UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA ANIMAL

Cuttlefish early development and behaviour
under future high CO_2 conditions

Érica Raquel Ferreira e Moura

Mestrado em Biologia da Conservação

Dissertação orientada por:
Doutora Marta Pimentel e Doutor Rui Rosa

2019

"The question is, are we happy to suppose that our grandchildren may never be able to see an elephant except in a picture book?"

David Attenborough

Agradecimentos

Desde pequeninos que somos incentivados a seguir os passos dos nossos educadores, eles são o nosso modelo e, normalmente, a intenção da criança é "eu quero ser como…".

Não, não segui esse caminho, pelo menos não em termos profissionais. Na família, sempre houve uma ligação aos animais e, desde que me lembro, sempre fui habituada a isso, ora porque os tínhamos em casa, ora porque os íamos ver a algum lado, ora porque íamos à pesca. Além disso, ainda mal sabia nadar e já tratava o mar como casa, e que peixinho que era… Como um peixe dentro de água, que gostava de ser todas as vezes que ia à praia, desenvolvi um carinho mais especial por aqueles que lá vivem. E, apesar de gostar bastante de amanhar o peixe, acho que essa paixão começou a estar ainda mais à flor da pele quando, num belo dia de pesca, tentei salvar uma tainha - ora, escusado será dizer, acabou por morrer no meio de tantos cuidados intensivos.

Digamos que isto foi só o início de uma bela história, que começou ainda nem eu era gente e que está muito longe do fim. À família, um gigante obrigada por me ajudarem a desenvolver esta paixão e por sempre acreditarem que seria um peixinho dentro de água, com olho de lince e memória de elefante, mesmo quando me davam as mil e uma sugestões sobre os cursos que eu deveria seguir. Um especial obrigada à família lisboeta por me ter recebido e albergado durante estes dois anos.

À Ciência Viva, deixo uma mensagem de agradecimento por me terem proporcionado experiências espetaculares com o programa "Ocupação Científica de Jovens nas Férias" e um obrigada a todos os intervenientes por me terem ajudado a confirmar que era isto que eu queria para o meu futuro.

Ao ICBAS (Instituto de Ciências Biomédicas Abel Salazar), por ter sido uma casa durante 3 anos e por me ter lançado neste mundo dos animais aquáticos. Que Ciências do Meio Aquático continue a marcar pontos por formar pessoas com paixão. Aos peixinhos que daí vieram, e aos demais, mas principalmente aos meus dois tristes tugas, um gigantesco obrigada por estarem sempre lá, mesmo que do "outro lado do mundo".

À Faculdade de Ciências da Universidade de Lisboa, mais especificamente ao Mestrado em Biologia da Conservação, agradeço por me relembrarem que quando queremos mesmo uma coisa, conseguimos, mesmo que para isso tenhamos de ultrapassar grandes obstáculos.

Ao Rui, obrigada por me teres recebido de braços abertos e por me teres dado a oportunidade de "voltar" aos bichinhos que me tinham encantado há 5 anos atrás, mesmo que na "concorrência". À Marta, à Catarina e ao Eduardo, um especial obrigada por terem caído nesta aventura de paraquedas e por terem feito com que tudo isto fosse possível, estando sempre lá, aturando os meus dramas, as minhas perfeições e todos os contratempos, arranjando sempre um plano B, C e D para tudo e mais alguma coisa e por me ensinarem um montão de coisas novas. À Cátia, ao Grilo e à Rita, obrigada por serem a minha "comfort food" e por tomarem conta da minha (in)sanidade mental e, mesmo que indiretamente, por me terem ajudado a manter o comboio nos carris. À Shark Team e à Kuka, obrigada por olharem pelos meus bichinhos como se fossem os vossos e obrigada também aos restantes membros do Laboratório Marítimo da Guia por todas as contribuições que foram dando para que esta dissertação se tornasse realidade. A

todos, obrigada por me terem recebido e integrado de tal forma que fez com que os 300 km que me separam de casa deixassem de existir.

Como não poderia deixar de ser, um obrigada super especial à companheira desta aventura, à pessoa que abriu as portas para que esta dissertação se tornasse possível, à nortenha mais brega que alguma vez conheci. Sim, amiga, pensavas que me esquecia de ti?! Além de partilharmos um segundo nome muito lindo, partilhamos uma paixão imensa pelo mundo aquático, o que levou a que as nossas vidas se cruzassem nesta experiência. Apesar do mundo não ser cor-de-rosa e de não correr tudo às mil maravilhas, quero agradecer-te do fundo do coração por toda ajuda que me deste, mesmo não fazendo parte do teu papel. Esta aventura não teria sido a mesma coisa sem ti e espero que, apesar de tudo, tenha valido a pena tanto para ti como para mim. Encontrar-nos-emos por aí em "mares nunca dantes navegados"!

Aos meus 4 patas, obrigada pela companhia e por animarem a minha vida de morcego. Aquelas noites sem vocês a fazerem corridas às 06:00 h da manhã, a passearem por cima do meu teclado ou a tentarem abrir portas e gavetas, não tinham sido a mesma coisa.

Com tudo isto aprendi que fazer ciência de laboratório não é justo. Não é justo termos de sacrificar seres indefeso em prol de algo maior. Não é justo, mas é o que tem de ser. A vocês, meus pequeninos, um pedido de desculpas por terem tido uma vida tão curta, por não vos ter deixado crescer e, ao mesmo tempo, obrigada por existirem e por terem sido a essência de tudo isto. As vossas vidas serão honradas da melhor maneira e espero que a vossa espécie demonstre a "inteligência" que possui combatendo todas as adversidades que o mundo vos irá trazer!

Abstract

Atmospheric CO_2 levels are rising since the beginning of the Industrial Era, and concomitantly, the uptake of CO_2 by the oceans is increasing and changing the seawater chemistry, a phenomenon known as ocean acidification (OA). These changes can compromise key biological traits of many marine organisms, with potential cascading effects to population and ecosystem levels. Besides the significant neurological/physiological impairments, there is increasing evidence of detrimental OA effects on the behavioural ecology of certain marine taxa, including in cephalopods. Yet, the previous behavioural studies performed in these highly-developed invertebrates were only focused on squids and the potential impacts in cuttlefish behaviour are still unknown.

Within this context, the main objectives of this dissertation were to investigate OA effects in the development and behaviour of the common cuttlefish (*Sepia officinalis*) early-life stages – from early embryogenesis until 20 days after hatching, namely by exposing them to either present-day ($\sim$ 400 µatm) and to the near-future levels of pCO_2 ($\sim$ 1000 µatm; $\Delta pH = 0.4$). A comprehensive assessment of OA effects on cuttlefish development was performed by gauging embryogenesis duration, hatching success, early survival rate and body size measures (e.g. weight and length). Furthermore, different aspects of the cuttlefish behavioural ecology, including shelter-seeking, hunting behaviour and response to a visual alarm cue, were analysed to achieve a holistic overview of the OA impacts in cuttlefish early development.

The present work did not find any evidence that OA future conditions compromise the cuttlefish embryonic development. The development time, hatching success, survival rate, and the length and weight of newly-hatched cuttlefish were similar between normocapnia and hypercapnia treatments. The Fulton's Index was the only parameter that showed significant differences, with higher values to the hypercapnia treatment, which may be related with a denser cuttlebone. Concerning to the behaviours analysed, and in contrast with previous cephalopod studies, the results suggest a certain behavioural resilience of the cuttlefish hatchlings towards near-future acidification conditions. The behaviours of hunting, shelter-seeking and response to a visual alarm cue did not show significant differences between treatments.

Their nekton-benthic (and active) lifestyle, their adaptability to the abiotic-fluctuating coastal environment and to the adverse conditions inside their eggs may favour the odds of the common cuttlefish recruits to endure the future acidified ocean. Nonetheless, this species is not only exposed to acidification in their natural environment, they may be also particularly susceptible to other anthropogenic pressure and other climate change-related variables. The cumulative effects of multiple stressors should be further addressed to accurately predict what the future reserves to this ecologically and economically important species.

Keywords: ocean acidification, common cuttlefish, early-life stages, embryogenesis, behaviour

Resumo

As alterações climáticas abrangem um conjunto de diferentes fatores, resultantes predominantemente da combustão de combustíveis fósseis, sendo o aumento global da temperatura um dos indicadores mais conhecidos. Para além deste, fazem também parte a subida do nível do mar, a perda de massa de gelo na Antártida, no Ártico e nas montanhas glaciares de todo o planeta, a ocorrência de eventos climáticos extremos e a acidificação dos oceanos.

Dada a dependência humana da energia obtida através dos combustíveis fósseis, a carga atmosférica dos gases de efeito estufa tem vindo a aumentar, desde o início da Era Industrial (1750), sendo o dióxido de carbono (CO_2) o GHG que regista o maior aumento. Os valores de CO_2 atmosférico observados hoje em dia são mais elevados do que aqueles que a Terra experienciou nos último 800.000 anos, no entanto, é esperado que continuem a aumentar. O oceano global é um dos grandes reservatórios de CO_2 atmosférico e, nos últimos séculos, tem vindo a absorver cerca de 30% do CO_2 gerado pelo Homem, o que desencadeou um aumento de 26% na concentração de iões de hidrogénio. Esta absorção de CO_2 atmosférico é considerada a causa principal da acidificação dos oceanos (nome dado ao processo de redução de pH gerado pelo aumento excessivo da pressão parcial de CO_2 (pCO_2) na superfície dos mesmos), tendo já instigado uma redução de 0,1 unidades de pH e, no final do século, espera-se uma descida de 0,2 a 0,4 unidades de pH. Quando o CO_2 reage com a água do mar desencadeia um conjunto de reações que levam à formação de ácido carbónico (H_2CO_3), ao aumento dos iões de bicarbonato (HCO_3^-), à redução dos iões de carbonato (CO_3^{2-}) e, consequentemente, à diminuição dos níveis de carbonato de cálcio ($CaCO_3$), o principal constituinte dos organismos com exosqueleto calcário ou concha. Este processo afeta a formação das estruturas calcárias, que necessitam dos iões de carbonato, e ainda outros processos biológicos e químicos fundamentais, devido à alteração no equilíbrio químico da água do mar.

A diminuição de pH e o aumento de CO_2 no meio aquático apresentam sérias consequências para a saúde dos ecossistemas, interferindo com a biogénese de estruturas calcárias e induzindo alterações fisiológicas, neurológicas e comportamentais nos organismos marinhos. Apesar destas implicações, os organismos marinhos que possuam sistemas excretor e regulador de pH eficazes (e.g. peixes adultos, crustáceos e cefalópodes) podem apresentar alguma tolerância quando expostos a concentrações elevadas de CO_2 durante períodos prolongados. Contudo, diferentes espécies de cefalópodes apresentam sensibilidades distintas a concentrações elevadas de pCO_2, sendo que estas diferenças poderão estar relacionadas com o tipo de estratégia de vida – as espécies pelágicas (i.e. mais ativas), tais como as lulas, demonstraram maior sensibilidade quando comparadas com animais bentónicos, tais como polvos e chocos.

O choco-comum, *Sepia officinalis*, é uma espécie necto-bentónica que realiza migrações sazonais para zonas de baixa profundidade, como os estuários, durante a época de reprodução. Trata-se de uma espécie com maior atividade durante a noite, passando o dia camuflada na areia. Tem uns olhos bastante desenvolvidos e depende das suas características visuais para aumentar a capacidade de sobrevivência, quer pela evasão de predadores, quer pela procura de alimento. Durante o desenvolvimento embrionário, os embriões estão protegidos do ambiente externo por camadas gelatinosas e por camadas de tinta existentes no ovo. Contudo, em condições naturais, o ambiente interno do ovo está sujeito a condições de hipercapnia (elevados níveis de pCO_2), que podem funcionar como uma pré-adaptação a uma futura exposição a condições de acidificação.

Os estudos existentes sobre o efeito da acidificação dos oceanos nestes organismos focam-se sobretudo no impacto relativamente à concha interna, desenvolvimento embrionário e

desenvolvimento de indivíduos recém-eclodidos/juvenis, sendo o efeito sobre as questões comportamentais praticamente desconhecido. Como tal, o presente estudo teve como objetivo descobrir e esclarecer as consequências da acidificação dos oceanos, prevista para o final do século (ΔpH = 0,4), sobre as fases iniciais de vida do choco-comum, *Sepia officinalis*. Neste sentido, ovos e indivíduos recém-eclodidos de choco-comum foram sujeitos a um cenário de acidificação, com valores estimados para o final do século (pH = 7,7; pCO$_2$ $\sim$ 1000 μatm) e a um cenário de controlo, com valores atuais (pH = 8,1; pCO$_2$ $\sim$ 400 μatm). Durante o tempo de exposição, que englobou o desenvolvimento embrionário e 20 dias pós eclosão, foi avaliado um conjunto de respostas biológicas, nomeadamente: 1) tempo médio de desenvolvimento embrionário, 2) sucesso de eclosão e 3) taxa de sobrevivência aos 20 dias pós eclosão. Aos 15 dias pós eclosão foi realizada uma bateria de testes comportamentais essenciais à sobrevivência nos primeiros estágios de vida, nomeadamente: a) escolha de abrigo, b) predatório e c) reação a um estímulo visual conspecífico (tinta). No final dos testes comportamentais foram recolhidos dados relativos à condição física dos indivíduos: i) peso, ii) comprimento total e iii) comprimento do manto, úteis para o cálculo do índice de condição (índice de Fulton).

O presente trabalho não encontrou evidências de que a acidificação dos oceanos, esperada num futuro próximo, possa provocar alterações no desenvolvimento embrionário, dada a inexistência de resultados estatisticamente significativos relativamente ao tempo de desenvolvimento, ao sucesso de eclosão e à taxa de sobrevivência, e ainda ao comprimento e peso dos animais recém-eclodidos entre os tratamentos aos quais foram expostos. Relativamente aos parâmetros comportamentais analisados, e em contraste com os resultados obtidos noutros estudos com cefalópodes, também não foram encontradas diferenças estatisticamente significativas nos comportamentos de procura de abrigo, de predação e reação a um estímulo visual conspecífico, demonstrando que a acidificação dos oceanos esperada para 2100 possivelmente não provocará alterações comportamentais (nos comportamentos aqui abordados) nos indivíduos recém-eclodidos. Contudo, foram observados resultados estatisticamente significativos relativamente ao fator de condição, apresentando um valor mais elevado nos indivíduos expostos às condições de hipercapnia. Tal facto, poderá estar relacionado com resultados obtidos por estudos anteriores que indicam que em condições de acidificação estes animais são capazes de hipercalcificar a sua concha interna, levando a que esta se torne mais densa. Todavia, permanecem desconhecidas as consequências desta hipercalcificação. Sendo a concha interna responsável pela flutuação neutra destes animais, alterações na sua estrutura poderão alterar comportamentos essenciais, como a natação e a predação. Permanece também por descobrir quais os efeitos desta exposição no desenvolvimento futuro dos indivíduos e o efeito quando expostos a concentrações mais elevadas de pCO$_2$.

Em suma, esta dissertação permitiu verificar que os estados iniciais de desenvolvimento de *Sepia officinalis* parecem ser resilientes a elevadas concentrações de pCO$_2$ ($\sim$ 1000 μatm), dado que não foram observadas diferenças significativas no desenvolvimento nem no comportamento destes indivíduos. Estes resultados estão de acordo com estudos realizados anteriormente que apontam para uma baixa sensibilidade desta espécie quando exposta a elevadas concentrações de pCO$_2$ que provocam respostas negativas na grande maioria dos invertebrados (e.g. corais e bivalves). Tal poderá estar relacionado com: i) o seu estilo de vida necto-bentónico, frequentemente exposto a um meio (costeiro e estuarino) já por si propenso a flutuações ambientais e ii) as condições naturais de hipercapnia experienciadas durante todo o desenvolvimento embrionário (i.e. no ambiente interno dos ovos). Contudo, o índice de Fulton foi a única variável resposta que demonstrou diferenças significativas entre os tratamentos, sublinhando a necessidade de compreender melhor quais as possíveis consequências de uma

concha interna potencialmente hipercalcificada. Adicionalmente, estudos futuros deverão ter em conta os efeitos da acidificação dos oceanos ao nível neurológico, de modo a corroborar a ausência de efeitos comportamentais.

Nesta dissertação apenas foi incluída a acidificação dos oceanos como fator das alterações climáticas. Porém, este não atua como um fator isolado, estando muitas vezes associado a outros, como por exemplo, aumento da temperatura, hipóxia ou poluição antropogénica. Como tal, é necessária uma pesquisa mais alargada (holística e multidisciplinar) e com base no efeito cumulativo de múltiplos fatores de stress para prever as consequências futuras das alterações climáticas nesta espécie ecologicamente e economicamente tão importante.

Palavras-chave: acidificação dos oceanos, choco-comum, estados iniciais de desenvolvimento, embriogénese, comportamento

List of Figures and Tables

List of Abbreviations and Symbols

GHGs Greenhouse gases

CO_2 Carbon dioxide

RCPs Representative Concentration Pathways

$CO_{2(atm)}$ Atmospheric CO_2

H_2CO_3 Carbonic acid

HCO_3^- Bicarbonate

CO_3^{2-} Carbonate ion

pH Power of hydrogen

H^+ Hydrogen ion

OA Ocean acidification

Ca^{2+} Calcium ion

$CaCO_3$ Calcium carbonate

pCO_2 Partial pressure of carbon dioxide

Cl^- Chloride ion

GABA γ-aminobutyric acid

GABA-A γ-aminobutyric acid receptor

CNS Central nervous system

ppm Parts per million

μatm Microatmospheres

TCO_2 Total carbon dioxide

TA Total alkalinity

L Length

W Width

H Height

GLM Generalized linear model

GLMM Generalized linear mixed model

AIC Akaike information criterion

DAH Days after hatching

DED Days of embryonic development

DML Dorsal mantle length

TBL Total body length

pO_2 Partial pressure of oxygen

PVF Perivitelline fluid

GGE Gross growth efficiencies

CF Concentration factor

$P_{c,ext}$ the point at which the rate of oxygen consumption was no longer maintained independent of ambient pO_2

$P_{c,in}$ internal critical pO_2

Ω_{ar} aragonite saturation state

NASOM Non-acid-soluble organic matrix

[x] Concentration

vs "*versus*"

etc "*etcetera*"

e.g. "for example"

i.e. "that is"

NS Not specified

NA Not applicable

1. Introduction

1.1. Climate change

The term "Climate change" is often identified by changes in the mean and/or in the variability of its properties that persist for an extended period (Cubasch et al., 2013; Hartmann et al., 2013). Yet, climate change is happening at an unprecedented rate, and is primarily caused by human activities, namely due to fossil fuels burning (Cubasch et al., 2013). Since the beginning of the Industrial Era, greenhouse gases (GHGs – carbon dioxide, methane, nitrous oxide, hydrofluorocarbons, perfluorocarbons and sulphur hexafluoride) concentrations have increased dramatically (Ciais et al., 2013; Cubasch et al., 2013), being the carbon dioxide (CO_2) the GHG with the major increase - from 278 (Ciais et al., 2013; Rhein et al., 2013) to 410.92 ppm (CO2.Earth, 2019).

The climate projections are crucial to understand the direction of climate change, helping to predict the problems for the future. The Representative Concentration Pathways (RCPs) describe four different 21st century pathways of GHGs emissions and atmospheric concentrations, air pollutant emissions and land use to project their consequences for the climate system. The RCPs include a stringent mitigation scenario (RCP2.6), two intermediate scenarios (RCP4.5 and RCP6.0) and one scenario with very high GHG emissions (RCP8.5) (IPCC, 2014). The present value observed for atmospheric CO_2 concentration is higher than those registered on Earth for, at least, in the past 800,000 years and, because of human dependence on fossil fuels combustion, it is expected (in the higher-emissions scenario) to continue rising until 950-1000 ppm by 2100 (see Figure 1.1.) (Rhein et al., 2013).
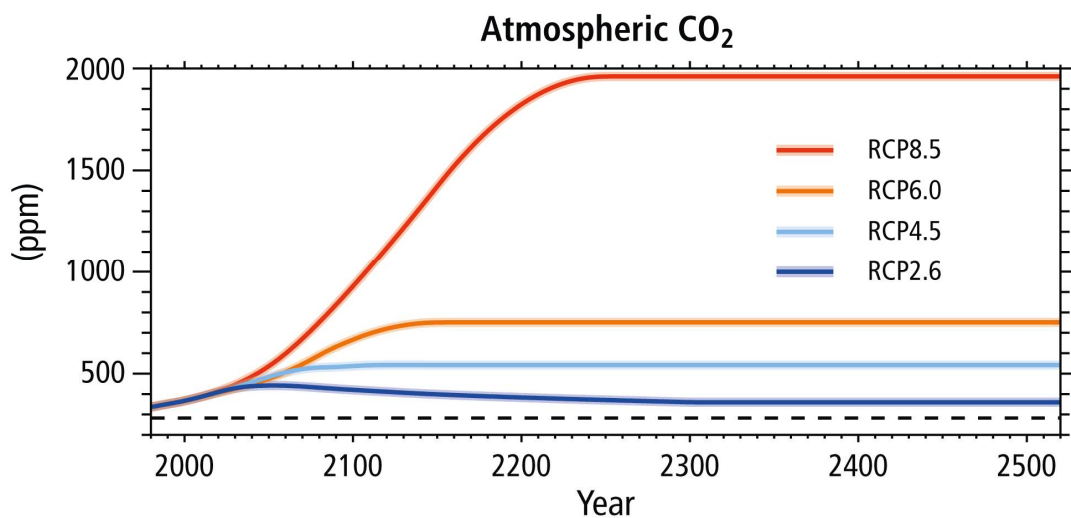
Figure 1.1.: Changes in global average atmospheric CO_2 given for all RCP scenarios – represented by coloured lines identified on the right side. From IPCC (2014).

After being emitted, the atmospheric CO_2 is rapidly dispersed within the atmosphere and it can absorb part of the solar radiation reflected by the earth's surface, radiating the absorbed energy back to the surface. As consequence of this process, and with the increase of the atmospheric CO_2, our planet is warming. Since 1880, the yearly global land and ocean temperature has increased at an average rate of 0.07 °C, per decade, with 2017 being the warmest year without El Niño (NOAA, 2018). Global warming has been described as one of the best-known indicator of climate change (Hartmann et al., 2013), but this phenomenon encompasses a set of other different factors, such as sea level rise, ice mass loss in Antarctica, Arctic and mountain glaciers worldwide, extreme weather events, changes in ocean salinity, and warming and acidification of the oceans (IPCC, 2014).

Carbon dioxide, besides being dispersed throughout the atmosphere, can have two fates, it can be absorbed by the terrestrial biosphere and/or by the upper oceans (see Figure 1.2.). The carbon reservoir in the ocean is roughly 50 times bigger than the atmospheric, which allows a store of large amounts of CO_2. Although, the global carbon uptake by land and oceans doubled from 1960 to 2010, it is likely that on a global scale both land and ocean sinks efficiency may decrease in a future, and generate climate disturbances (Rhein et al., 2013).
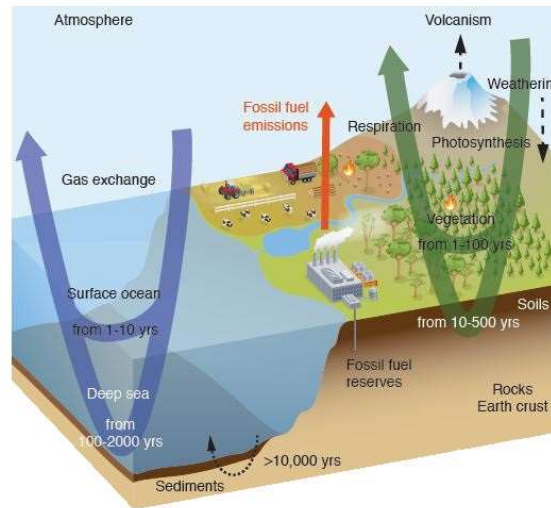
Figure 1.2.: General schematics of the global carbon cycle. From Ciais et al. (2013).

1.2. Ocean acidification

Over the past centuries, oceans have absorbed about 30% of the anthropogenic CO_2 from the atmosphere [$CO_{2(atm)}$]. This natural process of CO_2 absorption, due to differences in partial pressure of CO_2 between the air and the sea (Ciais et al., 2013), has benefited humankind by significantly reducing the greenhouse gas levels and reducing some of the global warming impacts'. However, when CO_2 reacts with seawater it increases the formation of carbonic acid (H_2CO_3), increases the amount of bicarbonate ions (HCO_3^-), reduces carbonate ions (CO_3^{2-}) (Rhein et al., 2013) and, consequently, changes the seawater chemistry (see Figure 1.3.).
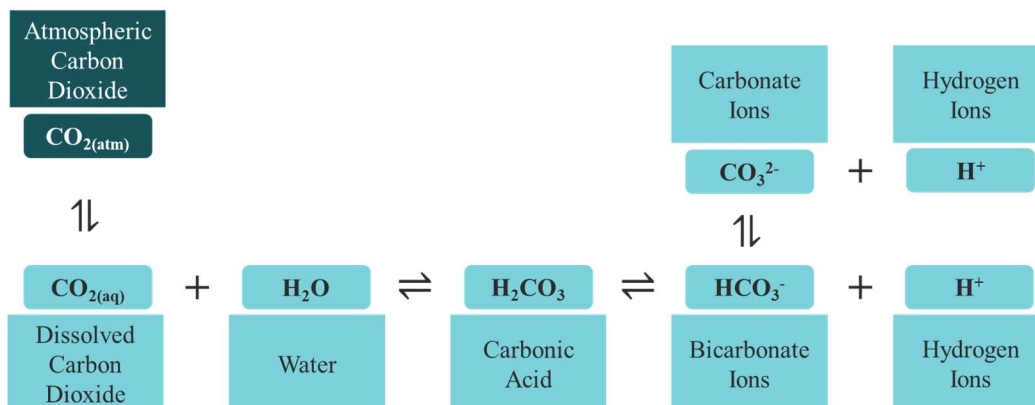
Figure 1.3.: The basic equations when CO_2 crosses the air-sea surface.

The increase in $CO_{2(atm)}$ is changing the chemical balance of seawater and increasing the production of hydrogen ions (H^+), being responsible for the reduction of seawater pH and for the increase in acidity. This pH reduction process, over an extended period of decades or longer, is known as ocean acidification (OA). In addition to the anthropogenic causes, ocean acidification can also be caused by other chemical additions or subtractions that are natural, such as increased volcanic activity, methane hydrate releases and long-term changes in net respiration (Rhein et al., 2013). The mean pH (total scale) of the ocean remains mildly basic at the present, near 8.1. However, since 1765, it has been observed a decrease of about 0.1 units on the ocean's pH, corresponding to a 26% increase of [H^+]. With the continuous increasing of CO_2 uptake and ocean acidification, future predictions indicate that, by the end of this century, the average ocean surface pH, that has been stable for more than 50 million years (Rhein et al., 2013), will be lowered 0.2 to 0.4 pH units than the actual levels if no action takes place (see Figure 1.4.). This drop in pH might represent serious threats to the health of the world's ocean ecosystems (Cubasch et al., 2013).
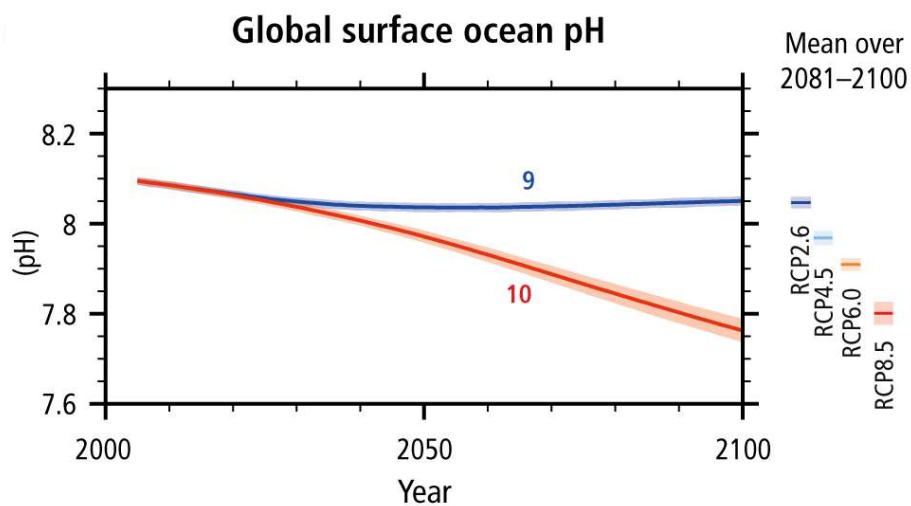
Figure 1.4.: Changes in ocean surface pH. Time series of projections and a measure of uncertainty (shading) are shown for scenarios RCP2.6 (blue) and RCP8.5 (red). The mean and associated uncertainties averaged over the 2081-2100 period are given for all RCP scenarios as coloured vertical bars on the right side of the figure. From IPCC (2014).

Under acidified conditions, the extra H^+ that is produced will combine with CO_3^{2-} to produce additional HCO_3^- and H_2CO_3. As carbonate ions bind with H^+, their availability decreases to combine with free calcium ions (Ca^{2+}) and to form calcium carbonate ($CaCO_3$) (Figure 1.5. – a) (Ciais et al., 2013). $CaCO_3$ is considered building block minerals, and its reduction (Figure 1.5. – b) is already affecting shell formation of many calcifying marine organisms (e.g. corals and bivalves) (Camp et al., 2017; Kurihara et al., 2007), and it may further affect fundamental biological processes of calcifying and non-calcifying organisms (Pimentel et al., 2014; Sui et al., 2017).

a) Ca^{2+} (Calcium Ions) $+$ CO_3^{2-} (Carbonate Ions) $\rightleftharpoons$ $CaCO_3$ (Calcium Carbonate)

b) $CaCO_3$ (Calcium Carbonate) $+$ $2H^+$ (Hydrogen Ions) $\rightleftharpoons$ Ca^{2+} (Calcium Ions) $+$ $CO_{2(aq)}$ (Dissolved Carbon Dioxide) $+$ H_2O (Water)
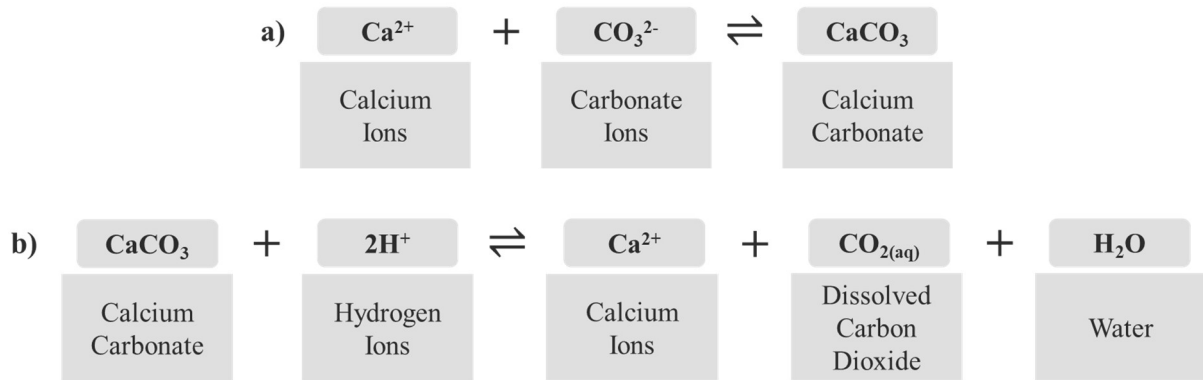
Figure 1.5.: a) Formation of calcareous structures and b) Dissolution in acidified waters.

Nonetheless, ocean warming and acidification, and many other environmental stressors (e.g. anthropogenic pression), do not act independently. Although the CO_2 that is taken up by the ocean mitigates the effects of greenhouse warming, ocean warming *per se* reduces the solubility of carbon dioxide in seawater, which changes the chemical carbon reactions and, consequently, reduces the CO_2 amount that oceans can absorb from the atmosphere (Ciais et al., 2013).

1.3. Biological responses of marine biota to ocean acidification

In the near-future, it is expected that ocean acidification will be more harmful to calcifying organisms and those with minimal physiological buffering capacities (e.g. calcareous sponges, corals and most echinoderms) (Knoll et al., 2007) than non-calcifying organisms and those equipped with a powerful capability to maintain their homeostasis and to compensate for extra and intracellular pH perturbations, such as adult fish and cephalopods (Hu et al., 2015). However, changes in the ocean chemistry have been also shown to impact some of these species that were thought to be more resilient.

One of the biggest problem caused by ocean acidification is the dissolution of calcium carbonate minerals, affecting some groups with $CaCO_3$ structures (Caldeira and Wickett, 2003; Fabry et al., 2008; Orr et al., 2005; Stocker et al., 2013), such as corals (Camp et al., 2017; Erez et al., 2011; Marubini et al., 2008), plankton (Riebesell et al., 2000) and molluscs (Kaplan et al., 2013; Kurihara et al., 2007; Maneja et al., 2011; Talmage and Gobler, 2010). But there are some species that are able to maintain or even increase their calcifying structures, like the gastropod *Patella vulgata* (Linnaeus, 1758), the mollusk *Mytilus edulis* (Linnaeus, 1758), the brittle star *Amphiura filiformis* (O. F. Muller, 1776) (Findlay et al., 2011), the fish *Solea senegalensis* (Kaup, 1858) (Pimentel et al., 2014) and the cephalopod *Sepia officinalis* (Linnaeus, 1758) (Dorey et al., 2013; Gutowska et al., 2010; Gutowska et al., 2008). Yet, it is not known what are the consequences of hypercalcification phenomenon on these organisms, but it is known that the metabolic costs increased with elevated seawater pCO$_2$ (Stumpp et al., 2011; Wood et al., 2008a), suggesting energetic trade-offs that impact, at least, the growth rate (Stumpp et al., 2011).

In addition to the above-mentioned consequences, there are other issues that can be affected by OA, for example, growth and survival (Baumann et al., 2012; Pimentel et al., 2014; Pimentel et al., 2016), acid-base imbalance (Fabry et al., 2008; Hu et al., 2015; Pimentel et al., 2014) and oxygen transport capacity/consumption (Fabry et al., 2008; Pimentel et al., 2015; Rosa et al., 2014) of different marine organisms. High CO_2 concentrations in the seawater have been shown to also induce neurological and behavioural deficiencies in a range of marine animals (McNeil and Sasse, 2016),

sometimes even endangering their survival. In many organisms, the exposure to elevated CO_2 levels (hypercapnia) may result in a proportional increase in the blood CO_2 levels (Tresguerres and Hamilton, 2017), and usually, the regulation of these acidosis occurs by upregulating active H^+ excretion and accumulating HCO_3^- in exchange for Cl^- (chloride ions) in physiological fluids (Regan et al., 2016; Tresguerres and Hamilton, 2017). These changes, resultant from acid-base regulation in high CO_2 environment, may interfere with the normal function of the primary inhibitory neurotransmitter in the vertebrates and invertebrates brain, the GABA-A receptor produced by GABAergic neurons (Kobayashi et al., 2013). In a normal state, GABA (γ-aminobutyric acid) binds to GABA-A, activating it and leading to an inflow of Cl^- and/or HCO_3^- into neurons and, consequently, to the hyperpolarization and inhibition of neural activity. With OA and changes in concentration of these ions, the process alters from inflow to outflow, resulting in depolarization and excitation. These changes may affect some neuronal functions and, consequently, may have implications on behaviour and in sensory preferences (Nilsson et al., 2012; Tresguerres and Hamilton, 2017). This neuronal activity plays an important role in a variety of states and physiological processes, including social behaviour (anxiety and aggression), physical movements (movement and feeding), circadian rhythms, emotional responses, learning and memory (Kobayashi et al., 2013).

Although it has been already demonstrated that acidification has consequences at different behavioural levels, e.g. foraging (Dixson et al., 2015; Pimentel et al., 2016), hunting (Dixson et al., 2015; Pistevos et al., 2017; Spady et al., 2018), predation vulnerability (Dixson et al., 2010; Munday et al., 2014; Munday et al., 2016) and response to olfactory cues (Dixson et al., 2010; Munday et al., 2009), more research is needed to know how this is underpinned by disruption in brain functions. But, beyond all the negative implications, some mostly active species with a good pH regulatory and excretory systems such as adult fishes, crustaceans and cephalopods can tolerate high CO_2 concentrations over long exposure times (Hu et al., 2015). However, there are differences within the cephalopods' species, since active pelagic squids show higher sensitivity to elevated pCO_2 compared with cuttlefish and octopods, which have a benthic lifestyle. Such differences suggest that different lifestyles and energetic limitations could represent a major feature, limiting the ability to mobilise energy resources to fuel acid-base compensatory processes in different cephalopods species (Hu et al., 2014).

1.4. General biology and ecology of the European common cuttlefish (*Sepia officinalis*)

Sepia officinalis, commonly known as common cuttlefish, is a nekto-benthic decapod that occurs predominantly on sandy and muddy bottoms, from the coastline (2-3 m depth) to approximately 200 m depth, with the greatest abundance in the upper 100 m (Guerra, 2006; Reid et al., 2005). It has a broad distribution from the North Atlantic, throughout the English Channel, and South into the Mediterranean Sea to the coast of West Africa (Vidal et al., 2014) (Figure 1.6.).
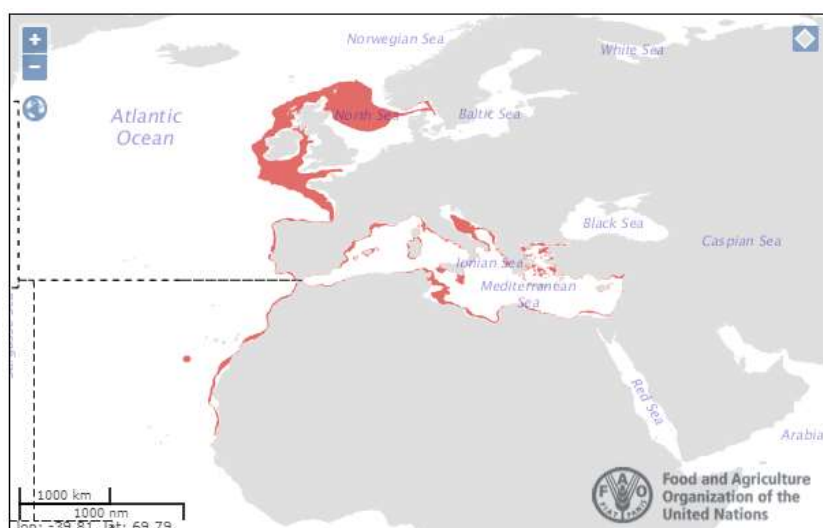
Figure 1.6.: Geographic distribution of *Sepia officinalis* (source FAO (2018)).

This species is relatively tolerant to environmental changes, including salinity (Guerra, 2006; Reid et al., 2005), but are the younger specimens that have a greater ecophysiological plasticity and can tolerate greater environmental instability, which allows them to colonise the upper zones, thus avoiding intraspecific competition (Reid et al., 2005). Unlike salinity tolerance, they are not very tolerant to low oxygen concentrations and their temperature limits range from 10 ºC to 30 ºC - with temperatures below 10 ºC, the individuals do not feed, stay inactive and die in a couple of days (Guerra, 2006).

Under natural conditions, these animals have a typical cephalopods' life cycle, known by the epithet "live fast, die young". "Live fast" because of their high growth rates, reaching the adult body size and breeding conditions at young age, as they have high efficiency of digestion and metabolism, thus they can successfully convert food intake to body growth. "Die young" because the adults die after breeding, leading to a mass mortality in those species with a highly synchronised breeding population (Boyle and Rodhouse, 2008). The common cuttlefish life cycle covers 12 to 24 months, but it can vary with environmental conditions (Reid et al., 2005). This species is gonochoristic (separated sexes) and the mating starts with an elaborate and ritualised courtship, which incorporates stereotyped visual displays and "mate guarding" (Reid et al., 2005). In the mating season, the cuttlefish undergoes seasonal migrations, between inshore waters (spring and summer) and offshore (autumn and winter) – to a depth of about 100 m (Reid et al., 2005). Usually, the large females are the first to leave deeper waters (early spring), followed by the larger males, and the first to spawn. Smaller individuals arrive and spawn later, throughout the summer. This situation of arrival by succession leads to two distinct spawning periods, as shown in Figure 1.7. (Boyle, 1987; Reid et al., 2005):

- **First** – the hatchlings of early broods will be exposed to favorable conditions, growing rapidly throughout the warm summer waters and go to deeper waters in autumn, where the low water temperature slows down the growth rate, but shorter days and low light intensity will accelerate the maturity. These females will be able to spawn in the next autumn (solid lines);
- **Second** – the hatchlings of late broods (autumn broods) will be exposed only for a short period of time to favorable conditions to grow, which does not allow them to be mature to the next spawning season. They will grow rapidly during the following summer, mature during the second winter and spawn early in spring of their second year of life (broken lines).
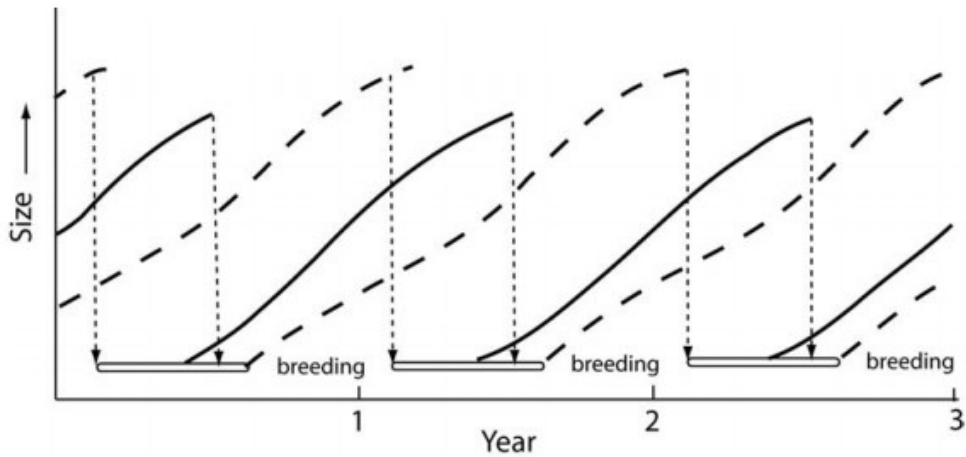
Figure 1.7.: Schematic representation of *Sepia officinalis* spawning periods. From Boyle and Rodhouse (2008).

In general, the spawning period of this species occurs between late March and early December, with peaks at water temperatures from 13 °C to 18 ºC (these parameters can vary depending on the location). After mating, the eggs are laid one by one through the funnel with a ring-shaped elongation of the envelope (Boyle, 1987) and in a grape-like clusters which simplifies the fixing process in seaweed, shells, debris and others (Reid et al., 2005), like drowned trees, cables or nets (Guerra, 2006). The encased eggs are normally black or dark brown due to the ink added to the gelatinous envelopes (Boletzky et al., 2006), but sometimes they can be translucent due the lack of this black pigment. This gelatinous and opaque capsule provides physical and chemical buffering between the embryo's microenvironment and the surrounding, ensuring protection against microbial attack and predation (Boletzky, 2003). When spawning ends, the females die, causing a massive postspawning mortality and male predominance (Reid et al., 2005). The remaining and the new individuals return to offshore (see Figure 1.8.), but the youngest animals only go to depths of about 50 to 80 m because their cuttlebones cannot withstand high water pressure (Reid et al., 2005).
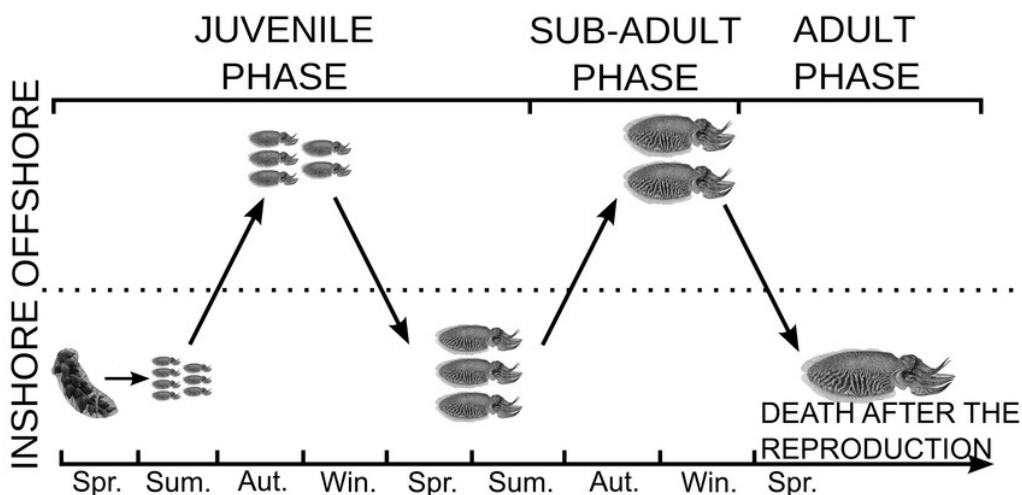
Figure 1.8.: An example of *Sepia officinalis* life cycle from egg to adult. Timings according with the English Channel breeders. From Gras (2013).

The embryogenesis is a temperature-dependent process with hatching occurring between 30 to 90 days after spawn (Boletzky et al., 2006; Boyle, 1987; Palmegiano and d'Apote, 1983; Reid et al., 2005; Rosa et al., 2013). During the embryonic development, energy needs are ensured through two yolk sacs, one internal and one external. When they hatch, either prematurely or in normal hatching, they always have the internal yolk sac that will last for 2-5 days and will be an energetic source, so they do not need to rely on external food supply. The absorption time of the internal yolk sac varies depending on the embryo development rate and the incubation temperature (Dickel et al., 1997). When the cuttlefish hatch prematurely, in addition to the internal yolk sac, it also has a remaining external yolk sac (O'Brien et al., 2017b; Wells, 1958), which drops rapidly, usually after few minutes of hatching (O'Brien et al., 2017b). Newly hatched individuals begin to feed before all internal reserves have been depleted (Dickel et al., 1997) and O'Brien et al. (2017b) explain this by the need that hatchlings have to start the hunting "training", while their energy reserves are not completely dependent on prey. This training period is in line with the observations of Dickel et al. (1997) that show a lower success rate of catches at this stage.

All cephalopods hatchlings have a highly developed central nervous system and most of them are strikingly similar to adults, both in morphology and basic behaviours, hatching with the basics for signaling and camouflage, e.g. the use of chromatophores and ink (Boyle, 1987). The common cuttlefish has complex behaviours (Budelmann, 1995) and the optic lobe is one of the most important part on *Sepia officinalis* brain, due its relevance on basic behaviours, such as predation (Messenger, 1968; Wells, 1958) and camouflage (Chichery and Chanelet, 1976). Most cephalopods have prominent and well developed eyes, that are important in their daily lives (Mather and Kuba, 2013), as well in their complex visual behaviour (Budelmann, 1995). The cuttlefish eyes are characterised by having a distinctive W-shaped pupil under bright light conditions, effective in balancing a vertically uneven light field (Mäthger et al., 2013), which probably is an adaptation to the way of life: living on the bottom, swimming directly backwards and preying on animals directly in front (Muntz, 1999). Under dark conditions, the pupil becomes larger and circular. Despite not having an instant 360° field of view, they can move their eyes to obtain a full field and a 3-dimensional information, essential to the camouflage patterns (Mäthger et al., 2013) by which these animals are highly known. They are colour blind (Mäthger et al., 2006; Muntz, 1999) but this do not restrain them to show and detect various camouflage patterns (Hanlon and Messenger, 1988).

From hatchlings to adults, *S. officinalis* exhibits very striking and variable body patterns, sometimes highly coloured to human, making these animals difficult to discriminate from the background (Guerra, 2006). Besides that, they have a light-induced burying behaviour, spending the daytime hidden in sand (Boyle, 1987; Reid et al., 2005). During the active time, typically during the night (Guerra, 2006), the individuals generally hover above the bottom, which is possible due to their cuttlebone, made by an aragonitic structure. The cuttlebone contributes approximately to 4% of the total body mass (Dorey et al., 2013; Gutowska et al., 2010), is chambered and gas-filled, giving them near-neutral buoyancy (Boyle, 1987). Yet, this buoyancy process consumes a lot of energy (Guerra, 2006).

The common cuttlefish is considered an active predator (Messenger, 1968; Wells, 1958) and a trophic opportunistic animal, feeding on a large range of diets, including crustaceans, fishes, mollusks and polychaetas (Boyle, 1987; Guerra, 2006; Mather and Scheel, 2014). Boal et al. (1999) suggest that this species is probably semi-solitary in natural conditions, however, Warnke (1994) argues that they are social animals, benefiting from this characteristic to increase the predation capacities. Beside the high range of live prey that cuttlefish can eat, under laboratory conditions, the individuals also eat processed and frozen food (Castro and Lee, 1994; Domingues et al., 2004; Sykes et al., 2013). These animals demonstrate cannibal behaviour, predating individuals of smaller size (Boyle, 1987; Ibánez and Keyl, 2010; Reid et al., 2005; Sykes et al., 2006), which may be a strategy to overcome temporary prey

shortages (Reid et al., 2005). At the beginning of development, they usually feed on smaller prey, like crustaceans, passing to larger prey during the growth, like fishes (Guerra, 2006).

Although feeding on a large variety of prey (Guerra, 2006), most attacks start when cuttlefish are partially buried and hidden in sand (Reid et al., 2005). Their attack strategy always follow the same sequence, beginning with the detection of a prey-shaped stimulus, followed by the orientation of the body to the position of the prey, and finally finishes with the capture (see Figure 1.9.) (Messenger, 1968; Wells, 1958). The capture can be performed in one of two ways: "tentacles attack" – usually used to catch faster and smaller prey; or "jumping attack" – a slower strategy chosen for larger prey (Duval et al., 1984; Messenger, 1968). When cuttlefish are younger, they are very vulnerable during the prey-capture moment because they have their own attention on the prey and nothing in the surrounding environment, which increases the predation risk. They can be preyed mostly by sharks, some demersal fishes and cephalopods (Reid et al., 2005).
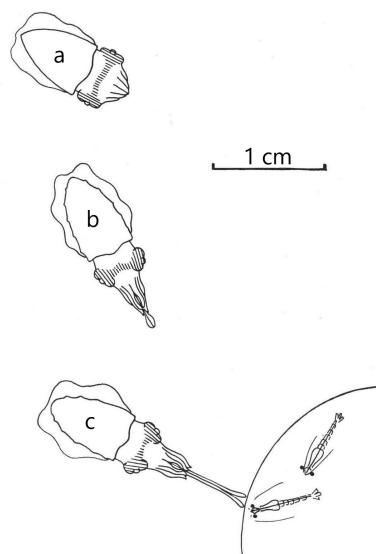
Figure 1.9.: The three attack phases according Wells (1958) and Messenger (1968): a) detection, b) orientation and c) capture. Ilustration from Wells (1958).

Hidden in sand, changing colours and changing its arms disposal, usually with the dorsal arms stretched upwards and ventral arms downwards, both more or less spread laterally (like a floating algae debris), are the primary responses that cuttlefish has to protect itself (Boyle, 1987) – process that encompasses a large number of chromatic, textural and postural components (Guerra, 2006). Another way of defense is by ink eject, creating a cloud as a "dummy" to distract the attacking element (Boyle, 1987), such as predators. Cephalopods ink, in addition to its function as a defensive element, (Boyle, 1987; Bush and Robison, 2007; Spady et al., 2014; Wood et al., 2010) also has been described to act as an alarm cue, both visual and chemical (Boal and Golden, 1999; Bush and Robison, 2007; Gilly and Lucero, 1992; Mezrai et al., 2018; Wood et al., 2008b). As shown by Boal and Golden (1999), *Sepia officinalis* species responds to ink from a conspecific, what shows the role of conspecifics' ink in defensive behaviour. The response to conspecifics decrease the predation risk, the animals are warned by others before being close to the danger, increasing their survival probability (Wood et al., 2008b).

1.5. *Sepia officinalis* and ocean acidification impacts

Ocean acidification has been proposed to constitute a significant stressor in benthic systems, amplifying hypercapnia conditions in naturally acidified and hypoxic habitats (Melzner et al., 2013). The common cuttlefish is an animal that experiences those characteristics in its habitat but, with its active high-metabolic lifestyle and the stressful abiotic conditions during the course of embryonic development, is pointed that it may be a pre-adaptation to cope better with future OA (Melzner et al., 2009). However, it is known that the early life stages of development are generally more vulnerable to climate changes, which may negatively affect species survival (Baumann et al., 2012; Hu, 2016; Kaplan et al., 2013; Rosa et al., 2014). According to Gutowska et al. (2008), *S. officinalis* embryos are expected to be more sensitive in terms of development and metabolism than juveniles and adults. Yet, it is worth noting that Reid et al. (2005) claim the opposite, i.e. that the early life stages reveal greater ecophysiological plasticity and tolerance to environmental instability in younger specimens.

Some works demonstrated the influence of OA in cuttlefish (see supplemental tables 9.2. – 9.4.), mainly in a physiological level and in body structures (Gutowska et al., 2010; Gutowska et al., 2008; Hu et al., 2011; Rosa et al., 2013). A non-influence of pH in hatchling and juvenile body measures was pointed in almost all studies (see Supplemental Table 9.2.), but a high effect of pH was noted in cuttlebone structure, trace elements and gene expression (see Supplemental Table 9.3. and 9.4.). Nevertheless, a hypercalcified cuttlebone, outcome of hypercapnia environment, significantly increased the weight of this structure, what may mask the reductions in body mass (Gutowska et al., 2010). The amplification of hypercapnia conditions was also seen inside the cuttlefish eggs exposed to high pCO_2, thus allowing the maintaining of diffusion CO_2 gradient (Dorey et al., 2013; Hu et al., 2011; Rosa et al., 2013). This acidified environment also increased eggs swelling, what could improve CO_2 permeability and reduces the partial pressure between the egg's inside environment and outside (Dorey et al., 2013; Lacoue-Labarthe et al., 2009).

Considering previous studies done with some species from Mollusca (Spady et al., 2018; Spady et al., 2014; Watson et al., 2017) and a preliminary study performed with the common cuttlefish by Maneja et al. (2011), it is expected the presence of some changes on the behaviour of this species when exposed to OA. However, it is also known that *S. officinalis* might be probably pre-adapted to some environmental fluctuations and that its short life cycle (1-2 years) may enhance the chance for evolutionary adaptation (Hu, 2016). These characteristics may only turn visible some OA effects in a very high $[CO_2]$ environment or in combination with other climate change factors such as temperature (Lacoue-Labarthe et al., 2009; Rosa et al., 2013), hypoxia (Rosa et al., 2013), and/or with exposition to contaminants.

1.6. Common cuttlefish and conservation approaches

In Portugal, one of the most important geographic distribution areas of common cuttlefish is the Sado estuary, a salt marsh site known by its resident population of common bottlenose dolphin (*Tursiops truncatus* - Montagu, 1821), one of the few places in Europe that has a resident population and the only known place on Portuguese coast (ICNF, 2007). This is a small population that has been declining at least during the past three decades (Augusto et al., 2012) and the *S. officinalis* is one of the most sought prey (Guerra, 2006; Santos et al., 2007). Besides being one of the main prey for an endangered population (Augusto et al., 2012; Guerra, 2006; Santos et al., 2007), it is also an important food resource for human population. The cuttlefish captures represent 55% of the total captures for vessels operating in the Sado river, which shows the importance for local economy and, consequently, for the country

fishing economy (ICNF, 2007). Thus, cuttlefish is an important species both ecologically, e.g. within trophic chain, and commercially, e.g. to the fishing economy.

Given the dependence of this species, for common bottlenose dolphins and humans, although its classification as a least concern species, it is crucial to realise what kind of negative implications its reduction can bring. With the increasing fisheries in Europe (Jereb et al., 2015) and the decrease in the ocean pH in the next few years (Stocker et al., 2013), it is necessary to enhance scientific knowledge on the acidification's role in this species. *S. officinalis* is not a priority species for management measures, but some measures have already been applied in Portugal and Galicia to reduce their fishing, as the implementation of minimum sizes (Jereb et al., 2015). However, the data about captures usually is not real (Pauly and Zeller, 2016), which turns essential to take action before the numbers become critical.

2. Objectives

The general objective of this dissertation was to evaluate a set of developmental and behavioural responses of the common cuttlefish (*Sepia officinalis*) early developmental stages to ocean acidification expected by the end of the century ($\Delta pH = 0.4$, ~ 1000 µatm). Specifically, the aim of this dissertation was to understand the effect of ocean acidification exposure on the embryonic development time, hatching success and on the survival rate and body measures (like weight and length) of cuttlefish exposed during embryonic development and 20 days post hatching to an acidified environment. Besides this development analysis, several behavioural trials were performed in cuttlefish after 15 days post hatching, to scrutinise the influence of acidification on the basic survival behaviours of early-life stages:

1) Shelter-seeking – to evaluate its preference for a black or white shelter;

2) Hunting behaviour – to evaluate the amount of prey that they can eat, the time that the hatchlings need to react and to catch the prey, and the capture effectiveness;

3) Visual detection of conspecific visual stimulus (ink) – to evaluate how cuttlefish react to a visual conspecific stimulus, usually used as an alarm cue.

For these behavioural tests it was expected:

1) Shelter-seeking – a higher preference, in the control scenario, for the black shelter because these animals are hidden during the day and active during the night (Guerra, 2006);

2) Hunting behaviour – a reduction of this behaviour in the animals exposed to an acidified treatment (Spady et al., 2018);

3) Visual detection of conspecific visual stimulus (ink) – responses with a higher severity level in the animals from control scenario (Wood et al., 2008b).

For shelter-seeking and visual detection of conspecific visual stimulus it was also expected a behavioural impairment in the animals exposed to ocean acidification scenario.

3. Materials and Methods

3.1. Egg collection

Sepia officinalis eggs, in the stage I-VI (initial stage of embryogenesis, Naef (1928); between 1-20 days after laying, Boletzky et al. (2006)), with a clear cleavage and differentiation of the germinal

disc, were collected during low tide in November of 2017, in Mitrena area located in Sado estuary (Figure 3.1.). This local is a salt marsh area, near the mouth of the estuary, where fishermen put craft traps (see Figure 3.2. – a and b) to pick up cuttlefish females. All the eggs were picked up by hand, placed in a bucket with aeration and transported to Laboratório Marítimo da Guia (LMG - FCUL), and immediately transferred to an isolated tank for an acclimatization period of 6 days.
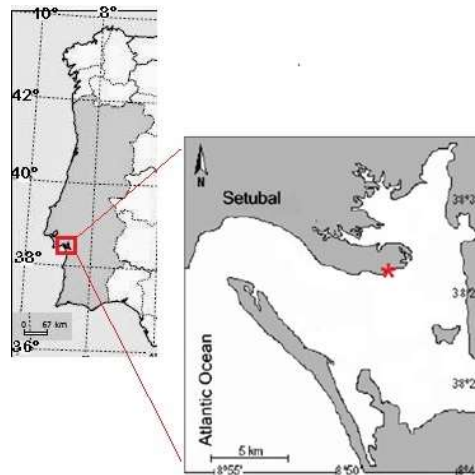
Figure 3.1.: Geographic location of the Sado estuary and the sampling site (Mitrena), indicated by a red star.
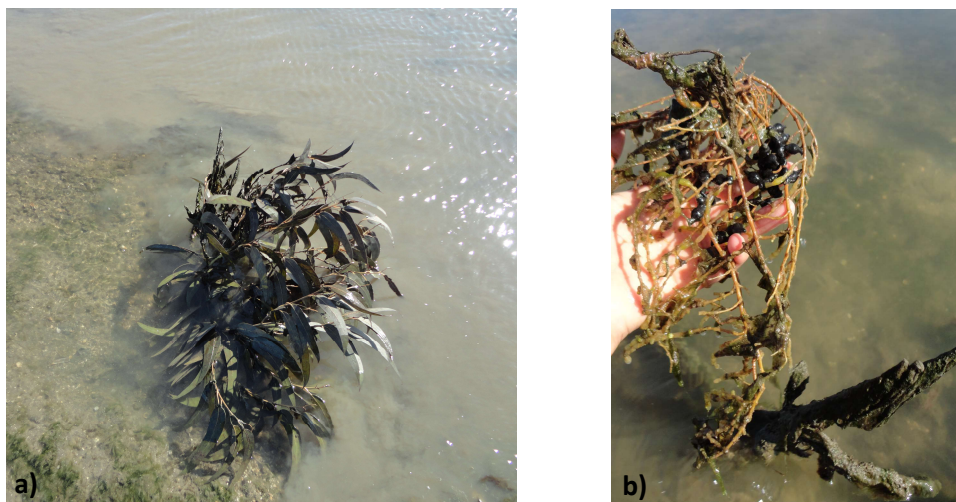
Figure 3.2.: a) Craft traps at Mitrena; b) Common cuttlefish eggs in a craft trap.

3.2. Egg incubation and life support systems

After the acclimatization period, the eggs were incubated under the different acclimation pH treatments (2 pH values × 3 replicates = 6 replicates; 6 replicates × 85 eggs = 510 eggs in total). The pH scenarios were chosen to reflect the following conditions: a) the annual present pH conditions (pH = 8.1, $pCO_2 \sim$ 400 ppm) and b) the near-future expected pCO_2 concentration (ΔpH = 0.4; 0.1%, $pCO_2 \sim$ 1000 ppm).

The acclimation period took place in a total of six independent experimental life support systems (bottom area = 49 cm L × 24 cm W, volume = 22 L; Figure 3.3. and Figure 3.4.) supplied by natural seawater pumped from the sea and filtered with 0.35 μm net and UV radiation, under a semi-closed system, to minimise bacterial activity (i.e. nitrifiers, denitrifiers) and dissolved inorganic carbon. The experimental tanks were also equipped with mechanical (glass wool) and biological (bioballs matured

with nitrifying bacteria) filtration. The systems' temperature was controlled by extra tanks, bain-marie system, and water chillers (Hailea, Guangdong, China), keeping the systems at 18 °C, the average temperature of the spawning season of *S. officinalis* in the western coast of Portugal. Room illumination was provided through overhead fluorescent lighting (MASTER TL-D Super 80, 4000K, 3350 lumen), under a photoperiod of 12 h light: 12 h dark. The experimental pH levels were adjusted automatically, via solenoid valves controlled through a Profilux system (Profilux 3, Kaiserslautern, Germany) connected to individual pH probes (Blueline 25 pH, SCHOTT Instruments, Mainz, Germany), immersed in each acidified sump, and to a powerbar that sends the order from the profilux to the solenoid valves, activating or deactivating them. The pH of natural seawater was reduced by the injection of a certified CO_2 gas mixture (Air Liquide, Miraflores, Algés, Portugal), via air stones, and balanced positively through aeration with CO_2-filtered air (using soda lime, Sigma-Aldrich, St Louis, MO, USA).
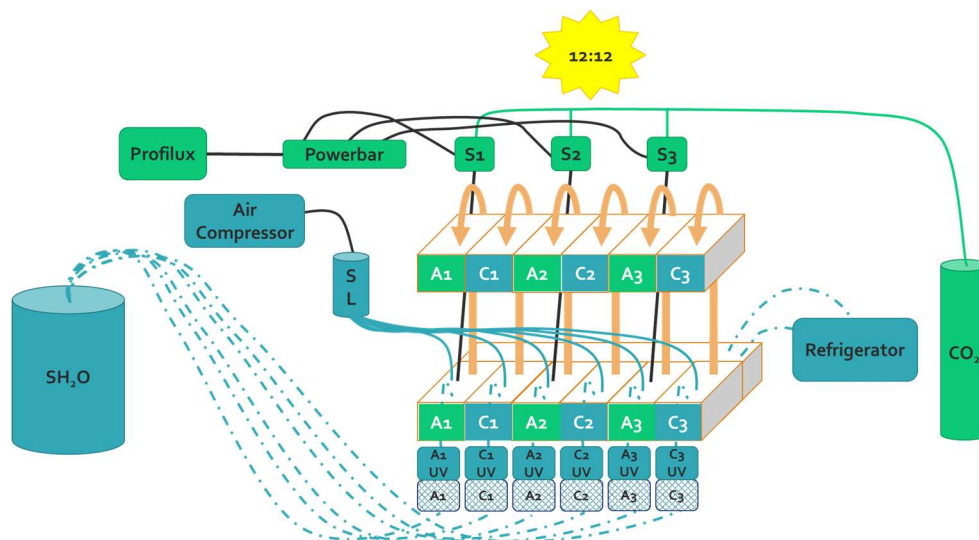
Figure 3.3.: Global experimental design – A1, A2 and A3 represent the replicates from the acidification treatment and C1, C2 and C3 the replicates from the control; the upper structures are the tanks where the individuals were kept and the lower structures are the corresponding sumps. SH_2O – seawater pumped from sea; SL: soda lime; S: solenoid valve; CO_2: bottle of certified CO_2 gas mixture; UV: filtration with ultraviolet radiation; ⊠: filtration with a 0.35 µm net; ▬ : tube with CO_2-filtered air; ▬: tubes with CO_2 gas mixture (only exists in acidification systems); ▬ ▬ ▪ : tubes with seawater.
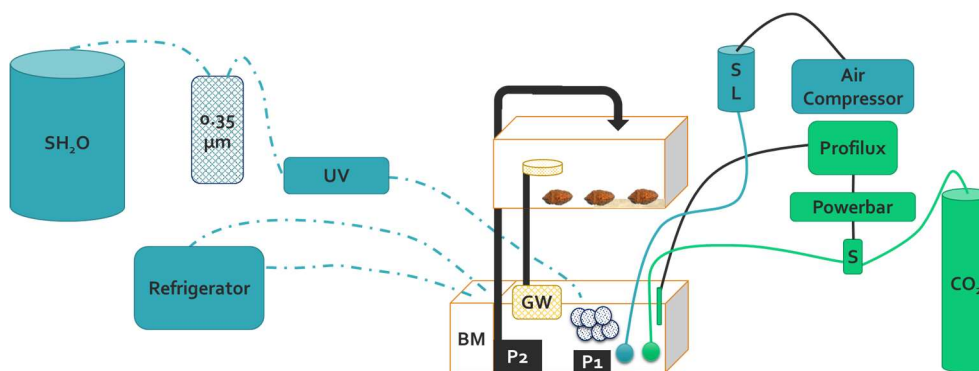
Figure 3.4.: Details from one of the acidification replicates'. Each replicate had an individual seawater supply with filtration of 0.35 µm net and UV radiation and an individual sump with glass wool (GW), bioballs (⊙), pH probe (▮) and one air stone to CO_2 gas mixture (●) – only exist in acidification systems – one air stone to CO_2-filtered air (●), a pump to ensure mixing/circulation of water (P1) and another to elevate the filtered water again to the system (P2). Besides that, each tank had a small sand shelter to enhance welfare and minimise stress, according to the Guidelines for the Care and Welfare of Cephalopods in Research (Fiorito et al., 2015), and a water outlet sealed with small mesh net to ensure that the individuals and the food did not leave the tank. BM – bain-marie system.

The eggs were randomly divided into 6 small boxes (16 cm L × 9.5 cm W), each one floating in each independent life support system described above (represented in Figure 3.3. and Figure 3.4.). Into

this small box, they were suspended in a grid 5 cm below the water surface, to ensure good aeration and an automatic separation between them and hatchlings (when they hatch, they could pass through the grid to the bottom of the box) (see Figure 3.5.). After hatching, the newborns were gently transferred to the outside tank and remained there under the different experimental pH conditions until the behavioural tests were performed. The cuttlefish newborns were fed *ad libitum* with frozen brine shrimp enriched with spirulina and the remained food was removed at the end of each day.

Figure 3.5.: Eggs' box floating into an independent life support system.

Along the 70 days of exposure (50 days during the embryonic development + 20 days after hatching), the daily routines included pH, salinity and temperature manual measurements, and hatching/mortalities counting. Seawater carbonate system speciation was calculated from total alkalinity (TA) (Sarazin et al., 1999) and pH (total scale) measurements using CO2SYS software (Lewis et al., 1998), with dissociation constants from Mehrbach et al. (1973) as refitted by Dickson and Millero (1987) (see Table 3.1.). The absorbance to calculate TA was measured in an UV spectrophotometer (UV-1800 spectrophotometer, Shimadzu, North America). pH was quantified manually with Metrohm pH meter (826 pH mobile, Metrohm, Filderstadt, Germany) connected to a glass electrode (± 0.001; Schott IoLine, SI analytics, Mainz, Germany) and calibrated against the seawater buffers Tris-HCl (Tris) and 2-aminopyridine-HCl (AMP) (Mare, Liège, Belgium) according to Dickson et al. (2007). Salinity was measured using a refractometer (V^2Refractometer, TMC, Iberia, Portugal) and maintained (~ 35) by adding 1 μm charcoal-filtered freshwater. Ammonia, nitrites and nitrates levels were monitored using colorimetric tests and maintained below detectable levels, lower than 0.5 mg/L, 0.2 mg/L and 80 mg/L (Fiorito et al., 2015), respectively.

Table 3.1.: Seawater physiochemical parameters in the support life systems. Values represent mean ± s.d..

Parameters	Control	Acidification
Temperature (°C)	17.8 ± 0.9	17.9 ± 0.8
Salinity	36 ± 1	36 ± 1
pH	8.05 ± 0.06	7.73 ± 0.07
TA (μmol/KgSW)	2584.7 ± 267.2	2522.0 ± 248.5
pCO_2 (μatm)	461.6 ± 85.1	1016.3 ± 180.7
TCO_2 (mmol/kgSW)	2316.7 ± 214.2	2406.4 ± 230.7
HCO_3^- (mmol/kgSW)	2101.1 ± 176.8	2266.9 ± 213.6

3.3. Hatchlings measurements

The dorsal mantle length (DML), total body length (TBL) and total body weight (TBW) were measured in all individuals, 20 days after hatching (DAH). Both mantle and total length were measured from photos taken to the newborns and the TBW was recorded with an analytical balance. The Fulton's Condition Index (K) was calculated according Fiorito et al. (2015) ($K = (TBW/DML^3) \times 100$), with TBW in gramme and DML in centimeter. These measurements were performed with two different groups, one had more five days of acclimation during the embryonic development than the other (represented as hatching date in the statistical analysis). Each day, the number of hatches and deaths were noted to further calculate the development time, the hatching success and the survival rate. At the end of the experiment, individuals were euthanised according with the Guidelines for the Care and Welfare of Cephalopods in Research (Fiorito et al., 2015) and saved for future biochemical and neurobiological research.

3.4. Behavioural analyses

3.4.1. Sampling

All behavioural tests were performed with 2 to 6 individuals per replicate, in a total of 12-16 individuals per treatment with about 15-20 DAH. Each individual was tested for two of the behavioural tests but performed in different days. The behavioural tests performed were: 1) shelter-seeking, 2) hunting behaviour and 3) visual detection of a conspecific stimulus. All the variables analysed can be seen in the Table 3.2.. Individual cuttlefish were carefully transferred from its holding tanks to the arena of each test and all arenas were designed according with the specific needs of each test. All the performed tests, described below, ran after a 10 minutes of acclimatization period (Darmaillacq et al., 2004; O'Brien et al., 2016; O'Brien et al., 2017a). In the end of each test, the individuals returned to the acclimation systems and the testing arenas were cleaned up.

Table 3.2.: Behavioural tests and the respective variables analysed.

Behavioural Tests	Variables analysed				
Shelter-seeking	No choice/ Choice	Black/White			
Hunting behaviour	Time to react to the 1st prey	Time to catch the 1st prey	Successful attacks	Unsuccessful attacks	Total attacks
Visual detection of conspecific stimulus	Reaction/ No reaction	Kind of reaction			

3.4.2. Shelter-seeking

A black and white arena (28.8 cm L (14.4 cm Black + 14.4 cm White) × 5 cm W; volume = 4000 mL; see Figure 3.6.) was built to test what kind of shelter (dark or light) individuals prefer. The arena was adapted from the scototaxis test (Maximino et al., 2010) and the neutral area was adjusted according Jutfelt et al. (2017). This behavioural test was performed at the same time with four individuals (visually separated from each other and from the observer) from the same treatment. Each cuttlefish was individually, gently and randomly (to prevent bias) placed on the arena, introduced in the middle of the upper zone, and the activity was filmed (Canon LEGRIA HF R56) for 10 minutes in a total of 20 minutes test. A fluorescent lamp was placed 48 cm above the arena and the camera below, to film all the behaviours performed. In total, 30 individuals were used to perform this test and the choice, black or white, was only considered when the individual left the neutral area and the permanence time in the choice was 70% of the total test time or higher (630 seconds or more). The no choice criteria was considered when the individuals did not make a choice (did not leave the neutral area – see Figure 3.6.) and did not remain in the choice for 630 seconds or more.
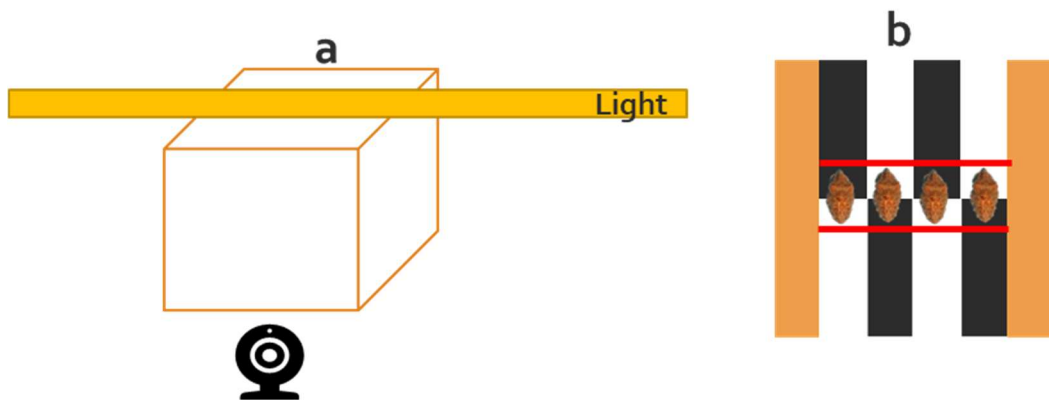
Figure 3.6.: a) Experimental design of shelter-seeking test with a camera (📷) and a light; b) The arena's view from below with Black and White sides, neutral area (between red lines) and four individuals.

3.4.3. Hunting behaviour

This test took place in an opaque arena (10 cm L × 8 cm W; volume = 500 mL, see Figure 3.7.) with a small sand shelter to minimise stress. A single cuttlefish and five prey were gently placed in each arena and each recording ran with two individual arenas (one individual per each arena) visually separated from each other and from the viewer. A camera and a lamp were placed in the holes of an opaque box, 30 cm above the arenas, to ensure enough and equally distributed lighting for both arenas. After acclimatization period and after prey introduction, cuttlefish activity was recorded (Canon LEGRIA HF R56) for 10 min, in a total test time of 20 minutes. Each cuttlefish had 5 prey (*Gammarus* sp.) available to hunt during the test - a pilot test revealed that 5 was the maximum number of prey that one individual could eat within the test period. In total, 28 cuttlefish and 140 prey were used within this test and, at the end of each test, the prey number was counted. The attack time was accounted between the reaction to the prey and its catch (successful attack).
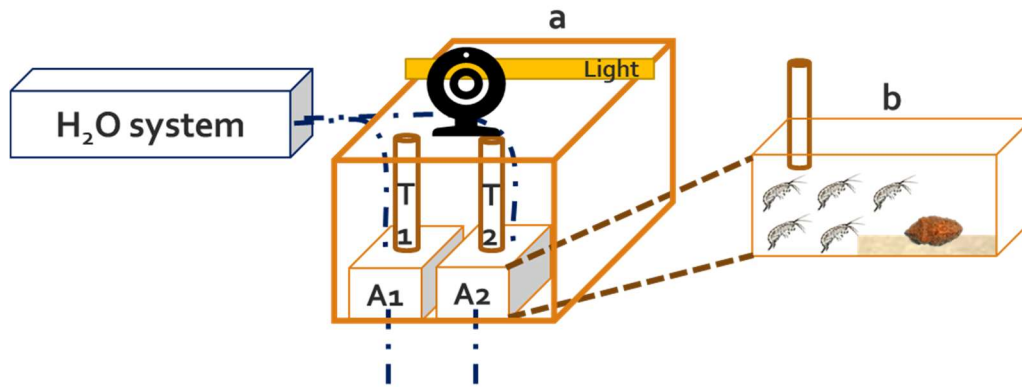
Figure 3.7.: a) Experimental design of hunting test – the outsider box is the opaque one and A1/A2 are the testing arenas. Each arena had one tube (T1/T2) to prey introduction and one to water system supply (▪ ━) – in the upper side – and another with a net to the water level maintenance – on the lateral side. 30 cm above both arenas, a camera (◉) and a fluorescent lamp were placed to record the tests and to illuminate the arenas' floor, respectively; b) An example of a testing arena with five prey and one cuttlefish.

3.4.4. *Visual detection of conspecific visual stimulus – ink*

A visual stimulus (commercial cuttlefish ink) was introduced in a small glass recipient placed in the center of a round and glass arena (area = 100 cm^2, volume = 500 mL; see Figure 3.8.) in order to allow visual stimulus and block the chemical stimulus. The arena was separated from the viewer and from the camera by an opaque material. Cuttlefish activity was filmed (Canon LEGRIA HF R56) during 5 minutes after the acclimatization period and after the introduction of the "white" stimulus, in a total of 20 minutes test. To be sure that the reaction of the individual was to the presence of the ink and not to other factors, a "white" stimulus (water) was introduced 5 minutes before the introduction of the stimulus in test, therefore, each cuttlefish had one "white" and one "ink" stimulus. To perform this test 28 individuals were used and the type of reaction was divided into 4 classes (0 = no reaction; 1 = ventilation or branchial movements; 2 = change of colour or stop of swimming; 3 = escape, attack or upper arms raised) adapted from Wood et al. (2008b).
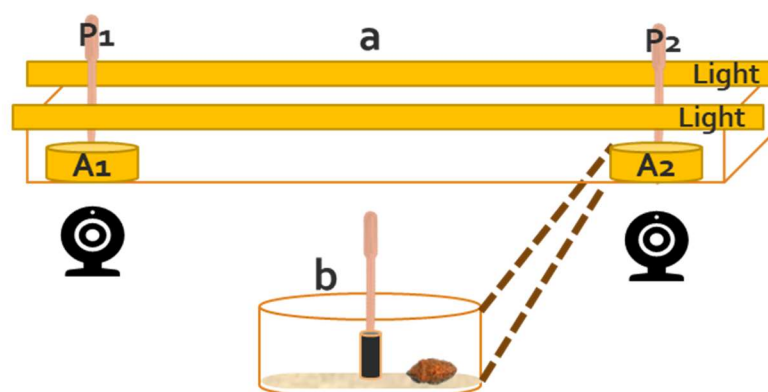
Figure 3.8.: a) Experimental design of visual test – A1/A2 are testing arenas. Each arena had one pipette (P1/P2) to white/ink introduction. The fluorescent lamps were placed 30 cm above both arenas and the cameras (◉) were set up below by each arena; b) An example of a testing arena with the small glass recipient and one hatchling.

4. Data analysis

From photograph and video records, all data were obtained with specific programs, i.e. ImageJ was used to obtain photography data (length measures) and BORIS software (Behavioural Observation Research Interactive Software v.6.0.5 – 2018-01-29) was used to analyses all video data. In the BORIS software were defined specific commands considering the specifics of each test. For the shelter-seeking test, four commands were defined to record the time spent in each area: a) start test, b) entry into the white area, c) entry into the black area and d) entry into the neutral area. In the hunting behaviour test, four commands were defined to acquire the timings and attack effectiveness of the individuals: a) prey introduction, b) reaction to the prey, c) attack and d) catch. For the visual detection test were defined six commands: reaction and no reaction, and one for each type of reaction (according to the aforementioned definitions).

After data visualisation, statistical analyses of the defined variables were performed with RStudio Software (Version 1.1.456 – © 2009-2018 RStudio, Inc.). All Generalized Linear Models (GLM) were performed with pH as factor. For all the variables analysed, replicates and hatching date were first included in generalized linear mixed models (GLMM) as random effect, to account for potential variability in the experimental design. However, random effects were not statistically significative. The best model for each output was chosen according to the calculation of Akaike Information Criterion (AIC), i.e. the best model was the one which featured the smallest AIC.

The Gaussian family of distribution was used to analyse weight (also with hatching date as factor and inverse link function); mantle length (also with replicate as factor) and total length. With the Gamma family and inverse link function were analysed reaction and catch time in the hunting behaviour test, and the Fulton's Index (also with hatching date as factor). The Binomial family of distribution was used to analyse the shelter-seeking test (choice/no choice and black/white) and the visual detection test for reaction variable. Count data were analysed with the Poisson family, i.e. hatching success and hatchlings survival, and the total of attacks observed in the hunting behaviour test. When a Poisson GLM was over dispersed (i.e. higher than 1.2), the family of distribution was changed to Negative Binomial and the model definitions were adjusted accordingly, as was the case for unsuccessful attacks from hunting behaviour test. All GLM models with Gamma family were adjusted according Sultan et al. (2008) and the Chi-square test was used in GLM models to obtain the p-value of factors with 3 or more levels (factor replicate in DML and DED models). To analyse the type of reaction in the visual detection test, a multinomial logistic regression model was performed with the 4 classes in test (already mentioned in chapter 3.4.4.). All statistical differences were considered when p-value < α, α = 0.05.

5. Results

5.1. Development Time, Survival and Body Measurements

Results for the development time, hatching success and survival rate analysis are presented in Figure 5.1. – a, b and c, respectively. Development time was similar in both treatments ($\sim 69 \pm 9$ days), i.e. there were no significant effects of ocean acidification ($p > 0.05$; GLM, Poisson family, more details in Supplemental Table 9.6.). Likewise, neither hatching success (73.33 ± 1.80 % under normocapnia and 70.20 ± 1.36 % under hypercapnia) nor survival rate (after 20 DAH; 66.86 ± 1.10 % and 69.30 ± 1.99 %, under normocapnia and hypercapnia, respectively) were significantly affected by high CO_2 ($p > 0.05$; GLM, Poisson family, see also Supplemental Table 9.5.).
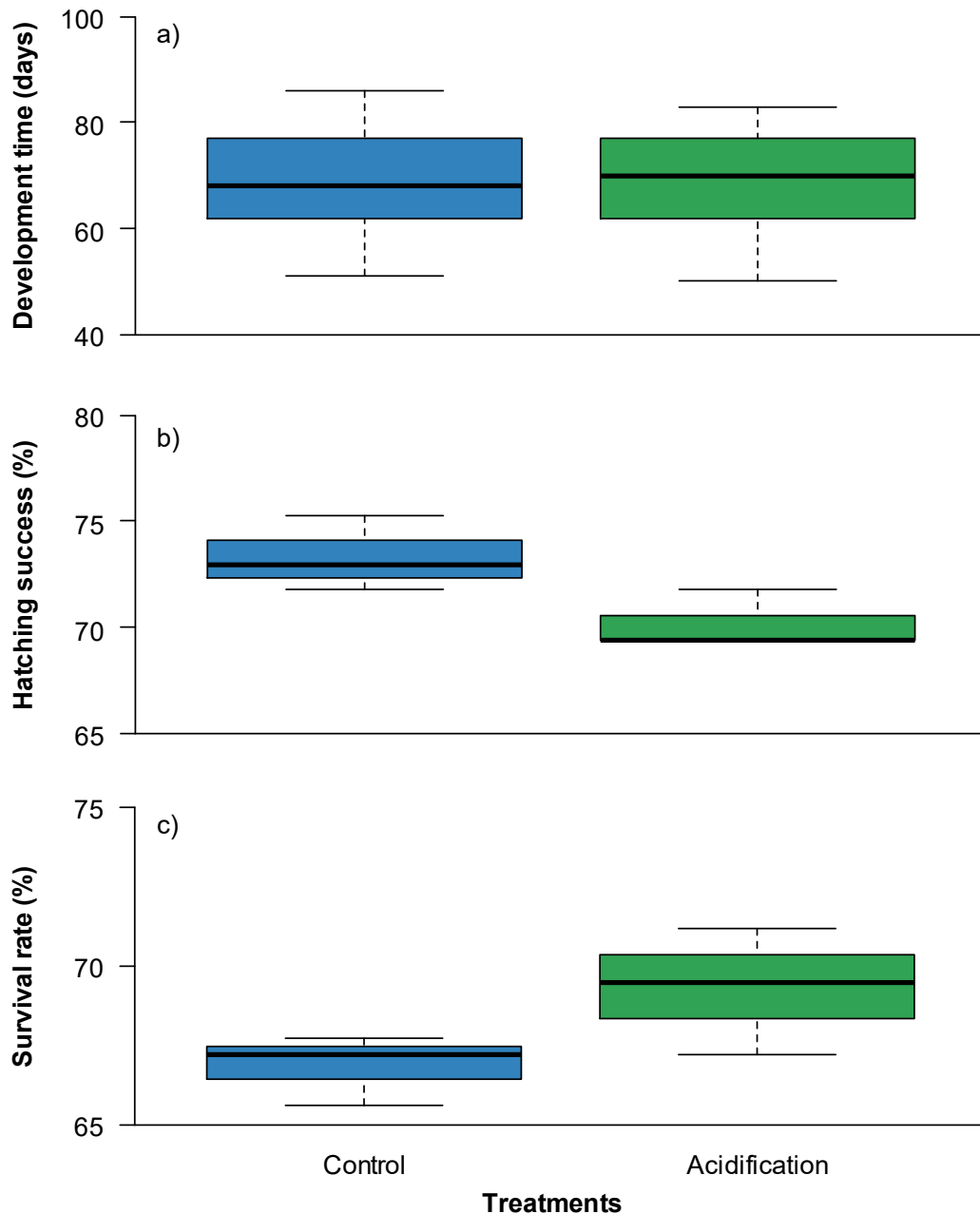
Figure 5.1.: Effect of ocean acidification (ΔpH = 0.4) on: a) development time, b) hatching success and c) survival rate (20 days after hatching) of the common cuttlefish *Sepia officinalis*. Boxplots illustrate median, upper and lower quartile, and inter-quartile range. Circles indicate individual outliers outside the inter-quartile range.

OA effects on the dorsal mantle length (DML), total body length (TBL), total body weight (TBW) and Fulton's condition index (K) are shown in Figure 5.1. – a, b, c and d, respectively. In opposition to DML ($p > 0.05$; GLM, Gaussian family, analysis in Supplemental Table 9.6.), TBL ($p > 0.05$; GLM, Gaussian family, analysis in Supplemental Table 9.5.) and TBW ($p > 0.05$; GLM, Gaussian family, analysis in Supplemental Table 9.5.), K values were significantly affected by ocean acidification ($p < 0.05$; GLM, Gamma family, analysis in Supplemental Table 9.5.).
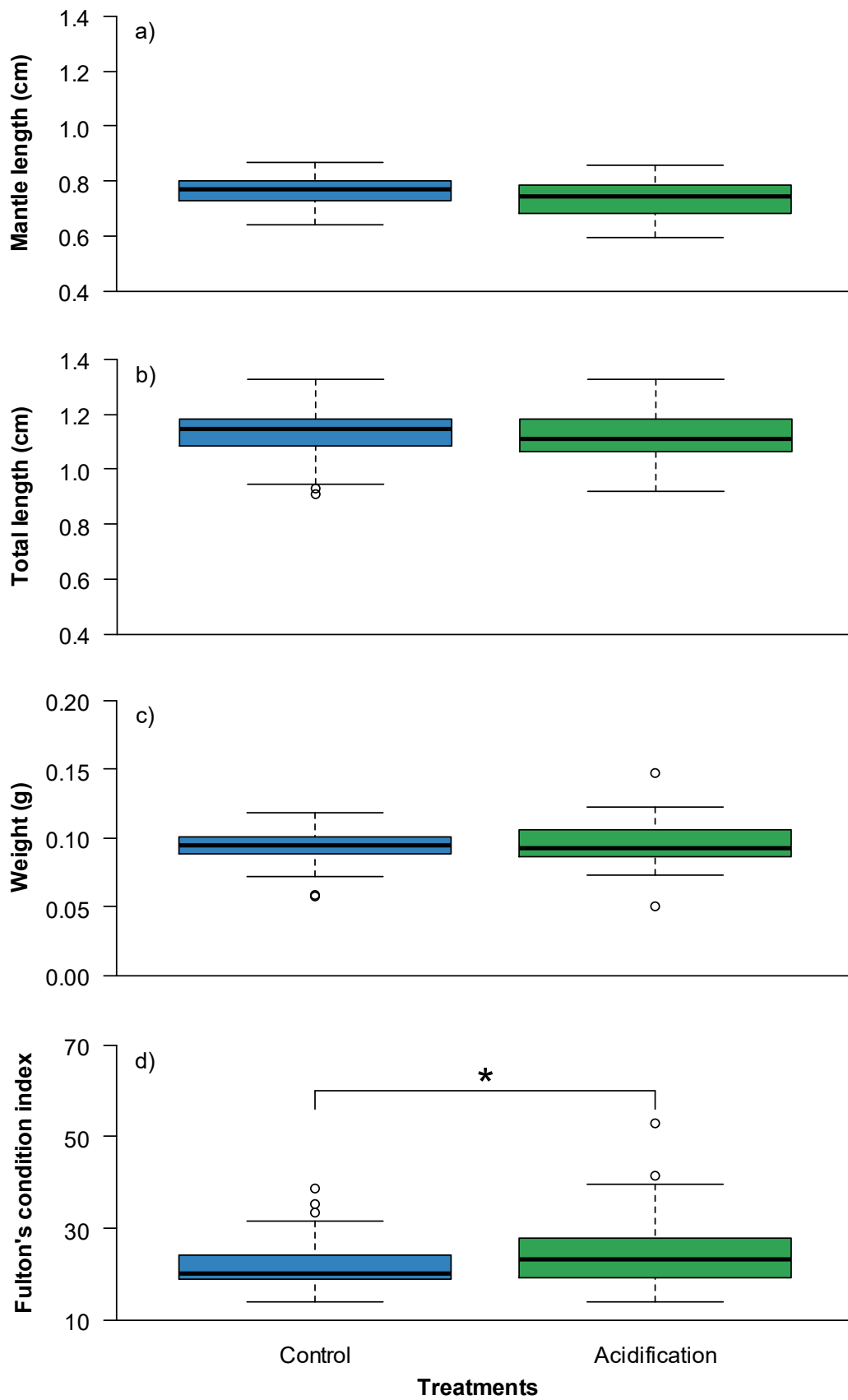
Figure 5.2.: Effect of ocean acidification (ΔpH = 0.4) on: a) dorsal mantle length (DML), b) total body length (TBL), c) total body weight (TBW) and d) Fulton's condition index (K) of the common cuttlefish *Sepia officinalis*. Boxplots illustrate median, upper and lower quartile, and inter-quartile range. Circles indicate individual outliers outside the inter-quartile range and asterisk represents significative differences between treatments ($p < 0.05$).

5.2. Behavioural Tests

5.2.1. *Shelter-seeking*

No significant differences were found between treatments in the choice rate of shelter neither in shelter preference (Figure 5.3. – a and b, respectively; $p > 0.05$; GLM, Binomial family, analysis in Supplemental Table 9.5.).
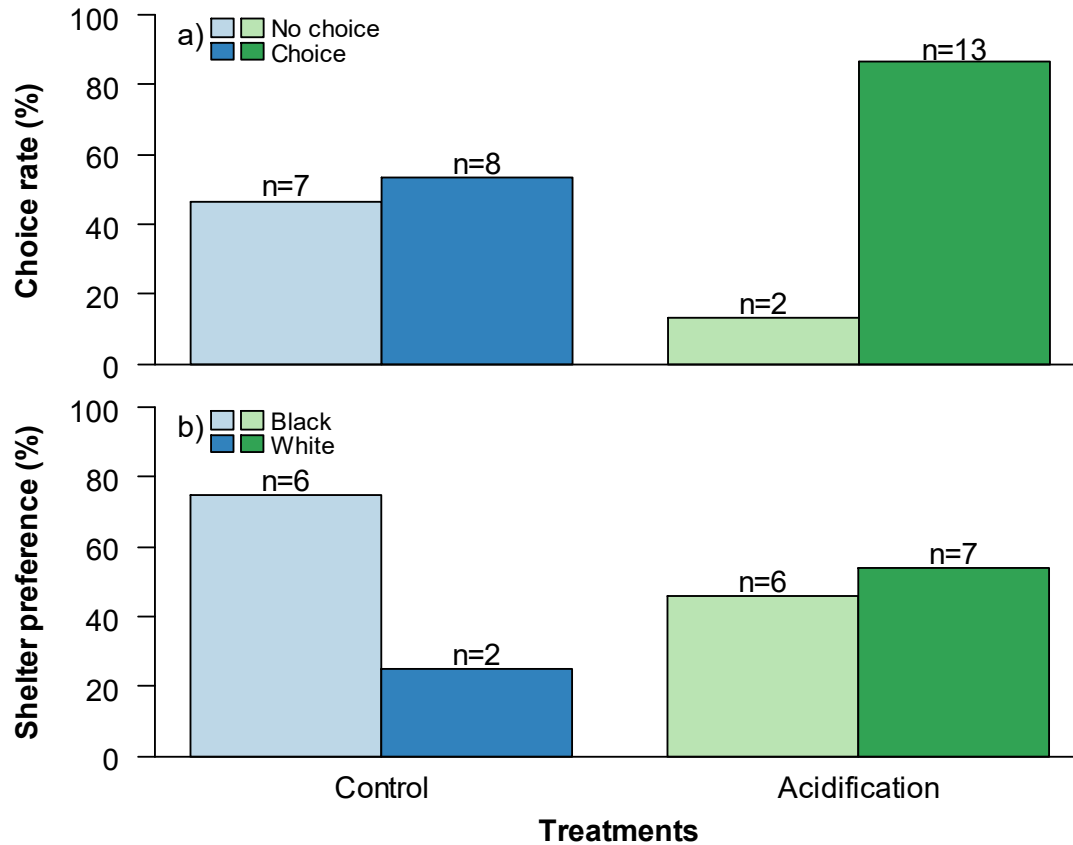
Figure 5.3.: Effect of ocean acidification ($\Delta pH = 0.4$) on the shelter-seeking behaviour of the common cuttlefish *Sepia officinalis* with 15-20 DAH: a) capacity to make a choice and b) shelter preference. Values represent the number of individuals in each treatment who made the respective choice.

5.2.2. Hunting Behaviour

No significant differences were seen in the hunting behaviour (namely in the reaction to prey and in the attack duration) between control and OA treatments (Figure 5.4. – a and b, respectively; $p > 0.05$; GLM, Gamma family, analysis in Supplemental Table 9.5.).
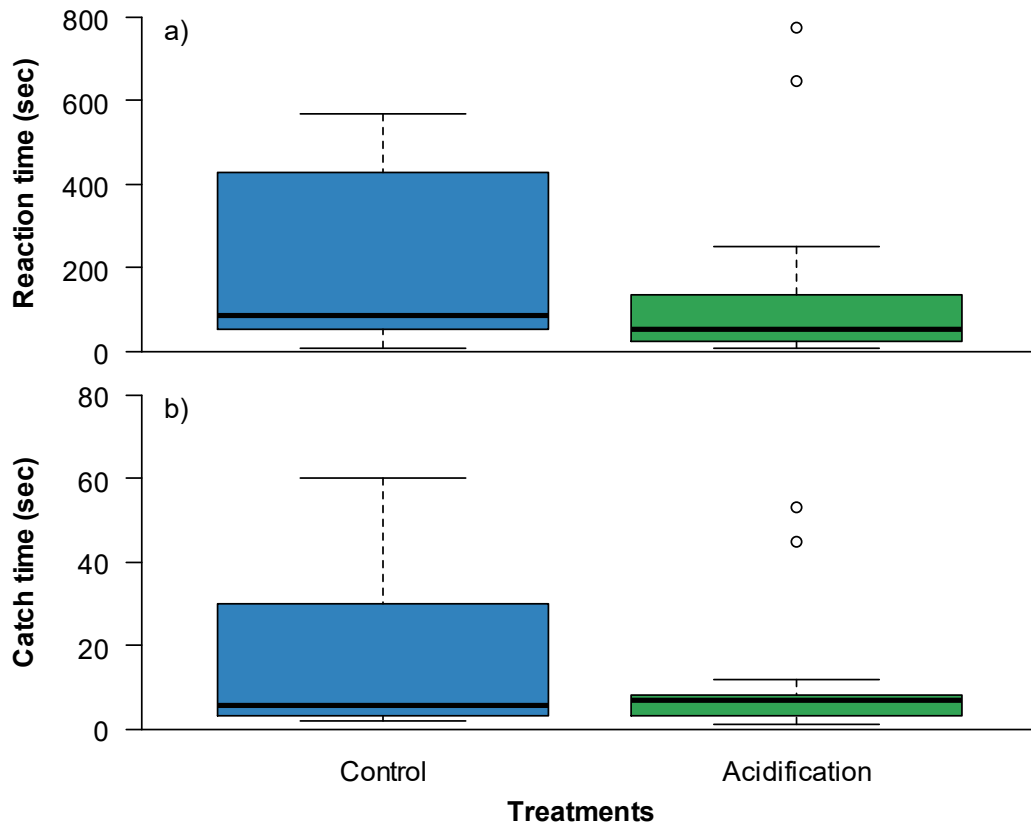
Figure 5.4.: Effect of ocean acidification ($\Delta pH = 0.4$) on: a) time of reaction to the prey and b) time to catch the prey of the common cuttlefish *Sepia officinalis* with 15-20 DAH. Boxplots illustrate median, upper and lower quartile, and inter-quartile range. Circles indicate individual outliers outside the inter-quartile range.

Moreover, similar findings were observed for the predatory success rate (Figure 5.5. – a; $p > 0.05$; GLM, Poisson family for the successful attacks and GLM.NB analysis for the unsuccessful attacks; more details in Supplemental Table 9.5.) and the total amount of captured prey (Figure 5.5. – b; $p > 0.05$; see successful attacks analysis in Supplemental Table 9.5.).
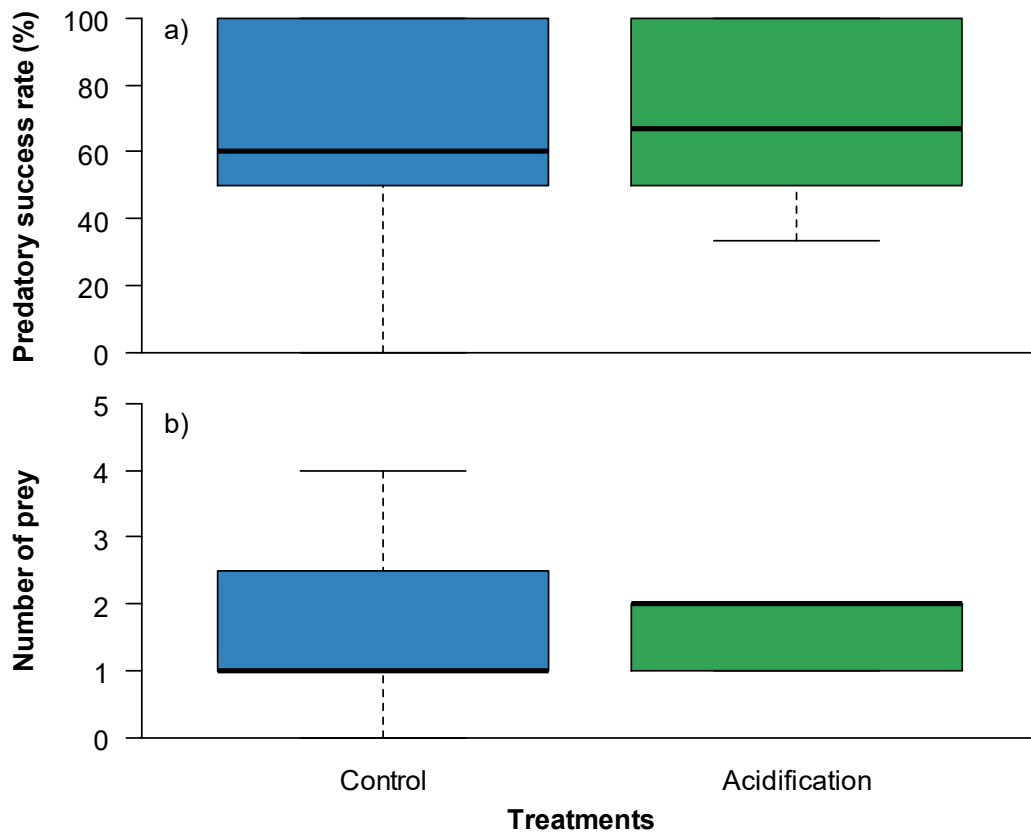
Figure 5.5.: Effect of ocean acidification ($\Delta pH = 0.4$) on: a) predatory success and b) the number of hunted prey of the common cuttlefish *Sepia officinalis* with 15-20 DAH. Boxplots illustrate median, upper and lower quartile, and inter-quartile range. Circles indicate individual outliers outside the inter-quartile range.

5.2.3. *Visual Detection of Conspecific Visual Stimulus – Ink*

Similar to the previous results, this type of behaviour was also not significantly affected by the experimental CO_2 treatments (Figure 5.6.; $p > 0.05$; GLM, Binomial family for the reaction/no reaction and multinomial logistic for the type of reaction, both analyses can be found in Supplemental Table 9.5.). A large proportion of individuals had no reaction (lighter colours) to the stimulus, 68.75% under normocapnia and 58.33% under hypercapnia. Regarding the individuals that had a reaction type 1 (increase of ventilation / increase of gills movement; less light colours), 12.50% had this reaction under normocapnia and 16.67% under hypercapnia. Near 18.75% and 16.67% had a reaction type 2 (change of colour / stop of swimming; less dark colours) under normocapnia and hypercapnia, respectively. Moreover, 8.33% of the individuals exposed to hypercapnia presented the more severe type of reactions – type 3 (escape from the stimulus / face the stimulus / upper arms raised; darker colours), contrary to normocapnia treatment where no individuals showed this type.
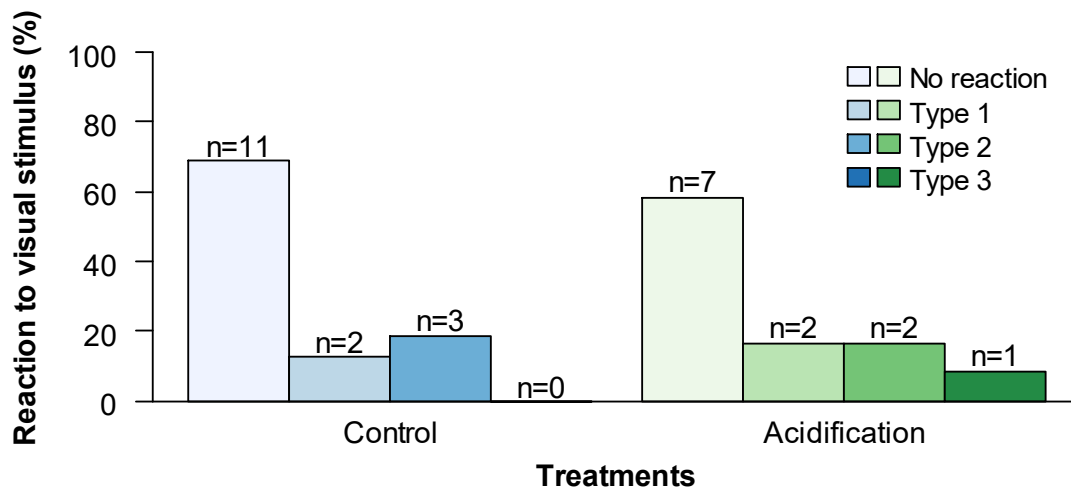
Figure 5.6.: Effect of ocean acidification ($\Delta pH = 0.4$) on the reaction to a conspecific visual stimulus of the common cuttlefish *Sepia officinalis* with 15-20 DAH. Values represent the number of individuals in each treatment who made the respective choice.

6. Discussion

The present study showed that future CO_2 levels may not elicit significant biological (and behavioural) responses during the early ontogeny (embryos and newborns) of the common cuttlefish *Sepia officinalis*. Such findings are probably related to the fact that this coastal species is naturally exposed to environmental hypercapnia during the course of its embryonic development and the fact that the latter stages display a nekton-benthic (and active) lifestyle, that may confer some pre-adaptation to near-future ocean acidification (Hu, 2016; Melzner et al., 2009). Gutowska et al. (2008) also supported this prediction, showing that *S. officinalis* did not exhibit sensitivity to elevated CO_2 levels within the range of concentrations that elicits a negative response in most other invertebrates (e.g. corals and bivalves).

Moreover, future ocean acidification conditions seem to promote hypercalcification in cuttlefish cuttlebone (Gutowska et al., 2010), which goes against the popular predictions of a decrease in the calcification rate as a result of changes in seawater carbonate chemistry (Findlay et al., 2011). The cuttlebone is an essential structure to achieve near-neutral buoyancy (Boyle, 1987) but the consequences of heavier cuttlebones in the daily life of cuttlefish is still poorly understood. The process to achieve a near-neutral buoyancy consumes a lot of energy (Guerra, 2006) and, as an active predator (Messenger, 1968; Wells, 1958) and in need of movement (e.g. for feeding and to reproduce), a heavier cuttlebone may increase energy expenditure and the necessary effort to reach a good buoyancy. According to Findlay et al. (2011) such hypercalcification may impose physiological trade-offs including increased metabolism, reduced health and changes in behavioural responses in association with this calcification upregulation, which may ultimately reduce organismal fitness.

6.1. Development Time, Survival and Body Measurements

Near-future CO_2 did not elicit major impacts on the development time, survival rates and size of *Sepia officinalis* early stages. Accordingly, there are also some studies that did not find effects of high CO_2 on the embryonic development rate (Dorey et al., 2013; Rosa et al., 2013) neither in survival (Dorey et al., 2013) or on the body size (Dorey et al., 2013; Hu et al., 2011; Lacoue-Labarthe et al., 2009; Rosa

24

et al., 2013; Sigwart et al., 2015). The abiotic conditions inside cuttlefish eggs have also been characterised as stressful conditions - with high levels of pCO_2 and HCO_3^-, and low pH (Melzner et al., 2009; Rosa et al., 2013). Giving these embryonic conditions, cuttlefish embryos may be consequently more adapted to develop in the future ocean pH conditions. Nonetheless, we found a significative difference in Fulton's index with exposure to high pCO_2 (control = 21.71 and acidification = 24.35). These findings suggest that cuttlefish were thicker when exposed to acidification than individuals from control. As mentioned above, *S. officinalis* may hypercalcify its cuttlebone under high CO_2 conditions (Dorey et al., 2013; Sigwart et al., 2015), despite its metabolic costs. This hypercalcification may mask a lower condition of these individuals and the animal's weight changes, as previously suggested by Gutowska et al. (2010), as more of the weight is allocated to a denser cuttlebone.

6.2. Behaviour

The common cuttlefish is a species with higher activity during the night (Guerra, 2006), staying usually hidden in the sand during daytime to avoid predation (Boyle, 1987; Reid et al., 2005). Thus, it would be expected a high percentage in the black side of the shelter, given that it would to be a safer place for them (and to be hidden). However, a higher percentage of animals did not make a choice under control conditions (46.67%), which may possibly be explained by the fact that these organisms use camouflage as their primary line of defense (Boyle, 1987) and do not need to choose an option regarding the type of shelter. Additionally, the option to use a non-bright white may have made these animals "comfortable" on the neutral area and in the white side, without necessity to move to a safer option.

The present findings about hunting behaviour support those obtained by Maneja et al. (2011), who also showed that cuttlefish early stages does not seem to be affected by near-future ocean acidification. These animals are active predators (Messenger, 1968; Wells, 1958), which was demonstrated by the short time between the first reaction to prey and the time to effectively catch it (control ~ 17 sec and acidification ~ 12 sec).

Here, we also observed that a high percentage of individuals did not react to the ink stimulus, either in control and acidification treatments. These results contrast to those observed with adult cuttlefish, that showed a high response to chemical ink stimulus (Boal and Golden, 1999) and to those obtained by Wood et al. (2008b) with the Caribbean reef squid when exposed to both chemical and visual stimulus. However, as found by Bedore et al. (2015), cuttlefish is able to reduce its bioelectric cues by a behavioural freeze response. Therefore, such high percentage of non-reacting animals may be hiding a freezing response, which indicates that more accurate studies are needed, specially focusing on this response.

7. Conclusion and Future Perspectives

Climate change encompasses a set of different factors created predominantly by burning fossil fuels, increasing the greenhouse gases concentration in the atmosphere (Cubasch et al., 2013). This problem may constitute a current and future threat to the conservation of many organisms due to its impact on ecological systems, which is possible to see through its effects on ice mass loss, extreme weather events (IPCC, 2014) and corals' health (Camp et al., 2017; Erez et al., 2011; Marubini et al., 2008). Unfortunately, this threat does not act alone, and it is intensified by the interaction with other stressors (McCarty, 2001), such as ocean exploitation, e.g. fishing and pollution. The carbon dioxide is the GHG with the major increase (Ciais et al., 2013; Rhein et al., 2013) and with this abrupt rise, and its

consequent uptake by the oceans, came the need to understand how it would affect marine wildlife. Subsequently, ocean acidification is one of the major subjects studied in the last decade (Browman, 2016; Williamson et al., 2017), but does not exist a standard for pCO$_2$ values, which makes it highly variant and difficult to compare between studies.

To fight the bad predictions about climate change is necessary an urge reduction of CO$_2$ emissions, mainly by decreasing the fossil fuels use, to return to a more stable situation. Nonetheless, that reduction may not be enough to avert serious stress and damage on Earth's life. Thus, some additional steps are needed to boost ecosystems' resilience, safeguard their wildlife and protect their capacity to supply vital goods and services. The creation of marine protected areas networks and more marine reserves (Roberts et al., 2017), as well the improvement and creation of new fishing management measures may be part of the solution. Furthermore, some global conservation efforts, applied mainly in land, can be extended to the marine area, as the CCS (climate change-integrated conservation strategies) that provide a framework in which biogeographers, ecologists and conservation managers can collaborate (Hannah et al., 2002).

Regarding the cuttlefish (*Sepia officinalis*), the present results indicate that its embryos and newborns may be quite resilient to near-future ocean acidification, since no significant differences were found in the early development neither in the behaviour. Nonetheless, Fulton's index was the only response variable that showed significant differences between treatments, which highlights the need to better understand the possible consequences of potential cuttlebone hypercalcification. Moreover, further research must be conducted, specially at the neurological level, to corroborate the lack of behavioural responses. The non-inclusion of any additional climate change-related stressors in this dissertation, to potentially explore cumulative effects, may be seen as one of its limitations. In fact, the environments that *S. officinalis* occupies, principally the shallow waters, are also particularly susceptible to anthropogenic pressures and to climate-change stressors. This makes the study of cumulative effects (i.e. of multiple stressors) of paramount importance to accurately predict what the future reserves to this ecologically and economically important species.

8. References[1]

Augusto, J. F., Rachinas-Lopes, P. and dos Santos, M. E. (2012). Social structure of the declining resident community of common bottlenose dolphins in the Sado Estuary, Portugal. *Journal of the Marine Biological Association of the United Kingdom* **92**, 1773-1782.

Baumann, H., Talmage, S. C. and Gobler, C. J. (2012). Reduced early life growth and survival in a fish in direct response to increased carbon dioxide. *Nature Climate Change* **2**, 38.

Bedore, C. N., Kajiura, S. M. and Johnsen, S. (2015). Freezing behaviour facilitates bioelectric crypsis in cuttlefish faced with predation risk. *Proceedings of the Royal Society B* **282**, 20151886.

Boal, J. and Golden, D. (1999). Distance chemoreception in the common cuttlefish, *Sepia officinalis* (Mollusca, Cephalopoda). *Journal of Experimental Marine Biology and Ecology* **235**, 307-317.

Boal, J. G., Hylton, R. A., Gonzalez, S. A. and Hanlon, R. T. (1999). Effects of crowding on the social behavior of cuttlefish (*Sepia officinalis*). *Journal of the American Association for Laboratory Animal Science* **38**, 49-55.

---

[1] The references follow the rules of JEB (Journal of Experimental Biology)

Boletzky, S. v. (2003). Biology of early life stages in cephalopod molluscs. *Advances in Marine Biology* **44**, 144-204.

Boletzky, S. v., Erlwein, B. and Hofmann, D. (2006). The Sepia egg: a showcase of cephalopod embryology. *Vie et Milieu* **56**, 191-201.

Boyle, P. and Rodhouse, P. (2008). Cephalopods: ecology and fisheries. John Wiley & Sons.

Boyle, P. R. (1987). Cephalopod life cycles. Volume II: Comparative reviews, (ed. P. R. Boyle). Academic Press: Harcourt Brace Jovanovich.

Browman, H. I. (2016). Applying organized scepticism to ocean acidification research. Oxford University Press.

Budelmann, B. (1995). Cephalopod sense organs, nerves and the brain: adaptations for high performance and life style. *Marine and Freshwater Behaviour and Physiology* **25**, 13-33.

Bush, S. L. and Robison, B. H. (2007). Ink utilization by mesopelagic squid. *Marine Biology* **152**, 485-494.

Caldeira, K. and Wickett, M. E. (2003). Oceanography: anthropogenic carbon and ocean pH. *Nature* **425**, 365.

Camp, E. F., Nitschke, M. R., Rodolfo-Metalpa, R., Houlbreque, F., Gardner, S. G., Smith, D. J., Zampighi, M. and Suggett, D. J. (2017). Reef-building corals thrive within hot-acidified and deoxygenated waters. *Scientific reports* **7**, 2434.

Castro, B. G. and Lee, P. G. (1994). The effects of semi-purified diets on growth and condition of *Sepia officinalis* L. (Mollusca: Cephalopoda). *Comparative Biochemistry and Physiology Part A: Physiology* **109**, 1007-1016.

Chichery, R. and Chanelet, J. (1976). Motor and behavioural responses obtained by stimulation with chronic electrodes of the optic lobe of *Sepia officinalis*. *Brain research* **105**, 525-532.

Ciais, P., Sabine, C., Bala, G., Bopp, L., Brovkin, V., Canadell, J., Chhabra, A., DeFries, R., Galloway, J., Heimann, M. et al. (2013). Carbon and Other Biogeochemical Cycles. In *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*, eds. T. F. Stocker D. Qin G.-K. Plattner M. Tignor S. K. Allen J. Boschung A. Nauels Y. Xia V. Bex and P. M. Midgley). Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press.

CO2.Earth. (2019). Earth's CO2.

Cubasch, U., Wuebbles, D., Chen, D., Facchini, M. C., Frame, D., Mahowald, N. and Winther, J.-G. (2013). Introduction. In *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*, eds. T. F. Stocker D. Qin G.-K. Plattner M. Tignor S. K. Allen J. Boschung A. Nauels Y. Xia V. Bex and P. M. Midgley). Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press.

Darmaillacq, A. S., Chichery, R., Poirier, R. and Dickel, L. (2004). Effect of early feeding experience on subsequent prey preference by cuttlefish, *Sepia officinalis*. *Developmental Psychobiology* **45**, 239-44.

Dickel, L., Chichery, M.-P. and Chichery, R. (1997). Postembryonic maturation of the vertical lobe complex and early development of predatory behavior in the cuttlefish (*Sepia officinalis*). *Neurobiology of learning and memory* **67**, 150-160.

Dickson, A. and Millero, F. (1987). A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. *Deep Sea Research Part A. Oceanographic Research Papers* **34**, 1733-1743.

Dickson, A. G., Sabine, C. L. and Christian, J. R. (2007). Guide to best practices for ocean CO_2 measurements. North Pacific Marine Science Organization.

Dixson, D. L., Jennings, A. R., Atema, J. and Munday, P. L. (2015). Odor tracking in sharks is reduced under future ocean acidification conditions. *Global Change Biology* **21**, 1454-1462.

Dixson, D. L., Munday, P. L. and Jones, G. P. (2010). Ocean acidification disrupts the innate ability of fish to detect predator olfactory cues. *Ecology letters* **13**, 68-75.

Domingues, P., Sykes, A., Sommerfield, A., Almansa, E., Lorenzo, A. and Andrade, J. P. (2004). Growth and survival of cuttlefish (*Sepia officinalis*) of different ages fed crustaceans and fish. Effects of frozen and live prey. *Aquaculture* **229**, 239-254.

Dorey, N., Melzner, F., Martin, S., Oberhänsli, F., Teyssié, J.-L., Bustamante, P., Gattuso, J.-P. and Lacoue-Labarthe, T. (2013). Ocean acidification and temperature rise: effects on calcification during early development of the cuttlefish *Sepia officinalis*. *Marine Biology* **160**, 2007-2022.

Duval, P., Chichery, M.-P. and Chichery, R. (1984). Prey capture by the cuttlefish (*Sepia officinalis* L): an experimental study of two strategies. *Behavioural processes* **9**, 13-21.

Erez, J., Reynaud, S., Silverman, J., Schneider, K. and Allemand, D. (2011). Coral Calcification Under Ocean Acidification and Global Change. In *Coral Reefs: An Ecosystem in Transition*, eds. Z. Dubinsky and N. Stambler), pp. 151-176. Dordrecht: Springer Netherlands.

Fabry, V. J., Seibel, B. A., Feely, R. A. and Orr, J. C. (2008). Impacts of ocean acidification on marine fauna and ecosystem processes. *ICES Journal of Marine Science* **65**, 414-432.

FAO. (2018). Species Fact Sheets – *Sepia officinalis* (Linnaeus, 1758).

Findlay, H. S., Wood, H. L., Kendall, M. A., Spicer, J. I., Twitchett, R. J. and Widdicombe, S. (2011). Comparing the impact of high CO_2 on calcium carbonate structures in different marine organisms. *Marine Biology Research* **7**, 565-575.

Fiorito, G., Affuso, A., Basil, J., Cole, A., de Girolamo, P., D'Angelo, L., Dickel, L., Gestal, C., Grasso, F., Kuba, M. et al. (2015). Guidelines for the Care and Welfare of Cephalopods in Research – A consensus based on an initiative by CephRes, FELASA and the Boyd Group. *Laboratory Animals* **49**, 1-90.

Gilly, W. F. and Lucero, M. T. (1992). Behavioral responses to chemical stimulation of the olfactory organ in the squid *Loligo opalescens*. *Journal of Experimental Biology* **162**, 209-229.

Gras, M. (2013). Contribution des frayères côtières au recrutement du stock de seiche *Sepia officinalis* de Manche: lien entre le succès de la phase pré-recrutée et l'abondance de la ressource. PhD Thesis. Caen.

Guerra, A. (2006). Ecology of *Sepia officinalis*. *Vie et Milieu* **56**, 97-107.

Gutowska, M. A., Melzner, F., Pörtner, H. O. and Meier, S. (2010). Cuttlebone calcification increases during exposure to elevated seawater pCO$_2$ in the cephalopod *Sepia officinalis*. *Marine Biology* **157**, 1653-1663.

Gutowska, M. A., Pörtner, H. O. and Melzner, F. (2008). Growth and calcification in the cephalopod *Sepia officinalis* under elevated seawater pCO$_2$. *Marine Ecology Progress Series* **373**, 303-309.

Hanlon, R. T. and Messenger, J. B. (1988). Adaptive coloration in young cuttlefish (*Sepia officinalis* L.): the morphology and development of body patterns and their relation to behaviour. *Philosophical Transactions of the Royal Society B* **320**, 437-487.

Hannah, L., Midgley, G. F. and Millar, D. (2002). Climate change-integrated conservation strategies. *Global Ecology and Biogeography* **11**, 485-495.

Hartmann, D. L., Klein Tank, A. M. G., Rusticucci, M., Alexander, L. V., Brönnimann, S., Charabi, Y., Dentener, F. J., Dlugokencky, E. J., Easterling, D. R., Kaplan, A. et al. (2013). Observations: atmosphere and surface. In *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*, eds. T. F. Stocker D. Qin G.-K. Plattner M. Tignor S. K. Allen J. Boschung A. Nauels

Y. Xia V. Bex and P. M. Midgley). Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press.

Hu, M. (2016). Effects of CO_2 driven ocean acidification on ontogenetic stages of the cutllefish *Sepia officinalis*. *Vie et Milieu* **66** (1): 57-63.

Hu, M. Y., Guh, Y.-J., Stumpp, M., Lee, J.-R., Chen, R.-D., Sung, P.-H., Chen, Y.-C., Hwang, P.-P. and Tseng, Y.-C. (2014). Branchial NH_4^+-dependent acid-base transport mechanisms and energy metabolism of squid (*Sepioteuthis lessoniana*) affected by seawater acidification. *Frontiers in zoology* **11**, 55.

Hu, M. Y., Hwang, P.-P. and Tseng, Y.-C. (2015). Recent advances in understanding trans-epithelial acid-base regulation and excretion mechanisms in cephalopods. *Tissue barriers* **3**, e1064196.

Hu, M. Y., Tseng, Y.-C., Stumpp, M., Gutowska, M. A., Kiko, R., Lucassen, M. and Melzner, F. (2011). Elevated seawater pCO_2 differentially affects branchial acid-base transporters over the course of development in the cephalopod *Sepia officinalis*. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **300**, R1100-R1114.

Ibánez, C. M. and Keyl, F. (2010). Cannibalism in cephalopods. *Reviews in Fish Biology and Fisheries* **20**, 123-136.

ICNF. (2007). ELABORAÇÃO DO PLANO DE ORDENAMENTO E GESTÃO PARA A RESERVA NATURAL DO ESTUÁRIO DO SADO. Instituto da Conservação da Natureza.

IPCC. (2014). Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change, eds. Core Writing Team, R.K. Pachauri and L.A. Meyer. IPCC, Geneva, Switzerland, pp. 151.

Jereb, P., Allcock, L. A., Lefkaditou, E., Piatkowski, U., Hastie, L. C. and Pierce, G. J. (2015). Cephalopod biology and fisheries in Europe: II. Species Accounts: ICES Cooperative Research Report **325**, pp. 360.

Jutfelt, F., Sundin, J., Raby, G. D., Krång, A. S. and Clark, T. D. (2017). Two-current choice flumes for testing avoidance and preference in aquatic animals. *Methods in Ecology and Evolution* **8**, 379-390.

Kaplan, M. B., Mooney, T. A., McCorkle, D. C. and Cohen, A. L. (2013). Adverse Effects of Ocean Acidification on Early Development of Squid (*Doryteuthis pealeii*). *PLoS ONE* **8**, e63714.

Knoll, A. H., Bambach, R. K., Payne, J. L., Pruss, S. and Fischer, W. W. (2007). Paleophysiology and end-Permian mass extinction. *Earth and Planetary Science Letters* **256**, 295-313.

Kobayashi, S., Takayama, C. and Ikeda, Y. (2013). Distribution of glutamic acid decarboxylase immunoreactivity within the brain of oval squid *Sepioteuthis lessoniana*. *Aquatic Biology* **19**, 97-109.

Kurihara, H., Kato, S. and Ishimatsu, A. (2007). Effects of increased seawater pCO_2 on early development of the oyster *Crassostrea gigas*. *Aquatic Biology* **1**, 91-98.

Lacoue-Labarthe, T., Martin, S., Oberhänsli, F., Teyssié, J.-L., Markich, S., Jeffree, R. and Bustamante, P. (2009). Effects of increased pCO_2 and temperature on trace element (Ag, Cd and Zn) bioaccumulation in the eggs of the common cuttlefish, *Sepia officinalis*. *Biogeosciences* **6**, 2561-2573.

Lewis, E., Wallace, D. and Allison, L. J. (1998). Program developed for CO_2 system calculations. Brookhaven National Lab., Dept. of Applied Science, Upton, NY (United States); Oak Ridge National Lab., Carbon Dioxide Information Analysis Center, TN (United States).

Maneja, R., Piatkowski, U. and Melzner, F. (2011). Effects of ocean acidification on statolith calcification and prey capture in early life cuttlefish, *Sepia officinalis*. *Journal of Shellfish Research* **30**, 1011.

Marubini, F., Ferrier-Pagès, C., Furla, P. and Allemand, D. (2008). Coral calcification responds to seawater acidification: a working hypothesis towards a physiological mechanism. *Coral Reefs* **27**, 491-499.

Mather, J. and Scheel, D. (2014). Behaviour. In *Cephalopod culture*, pp. 17-39: Springer Science & Business Media.

Mather, J. A. and Kuba, M. J. (2013). The cephalopod specialties: complex nervous system, learning, and cognition. *Canadian journal of zoology* **91**, 431-449.

Mäthger, L. M., Barbosa, A., Miner, S. and Hanlon, R. T. (2006). Color blindness and contrast perception in cuttlefish (*Sepia officinalis*) determined by a visual sensorimotor assay. *Vision research* **46**, 1746-1753.

Mäthger, L. M., Hanlon, R. T., Håkansson, J. and Nilsson, D.-E. (2013). The W-shaped pupil in cuttlefish (*Sepia officinalis*): functions for improving horizontal vision. *Vision research* **83**, 19-24.

Maximino, C., De Brito, T. M., de Mattos Dias, C. A. G., Gouveia Jr, A. and Morato, S. (2010). Scototaxis as anxiety-like behavior in fish. *Nature protocols* **5**, 209.

McNeil, B. I. and Sasse, T. P. (2016). Future ocean hypercapnia driven by anthropogenic amplification of the natural CO_2 cycle. *Nature* **529**, 383.

Mehrbach, C., Culberson, C., Hawley, J. and Pytkowicx, R. (1973). Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. *Limnology and Oceanography* **18**, 897-907.

Melzner, F., Gutowska, M., Langenbuch, M., Dupont, S., Lucassen, M., Thorndyke, M., Bleich, M. and Pörtner, H.-O. (2009). Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? *Biogeosciences* **6**, 2313-2331.

Melzner, F., Thomsen, J., Koeve, W., Oschlies, A., Gutowska, M. A., Bange, H. W., Hansen, H. P. and Körtzinger, A. (2013). Future ocean acidification will be amplified by hypoxia in coastal habitats. *Marine Biology* **160**, 1875-1888.

Messenger, J. B. (1968). The visual attack of the cuttlefish, *Sepia officinalis*. *Animal Behaviour* **16**, 342-357.

Mezrai, N., Arduini, L., Dickel, L., Chiao, C.-C. and Darmaillacq, A.-S. (2018). Awareness of danger inside the egg? Evidence of innate and learned predator recognition in cuttlefish embryo. *bioRxiv*, 508853.

Munday, P. L., Cheal, A. J., Dixson, D. L., Rummer, J. L. and Fabricius, K. E. (2014). Behavioural impairment in reef fishes caused by ocean acidification at CO_2 seeps. *Nature Climate Change* **4**, 487.

Munday, P. L., Dixson, D. L., Donelson, J. M., Jones, G. P., Pratchett, M. S., Devitsina, G. V. and Døving, K. B. (2009). Ocean acidification impairs olfactory discrimination and homing ability of a marine fish. *Proceedings of the National Academy of Sciences* **106**, 1848-1852.

Munday, P. L., Welch, M. J., Allan, B. J., Watson, S.-A., McMahon, S. J. and McCormick, M. I. (2016). Effects of elevated CO_2 on predator avoidance behaviour by reef fishes is not altered by experimental test water. *PeerJ* **4**, e2501.

Muntz, W. (1999). Visual systems, behaviour, and environment in cephalopods. In *Adaptive mechanisms in the ecology of vision*, pp. 467-483: Springer.

Naef, A. (1928). Die Cephalopoden. Embryologie. *Die Fauna Flora Golf Neapel* **35**, 1-357.

Nilsson, G. E., Dixson, D. L., Domenici, P., McCormick, M. I., Sørensen, C., Watson, S.-A. and Munday, P. L. (2012). Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function. *Nature Climate Change* **2**, 201.

NOAA. (2018). State of the Climate: Global Climate Report for Annual 2017.

O'Brien, C. E., Bowie, M., Billard, P., Darmaillacq, A. S., Jozet-Alves, C., Benhaïm, D., Basuyaux, D. O. and Dickel, L. (2016). The effect of an artificial incubation environment on hatchling size and behavior in the cuttlefish, *Sepia officinalis*. *Vie et Milieu* **66**, 1-9.

O'Brien, C. E., Jozet-Alves, C., Mezrai, N., Bellanger, C., Darmaillacq, A.-S. and Dickel, L. (2017a). Maternal and Embryonic Stress Influence Offspring Behavior in the Cuttlefish *Sepia officinalis*. *Frontiers in physiology* **8**.

O'Brien, C. E., Mezrai, N., Darmaillacq, A. S. and Dickel, L. (2017b). Behavioral development in embryonic and early juvenile cuttlefish (*Sepia officinalis*). *Developmental Psychobiology* **59**, 145-160.

Orr, J. C., Fabry, V. J., Aumont, O., Bopp, L., Doney, S. C., Feely, R. A., Gnanadesikan, A., Gruber, N., Ishida, A. and Joos, F. (2005). Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature* **437**, 681.

Palmegiano, G. and d'Apote, M. (1983). Combined effects of temperature and salinity on cuttlefish (*Sepia officinalis* L.) hatching. *Aquaculture* **35**, 259-264.

Pauly, D. and Zeller, D. (2016). Catch reconstructions reveal that global marine fisheries catches are higher than reported and declining. *Nature communications* **7**, ncomms10244.

Pimentel, M. S., Faleiro, F., Diniz, M., Machado, J., Pousão-Ferreira, P., Peck, M. A., Pörtner, H. O. and Rosa, R. (2015). Oxidative stress and digestive enzyme activity of flatfish larvae in a changing ocean. *PLoS ONE* **10**, e0134082.

Pimentel, M. S., Faleiro, F., Dionísio, G., Repolho, T., Pousão, P., Machado, J. and Rosa, R. (2014). Defective skeletogenesis and oversized otoliths in fish early stages in a changing ocean. *Journal of Experimental Biology*, jeb. 092635.

Pimentel, M. S., Faleiro, F., Marques, T., Bispo, R., Dionísio, G., Faria, A. M., Machado, J., Peck, M. A., Pörtner, H., Pousão-Ferreira, P. et al. (2016). Foraging behaviour, swimming performance and malformations of early stages of commercially important fishes under ocean acidification and warming. *Climatic Change* **137**, 495-509.

Pistevos, J. C., Nagelkerken, I., Rossi, T. and Connell, S. D. (2017). Antagonistic effects of ocean acidification and warming on hunting sharks. *Oikos* **126**.

Regan, M. D., Turko, A. J., Heras, J., Andersen, M. K., Lefevre, S., Wang, T., Bayley, M., Brauner, C. J., Phuong, N. T. and Nilsson, G. E. (2016). Ambient CO_2, fish behaviour and altered GABAergic neurotransmission: exploring the mechanism of CO_2-altered behaviour by taking a hypercapnia dweller down to low CO_2 levels. *Journal of Experimental Biology* **219**, 109-118.

Reid, A., Jereb, P. and Roper, C. F. E. (2005). Family sepiidae. In *Cephalopods of the world. An annotated and illustrated catalogue of species known to date. In: Chambered Nautiluses and Sepioids (Nautilidae, Sepiidae, Sepiolidae, Sepiadariidae, Idiosepiidae, and Spirulidae)*, eds. P. Jereb and C. F. E. Roper), pp. 57–152. Rome, Italy: FAO.

Rhein, M., Rintoul, S. R., Aoki, S., Campos, E., Chambers, D., Feely, R. A., Gulev, S., Johnson, G. C., Josey, S. A., Kostianoy, A. et al. (2013). Observations: Ocean. In *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*, eds. T. F. Stocker D. Qin G.-K. Plattner M. Tignor S. K. Allen J. Boschung A. Nauels Y. Xia V. Bex and P. M. Midgley). Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press.

Riebesell, U., Zondervan, I., Rost, B., Tortell, P. D., Zeebe, R. E. and Morel, F. M. (2000). Reduced calcification of marine plankton in response to increased atmospheric CO_2. *Nature* **407**, 364.

Roberts, C. M., O'Leary, B. C., McCauley, D. J., Cury, P. M., Duarte, C. M., Lubchenco, J., Pauly, D., Sáenz-Arroyo, A., Sumaila, U. R. and Wilson, R. W. (2017). Marine reserves can mitigate and promote adaptation to climate change. *Proceedings of the National Academy of Sciences*, 201701262.

Rosa, R., Trubenbach, K., Pimentel, M. S., Boavida-Portugal, J., Faleiro, F., Baptista, M., Dionisio, G., Calado, R., Portner, H. O. and Repolho, T. (2014). Differential impacts of ocean acidification and warming on winter and summer progeny of a coastal squid (*Loligo vulgaris*). *Journal of Experimental Biology* **217**, 518-25.

Rosa, R., Trubenbach, K., Repolho, T., Pimentel, M., Faleiro, F., Boavida-Portugal, J., Baptista, M., Lopes, V. M., Dionisio, G., Leal, M. C. et al. (2013). Lower hypoxia thresholds of cuttlefish early life stages living in a warm acidified ocean. *Proceedings of the Royal Society B* **280**, 20131695.

Santos, M. E. d., Coniglione, C. and Louro, S. (2007). Feeding behaviour of the bottlenose dolphin, *Tursiops truncatus* (Montagu, 1821) in the Sado estuary, Portugal, and a review of its prey species. *Revista Brasileira de Zociências*, 31-40.

Sarazin, G., Michard, G. and Prevot, F. (1999). A rapid and accurate spectroscopic method for alkalinity measurements in sea water samples. *Water Research* **33**, 290-294.

Sigwart, J. D., Lyons, G., Fink, A., Gutowska, M. A., Murray, D., Melzner, F., Houghton, J. D. and Hu, M. Y.-a. (2015). Elevated pCO$_2$ drives lower growth and yet increased calcification in the early life history of the cuttlefish *Sepia officinalis* (Mollusca: Cephalopoda). *ICES Journal of Marine Science* **73**, 970-980.

Spady, B. L., Munday, P. L. and Watson, S. A. (2018). Predatory strategies and behaviours in cephalopods are altered by elevated CO$_2$. *Global Change Biology*, **24**(6), 2585-2596.

Spady, B. L., Watson, S.-A., Chase, T. J. and Munday, P. L. (2014). Projected near-future CO$_2$ levels increase activity and alter defensive behaviours in the tropical squid *Idiosepius pygmaeus*. *Biology open* **3**, 1063-1070.

Stocker, T. F., Qin, D., Plattner, G., Tignor, M., Allen, S., Boschung, J., Nauels, A., Xia, Y., Bex, V. and Midgley, P. (2013). Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press.

Stumpp, M., Wren, J., Melzner, F., Thorndyke, M. and Dupont, S. (2011). CO$_2$ induced seawater acidification impacts sea urchin larval development I: elevated metabolic rates decrease scope for growth and induce developmental delay. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **160**, 331-340.

Sui, Y., Liu, Y., Zhao, X., Dupont, S., Hu, M., Wu, F., Huang, X., Li, J., Lu, W. and Wang, Y. (2017). Defense responses to short-term hypoxia and seawater acidification in the thick shell mussel *Mytilus coruscus*. *Frontiers in physiology* **8**, 145.

Sultan, K., Al-Dayian, G. and Mohammad, H. (2008). Estimation and prediction from gamma distribution based on record values. *Computational Statistics & Data Analysis* **52**, 1430-1440.

Sykes, A., Domingues, P., Correia, M. and Andrade, J. (2006). Cuttlefish culture – State of the art and future trends. *Vie et Milieu* **56**, 129-138.

Sykes, A. V., Gonçalves, R. A. and Andrade, J. P. (2013). Early weaning of cuttlefish (*Sepia officinalis*, L.) with frozen grass shrimp (*Palaemonetes varians*) from the first day after hatching. *Aquaculture Research* **44**, 1815-1823.

Talmage, S. C. and Gobler, C. J. (2010). Effects of past, present, and future ocean carbon dioxide concentrations on the growth and survival of larval shellfish. *Proceedings of the National Academy of Sciences* **107**, 17246-17251.

Tresguerres, M. and Hamilton, T. J. (2017). Acid-base physiology, neurobiology and behaviour in relation to CO$_2$-induced ocean acidification. *Journal of Experimental Biology* **220**, 2136-2148.

Vidal, E. A., Villanueva, R., Andrade, J. P., Gleadall, I. G., Iglesias, J., Koueta, N., Rosas, C., Segawa, S., Grasse, B. and Franco-Santos, R. M. (2014). Cephalopod culture: current status of

main biological models and research priorities. In *Advances in Marine Biology*, vol. 67, pp. 1-98: Elsevier.

Warnke, K. (1994). Some aspects of social interaction during feeding in *Sepia officinalis* (Mollusca: Cephalopoda) hatched and reared in the laboratory. *Vie et Milieu* **44**, 125-131.

Watson, S. A., Fields, J. B. and Munday, P. L. (2017). Ocean acidification alters predator behaviour and reduces predation rate. *Biology Letters* **13**.

Wells, M. (1958). Factors affecting reactions to Mysis by newly hatched *Sepia*. *Behaviour* **13**, 96-111.

Williamson, P., Turley, C. and Ostle, C. (2017). Ocean acidification. *MCCIP Science Review*.

Wood, H. L., Spicer, J. I. and Widdicombe, S. (2008a). Ocean acidification may increase calcification rates, but at a cost. *Proceedings of the Royal Society of London B* **275**, 1767-1773.

Wood, J. B., Maynard, A. E., Lawlor, A. G., Sawyer, E. K., Simmons, D. M., Pennoyer, K. E. and Derby, C. D. (2010). Caribbean reef squid, *Sepioteuthis sepioidea*, use ink as a defense against predatory French grunts, *Haemulon flavolineatum*. *Journal of Experimental Marine Biology and Ecology* **388**, 20-27.

Wood, J. B., Pennoyer, K. E. and Derby, C. D. (2008b). Ink is a conspecific alarm cue in the Caribbean reef squid, *Sepioteuthis sepioidea*. *Journal of Experimental Marine Biology and Ecology* **367**, 11-16.

9.   Appendix (supplemental material)

Supplemental Table 9.1.: State of art of OA influence in *Sepia officinalis*. Parameters of the presented studies.

Reference	*p*CO$_2$	pH	TA	Temperature (°C)	Salinity	Incubation Period (stage)	Additional Parameters
Gutowska et al. (2008)	Control 1: 705 ± 101 ppm	7.94 ± 0.06	NS	16.32 ± 0.12	32.8 ± 0.5	Juvenile during 6 weeks	NA
	~ 4000 ppm: 4271 ± 373 ppm	7.23 ± 0.04		16.37 ± 0.12	32.9 ± 0.4		
	Control 2: 628 ± 60 ppm	8.01 ± 0.04		17.45 ± 0.16	31.4 ± 0.4		
	~ 6000 ppm: 6068 ± 389 ppm	7.10 ± 0.03		17.43 ± 0.15	32.3 ± 0.6		
Lacoue-Labarthe et al. (2009)	Control 1 8.10: 404 ppm	8.09 ± 0.04	2597 ± 0.012 mmol kg^{-1}	16 ± 0.1	38	Embryo (stage 1) until the end of development	800, 140 and 1000x10^{-3} pmol L^{-1} of Ag, Cd and Zn
	7.85; 900 ppm: 781 ppm	7.84 ± 0.04					
	7.60; 1400 ppm: 1399 ppm	7.61 ± 0.11					
	Control 2 8.10: 399 ppm	8.09 ± 0.09		18.9 ± 0.3			
	7.85; 900 ppm: 799 ppm	7.84 ± 0.04					
	7.60; 1400 ppm: 1440 ppm	7.61 ± 0.08					
Gutowska et al. (2010)	Control 64Pa: 63.6 ± 6.1 Pa	8.01 ± 0.04	NS	17.45 ± 0.16	31.4 ± 0.4	Juvenile during 6 weeks	NA
	615 Pa: 614.8 ± 39.4 Pa	7.10 ± 0.03		17.43 ± 0.15	32.3 ± 0.6		

Supplemental Table 9.1.: Continued.

Reference	pCO_2	pH	TA	Temperature (°C)	Salinity	Incubation Period (stage)	Additional Parameters
Hu et al. (2011)	Control 1: 70.97 ± 4.13 Pa	7.96 ± 0.02	2,413.81 ± 49.10	14.60 ± 0.47	34.94 ± 0.10	Embryo (stage 17-18) + Hatchling during 8 weeks	NA
	~ 0.14 kPa: 164.04 ± 24.48 Pa	7.63 ± 0.02	2,431.89 ± 56.12	14.62 ± 0.47	34.92 ± 0.07		
	~ 0.4 kPa: 371.35 ± 28.52 Pa	7.28 ± 0.03	2,415.16 ± 43.00	14.62 ± 0.45	34.90 ± 0.05		
	Control 2: 50.255 ± 8.757 Pa	8.21 ± 0.08	2,827.00 ± 129.08	14.81 ± 0.20	33.08 ± 0.20	Juvenile during 6 weeks	
	~ 0.4 kPa: 308.936 ± 37.401 Pa	7.39 ± 0.09	3,127.53 ± 141.91	14.83 ± 0.21	33.09 ± 0.21		
Maneja et al. (2011)	Control: 700 µatm	NS	NS	15	35	Embryo + Hatchling during 63 days	NA
	1400 µatm						
	4000 µatm						
Dorey et al. (2013) *Exp (experiment)	Exp 1 - Control 1 8.10; ~ 390 µatm: 378 ± 65 µatm	8.11 ± 0.08	2597 ± 12 mmol kg^{-1}	16.0 ± 0.1	38	Embryo (stage 1) until the end of development	$\Omega_{ar} > 1.15$
	Exp 1 7.85; ~ 800 µatm: 775 ± 83 µatm	7.84 ± 0.05					
	Exp 1 7.60; ~ 1400 µatm: 1433 ± 208 µatm	7.60 ± 0.08					

Supplemental Table 9.1.: Continued.

Reference	pCO$_2$	pH	TA	Temperature (°C)	Salinity	Incubation Period (stage)	Additional Parameters
Dorey et al. (2013) *Exp (experiment)	Exp 1 - Control 2 8.10; ~ 390 µatm: 393 ± 83 µatm	8.14 ± 0.09	2597 ± 12 mmol kg^{-1}	18.9 ± 0.3	38	Embryo (stage 1) until the end of development	$\Omega_{ar} > 1.15$
	Exp 1 7.85; ~ 800 µatm: 766 ± 181 µatm	7.58 ± 0.08					
	Exp 1 7.60; ~ 1400 µatm: 1517 ± 273 µatm	7.86 ± 0.12					
	Exp 2 - Control 1 8.10; ~ 390 µatm: 376 ± 11 µatm	8.06 ± 0.08	2549 ± 65 mmol kg^{-1}	16.0 ± 0.4		Embryo (stage 1) + Hatchling 19 DAH	
	Exp 2 7.85; ~ 800 µatm: 739 ± 15 µatm	7.81 ± 0.04					
	Exp 2 7.60; ~ 1400 µatm: 1381 ± 30 µatm	7.60 ± 0.09					
	Exp 2 - Control 2 8.10; ~ 390 µatm: 370 ± 7 µatm	8.03 ± 0.06		19.1 ± 0.5			
	Exp 2 7.85; ~ 800 µatm: 741 ± 22 µatm	7.83 ± 0.02					
	Exp 2 7.60; ~ 1400 µatm: 1395 ± 38 µatm	7.60 ± 0.03					

Supplemental Table 9.1.: Continued.

Reference	pCO_2	pH	TA	Temperature (°C)	Salinity	Incubation Period (stage)	Additional Parameters
Rosa et al. (2013)	Control 1: 500 ppm	8.1 ± 0.1	NS	18.0 ± 0.28	35.0 ± 1.0	Embryo (stage I) until the end of development	NA
	1000 - 1200 ppm	7.5 ± 0.1					
	Control 2: 500 ppm	8.1 ± 0.1		22.0 ± 0.2			
	1000 - 1200 ppm	7.5 ± 0.1					
Sigwart et al. (2015)	Control ~ 0.05kPa: 0.052 ± 0.008 kPa	8.02 ± 0.04	2567 ± 80	16 during development; 18 after hatching	32.2 ± 2	Embryo (stage10) + Hatchling 19 DAH + Hatchling 34 DAH + Hatchling 47 DAH	NA
	~ 0.14 kPa: 0.135 ± 0.020 kPa	7.76 ± 0.05	2557 ± 116				
	~ 0.37 kPa: 0.368 ± 0.039 kPa	7.27 ± 0.03	2595 ± 132				

Supplemental Table 9.2: State of art of OA influence in *Sepia officinalis*. Parameters of the presented studies.

Reference	PVF	Egg Measures	Embryo Measures	Development time / Hatching Success	Premature Hatching	Survival	Hatchling / Juvenile Measures
Gutowska et al. (2008)	NA	NA	NA	NA	NA	NA	pH did not affect soft-tissue growth performance
							pH did not affect the exponential curves used to calculate daily growth
							pH did not affect the mantle length
							pH did not affect the gross growth efficiencies (GGE)
							pH did not affect the final wet mass gained
Lacoue-Labarthe et al. (2009)	NA	higher egg weight at the end of development with decrease with pH	NA	NA	NA	NA	pH had no significant impact on the juvenile weight at hatching time
Hu et al. (2011)	$p\mathrm{CO}_2 = 0.16$ kPa: significantly decreased PVF pH	NA	$p\mathrm{CO}_2 = 0.16$ kPa: did not cause growth delays	NA	NA	NA	juvenile and $p\mathrm{CO}_2 = 0.37$ kPa: had no significant influence in growth rates
							hatchling and $p\mathrm{CO}_2 = 0.16$ kPa: did not cause growth delays

Reference	PVF	Egg Measures	Embryo Measures	Development time / Hatching Success	Premature Hatching	Survival	Hatchling / Juvenile Measures
Hu et al. (2011)	$pCO_2 = 0.37$ kPa: significant decreased PVF pH	NA	$pCO_2 = 0.37$ kPa: significantly changed the DML of late-stage embryos	NA	NA	NA	$pCO_2 = 0.37$ kPa: significantly reduced hatchlings (2 days post hatch) body mass and DML
Dorey et al. (2013)	Exp 2: pH did not significantly influence PVF pO_2 in eggs close to hatching Exp 2: pH had a significant combined effect with the temperature (16 °C) decreasing PVF pO_2 in eggs close to hatching with decreasing pH Exp 2: PVF pCO_2 increased with lower pH Exp 2: CO$_2$ gradient between PVF and seawater increased with higher seawater pCO_2	pH influenced significantly the egg weight	Exp 1: pH did not affect the whole body dry weight at the end of the development	Exp 2: pH did not cause hatching delay Exp 1: pH did not affect hatching success	NA	Exp 2: pH did not affect the survival rates of juveniles	Exp 2: pH had no effect on body dry weight after 19 days of incubation

Supplemental Table 9.2: Continued.

Reference	PVF	Egg Measures	Embryo Measures	Development time / Hatching Success	Premature Hatching	Survival	Hatchling / Juvenile Measures
Rosa et al. (2013)	NA	NA	NA	pH did not change the development time	decrease in pH significantly increased premature hatching	pH had significant combined effect with temperature (22 °C), leading to lower survival	NA
Sigwart et al. (2015)	NA	pH was not significative in exponential decrease of yolk wet mass with increasing of embryo wet mass	NA	pH had significative effect on development time	NA	NA	intermediate pH had no effect in hatchling wet mass
		yolk wet mass exponentially decreased over incubation time with higher values in high pCO_2					hatchling wet mass significantly decreased in high pCO_2

Supplemental Table 9.3: State of art of OA influence in *Sepia officinalis*. Parameters of the presented studies.

Reference	Oxygen Consumption / Metabolism	Cuttlebone	Statolith	Hunting Behaviour
Gutowska et al. (2008)	$pCO_2 \sim 6000$ ppm: no significative effect on standard metabolic rates	pH did not significantly affect the formation rate, determined from mantle length measurements	NA	NA
		$pCO_2 \sim 6000$ ppm: total calcium carbonate accumulation, when directly measured, significantly higher than in the control		
Gutowska et al. (2010)	NA	morphology was not affected by pH	NA	NA
		pH did not affect the slopes of the cuttlebone length to mass relationships		
		pH had no effect on the relationship between length and width		
		pH had no effect on the average heights of the structures, due to the large variability of sizes		
		pH had significative effect on width with the average of high CO_2 treatment being slightly narrower compared to the control group		
		high CO_2 treatment shown significantly shorter final length, despite it was minimally		
		significant reduction in average height in high CO_2 treatment		
		length and height were no longer linearly related in high CO_2 treatment		
		average mass of CO_2 cuttlebones was significantly greater with decreasing pH		
		significant decrease in distance between lamellae with decreasing pH		
		lamellar and pillar thickness increased with decrease of pH		
		higher occurrence of irregular $CaCO_3$ deposition (spherical structures) in high CO_2 treatment		
		average number of spherical structures visible along the midline fracture was significantly greater in high CO_2 treatment		
		cuttlebone composition, in terms of the ratio between $CaCO_3$ and non-acid-soluble organic matrix (NASOM), was significantly smaller under elevated pCO_2		

Supplemental Table 9.3: Continued.

Reference	Oxygen Consumption / Metabolism	Cuttlebone	Statolith	Hunting Behaviour
Maneja et al. (2011)	NA	NA	pCO_2 = 1400 µatm: did not show alterations in statolith calcification embryo and hatchling and pCO_2 = 4000 µatm: significative reduction of statolith calcification statolith morphometrics that showed the most remarkable changes with high pCO_2: total length, rostrum transects, wing area and weight	pCO_2 = 4000 µatm: reduced ability to capture prey and annulled the ability to successfully launch attacks against prey organisms
Dorey et al. (2013)	NA	Exp 1: pH did not significantly affect the weight of embryo's cuttlebone in the end of development Exp 2: decreasing pH dropped the Ω_{ar} values; lower values with pH = 7.60 Exp 2: acidification rapidly worsen conditions advantageous to calcium precipitation in the embryonic fluid, while this appeared to be counterbalanced by seawater warming embryo and juvenile: decreasing pH had greater accumulation of $CaCO_3$ embryo and juvenile: lowest pH led to an increase of calcium incorporation	NA	NA

Supplemental Table 9.3: Continued.

Reference	Oxygen Consumption / Metabolism	Cuttlebone	Statolith	Hunting Behaviour
Rosa et al. (2013)	pH did not vary significantly the $P_{c,ext}$ (the point at which the rate of oxygen consumption was no longer maintained independent of ambient oxygen partial pressure) and the $P_{c,in}$ (internal critical oxygen partial pressures) of intermediate embryos the pre-hatchlings' $P_{c,ext}$ and $P_{c,in}$ increased significantly with high CO_2 and warming scenario high CO_2 significantly lower pre-hatchings RMR (oxygen consumption measurements) independently of temperature	NA	NA	NA
Sigwart et al. (2015)	pCO_2 did not affect oxygen consumption rates of embryos and hatchlings	pH did not affect the number of minor laminae ranged, the embryonic shell size and the larval lamellar interspace $pCO_2 \sim 0.37$ kPa: significant on the ratio of the cuttlebone dry mass to animal wet mass on hatchlings with 19 DAH, but $pCO_2 \sim 0.14$ kPa was not mean dry-shell contribution to animal mass was significantly larger in both high pCO_2 on hatchlings with 34 DAH $pCO_2 \sim 0.37$ kPa: slightly but significantly shorter, relative to their width $pCO_2 \sim 0.37$ kPa: significant on the aspect ratio (length/width), but $pCO_2 \sim 0.14$ kPa was not pH was significant in overall length of hatchlings with 47 DAH pH varied significantly the dry mass of hatchlings with 47 DAH the interspace in the ventral lamellae of hatchlings with 47 DAH was increasingly more compact in both high pCO_2 treatments	NA	NA

Supplemental Table 9.4.: State of art of OA influence in *Sepia officinalis*. Parameters of the presented studies.

Reference	Gene Expression	Trace Elements
Lacoue-Labarthe et al. (2009)	NA	pH had no significative effect on ^{109}Cd CF in the perivitelline fluid
		hatchlings: decreasing pH enhanced the ^{110m}Ag accumulation in the tissues and low temperature amplified this effect
		^{110m}Ag CF in the perivitelline fluid varied with pH at 19 °C, but did not at 16 °C
		embryo: ^{110m}Ag was more effectively taken up from the perivitelline fluid with decreasing pH, with the highest CF values in high pCO$_2$
		19 °C: ^{110m}Ag uptake rate in their eggshell was higher at control pH
		19 °C: ^{110m}Ag CF values reached at the end of development were lower at lower pHs
		16 °C: similar patterns of ^{110m}Ag at 19 °C, although ^{110m}Ag CF reached a steady-state equilibrium at pH = 8.10 and the elimination rate at lowest pH were lower than at 19°C
		embryonic growth and hatchlings: ^{110m}Ag were more efficiently accumulated at pH = 7.60 and ^{65}Zn at pH = 7.85
		pH = 7.60: significant accumulation of ^{65}Zn and ^{110m}Ag in the pooled vitellus and embryo determined earlier
		^{65}Zn CF showed the maximal values in the juveniles hatched at pH = 7.85, independent of temperature, and the CF at pH = 7.60 was lower than at pH = 8.10
		^{65}Zn: CF$_{PVF/sw}$ perivitelline fluid decreased with decreasing pH; CF$_{emb/PVF}$ were maximal at pH = 7.85 leading to the highest Zn accumulation in the hatchlings
		pH = 7.60: lower accumulation of ^{65}Zn in the eggshell
		pH = 7.60: pattern of ^{109}Cd accumulation changed after only 7 DED
		pH and temperature showed a combined effect on the maximal ^{109}Cd CF values in the eggshell
		^{109}Cd CF decreased with increasing pCO$_2$
		Cd accumulation from the perivitelline fluid to the embryo increased with decreasing pH at 16 °C

Supplemental Table 9.4.: Continued.

Reference	Gene Expression	Trace Elements
Hu et al. (2011)	pH did not affect the mRNA expression of all genes tested in juveniles	NA
	pH did not affect the NKA maximum activity in juveniles at 42 days of incubation	
	gill tissues of late-stage embryos: pH had no effect in *soNDCBE* expression	
	juveniles and $pCO_2 = 0.37$ kPa: significative changes in *NKA* maximum activity after 48 h of incubation	
	juveniles: *NKA* maximum activity was significantly increased in CO_2 treatments from 48 h to 11 days of incubation	
	juveniles: no transcriptional response was detected for *soNKA*, in both high pCO_2 treatments	
	gill tissues of late-stage embryos and $pCO_2 = 0.37$ kPa: significant downregulation for the transcripts of ion-transporting proteins *NKA*, *NBCe*, and *cCAII*	
	embryos and $pCO_2 = 0.16$ kPa: only two significant changes - *NBCe* was downregulated and *cCAII* was upregulated	
	hatchlings and $pCO_2 = 0.37$ kPa: similar general expression pattern with strong downregulation of all acid-base regulatory candidate genes (e.g., *NKA*, *NBCe* and *cCAII*)	
	hatchlings and $pCO_2 = 0.37$ kPa: significative downregulation of ATP-synthase, cytochrome-*c* oxidase subunit 1 (*COX*) and cytochrome *P*-450 (*CYP-450*)	
	hatchlings and $pCO_2 = 0.16$ kPa: only two significant changes - *NBCe* was downregulated and *COX* was upregulated	

Supplemental Table 9.5.: Results of the statistical models applied for analysis of ocean acidification effects in *Sepia officinalis*. Codes: *** when p-value < 0; ** when p-value < 0.001; * when p-value < 0.05; . when p-value < 0.1.

	Model	Fixed effects	Terms	Estimate	Std. error	Z value	Pr(>|z|)	
Hatching success	GLM, family=Poisson AIC=39.826	Final eggs number~pH	(Intercept)	-0.31015	0.07313	-4.241	2.22E-05	***
			pH	0.04372	0.10457	0.418	0.676	
Survival	GLM, family=Poisson, link=identity AIC=37.437	Final hatchlings number~pH	(Intercept)	37.5342	3.7268	10.072	<2E-16	***
			pH	-0.2894	5.2599	-0.055	0.956	
TBL	GLM, family=Gaussian AIC=-200.79	Total length~pH	(Intercept)	1.13948	0.01231	92.58	<2E-16	***
			pH	-0.01836	0.01792	-1.025	0.308	
Weight	GLM, family=Gaussian, link=inverse AIC=-613.3497	Weight~pH+Hatching date	(Intercept)	9.9822	0.254	39.301	<2E-16	***
			pH	-0.2223	0.2827	-0.786	0.434	
			Hatching date	1.1712	0.2828	4.142	7.08E-05	***
Fulton's index	GLM, family=Gamma, link=inverse AIC=648.1903	Fulton Index~pH+Hatching date	(Intercept)	0.039881	0.001703	23.424	<2E-16	***
			pH	-0.004093	0.001869	-2.19	0.0286	*
			Hatching date	0.010173	0.001866	5.452	4.98E-08	***
Shelter choice	GLM, family=Binomial AIC=36.50793	Choice/No choice~pH	(Intercept)	0.1335	0.5175	0.258	0.7964	
			pH	1.7383	0.9191	1.891	0.0586	.
Shelter preference	GLM, family=Binomial AIC=30.94219	Black/White~pH	(Intercept)	-1.0986	0.8165	-1.346	0.178	
			pH	1.2528	0.988	1.268	0.205	
Reaction time	GLM, family=Gamma, link=inverse AIC=342.2580	Reaction~pH	(Intercept)	0.004788	0.001474	3.248	0.00116	**
			pH	0.001052	0.002378	0.442	0.65816	
Catch time	GLM, family=Gamma, link=inverse AIC=203.3067	Catch~pH	(Intercept)	0.06034	0.01729	3.491	0.000481	***
			pH	0.02299	0.03021	0.761	0.446625	
Successful attacks	GLM, family=Poisson AIC=83.24594	Successful attacks~pH	(Intercept)	0.5878	0.1925	3.054	0.00226	**
			pH	-0.1082	0.291	-0.372	0.70995	
Unsuccessful attacks	GLM.NB AIC=79.99485	Unsuccessful attacks~pH	(Intercept)	0.06454	0.3214	0.201	0.841	
			pH	-0.3269	0.50037	-0.653	0.514	

Supplemental Table 9.5.: Continued.

	Model	Fixed effects	Terms	Estimate	Std. error	Z value	Pr(>|z|)	
Total attacks	GLM, family=Poisson AIC=105.1945	Total attacks~pH	(Intercept)	1.0531	0.1525	6.906	4.99E-12	***
			pH	-0.1841	0.2356	-0.781	0.435	
Visual reaction	GLM, family=Binomial AIC=40.1754	Response to stimulus~pH	(Intercept)	-0.7885	0.5394	-1.462	0.144	
			pH	0.452	0.7961	0.568	0.57	
Visual type of reaction	Multinomial Logistic Regression AIC=44.33269	pH~Kind of reaction	(Intercept)	0.45245647	0.4835191	0.93575716	0.3493982	
			Reaction type 1	-0.4521759	1.1107613	-0.40708645	0.6839445	
			Reaction type 2	-0.04648618	1.0330578	-0.04499862	0.9641084	
			Reaction type 3	-8.84852701	66.5722199	-0.13291621	0.8942596	

Supplemental Table 9.6.: Results of the statistical models, with chi-square test, applied for analysis of ocean acidification effects in *Sepia officinalis*. Codes: *** when p-value < 0; ** when p-value < 0.001; * when p-value < 0.05; . when p-value < 0.1.

	Model	Fixed effects	Terms	Deviance Resid.	Resid. Dev	Pr(>Chi)	
DED	GLM, family=Poisson AIC=2631.284	Day~pH+Replicate	NULL		561.26		
			pH	0.0379	395.44	0.84561	
			Replicate	10.8574	384.58	0.02821	*
DML	GLM, family=Gaussian AIC=-298.9998	Mantle length~pH+Replicate	NULL		0.37639		
			pH	0.00884	0.36755	0.098537	.
			Replicate	0.043636	0.32392	0.009188	**