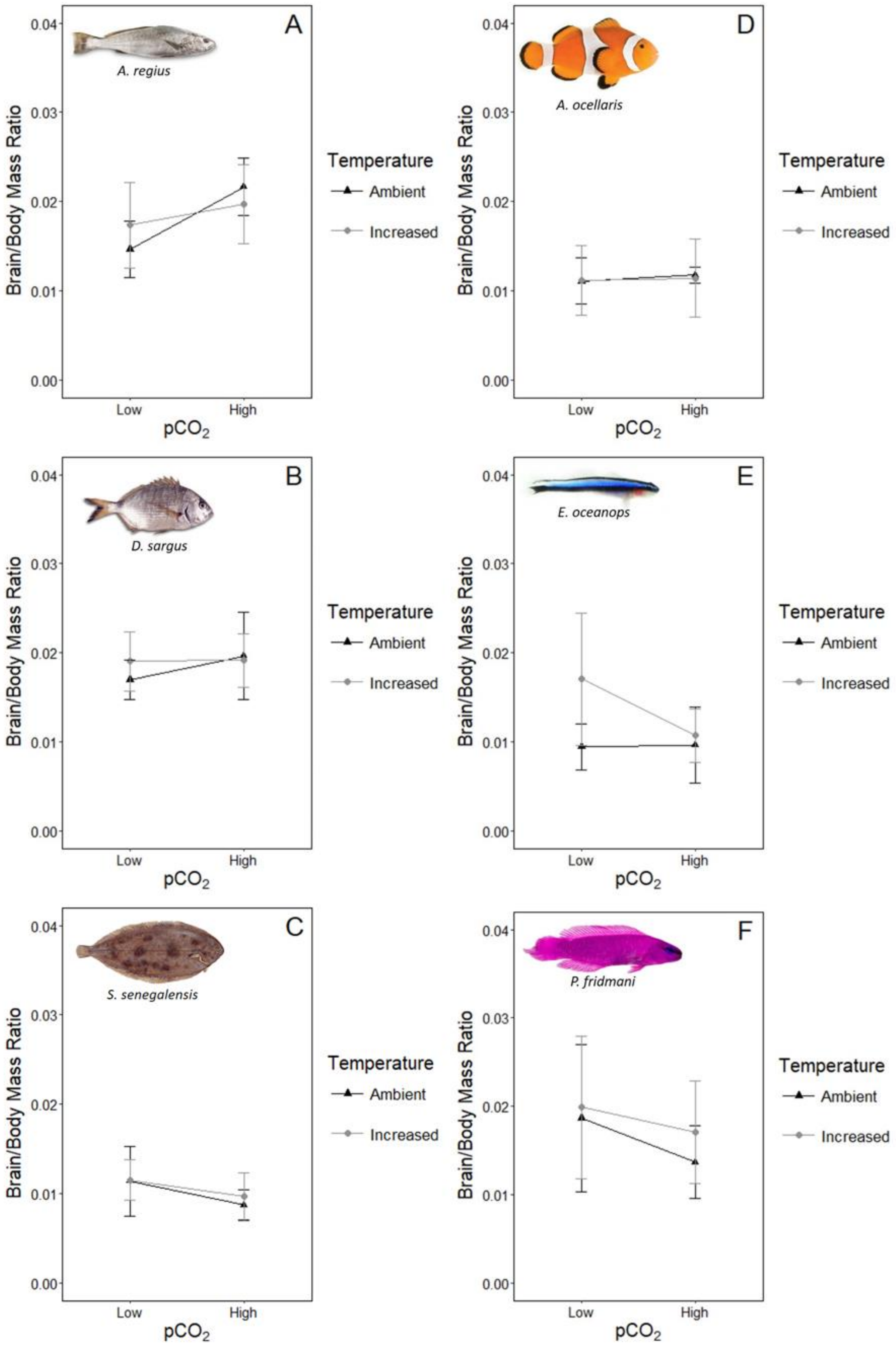


Figure 4.1 – Impact of OW and OA in the brain/body mass ratio (B/Bo) of six teleost species For temperate fishes – meagre *Argyrosomus regius* (A) seabream *Diplodus sargus* (B) and flatfish *Solea senegalensis* (C), and ii) for tropical fishes – clown anemonefish *Amphiprion ocellaris* (D) neon goby *Elacatinus oceanops* (E) and orchid dottyback *Pseudochromis fridmani* (F). The dots represents the arithmetic mean; the whiskers represents the standard deviation.

4.2 Brainstem/brain mass ratio

From the six studied-species only *D. sargus* showed a significant (positive) response in brainstem/brain mass ratio (BS/B) to ocean acidification (Fig.4.2B, p<0.001). Moreover, temperature only elicited a significant positive effect on the BS/B of *A. ocellaris* (Fig.4.2D, p<0.05) and a significant negative one in *E. oceanops* (Fig.4.2E, p<0.05). For the other three species studied here (*A. regius, S. senegalensis* and *P. fridmani*) there were no changes in BS/B under warming and acidification (Fig.4.2A, C and F, p>0.05).

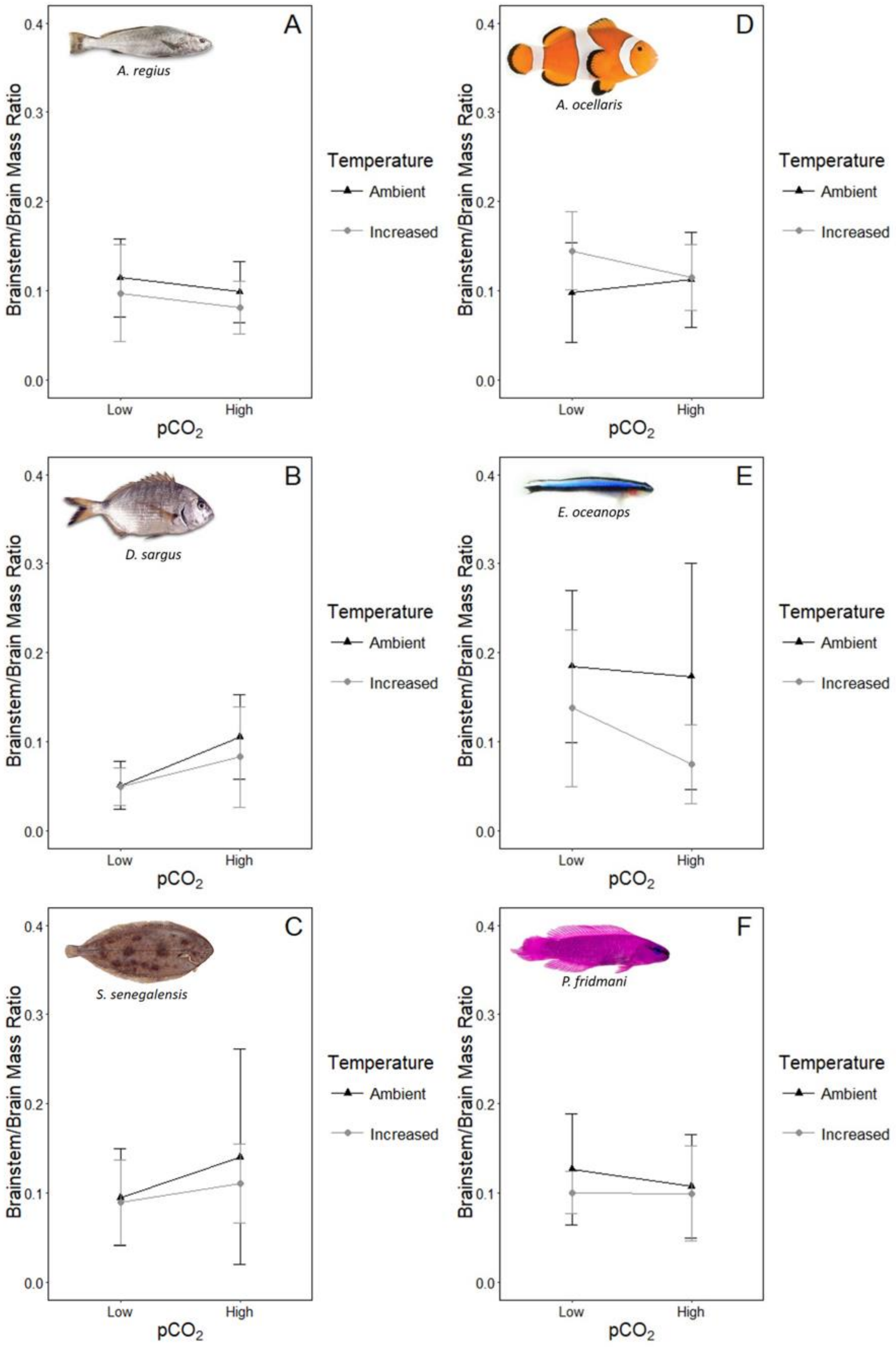


Figure 4.2 – Impact of OW and OA in the brainstem/brain mass ratio (Bs/B) of six teleost species. For temperate fishes – meagre *Argyrosomus regius* (A) seabream *Diplodus sargus* (B) and flatfish *Solea senegalensis* (C), and ii) for tropical fishes – clown anemonefish *Amphiprion ocellaris* (D) neon goby *Elacatinus oceanops* (E) and orchid dottyback *Pseudochromis fridmani* (F). The dots represents the arithmetic mean; the whiskers represents the standard deviation.

4.3 Cerebellum/brain mass ratio

Significant increases in the cerebellum/brain mass ratio (C/B) under high pCO_2 were observed in the temperate *D. sargus* (Fig. 4.3B, $p<0.05$) and tropical *E. oceanops* (Fig. 4.3E, $p<0.01$) and *P. fridmani* (Fig. 4.3F, $p<0.05$). Regarding other three studied species, there were no OA-induced changes ($p>0.05$). Additionally, the warming effect on C/B was not significant in all six species ($p>0.05$)

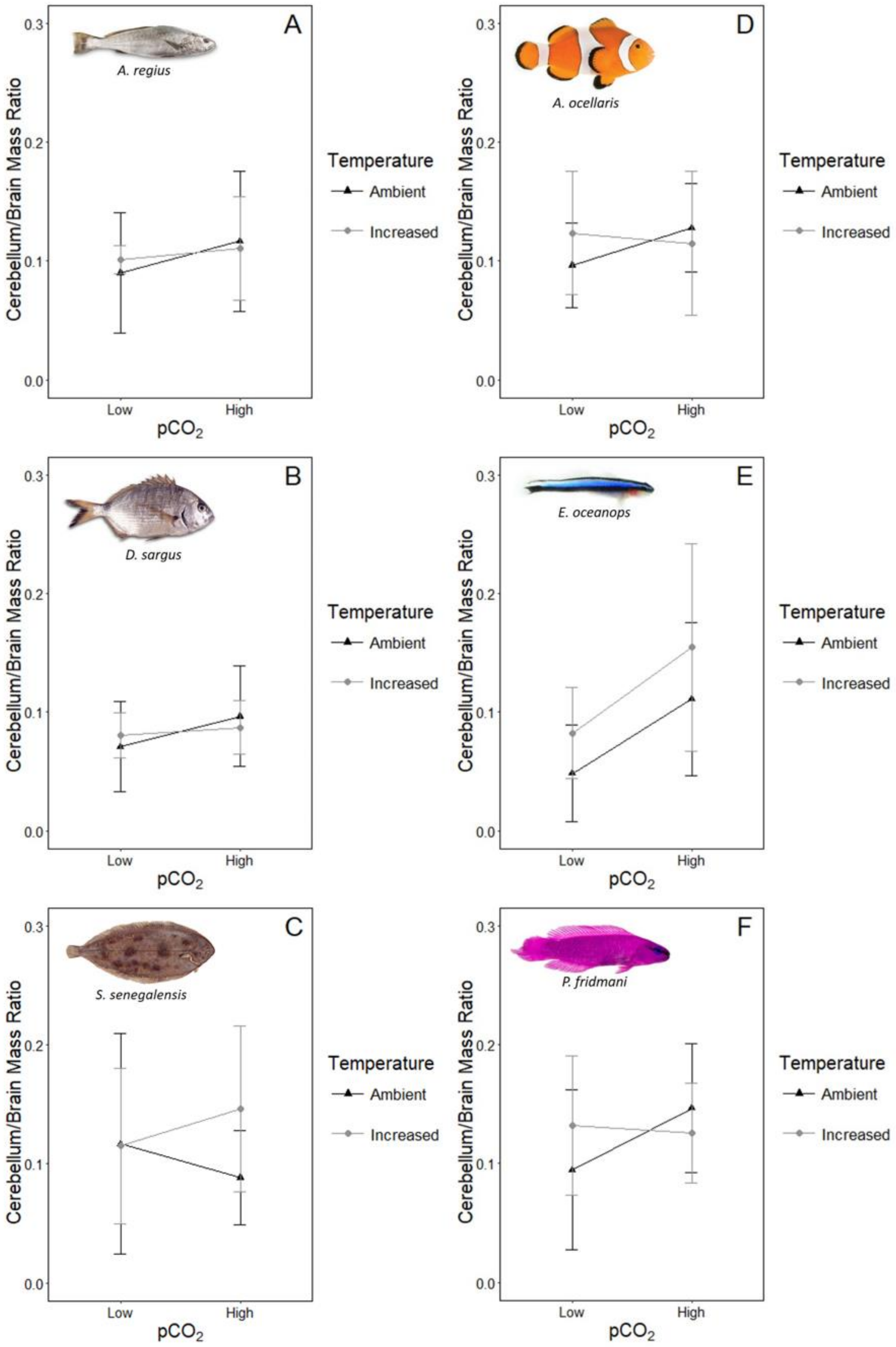


Figure 4.3 – Impact of OW and OA in the cerebellum/brain mass ratio (C/B) of six teleost species. For temperate fishes – meagre *Argyrosomus regius* (A) seabream *Diplodus sargus* (B) and flatfish *Solea senegalensis* (C), and ii) for tropical fishes – clown anemonefish *Amphiprion ocellaris* (D) neon goby *Elacatinus oceanops* (E) and orchid dottyback *Pseudochromis fridmani* (F). The dots represents the arithmetic mean; the whiskers represents the standard deviation.

4.4 Diencephalon/brain mass ratio

Regarding the diencephalon/brain mass ratio (D/B), there were significant decreases under high pCO_2 in *D. sargus* at both thermal scenarios (Figure 4.4B, $p<0.01$). The other two temperate species (*A. regius* and *S. senegalensis*) did not reveal any changes in D/B under acidification or warming (Figure 4.4A and C, $p>0.05$). On the other hand, the tropical species displayed contrasting OA-responses under ambient and warming conditions. In *A. ocellaris, E. oceanops*, and *P. fridmani* increased temperature and high pCO_2 had a significant interaction in D/B (Figure 4.4D, E and F, $p<0.01$, $p<0.01$ and $p<0.05$, respectively). While regarding *A. ocellaris,* OA and OW alone promoted a significant reduction in the D/B (Figure 4.4 D, $p<0.05$ in both OA and OW), in *P. fridmani*, OW alone endorsed a significant increase n D/B (Figure 4.4 F, $p<0.05$).

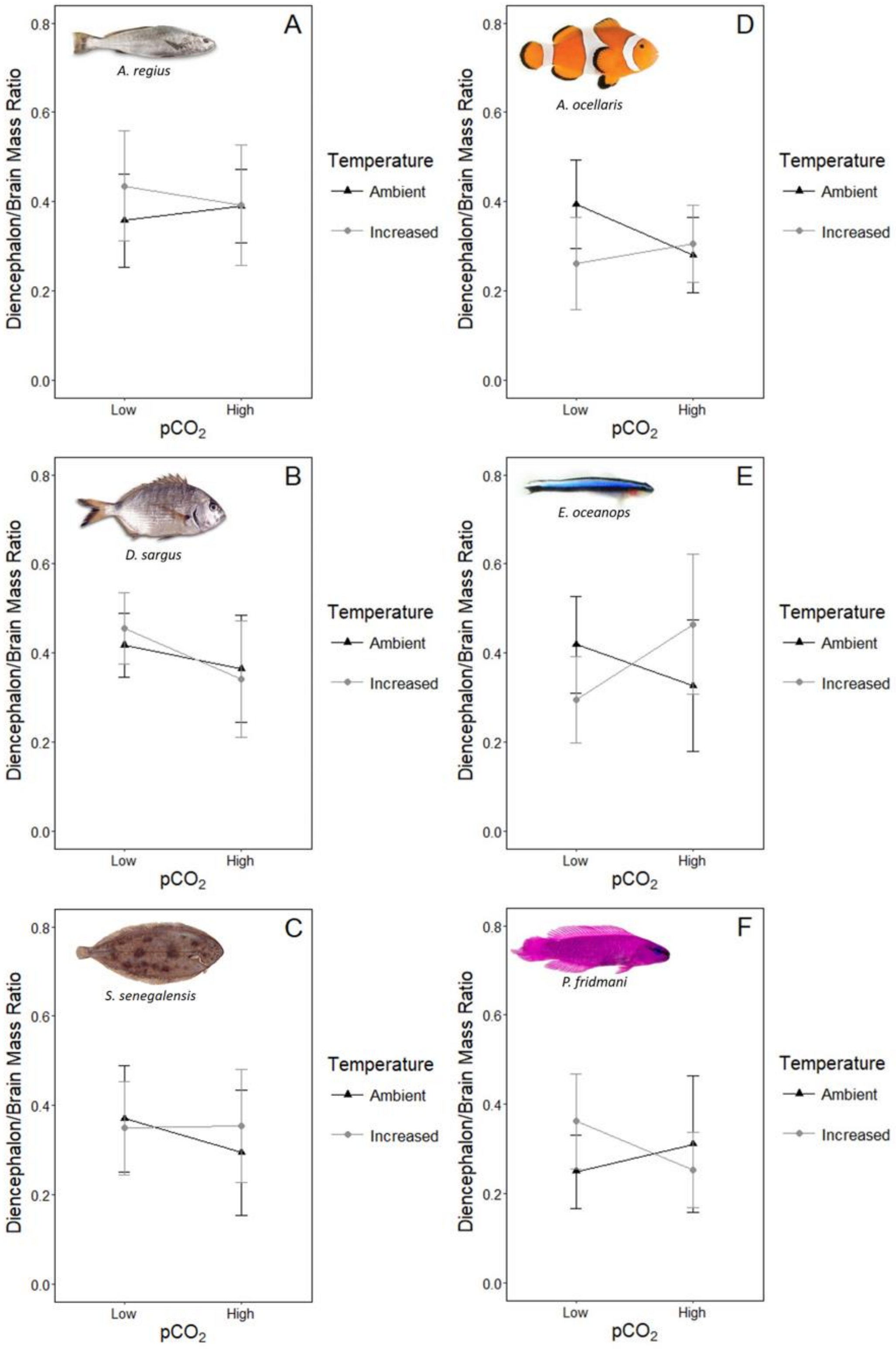


Figure 4.4 – Impact of OW and OA in the diencephalon/brain mass ratio (D/B) of six teleost species. For temperate fishes – meagre *Argyrosomus regius* (A) seabream *Diplodus sargus* (B) and flatfish *Solea senegalensis* (C), and ii) for tropical fishes – clown anemonefish *Amphiprion ocellaris* (D) neon goby *Elacatinus oceanops* (E) and orchid dottyback *Pseudochromis fridmani* (F). The dots represents the arithmetic mean; the whiskers represents the standard deviation.

4.5 Optic tectum/brain mass ratio

As far as optic tectum/brain mass ratio (O/B) is concerned, there were no significant changes under warming or acidification in five of the six studied species (*A. regius, D. sargus, S. senegalensis, A. ocellaris* and *E. oceanops*; Fig. 4.5, $p>0.05$). Only *P. fridmani* revealed significant (and constrasting) OA-responses under ambient and warming conditions. While increased temperature and high pCO_2 led to a significant increase in O/B (Fig. 4.5F, $p<0.001$), high pCO_2 under present-day temperature elicited a significant decrease in O/B compared to the control ($p<0.001$).

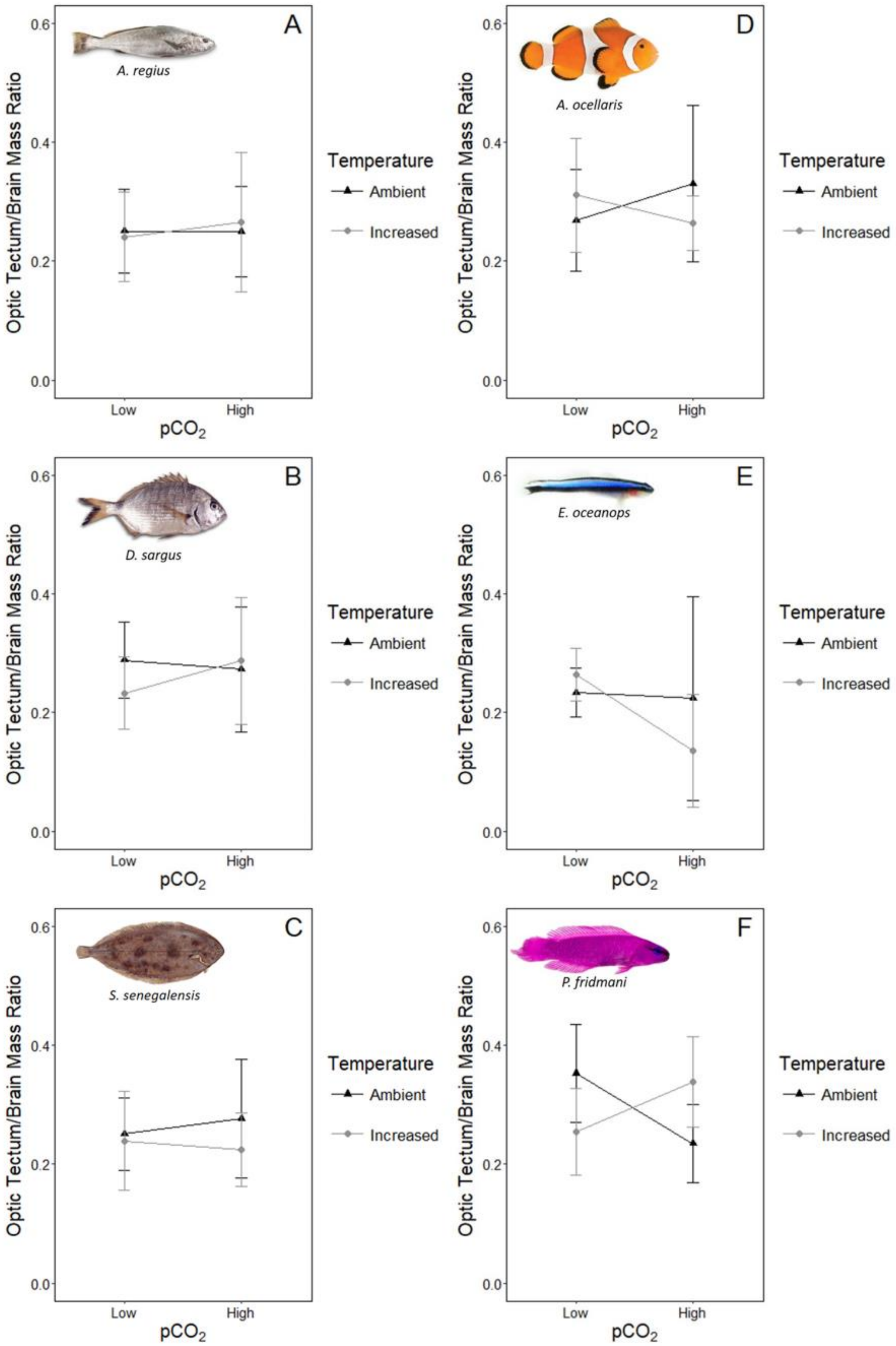


Figure 4.5 – Impact of OW and OA in the optic tectum/brain mass ratio (O/T) of six teleost species. For temperate fishes – meagre *Argyrosomus regius* (A) seabream *Diplodus sargus* (B) and flatfish *Solea senegalensis* (C), and ii) for tropical fishes – clown anemonefish *Amphiprion ocellaris* (D) neon goby *Elacatinus oceanops* (E) and orchid dottyback *Pseudochromis fridmani* (F). The dots represents the arithmetic mean; the whiskers represents the standard deviation.

4.6 Telencephalon/brain mass ratio

Regarding telencephalon/brain mass ratio (T/B), there were no significant changes under warming or acidification in all the six studied species (Fig. 4.6, $p>0.05$).

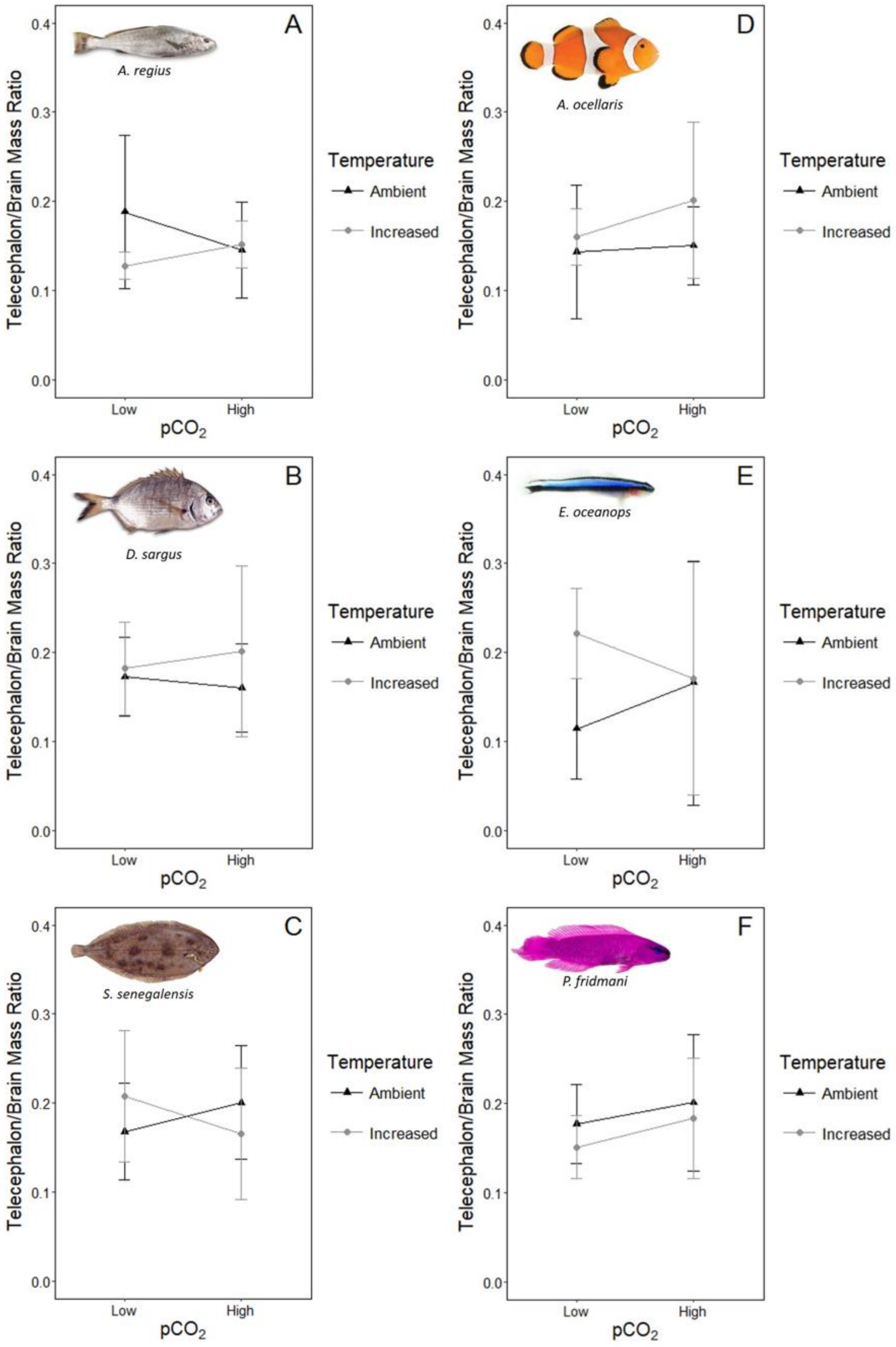


Figure 4.6 – Impact of OW and OA in the telecephalon/brain mass ratio (T/B) of six teleost species. For temperate fishes – meagre *Argyrosomus regius* (A) seabream *Diplodus sargus* (B) and flatfish *Solea senegalensis* (C), and ii) for tropical fishes – clown anemonefish *Amphiprion ocellaris* (D) neon goby *Elacatinus oceanops* (E) and orchid dottyback *Pseudochromis fridmani* (F). The dots represents the arithmetic mean; the whiskers represents the standard deviation.

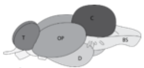
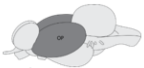
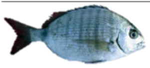
5 Discussion

First, I would like to highlight that comparing brain size differences among species, and relating them to behavioural and ecological traits, is not accurate due to a multitude of factors, but especially because: i) brain size and body size do not scale linearly across species (Montgomery et al. 2010; Fitzpatrick et al. 2012; Montgomery et al. 2013) ii) brain size and neuronal count do not scale uniformly across species (Herculano-Houzel et al. 2015; Olkowicz et al. 2016) and iii) differences in brain size across species do not necessarily render into cognitive ability differences among species (Logan et al. 2017). Therefore, here I will avoid speculative comparisons among the studied species, and will only be focused on the OW and OA related responses. Within this context, it is known that neurobiological functioning and fish behaviour can be widely affected by OW and OA (Munday et al. 2012; Nilsson et al. 2012; Chivers et al. 2014; Heuer and Grosell 2014; Lai et al. 2017; Schunter et al. 2018), and here I also showed that these two environmental stressors differentially affected the total brain and distinct brain areas of the different tropical and temperate species.

5.1 Temperate species under OA

Regarding the effects of OA over the temperate species, there is no noticeable pattern, and are different to each other among species and brain macro-regions (see summary in Table 5.1).

Table 5.1 – Summary of the effects of ocean acidification on the B/Bo, BS/B, C/B, D/B, O/B and T/B of meagre *Argyrosomus regius*, seabream *Diplodus sargus*, flatfish *Solea senegalensis*

	 B/Bo	 BS/B	 C/B	 D/B	 OT/B	 T/B
 *A. regius*	↑	-	-	-	-	-
 *D. sargus*	-	↑	↑	↓	-	-
 *S. senegalensis*	↓	-	-	-	-	-

↑↓ - Positive or negative effect of OA; brain to body mass ratio (B/Bo), brainstem to brain mass ratio Bs/B, cerebellum to brain mass ratio C/B, diencephalon to brain mass ratio D/B, optic tectum/brain mass ratio OT/B and telencephalon/brain mass ratio T/B

A. regius, a benthopelagic species presenting migratory and predatory behaviour and facing a complex habitat (Chao and Trewavas 1990; Pollard et al. 2011), may adapt better to future conditions of OA, developing an overall larger brain when faced with a higher pCO_2 habitat. However, Pimentel et al. (2016) found that ocean acidification is responsible for behavioural changes in larvae from this species, namely reducing swimming capacities. Concomitantly, recent studies also show a higher body growth rate in *A. regius* larvae with higher pCO_2 treatments (Faria et al. 2017). Since there were no significant differences in any specific brain-region ratio, those traces cannot be related to any of those ratios differences.

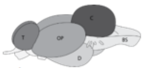
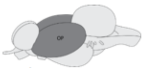
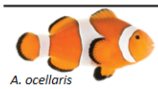
Concerning *D. sargus*, although, there were some variations in different brain regions, the overall brain to body mass ratio was the same in all treatments. The fact that this species has a large distribution area (Bauchot 1987) and the variations in pCO_2 in this species distribution (IPCC 2013) explains its ability to adapt to different conditions of pCO_2 with minor changes in their overall brain development. The positive response of BS/B to OA may represent an over-investment in this brain region. Being this brain-region responsible for the communication between the brain and the spinal cord the majority of the sensorial systems and muscle control, this over enlargement may promote faster responses to external stimuli - assuming that a bigger brainstem has a better function than a smaller one. The positive response of the C/B ratio to OA, may also suggest a compensatory growth increase in response to impaired behavioural traits, as there are empirical evidences showing the behavioural impairment in many fish species under OA (Domenici et al. 2012; Lönnstedt et al. 2013), and the cerebellum is responsible for many cognitive behaviour traits. Being a reef-associated species, *D. sargus* (Bauchot 1987) developed a larger cerebellum probably because of the high demanding cognitive capacities required when living in a more complex habitat (Shumway 2008). As the diencephalon is responsible for the fish homeostasis and endocrine system, processing environmental changes and the relaying of motor and sensory signals (Helfman et al. 2009; Vanderah 2016), one may infer that the evident reduction in the investment of the diencephalon may disruptions such functions. Also, pathologies related to a reduction in the diencephalon are related to memory deficiencies (Dzieciol et al. 2017)

The results regarding *S. senegalensis*, suggest a lower energy investment in brain development, as the brain suffered an overall reduction with OA. This reduction may be related to the highly energetic demanding metamorphosis (Cueto et al. 2000; Geffen et al. 2007) allocating a smaller proportion of the total energy to brain growth. Despite this overall reduction in the brain to body mass ratio, the relative proportions of each brain region were maintained. Results from Sampaio et al. (2018) shows that ocean acidification relates to behavioural changes, namely, choosing habitats in which their camouflage is less effective. This behavioural traces may imply lower predator avoidance by this species and consequently ocean acidification effects may lead to a reduction in this species' population.

5.2 Tropical species under OA

Similarly to what happens with the effects of OA in temperate species, there isn't also a noticeable pattern and the results are different among species and brain macro-regions (see summary in Table 5.2).

Table 5.2 – Summary of the effects of ocean acidification on the B/Bo, BS/B, C/B, D/B, O/B and T/B of clownfish *Amphiprion ocellaris*, neon goby *Elacatinus oceanops* and orchid dottyback *Pseudochromis fridmani*

	 B/Bo	 BS/B	 C/B	 D/B	 OT/B	 T/B
 *A. ocellaris*	-	-	-	↓	-	-
 *E. oceanops*	-	-	↑	-	-	-
 *P. fridmani*	-	-	↑	-	↓	-

↑↓ - Positive or negative effect of OA; brain to body mass ratio (B/Bo), brainstem to brain mass ratio Bs/B, cerebellum to brain mass ratio C/B, diencephalon to brain mass ratio D/B, optic tectum/brain mass ratio OT/B and telencephalon/brain mass ratio T/B

In my results, *A. ocellaris* did not revealed significant changes in its total brain weight, however, the significant reduction in the diencephalon observed may induce ecological challenges as it may pose impediments in sensorial acuity and motor signalling, homeostasis, and autonomic nervous system, in regulating circadian and seasonal cycles and in processing environmental changes (Helfman et al. 2009; Vanderah 2016).

Concerning the C/B ratio, it was positively affected by OA in two tropical species (*E. oceanops* and *P. fridmani)*. Again, I argue that these findings suggest a compensatory growth to avoid putative cognitive disruptions, as the cerebellum is responsible for many cognitive behaviour traits (Sullivan 2010), and also because there are already some evidences showing the impairment of cognitive behaviours in many fish species under OA (Domenici et al. 2012; Lönnstedt et al. 2013).

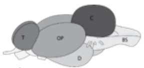
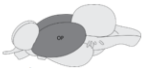
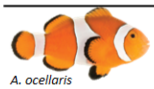
Reef-associated species (*E. oceanops* and *P. fridmani*) (Randall 1986; Robins and Ray 1986; Bauchot 1987), with exception of *A. ocellaris* (Allen 1991), developed larger cerebella, probably related to the high demanding cognitive capacities required by a more complex habitat, as was mentioned for the temperate *D. sargus*. The reduction in the investment of the D/B may cause disruptions in the fish homeostasis and endocrine system, impairing the response to environmental changes and the relaying of motor and sensory signals (Helfman et al. 2009; Vanderah 2016). The fact that *E. oceanops* is also frequently engaged in cleaning interactions, and is monogamous (Whiteman and Côté 2004), may also explain the higher investment in the cerebella, as those are complex inter- and intra-specific relationships that require relatively high cognitive capacities (Breed and Moore 2012; Soares 2017). Similarly, to the results obtained for *E. oceanops*, OA increased C/B on the brain of *P. fridmani*, which also may represent an advantage to a reef-associated species inhabiting a complex habitat. Concomitantly, the same stressor produces the opposite effect on the OT/B of the same species. This fact is prone to affect the reception and processing of visual stimuli and the spatial perception, as the OT is responsible for those abilities (Helfman et al. 2009; Northmore 2011). Moreover, it is also prone to affect rapid decision-making behaviours as those needed for predator avoidance (Manríquez et al. 2014) - and the coordination between sensory systems (Ashur et al. 2017).

Within the OA context, it is important to highlight the excitatory role of GABA signalling in immature neurons (Sernagor et al. 2010). As it does not turn into inhibitory signalling, it may promote an over enlargement of different brain regions, related to the integration and maturation of new forming neurons promoted by GABAergic excitation (Tozuka et al. 2005). This may have been the case for what happened for the increase registered in the B/Bo of *A. regius,* in the BS/B of *D. sargus,* and in the C/B of *D. sargus,* both from temperate habitats and of *E. oceanops* and of *P. fridmani,* from tropical habitats. Among the causes for the decrease of total brains and parts of it in the different fish species tested, it is also interesting to highlight that the results observed may be justified with the fact that high pCO_2 reduces the aerobic capacity in some species (Munday et al. 2009a), hence, the energy available for the brain development.

5.3 Tropical species under OW

In the case of the effect of OW over the three temperate species, there is also no noticeable pattern, and the results are different among species and brain macro-regions (Table 5.3).

Table 5.3 – Summary of the effects of ocean warming on the B/Bo, BS/B, C/B, D/B, O/B and T/B of clownfish *Amphiprion ocellaris*, neon goby *Elacatinus oceanops* and orchid dottyback *Pseudochromis fridmani*

	 B/Bo	 BS/B	 C/B	 D/B	 OT/B	 T/B
 *A. ocellaris*	-	↑	-	↓	-	-
 *E. oceanops*	↑	↓	-	-	-	-
 *P. fridmani*	-	-	-	↑	↓	-

↑↓ - Positive or negative effect of OW brain to body mass ratio (B/Bo), brainstem to brain mass ratio Bs/B, cerebellum to brain mass ratio C/B, diencephalon to brain mass ratio D/B, optic tectum/brain mass ratio OT/B and telencephalon/brain mass ratio T/B

Faced with the future warming conditions, *A. ocellaris* positively developed its brainstem. Being the brainstem responsible for somatic and visceral functions, and responsible for the connections between the brain and most of the sensory systems, its development in this condition of warming may, for the case of this species, let them be less vulnerable to problems related with these functions. Future conditions of OW promoted a reduction in the diencephalon of *A. ocellaris*. Since the diencephalon is responsible for the control of the autonomic nervous system, the coordination of homeostasis and the endocrine system, processing environmental changes and regulating circadian and seasonal cycles (Helfman et al. 2009), relaying motor and sensory signals and regulating consciousness states (Vanderah 2016), its reduction may impair these functions implying a less successful adaptation of this species to the habitats in the future condition of climate change here predicted.

As far as the *E. oceanops* species is concerned, the OW related B/Bo increase may be related to an absolute growth in all the brain-regions (keeping the same ratio to the total brain) except for brainstem. This overall enlargement may benefit the species in what concerns features related to a bigger brain, such as cognitive capacities (Kotrschal et al. 2013). However, the relative decrease of the brainstem in *E. oceanops* may reveal that this species will probably be less well succeeded in what concerns sensorial perceptions. Accordingly, *E. oceanops* may in this condition be more vulnerable to problems related to somatic and visceral functions.

For *P. fridmani,* I propose that the relative increase of the diencephalon may imply benefits for this species. The development of this brain-region, being responsible for the autonomic nervous system, the coordination and regulation of homeostasis and the endocrine system, processing environmental changes and regulating circadian and seasonal cycles, changing in coloration (Helfman et al. 2009), relaying motor and sensory signals and regulating consciousness states (Vanderah 2016), seems to indicate better fitness to the habitat. However, as the results are expressed in ratios, this increase may be a consequence of the relative decrease of the OT/B.

Finally, for the relative reduction of the optic tectum, with OW, the *P. fridmani* will probably be affected by reduced visual acuity, a worse topographical perception of external ambient and impaired decision-making behaviours as predator avoidance (Northmore 2011).

5.4 Temperate vs tropical species under OW

Regarding warming, and contrarily to what was found for OA, here I show that warming affected only specific brain investment in tropical species. This may be explained by the fact that specific thermal ranges evolved to be as narrow as possible, to reduce maintenance costs (Fangue et al. 2006; Pörtner 2006). The temperate species are adapted to a wider range of temperature (Pörtner and Farrell 2008) and the experimental temperature increase was probably within their optimal temperature range. OW affected either the total brain or some of the brain regions of the temperate species in different ways (see summary in Table 5.3).

5.5 Interaction between OA and OW

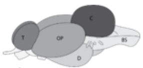
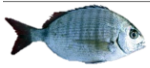
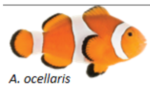
Regarding the interaction between ocean warming and ocean acidification, my results suggest that only tropical species were affected. The fact that temperate species are adapted to a wider range of temperature is once again appointed as the main responsible for this absence of significant effects of the combination of both stressors in temperate species. Table 5.4 summarises the interaction of both stressors, and it is clear that diencephalon was the brain region with more significant interactions, namely in the tropical species. The joint effects of these stressors on other brain regions were neglectable namely in BS/B, C/B, and T/B.

The significant interactions that occurred regarding the diencephalon in the three tropical species occurred all in a way in which the addition of a second stressor attenuates the effect of the first one. Meaning that there is a cross-tolerance effect on the interaction of both stressors.

From these tables, one may also infer that the only species in which there was a significant interaction between both stressors regarding total brain weight was *E. oceanops*, where, in individuals exposed to OW, the total brain weight increased. However, when OW was associated with OA, the response was different: in this case, the total brain weight reduced back to values closer to the control level. Meaning that there was an antagonistic effect, with a cross-tolerance mechanism, in which the addition of high pCO_2 elevated the tolerance of this species to warmer waters. This Cross-tolerance mechanism may imply that future conditions of climate change (OW and OA) will not produce differences in the total brain weight of this species.

Also, the only species where the OT/B was affected by the interaction of both stressors was *P. fridmani*. This is also a case of an antagonistic interaction associated with a cross-tolerance mechanism, where there was an adverse effect of each stressor separately, but when both stressors were combined, that adverse effect was attenuated.

Table 5.4 – Compiled table with the summary of the effects of ocean warming (OW), acidification (OA) and the combined summary of the effects effect of OW and OA on the B/Bo, Bs/B, C/B, D/B, O/B and T/B of the six teleost species tested: meagre Argyrosomus regius, seabream Diplodus sargus, flatfish Solea senegalensis, clownfish Amphiprion ocellaris, neon goby Elacatinus oceanops and orchid dottyback Pseudochromis fridmani

	 B/Bo	 BS/B	 C/B	 D/B	 OT/B	 T/B
 *A. regius*	↑	-	-	-	-	-
 *D. sargus*	-	↑	↑	↓	-	-
 *S. senegalensis*	↓	-	-	-	-	-
 *A. ocellaris*	-	↑	-	↓↓↑	-	-
 *E. oceanops*	↑↓	↓	↑	↑	-	-
 *P. fridmani*	-	-	↑	↑↓	↓↓↑	-

↑↓ - Positive or negative effect of OW ↑↓ - Positive or negative effect of OA

↑↓ - Positive or negative effect of OW + OA; brain to body mass ratio (B/Bo), brainstem to brain mass ratio Bs/B, cerebellum to brain mass ratio C/B, diencephalon to brain mass ratio D/B, optic tectum/brain mass ratio OT/B and telencephalon/brain mass ratio T/B

Here I state that in every case in which there was an interaction between the two stressors, it was an antagonistic interaction with a cross-tolerance mechanism. This means that there must be one or more alternative mechanisms by which those brain regions can grow in a way more similar to the way they grow in control conditions. However, this does not mean *per se* that there are no differences in the brain function.

T/B is not affected by OW and OA in none of the studied species. Once the telencephalon is responsible for many behavioural traits, evidences point to the fact that behavioural impacts due to climate change, in particular, OA, are related more to the function of telencephalon than to its relative weight.

6 Concluding remarks and future directions

This study shows that OA and OW are prone to exert different effects on brain development in marine fish species from both temperate and tropical habitats. The quite different results among the studied species highlight the idea of species-specific phenotypic responses to these climate change-related stressors. As future directions, research focused on brain neurotransmitters quantification under such climate change scenarios should be pursuit, to further connect to eventual variations with species-specific behaviours. Other life-cycle stages should also be studied, namely larval ones because it is during the early ontogeny that the brain, and its parts, develop faster and with greater differentiation.

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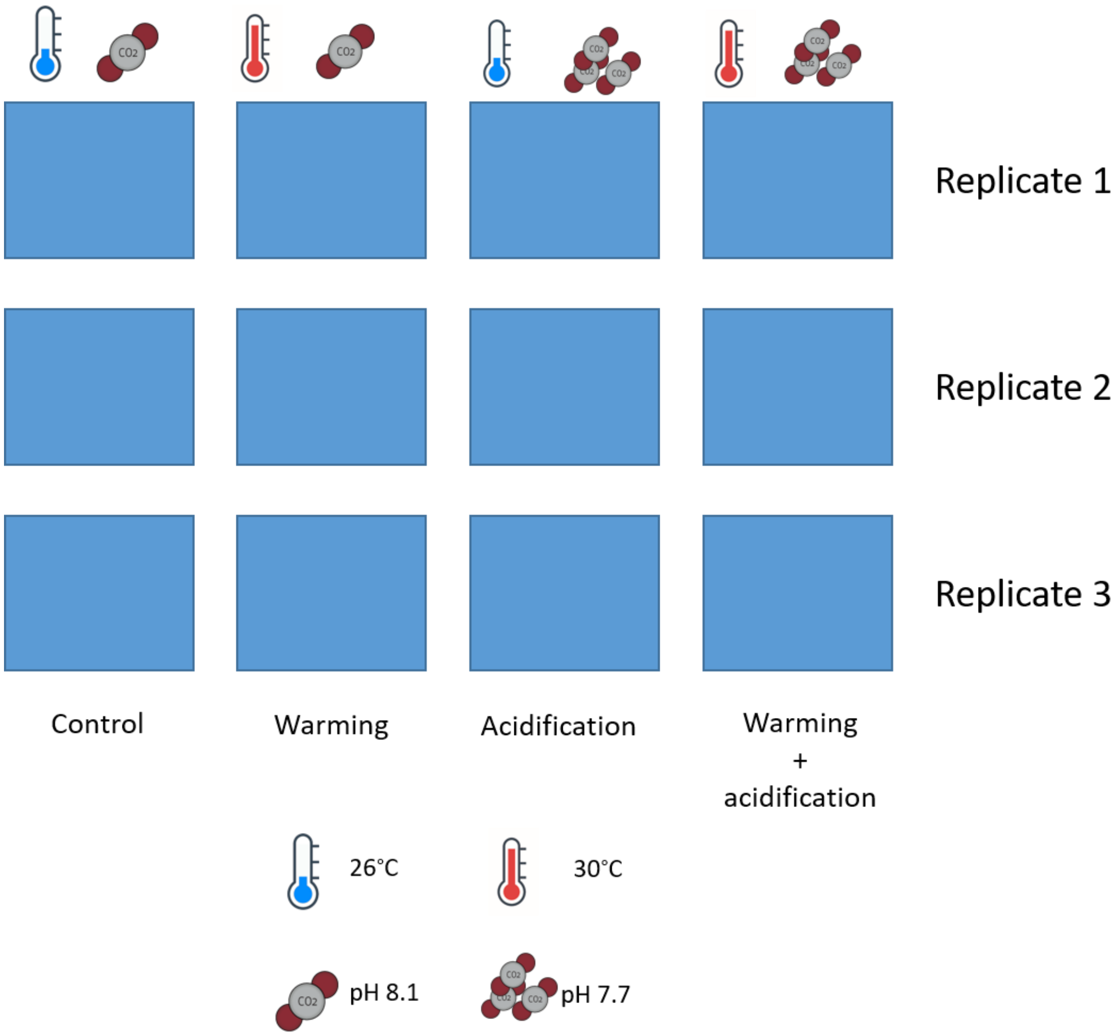
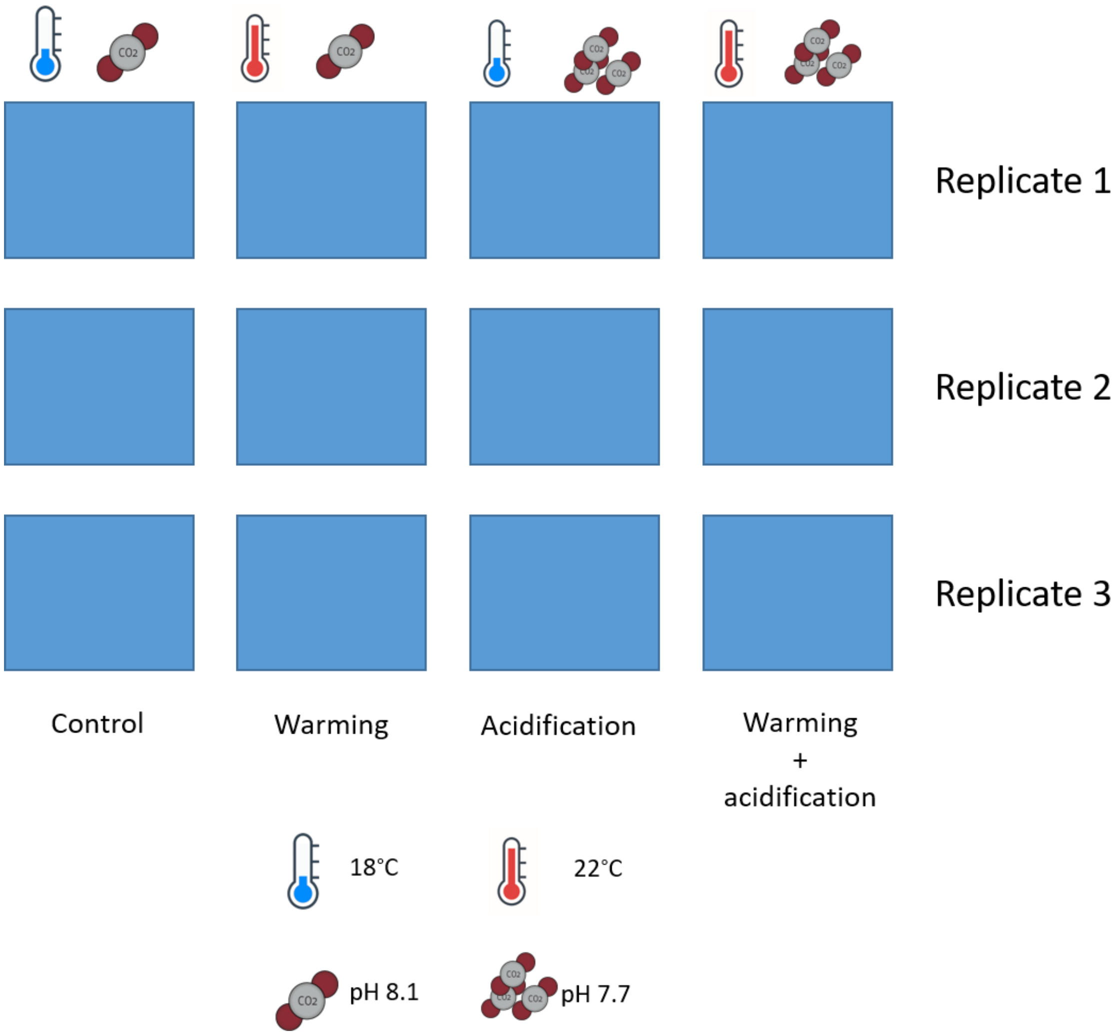
8 Supplemental material

8.1 Experimental setup

Tropical systems



Temperate systems



8.2 Models summary tables

Table 1. GLMM analysis of *A. regius* brain/body mass ratio

*Initial Model: GLMM: brain/body mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.014680	0.001177	12.477	1.42e-08
Temperature	0.002755	0.002058	1.339	0.189046
pCO_2	0.006950	0.001583	4.392	**0.000105**
Temperature:pCO_2	-0.004712	0.002575	-1.830	0.075561

Table 2. GLMM analysis of *D. sargus* brain/body mass ratio

*Initial Model: GLMM: brain/body mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.016971	0.001011	16.787	<2e-16
Temperature	0.002057	0.001462	1.407	0.1666
pCO_2	0.002660	0.001430	1.861	0.0696
Temperature:pCO_2	-0.002556	0.002045	-1.250	0.2181

Table 3. GLMM analysis of *S. senegalensis* brain/body mass ratio

*Initial Model: GLMM: brain/body mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.0114007	0.0008424	13.534	1.39e-09
Temperature	0.0001130	0.0011196	0.101	0.9201
pCO_2	-0.0026532	0.0011451	-2.317	**0.0256**
Temperature:pCO_2	0.0007710	0.0016015	0.481	0.6328

Final Model: GLMM: brain/body mass ratio in function of pCO_2 + (1|tank) (Family: gaussian)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.0114572	0.0006313	18.150	1e-05
pCO_2	-0.0022468	0.0007867	-2.856	**0.00657**

Table 4. GLMM analysis of *A. ocellaris* brain/body mass ratio

*Initial Model: GLMM: brain/body mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.0110657	0.0010346	10.696	<2e-16
Temperature	0.0001176	0.0015518	0.076	0.940
pCO_2	0.0007027	0.0015032	0.467	0.643
Temperature:pCO_2	-0.0004885	0.0021378	-0.228	0.821

Table 5. GLMM analysis of *E. oceanops*brain/body mass ratio

*Initial Model: GLMM: brain/body mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.008697	0.002165	4.018	0.01246
Temperature	0.007586	0.002252	3.368	**0.00304**
pCO_2	0.001425	0.001874	0.761	0.45563
Temperature:pCO_2	-0.007417	0.002866	-2.588	**0.01752**

Table 6. GLMM analysis of *P. fridmani* brain/body mass ratio

*Initial Model: GLMM: brain/body mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.018662	0.002012	9.276	4.43e-11
Temperature	0.001200	0.003226	0.372	0.712
pCO_2	-0.004595	0.002916	-1.576	0.124
Temperature:pCO_2	0.001807	0.004309	0.419	0.677

Final Model: GLMM: brain/body mass ratio in function of pCO_2 + (1|tank) (Family: gaussian)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.019128	0.001557	12.29	8.44e-15
pCO_2	-0.003421	0.002100	-1.63	0.111

Table 7. GLMM analysis of *A. regius* brainstem/brain mass ratio

*Initial Model: GLMM: brainstem/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.1144816	0.0115927	9.875	8.67e-12
Temperature	-0.0174650	0.0207376	-0.842	0.405
pCO_2	-0.0159946	0.0160493	-0.997	0.326
Temperature:pCO_2	-0.0002176	0.0260083	-0.008	0.993

Final Model: GLMM: brainstem/brain mass ratio in function of temperature + (1|tank) (Family: gaussian)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.106046	0.008375	12.661	0.00012
temperature	-0.020854	0.012192	-1.711	0.09560

Table 8. GLM analysis of *D. sargus* brainstem/brain mass ratio

*Initial Model: GLMM: brainstem/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gamma)*

	Estimate	Std. Error	t value	Pr(>|z|)
(Intercept)	-2.97862	0.13858	-21.494	< 2e-16
Temperature	-0.02159	0.20039	-0.108	0.914183
pCO_2	0.72954	0.19598	3.722	**0.000197**
Temperature:pCO_2	-0.21954	0.28029	-0.783	0.433484

Final Model: GLMM: brainstem/brain mass ratio in function of pCO_2 + (1|tank) (Family: gamma)

	Estimate	Std. Error	t value	Pr(>|z|)
(Intercept)	-2.9889	0.1016	-29.422	< 2e-16
pCO_2	0.6265	0.1422	4.407	**1.05e-05**

Table 9. GLM analysis of *S. senegalensis* brainstem weight ratio

*Initial Model: GLMM: brainstem weight ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.094931	0.022725	4.177	0.00138
Temperature	-0.005491	0.028929	-0.190	0.85038
pCO_2	0.045193	0.029593	1.527	0.13438
Temperature:pCO_2	-0.024035	0.041384	-0.581	0.56456

Final Model: GLMM: brainstem weight ratio in function of pCO_2 + (1|tank) (Family: gaussian)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.09218	0.01750	5.269	0.00461
pCO_2	0.03252	0.02044	1.590	0.11904

Table 10. GLMM analysis of *A. ocellaris* brainstem/brain mass ratio

*Initial Model: GLMM: brainstem/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.09780	0.01510	6.479	2.07e-07
Temperature	0.04678	0.02264	2.066	**0.0465**
pCO_2	0.01445	0.02193	0.659	0.5146
Temperature:pCO_2	-0.04444	0.03119	-1.425	0.1634

Table 11. GLMM analysis of *E. oceanops* brainstem/brain mass ratio

*Initial Model: GLMM: brainstem/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gamma)*

	Estimate	Std. Error	t value	Pr(>|z|)
(Intercept)	-1.68861	0.25325	-6.668	2.6e-11
Temperature	-0.29563	0.40042	-0.738	0.460
pCO_2	-0.06553	0.32694	-0.200	0.841
Temperature:pCO_2	-0.54250	0.50800	-1.068	0.286

Final Model: GLMM: brainstem/brain mass ratio in function of temperature + (1|tank) (Family: gamma)

	Estimate	Std. Error	t value	Pr(>|z|)
(Intercept)	-1.7274	0.1670	-10.344	<2e-16
temperature	-0.5992	0.2567	-2.334	**0.0196**

Table 12. GLM analysis of *P. fridmani* brainstem weight ratio

*Initial Model: GLMM: brainstem weight ratio in function of temperature * pCO_2 + (1|tank) (Family: gamma)*				
	Estimate	Std. Error	t value	Pr(>|z|)
(Intercept)	-2.0683	0.1593	-12.983	<2e-16
Temperature	-0.2293	0.2555	-0.898	0.369
pCO_2	-0.2591	0.2309	-1.122	0.262
Temperature:pCO_2	0.2479	0.3412	0.726	0.468

Table 13. GLMM analysis of *A. regius* cerebellum/brain mass ratio

*Initial Model: GLMM: cerebellum/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gamma)*				
	Estimate	Std. Error	t value	Pr(>|z|)
(Intercept)	-2.4077	0.1363	-17.660	<2e-16
Temperature	0.1139	0.2439	0.467	0.640
pCO_2	0.2601	0.1887	1.378	0.168
Temperature:pCO_2	-0.1704	0.3059	-0.557	0.578

Table 14. GLMM analysis of *D. sargus* cerebellum/brain mass ratio

*Initial Model: GLMM: cerebellum/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*				
	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.070816	0.012521	5.656	0.00342
Temperature	0.007963	0.012199	0.653	0.51756
pCO_2	0.025680	0.011921	2.154	**0.03715**
Temperature:pCO_2	-0.017219	0.017057	-1.010	0.31864
Final Model: GLMM: cerebellum/brain mass ratio in function of pCO_2 + (1|tank) (Family: gaussian)				
	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.074612	0.011140	6.698	0.00893
pCO_2	0.017256	0.008424	2.048	**0.04665**

Table 15. GLMM analysis of *S. senegalensis* cerebellum/brain mass ratio

*Initial Model: GLMM: cerebellum/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.116811	0.020726	5.636	2.73e-05
Temperature	-0.001549	0.028371	-0.055	0.957
pCO_2	-0.028665	0.029015	-0.988	0.329
Temperature:pCO_2	0.059690	0.040580	1.471	0.149

Table 16. GLMM analysis of *A. ocellaris* cerebellum/brain mass ratio

*Initial Model: GLMM: cerebellum/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.09682	0.01561	6.204	2.37e-05
Temperature	0.02547	0.02261	1.126	0.268
pCO_2	0.03184	0.02180	1.460	0.154
Temperature:pCO_2	-0.03924	0.03104	-1.264	0.215

Table 17. GLMM analysis of *E. oceanops* cerebellum/brain mass ratio

*Initial Model: GLMM: cerebellum/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gamma)*

	Estimate	Std. Error	t value	Pr(>|z|)
(Intercept)	-3.0275	0.2502	-12.099	<2e-16
Temperature	0.5320	0.3956	1.345	0.1787
pCO_2	0.8305	0.3230	2.571	**0.0101**
Temperature:pCO_2	-0.2015	0.5019	-0.401	0.6881

Final Model: GLMM: cerebellum/brain mass ratio in function of temperature+ pCO_2 + (1|tank) (Family: gamma)

	Estimate	Std. Error	t value	Pr(>|z|)
(Intercept)	-2.9759	0.2209	-13.472	< 2e-16
Temperature	0.4076	0.2440	1.671	0.09480
pCO_2	0.7458	0.2478	3.010	**0.00261**

Table 18. GLMM analysis of *P. fridmani* cerebellum/brain mass ratio

*Initial Model: GLMM: cerebellum/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*				
	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.15289	0.01914	7.990	5.7e-06
Temperature	-0.02726	0.02342	-1.164	0.2526
pCO_2	-0.05920	0.02390	-2.477	**0.0184**
Temperature:pCO_2	0.06729	0.03535	1.904	0.0654

Table 19. GLMM analysis of *A. regius* diencephalon/brain mass ratio

*Initial Model: GLMM: diencephalon/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*				
	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.35783	0.03821	9.365	1.32e-05
Temperature	0.06334	0.05900	1.074	0.290
pCO_2	0.03196	0.04468	0.715	0.479
Temperature:pCO_2	-0.06111	0.07340	-0.833	0.411

Table 20. GLMM analysis of *D. sargus* diencephalon/brain mass ratio

*Initial Model: GLMM: diencephalon/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*				
	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.36499	0.03252	11.223	1.14e-07
Temperature	-0.02353	0.04187	-0.562	0.577
pCO_2	0.05242	0.04187	1.252	0.218
Temperature:pCO_2	0.06329	0.05990	1.057	0.297
Final Model: GLMM: diencephalon/brain mass ratio in function of pCO_2 + (1|tank) (Family: gaussian)				
	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.35322	0.02444	14.451	5.27e-05
pCO_2	0.08315	0.02968	2.802	**0.00759**

Table 21. GLMM analysis of *S. senegalensis* diencephalon/brain mass ratio

*Initial Model: GLMM: diencephalon/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.37015	0.04353	8.503	0.000113
Temperature	-0.02107	0.04741	-0.444	0.659104
pCO_2	-0.07569	0.04851	-1.560	0.126356
Temperature:pCO_2	0.08062	0.06782	1.189	0.241396

Table 22. GLMM analysis of *A. ocellaris* diencephalon/brain mass ratio

*Initial Model: GLMM: diencephalon/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.39393	0.02947	13.367	4.44e-15
Temperature	-0.13310	0.04420	-3.011	**0.00488**
pCO_2	-0.11413	0.04282	-2.665	**0.01168**
Temperature:pCO_2	0.15851	0.06090	2.603	**0.01360**

Table 23. GLMM analysis of *E. oceanops* diencephalon/brain mass ratio

*Initial Model: GLMM: diencephalon/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.41897	0.05570	7.522	1.62e-07
Temperature	-0.12421	0.08807	-1.410	0.1724
pCO_2	-0.09266	0.07191	-1.289	0.2109
Temperature:pCO_2	0.26213	0.11173	2.346	**0.0284**

Table 24. GLMM analysis of *P. fridmani* diencephalon/brain mass ratio

*Initial Model: GLMM: diencephalon/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.24926	0.03194	7.803	2.99e-09
Temperature	0.11220	0.05123	2.190	**0.0351**
pCO_2	0.06450	0.04629	1.393	0.1721
Temperature:pCO_2	-0.17297	0.06842	-2.528	**0.0160**

Table 25. GLMM analysis of *A. regius* optic tectum/brain mass ratio

*Initial Model: GLMM: optic tectum/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	2.505e-01	3.526e-02	7.105	0.00121
Temperature	1.564e-02	4.467e-02	0.350	0.72832
pCO_2	-9.156e-04	3.357e-02	-0.027	0.97840
Temperature:pCO_2	4.128e-05	5.541e-02	0.001	0.99941

Table 26. GLMM analysis of *D. sargus* optic tectum/brain mass ratio

*Initial Model: GLMM: optic tectum/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.28833	0.02721	10.596	1.24e-07
Temperature	-0.05615	0.03602	-1.559	0.127
pCO_2	-0.01557	0.03521	-0.442	0.661
Temperature:pCO_2	0.07052	0.05037	1.400	0.169

Table 27. GLMM analysis of *S. senegalensis* optic tectum/brain mass ratio

*Initial Model: GLMM: optic tectum/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.25045	0.02237	11.198	2.49e-14
Temperature	-0.01162	0.03163	-0.367	0.715
pCO_2	0.02644	0.03234	0.817	0.418
Temperature:pCO_2	-0.04123	0.03234	-0.911	0.367

Final Model: GLMM: optic tectum/brain mass ratio in function of temperature + (1|tank) (Family: gaussian)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.26310	0.01595	16.491	<2e-16
Temperature	-0.03165	0.02233	-1.418	0.163

Table 28. GLMM analysis of *A. ocellaris* optic tectum/brain mass ratio

*Initial Model: GLMM: optic tectum/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.26848	0.02914	9.214	9.09e-11
Temperature	0.04267	0.04371	0.976	0.3358
pCO_2	0.06122	0.04234	1.446	0.1573
Temperature:pCO_2	-0.10797	0.06021	-1.793	0.0819

Table 29. GLMM analysis of *E. oceanops* optic tectum/brain mass ratio

*Initial Model: GLMM: optic tectum/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.24278	0.05349	4.539	0.00193
Temperature	0.02443	0.07116	0.343	0.73496
pCO_2	-0.01691	0.05892	-0.287	0.77700
Temperature:pCO_2	-0.11302	0.09048	-1.249	0.22605

Table 30. GLMM analysis of *P.fridmani* optic tectum/brain mass ratio

*Initial Model: GLMM: optic tectum/brain mass ratio in function of temperature * pCO_2+ (1|tank) (Family: gaussian)*				
	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.35269	0.02237	15.770	2e-16
Temperature	-0.09795	0.03586	-2.731	**0.009712**
pCO_2	-0.12040	0.03241	-3.715	**0.000687**
Temperature:pCO_2	0.20393	0.04791	4.257	**0.000142**

Table 31. GLMM analysis of *A. regius* telencephalon/brain mass ratio

*Initial Model: GLMM: telencephalon/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*				
	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.18805	0.01699	11.069	3.85e-13
Temperature	-0.06061	0.03039	-1.994	0.0537
pCO_2	-0.04270	0.02352	-1.815	0.0778
Temperature:pCO_2	0.06679	0.03812	1.752	0.0883

Table 32. GLMM analysis of *D. sargus* telencephalon/brain mass ratio

*Initial Initial Model: GLMM: telencephalon/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*				
	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.172585	0.019514	8.844	3.87e-07
Temperature	0.008772	0.026404	0.332	0.741
pCO_2	-0.012327	0.025814	-0.478	0.636
Temperature:pCO_2	0.032241	0.036926	0.873	0.388

Table 33. GLMM analysis of *S. senegalensis* telencephalon/brain mass ratio

*Initial Model: GLMM: telencephalon/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.16765	0.01937	8.656	5.69e-11
Temperature	0.03972	0.02739	1.450	0.1543
pCO_2	0.03276	0.02801	1.170	0.2485
Temperature:pCO_2	-0.07509	0.03917	-1.917	0.0619

Table 34. GLMM analysis of *A. ocellaris* telencephalon/brain mass ratio

*Initial Model: GLMM: telencephalon/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.143367	0.020976	6.835	7.25e-08
Temperature	0.016648	0.031464	0.529	0.600
pCO_2	0.006984	0.030478	0.229	0.820
Temperature:pCO_2	0.034169	0.043346	0.788	0.436

Final Model: GLMM: telencephalon/brain mass ratio in function of temperature + (1|tank) (Family: gaussian)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.14668	0.01518	9.661	1.55e-11
Temperature	0.03716	0.02147	1.731	0.092

Table 35. GLMM analysis of *E. oceanops* telencephalon/brain mass ratio

*Initial Model: GLMM: telencephalon/brain mass ratio in function of temperature * pCO_2 + (1|tank) (Family: gaussian)*

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.10901	0.04851	2.247	0.047
Temperature	0.11030	0.07019	1.572	0.132
pCO_2	0.05564	0.05787	0.961	0.347
Temperature:pCO_2	-0.10527	0.08919	-1.180	0.252

Table 36. GLMM analysis of *P. fridmani* telencephalon/brain mass ratio

*Initial Model: GLMM: telencephalon/brain mass ratio in function of temperature * pCO$_2$ + (1|tank) (Family: gaussian)*				
	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.177161	0.020737	8.543	5.38e-05
Temperature	-0.026347	0.027213	-0.968	0.340
pCO$_2$	0.024654	0.024517	1.006	0.322
Temperature:pCO$_2$	0.008247	0.036270	0.227	0.821

Final Model: GLMM: telencephalon/brain mass ratio in function of pCO$_2$ + (1|tank) (Family: gaussian)				
	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.16692	0.01791	9.318	0.000731
pCO$_2$	0.02498	0.01769	1.412	0.166547