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Impact of warming and acidification on anxiety levels and camouflage abilities of cuttlefish early stages

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2021

Agradecimentos

Em primeiro lugar, gostaria de agradecer profundamente aos meus orientadores por me terem proporcionado esta experiência. Obrigada pelos conselhos e pela oportunidade de trabalhar no laboratório sob a vossa orientação. À Kuka, obrigada pela paciência, pelo apoio constante, tempo e atenção. Ao Rui, uma pessoa com uma energia contagiante, agradeço pela inspiração que me deste, motivando-me a seguir a minha paixão.

À Marta por todos os bons momentos, pelo apoio e por estares sempre disponível para ouvir os meus desabafos, pela luta e entreajuda constantes que tivemos ao longo deste ano.

Special thanks to Zé, Tiago and Eve, for having participated in this project, for helping to solve problems, providing equipment, and teaching me invaluable knowledge on aquarium set up and maintenance, as well as answering all my questions and stimulating critical thinking.

À Kuka, Zé, Marta, Martim, Beatriz, obrigada por participarem no Journal Club, pela troca de conhecimento, pelas críticas construtivas e pela paciência com que me ouviram.

Obrigada aos colegas da Guia, que demonstraram um grande espírito de colaboração, e que me permitiram viver um ano empolgante, apesar da pandemia.

Obrigada à Bia, pelo apoio emocional, amor e carinho. Por me mostrares que a vida não é só trabalho. Obrigada pela tua alegria e entusiasmo, mesmo quando o mundo está contra ti.

Para além de agradecer, dedico esta tese aos meus pais que tanto trabalharam para que eu pudesse ter acesso a um ensino de qualidade, sem nunca me pressionarem para ser algo diferente daquilo que sou. Obrigada do fundo do coração por todo o apoio que me deram ao longo deste percurso. Por me aturarem nos momentos bons e nos menos bons, mostrando-se sempre disponíveis para escutar até os detalhes mais aborrecidos do meu trabalho. Agradeço também ao meu irmão, por ouvir os meus desabafos e me motivar, mostrando-me que sou capaz de ultrapassar as adversidades naturais da vida.

Por fim, agradeço à FCT, por ter financiado este projeto, bem como por ter fornecido as condições necessárias à sua conclusão.

Abstract

Ocean warming and acidification have been shown to elicit deleterious biological effects on cephalopod molluscs, especially during early ontogeny. Yet, little is known about the impacts of these climate change-related stressors on the camouflage abilities and anxiety-related behaviours of cephalopod early life stages. The aim of this dissertation was to evaluate, for the first time, the effect of end-of-the-century projected levels of ocean warming (W; + 3ºC) and acidification (A; 980 µatm pCO_2) on *Sepia officinalis* hatchlings' anxiety levels and ability to camouflage in different substrate complexities (sand and black and white gravel). Latency to maximum camouflage was registered remotely, and pixel intensity along body planes was gauged and compared with background intensities in gravel to evaluate disruptiveness through photographs. Furthermore, camouflage scores were attributed to each cuttlefish. To infer anxiety levels, novel object tests were performed. Average acceleration, immobility and time spent near the object were extracted from video recordings. Hatching success was lowered under A (95%) compared to control conditions (98.8%), and this effect was exacerbated under A and W combined (AW; 72.7%). Embryonic period was prolonged by A (50 days) and was reduced by W (35 days) compared to 45 days under control conditions. Camouflage scores were higher in sand compared to gravel, denoting a better adaptation to sandy substrates. AW significantly delayed camouflage response in the gravel substrate compared to W alone. Moreover, cuttlefish exhibited a higher contrast and consequently a stronger disruptive pattern under W (but to a lesser extent under AW), with no changes in background matching. Anxiety behaviours were not affected by the different treatments. These findings suggest that, although climate change may elicit relevant physiological challenges to cuttlefish (associated with decreasing fitness and survival), these invertebrate molluscs do not show significant behavioural changes under the predicted conditions for the ocean of tomorrow.

Keywords: cephalopod, embryogenesis, climate change, crypsis, novel object

Resumo alargado

Devido à sua alta densidade e baixo calor específico, o oceano atua como um reservatório de calor, mitigando assim mudanças no clima. De fato, estima-se que o oceano tenha absorvido 93% da energia libertada pelo aquecimento global entre 1971 e 2010. Simultaneamente, pela elevada solubilidade do CO_2 na água, o oceano representa o maior depósito de emissões antropogénicas de CO_2. Isto levou a uma diminuição progressiva de pH da água do oceano, tendência que deverá persistir. Esta alteração no pH do oceano pode também causar bioerosão e menor disponibilidade em oxigénio para os organismos aquáticos. É esperado que muitas espécies atinjam o seu limite térmico a níveis relativamente baixos de aquecimento global, quando expostas aos níveis projetados de acidificação. O aquecimento e acidificação dos oceanos provocam efeitos biológicos adversos nos moluscos cefalópodes, especialmente em estados iniciais de vida. No entanto, os impactos desses fatores na capacidade de camuflagem e na ansiedade dos cefalópodes juvenis permanecem desconhecidos. Chocos (*S. officinalis)* recém-nascidos dependem quase exclusivamente da camuflagem para proteção contra os predadores. Por outro lado, apesar de a ansiedade ser usualmente vista como uma ferramenta para a sobrevivência em ambientes de alta predação, também pode ser uma resposta prejudicial a fatores relacionados com as alterações climáticas, dado que não representam uma ameaça imediata, como é o caso da presença de predadores. Este estado afetivo pode potencialmente limitar a procura de alimento e lesar estratégias defensivas como a camuflagem. Desta maneira, pretendeu-se com esta dissertação testar a tolerância comportamental de chocos recém-nascidos à exposição embrionária a níveis projetados para o final do século de aquecimento (Aq; + 3ºC) e acidificação (Ac; 980 µatm pCO_2). Para este fim, foram avaliados os níveis de ansiedade e capacidade de camuflagem em dois substratos de complexidades diferentes (areia e gravilha preta e branca).

Entre dois a cinco dias pós-eclosão, os animais foram sujeitos aos dois ensaios, começando pelo teste de ansiedade, e com pelo menos quatro horas de intervalo entre testes. Relativamente à ansiedade, os chocos foram colocados numa arena branca circular com um objeto desconhecido (tampa roxa) no centro, e filmados durante vinte minutos. Indicadores de comportamento ansiogénico (aceleração e imobilidade médias e tempo despendido na proximidade do objeto) foram extraídos dos vídeos através de um *software* de rastreamento do movimento dos animais (ToxTrac). Após este ensaio, e para avaliar a sua capacidade de camuflagem, os chocos foram colocados numa arena branca com fundo arenoso ou mistura de gravilha durante dez minutos. O processo foi repetido sem interrupção para o substrato seguinte. Fotografias foram tiradas remotamente, e, simultaneamente, foram registados tempo de aclimatação, latência para a camuflagem máxima e tentativas de enterro na areia. A partir das fotografias de camuflagem máxima foi possível aferir a intensidade de pixéis nos planos corporais do choco e compará-la com as intensidades da gravilha preta no substrato, através do software ImageJ. Para além disso, foi atribuída uma pontuação de camuflagem a cada choco. Foram também recolhidos dados de condição física, como o comprimento do manto, sucesso de eclosão e tempo de desenvolvimento desde que os ovos começaram a ser aclimatados. Para determinar a influência dos tratamentos nas variáveis-resposta associadas à qualidade de camuflagem e comportamentos de ansiedade, foram ajustados modelos lineares generalizados.

O sucesso de eclosão foi inferior perante o tratamento de Ac (95%) relativamente ao controlo (98.8%), embora não tenham sido verificadas diferenças com o Aq (98.8%). Observou-se um efeito interativo demarcado sob a combinação dos tratamentos (72.7%). A duração do período embrionário foi prolongada pela Ac (50 dias) e reduzida pelo Aq (35 dias) relativamente a 45 dias no controlo. A combinação dos tratamentos resultou em períodos embrionários intermédios. Embora não tenha sido observada influência dos tratamentos na pontuação atribuída à camuflagem, esta foi superior na areia relativamente à gravilha, revelando uma melhor adaptação do choco a substratos arenosos. Por outro lado, também não houve efeito dos tratamentos na tentativa, por parte dos chocos, de se enterrarem na areia. O efeito combinado de Aq e Ac atrasou significativamente a resposta de camuflagem na gravilha relativamente ao Aq por si só, evidenciando a existência de um custo subjacente associado a estes fatores. Este efeito pode ter origem num desenvolvimento diminuído do lobo ótico, que desempenha um papel central na camuflagem, e que apenas se desenvolve no final da embriogénese. Por outro lado, este resultado poderá ser explicado pela ausência de predadores, e assim, os chocos evitarem incorrer os elevados custos de manutenção da camuflagem e os custos energéticos acrescidos sob o Aq. Para além disso, os chocos exibiram um padrão mais disruptivo sob Aq e Aq + Ac. Isto não se refletiu numa melhor correspondência com as intensidades do substrato, possivelmente pelos chocos por vezes recorrerem a padrões uniformes escuros, ao invés de disruptivos. Este resultado foi verificado noutros trabalhos da mesma forma com outras fontes de *stress*, como a exposição a predadores ou *stress* materno. Por conseguinte, o aprimoramento da camuflagem disruptiva constitui presumivelmente uma adaptação comportamental a níveis superiores de *stress*, embora o custo energético e metabólico desta não seja atualmente conhecido. Os comportamentos associados a elevados níveis de ansiedade, isto é, atividade elevada, tempo imóvel reduzido, e ausência de exploração (distanciamento do objeto desconhecido) não foram afetados pelos tratamentos. Este resultado é consistente com os de trabalhos anteriores, pois outras fontes de *stress* embrionárias (ex., acidificação, sinais de presença de predadores) também não afetaram o comportamento de chocos juvenis. Logo, as diferenças verificadas entre chocos podem ser atribuídas a diferenças de temperamento. Estes resultados demonstram que os chocos são fisiologicamente e comportamentalmente resistentes à acidificação do oceano projetada para o final do século *per se*, tendo os chocos sofrido apenas um prolongamento da embriogénese. Apesar do aquecimento ter efeitos marcados na fisiologia dos chocos, a combinação destas duas alterações físico-químicas aparenta ter efeitos negativos diretos exacerbados, como a sucesso de eclosão, embora a Ac possa mitigar certos efeitos adversos do Aq. Os efeitos sub-letais (latência de camuflagem, contraste do padrão disruptivo e ansiedade) destes fatores no comportamento são reduzidos. De fato, se a camuflagem ótima for atrasada, apenas pode aumentar moderadamente a probabilidade de deteção por predadores. Sendo assim, é expectável que estes animais se adaptem às futuras condições do oceano, visto apresentarem uma grande plasticidade fenotípica e habitarem zonas costeiras, de grande variabilidade ambiental inerente. Todavia, existem lacunas importantes no conhecimento do comportamento dos cefalópodes no que diz respeito a outras fontes de *stress* observadas e previstas para o final do século. Especial atenção deve ser concedida à desoxigenação, que, juntamente com o aquecimento e a acidificação, forma o “trio letal”, responsável por grande parte das extinções em massa do passado. Outros fenómenos pontuais podem ter efeitos irreversíveis na fauna marinha, por exemplo, a hipóxia ou as ondas de calor marinhas. Por outro lado, outros estados de vida vulneráveis, como os gâmetas, zigotos, e embriogénese inicial, podem não estar equipados para lidar com estas ameaças.

O choco ocupa um lugar importante nas teias tróficas, atuando como predador e como presa, pelo que impactos no *fitness* deste animal terão consequências a nível do ecossistema. Estes resultados têm implicações diretas na gestão de mananciais do choco, assistindo na tomada de decisões baseadas nos efeitos – pontuais ou a longo termo – das alterações climáticas nesta espécie. Simultaneamente, contribuirão para esforços prospetivos de conservação, com vista nos Objetivos de Desenvolvimento Sustentável (ONU) 13, de tomar ação urgente para combater as alterações climáticas e os seus impactos; e 14, de conservar e usar de modo sustentável o oceano, mares e recursos marinhos.

Palavras-chave: cefalópode, embriogénese, alterações climáticas, cripsis, exploração

Acronyms and abbreviations

A - Acidification

AW – Acidification and warming combined

C - Control

CO_2 – Carbon dioxide

ERF – Effective radiative forcing

GLM – Generalized linear model

IPCC – Intergovernmental Panel for Climate Change

LM – Linear model

OA – Ocean acidification

OHC – Ocean heat content

OW – Ocean warming

$p\mathrm{CO}_2$ – Carbon dioxide partial pressure

$p\mathrm{O}_2$ – Oxygen partial pressure

pH – Power of hydrogen

SSP – Shared Socioeconomic Pathway

SST – Sea surface temperature

SW – Seawater

W - Warming

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1. Introduction

The world's climate has undergone extensive changes since the pre-industrial era, a process described by Duarte (2014) as Anthropogenic Global Change, *i.e.*, "the global-scale changes resulting from the impact of human activity on the major processes that regulate the functioning of the Biosphere*"*. The most noticeable cause is an enhanced greenhouse effect, derived from the abnormally high emission of gases which absorb infrared radiation, causing the Earth to warm (Albritton *et al.*, 2001). For instance, significant recent changes in levels of nitrous oxide (N_2O), methane (CH_4) and carbon dioxide (CO_2) are indicative of increased human pressure (Ehhalt & Prather, 2001). The rise in CO_2 concentration in the atmosphere leads to ocean acidification (OA) (Caldeira & Wickett, 2003), while the warming effect hinders the ocean's oxygenation. Jointly, warming, acidification and deoxygenation are viewed as the "deadly trio". This is due to their central role in most historical global mass extinctions, and their alarming importance in modern world change (Knoll *et al*., 1996; Bijma *et al*., 2013). Further, it is believed that the repercussions of anthropogenic activities on climate will prevail long after the emissions are suspended (Rabalais *et al*., 2014).

1.1. Ocean warming

Due to its high density and low specific heat, the ocean acts as a heat reservoir, and consequently buffers changes in climate (Reid, 2016). Indeed, it is estimated that the ocean has absorbed 91% of the energy released by global warming between 1971 and 2018 (Fox-Kemper *et al.*, 2021), most of it accumulating in the upper 700 m of the ocean (Rhein *et al.*, 2013). Hence, the Ocean Heat Content (OHC) has been increasing since 2005, and the Intergovernmental Panel on Climate Change (IPCC) considers *likely* that the warming rate has increased since 1993, by at least two-fold. Additionally, the ocean is expected to warm at least twice as much as it has to date under a low emissions scenario (global Effective Radiative Forcing [ERF] of 2.6), and five to seven-fold under a high emissions scenario (ERF 8.5) between the depths of 0 m and 2000 m. The latter represents a projected increment of approximately 2150 ZJ (Bindoff *et al*., 2019). End-of-the century sea surface temperature change projections range from stabilization at current temperatures to a ~ 5ºC temperature increment (Fox-Kemper *et al.*, 2021), depending on the Shared Socioeconomic Pathway (SSP) and the ERF (**Figure 1**).

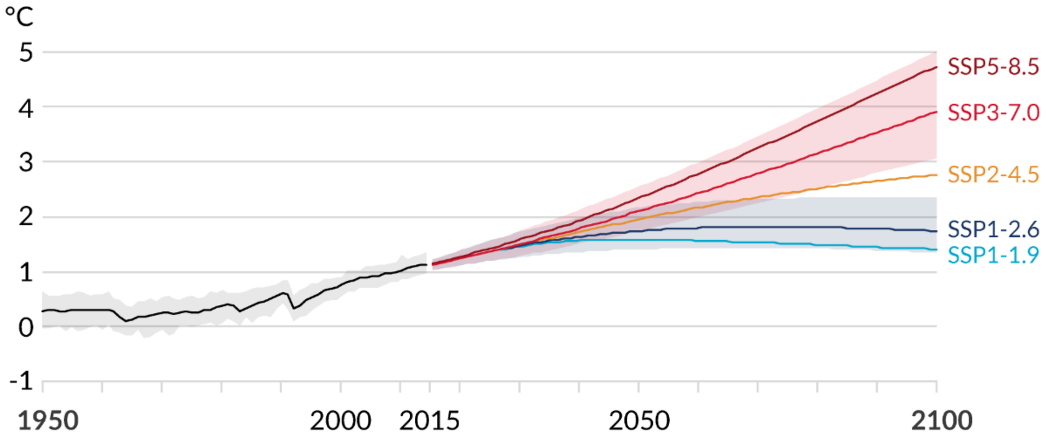

Figure 1 *– Global Ocean surface temperature change relative to 1850-1900. Simulated historical changes since 1950 (black curve) and projected pH under five Shared Socioeconomic Pathways (in five colours). Shades represent uncertainty ranges. From IPCC (2021).*

An increase in OHC precipitates changes such as melting of the cryosphere and expansion of water molecules, which in turn lead to a rise in sea level; and strengthening of the El Niño Southern Oscillation, and thus extreme heat events (Reid, 2016). It further contributes to an increased stratification of the ocean's upper layer, deoxygenation and salinisation (Bindoff *et al.*, 2019). The direct effects of Ocean Warming (OW) on organisms include increased metabolism costs, hindered oxygen delivery to tissues and consequent narrower ecological niche (Deutsch *et al.*, 2020), and increased vulnerability to disease, which often results in mass mortalities (Clemente *et al.*, 2014; Rivetti *et al.*, 2014; Genin *et al.*, 2020). Accordingly, fisheries are susceptible to a decrease in the maximum catch potential of many fish stocks, averaging a *ca.* 4% decrease per decade (Bindoff *et al.*, 2019). Nevertheless, OW also induces lasting changes in communities through altered species interactions, for instance, changes in bottom-up forcing owing to a predominance of resistant plankton species (Southward *et al.*, 1995).

1.2. Ocean acidification

Due to CO_2's high solubility in water, the ocean is the largest sink for anthropogenic CO_2 emissions (Bauer *et al.*, 2013). It is estimated to have absorbed from 20% to 30% of anthropogenic CO_2 emissions since the late 1980s, corresponding to a decrease in ocean pH of 0.1 units. According to the IPCC, this drop is expected to persist, with pH declining as far as an additional 0.4 units by 2100, under a fossil-fuel-based development and high emissions scenario (SSP5-8.5; Lee *et al.*, 2021; **Figure 2**).

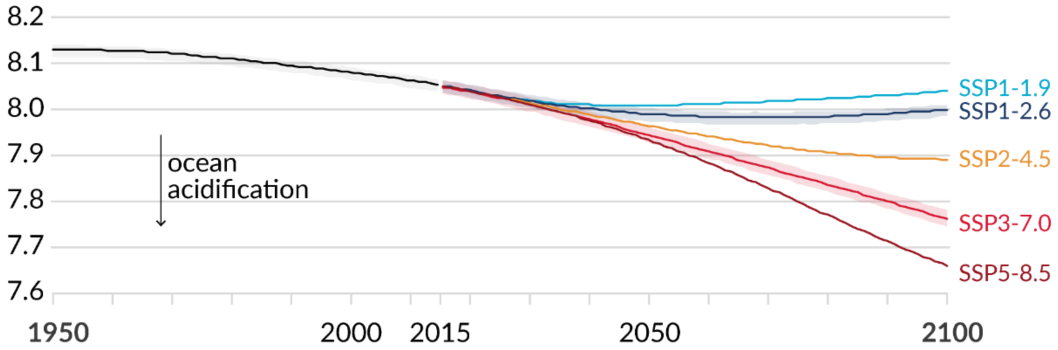

Figure 2 *- Global Ocean surface pH. Simulated historical changes since 1950 (black curve) and projected pH under five Shared Socioeconomic Pathways (in five colours). Shades represent uncertainty ranges. From IPCC (2021).*

Ocean acidification occurs as CO_2 dissolves in the water, forming sequentially carbonic acid (H_2CO_3), bicarbonate ions (HCO_3^-) and carbonate ions (CO_3^{2-}) while releasing a proton (H^+) in the course of each reaction, hence lowering pH in the upper ocean layers (**Figure 3**). These reactions are reversible and near equilibrium. Thus, with an increased input of CO_2, and consequent rise in bicarbonate and hydrogen ion concentrations, carbonate ion concentration declines (Doney *et al.*, 2009). Upon reaching the ocean's surface layer, atmospheric CO_2 dissolves and is transported to deeper waters by the ocean's currents. Surface layers are then available to take up CO_2 again, allowing for its accumulation in the ocean (Pörtner, 2008), and resulting in hypercapnic waters, *i.e.*, with increased CO_2 partial pressure (pCO_2). Concurrently, carbon is sequestered through photosynthesis and the building of calcareous structures by organisms, such that the decrease in pCO_2 allows for more CO_2 to be absorbed by the ocean, as per Henry's Law.

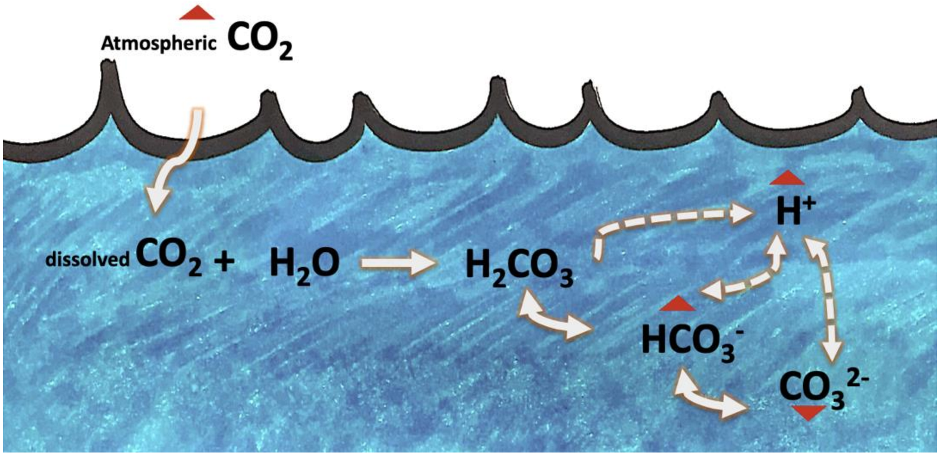

Figure 3 *– Chemical reactions following an increase in atmospheric carbon dioxide (CO_2), resulting in ocean acidification. Triangles represent a net increase (pointing upward) and decrease (pointing downward) in total molecule concentration.*

Ocean acidification has several detrimental effects on marine biota. With respect to negative effects, it compromises the ability of certain organisms to develop skeletons; namely corals, calcareous plankton and other calcium carbonate shell-forming groups (Caldeira & Wickett, 2003). This is a result of the decline in carbonate ions, which reduces the stability ("saturation state") of the mineral forms of calcium carbonate ($CaCO_3$) (Watson *et al.*, 2012). Furthermore, elevated CO_2 and subsequent acidification result in higher energetic costs for animals, as consumers require additional energy for acid-base regulation, ultimately affecting growth, survival and reproduction (Gaylord *et al*., 2015). However, severe repercussions of OA are expected to also alter species interactions, for example, through increased competition from organisms that are more resilient to or benefit from OA (Wootton *et al.*, 2008; Diaz-Pulido *et al.*, 2010). Indeed, many seagrasses and fleshy algae respond positively to hypercapnia. In addition to supplementing photosynthesis with CO_2, greater concentrations of this gas allow them to bypass mechanisms of bicarbonate conversion, thus leading to lower energy requirements (Gaylord *et al*., 2015). Nevertheless, organismic responses to OA are often species-specific and vary according to the environment. Species in naturally low pH environments, *e.g.*, hydrothermal vents, oxygen-minimum zones and upwelling sites; or environments with high pH spatial and temporal variability, such as coastal zones and shelf-seas, seem less affected by OA. Yet, as they are often near their biological limits, these species might be exposed sooner to harmful thresholds (Hennige *et al*., 2014).

1.3. Cephalopods

Cephalopods are molluscs from the class Cephalopoda, and comprise approximately 800 species of octopuses, squid, cuttlefish, nautiluses and vampire squid (Boyle & Rodhouse, 2005; Rodhouse *et al*., 2014). These abundant marine organisms are important partakers in trophic webs, as both prey and predators (Guerra, 2006). They exhibit fast growth rates and attain large body sizes, resulting in short life spans. Indeed, most cephalopods live from a few months to one year (Nixon &

Young, 2003). Due to their high metabolic requirements, they are prevailingly voracious predators, and display high activity levels. Although they can adopt a benthic lifestyle (*e.g.*, octopuses) or use undulatory fin movements to hover or move slowly, they also employ jet propulsion, an energetically inefficient method of rapid locomotion, during escape or prey capture attempts. To achieve this, water is expelled from the mantle cavity, propelling the animal in the opposite direction. Thus, in conjunction with their high life-histories plasticity, they are considered ecological opportunists (Rodhouse *et al*., 2014). For example, as fishing pressure increased on cephalopod and similar trophic level-fish stocks over a 25-year period and fifteen FAO areas, predatory fish stocks declined, whilst cephalopod stocks thrived (Caddy & Rodhouse, 1998), a phenomenon also observed by Doubleday *et al.* (2016). Contrarily to other molluscs, cephalopods are gonochoristic, *i.e.*, they present distinct sexes, and most known species are semelparous, as they die after the first reproduction (Nixon & Young, 2003; Boyle & Rodhouse, 2005). However, this group displays both r- and k-selected traits, producing from 2500 to hundreds of thousands of eggs depending on the species (Rodhouse *et al*., 2014).

Cephalopods possess characteristic features associated with various advanced behaviour patterns. The most prominent are their highly developed circumesophageal brain and highly sensitive visual system. Moreover, they have shown a capacity to learn from experience and to discriminate between forms (Nixon & Young, 2003). These features are conducive to an exceptional ability to camouflage, which is dependent upon effective and dynamic neural control and sensory information processing (Boyle & Rodhouse, 2005). It is a pivotal anti-predator strategy in an evolutionary arms race centred on the visual system (Hanlon *et al*., 2009). Oppositely to conspicuousness or signalling other animals, wherein the animal's body pattern diverges from its background (Zylinski *et al*., 2011), camouflage is the capability of an animal to blend in the environment and prevent detection from visual predators (Zylinski *et al*., 2011; Zylinski & Osorio, 2014). Coleoid cephalopods (octopuses, squid and cuttlefish), which are devoid of an external shell (Boyle & Rodhouse, 2005), are uniquely equipped to dynamically camouflage, as they control their skin coloration and texture neurologically, based solely on their vision (Hanlon & Messenger, 1988). Apart from exhibiting an impressive repertoire of skin patterns and a sophisticated visual system, this characteristic allows them to change skin patterning instantly (Hanlon & Messenger, 1988; - 2018).

1.4. Impact of climate on cephalopods

Most cephalopod species are stenohaline, hence sea surface salinity is considered a good predictor of many cephalopod species' distributions (Lauria *et al*., 2016). However, naturally occurring salinity levels do not seem to impact cephalopod survivorship, provided changes are gradual (Rodhouse *et al*., 2014). Similarly, sea surface temperature (SST) greatly influences distribution. As ectotherms, cephalopods are particularly vulnerable to changes in temperature. Whilst low temperatures decrease metabolism and survival rates, high temperatures cause marked mortality above the thermal optimum range, most likely due to the added difficulty of transporting oxygen to tissues and increased food requirements to sustain an accelerated metabolism (Brix *et al*., 1989). Caballero-Alfonso *et al*. (2010), for instance, found that octopus landings were negatively correlated with high SSTs. Concurrently, changes in dissolved CO_2 content and consequent OA might be detrimental to the survival of cephalopods, considering that CO_2 interferes with O_2 binding to the gills (Rodhouse *et al*., 2014). Further, there is evidence of synergistic effects between these stressors on marine organisms (Rosa & Seibel, 2008; Araújo *et al*., 2018). Despite this, cephalopods were observed by Doubleday *et al*. (2016) to have undergone a population expansion between 1953 and 2013, whilst most fish populations were declining. This phenomenon co-occurs with large-scale processes, such as overfishing- and climate change-driven competition and predation relaxation and anthropogenic climate change. Regarding the latter, the most likely change responsible for this growth is ocean warming, as it leads to faster growth rates and shorter life cycles, providing a competitive edge relative to longer-lived species.

Tolerance to climate-related changes is likely to differ between juveniles and adult cephalopods. On one hand, hatchlings must allocate considerable energy to rapid growth. On the other, hatchlings that have exhausted their maternal yolk supply must contend with high food intake requirements, as a result of a faster metabolism (Boyle & Boletski, 1996; Pimentel *et al*., 2012). Salinities in the ocean are relatively stable, however, fluctuations are frequent in cephalopod breeding grounds (*e.g*., coastal areas, sheltered bays). Although high salinities (40 ppt and higher) seem to impede hatching and low salinities (20 ppt and lower) to cause abnormalities in neritic newly-born cephalopods, their optimum salinity range is broad (> 15 ppt) (Nabhitabhata *et al*., 2001). Similarly, as hatchlings undergo an exponential growth phase, with higher metabolic rates than adults, they are more vulnerable to temperature fluctuations (Leporati *et al*., 2007). Cuttlefish embryonic stages were found to have lower survival rates and higher premature hatching under warming and acidification combined (AW; + 4ºC, Δ 0.5 pH, ~ 1600 μatm CO_2), as well as under warming (W) alone (*Sepia officinalis*; Rosa *et al*., 2013). Further, pre-hatching eggs displayed lower hypoxic thresholds when exposed to AW (Rosa *et al*., 2013).. In this instance, low O_2 partial pressures (pO_2) led to a limited oxygen supply, thereby lowering respiration rates below optimal rates and incurring sub-lethal effects on the organism (Roman et al., 2019). Concomitantly, high temperatures were found to accelerate octopus (*Octopus vulgaris*) embryo growth rates and decrease survival rates, as well as engender premature paralarvae, and small hatchling size (Repolho *et al*., 2014). Shortened embryonic periods caused by high temperatures were also found in the European squid (*Loligo vulgaris)*, in concert with premature hatching and a more frequent occurrence of malformations (Rosa *et al*., 2012; - 2014). However, A (acidification) was found to have no impacts on cuttlefish hatchlings' fitness (Rosa *et al*., 2013; Moura *et al*., 2019), with the exception of increased rates of calcification in the cuttlebone at 980 μatm (assuming cuttlefish are fed; Otjacques *et al*., 2020), or starting from a decrease of 0.25 pH units (Dorey *et al*., 2012) relative to current pH levels. With respect to behaviour, Moura *et al*. (2019) observed no effects of A on shelter-seeking, hunting or detection of conspecifics in cuttlefish hatchlings. In adult pygmy squid, however, elevated CO_2 resulted in increased activity levels and attraction and aggression toward conspecifics (Thomas *et al*., 2021). Concomitantly, prey attack latency of pygmy squid and bigfin reef squid was increased under A. To date, effects of warming on cephalopod behaviour are unknown, including in the European cuttlefish.

1.5. The European cuttlefish, *Sepia officinalis* Linnaeus, 1758

The European cuttlefish, *Sepia officinalis* Linnaeus, 1758, is a cephalopod mollusc of the Sepiidae family, and is one of the most studied cephalopods in the world, primarily due to its economic importance and ease of husbandry. In view of its relevance for world fisheries, aquaculture production of this species is seeing important advancements, despite the reliance of this species on live prey during its early life stages as a current drawback (Sykes *et al*., 2014). *S. officinalis'* distribution includes the Northeast Atlantic and the Mediterranean Sea. It is a nekto-benthic species, which can be encountered in the upper 200 m of the continental shelf (Reid *et al*., 2005). Apart from its characteristic seasonal migration between deep and shallow waters for reproduction (Guerra, 2006), this species exhibits nocturnal activity and performs daily vertical migrations (Webber *et al*., 2000). It is found camouflaged or buried in sandy or muddy bottoms during the day (Reid *et al*., 2005).

Similarly to most cephalopods, *S. officinalis* are highly adaptable to environmental conditions, considering their high fecundity and short life span (Guerra & Castro, 1988; Bettencourt & Guerra, 1999). Yet, as ectothermic organisms, cuttlefish growth rate is highly dependent upon temperature (Bettencourt & Guerra, 1999), in as much as their energy demand increases with activity and temperature, as does their need for oxygen and food supply (Brey, 2010). Spawning period in the Iberian Peninsula is biannual, occurring between early spring and late summer, and, to a lesser extent, in winter (Guerra, 2006). Newly-hatched cuttlefish, referred to as juveniles (Naef, 1928 *in* Robin *et al*., 2014), are morphologically and behaviourally similar to adults, and adopt a benthic lifestyle upon hatching (Young & Harman, 1988; Guerra, 2006).

1.6. Anxiety and camouflage in *Sepia officinalis*

Cuttlefish display an array of social, anti-predator, reproductive behaviours, among others. From a survivorship standpoint, camouflage is arguably the most relevant anti-predator behaviour (Hanlon *et al*., 2009), and putative anxiety provides a proxy for foraging and shelter-seeking activities (Hamilton *et al*., 2014).

Although proneness to anxiety has a strong genetic basis, interactions with conspecifics and environmental conditions are decisive in its development (Boissy, 1995). For example, repeated suffering is likely to result in suffering-avoidance behaviour, and therefore anxiety (Nettle & Bateson, 2012). Negative experiences (as early as late gestation) were found to lead to anxiety-like behaviour in rats, *i.e*., avoidance of novelty (Vallée *et al*., 1997). Anxiety is a generally unpleasant affective state, that is viewed by most evolutionary biologists as a necessary adaptive response that increases attention and prompts risk-assessment, in order to detect and avoid danger (Mendl *et al*., 2010). The 'smoke-detection principle' ascribes to this: the trade-off between risking death and losing a few calories in vigilance favours minimising false negatives, whilst accepting numerous false positives (Nettle & Bateson, 2012). However, if the threshold is set too low, anxiety implies unnecessary physiological stress (DeGrazia & Rowan, 1991; Barlow *et al*., 2015). Indeed, it is often considered a form of suffering or distress (Lang *et al*., 2000; Fiorito *et al*., 2015). Aside from being a source of suffering, excessive anxiety may affect survival, as it can hinder physiological functioning (McEwen, 2003) and inhibit exploratory behaviour (Pellow & File, 1986; Hamilton *et al*., 2014), potentially limiting foraging opportunities. Research on anxiety in invertebrates is still in its infancy. Anxiety in vertebrates is regulated by the HPA (hypothalamic–pituitary–adrenal gland) axis, which does not exist in invertebrates, and is tightly linked to the serotonin pathway, present in both groups (Curran & Chalasani, 2012). Anxiety-like behaviours, similar to those found in rats associated with the serotoninergic pathway have been reported in the nematode worm *Caenorhabditis elegans* (Dierick & Greenspan, 2007), in the fruit-fly *Drosophila melanogaster* (Mohammad *et al*., 2016) and in the crayfish *Procambarus clarkii* (Fossat *et al*., 2014). Although only representing a facet of anxiety, behaviours displayed by these animals, such as avoidance of light and aggression, are indicative of anxiety in vertebrates (Arrant *et al*., 2013; Hamilton *et al*., 2014; Patki *et al*., 2015). Squid have been shown to possess regions of the brain which are analogous to the hypothalamus in vertebrates, *i.e*., neurosecretory cells distributed across the buccal, sub-pedunculate, and in part of the supra-esophageal mass (Young, 1970), and the neuromodulator serotonin was reported in pygmy squid (Wollesen *et al*., 2010). Additionally, octopuses have been shown to experience pain (Crook, 2021), a negative affective response. *S. officinalis* was found to increase fin beats and ink ejection upon serotonin injection to the optic lobe (Chichery & Chichery, 1985). Although it can potentially experience anxiety, knowledge on affective responses of this species is lacking.

Contrarily to anxiety-related neurotransmitter pathways and behaviours, camouflage mechanisms were extensively studied. *S. officinalis* possesses motor nerves that extend directly from the brain to the chromatophore organs, which control pigmentary colours, thus preventing any synaptic delays. These organs are discrete sacs of pigment ringed with muscles that contract or expand to hide or expose the pigments. Pigmentary organs are further complemented by short wavelength colour-reflecting cells termed iridophores and leucophores (Hanlon & Messenger, 1988). Despite being colour-blind (Marshall & Messenger, 1996), cuttlefish are able to closely emulate background colours (Mäthger *et al*., 2008), perhaps aided by opsin proteins present in their skin, which detect light (Mäthger *et al*., 2010). The European cuttlefish is particularly responsive to edges and differences in contrast (Kelman *et al*., 2007; Zylinski *et al*., 2009a) and employs two main tactics to achieve camouflage. On one hand, it matches the background, *i.e*., resembles the colour, contrast and pattern of the background in order to hinder detection; and on the other, it disrupts the body outline by creating false edges to impede recognition (Hanlon & Messenger, 1988; Troscianko *et al*., 2017). Although this species displays a continuum of chromatic body patterns, three types are commonly recognizable based on the size of light and dark spots, namely: i) uniform (small spots), ii) mottle (medium-sized spots) and, iii) disruptive patterns (large spots, and other forms) (Hanlon & Messenger, 1988; Kelman *et al*., 2007; Merilaita *et al*., 2017) (**Figure 4**). The latter prevents recognition by creating the appearance of false edges and boundaries and hiding the animal's true outline and shape (Stevens & Merilaita, 2009). To produce these patterns, cuttlefish control the expression of approximately forty behavioural components (including chromatic, textural and postural components), independently from each other (Zylinski & Osorio, 2014).

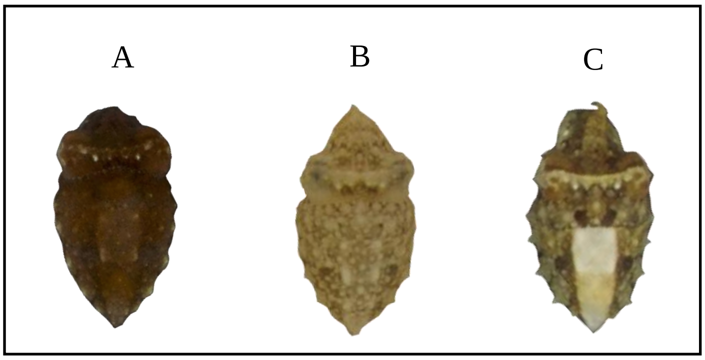

Figure 4 *– Cuttlefish body patterns commonly described as A) uniform, B) mottle and C) disruptive. Photo credits: Mélanie Marques*

Camouflage in juveniles is crucial for survival, as predation rates are high. Whilst adult cuttlefish use body patterning mostly in reproductive behaviours, juveniles use it primarily for concealment, wherein light mottle and disruptive patterns are most useful. Nevertheless, the brain regions responsible for body patterning are already well developed in hatchlings, and they are able to produce most patterns observed in adult individuals (Hanlon & Messenger, 1988).

1.7. Objectives

The aim of this dissertation was to evaluate the effect of end-of-the-century projected levels of ocean warming (+ 3ºC; SSP2-4.5) and acidification (980 µatm pCO_2; SSP3-7.0) on *S. officinalis* early development, and hatchlings' anxiety levels and ability to camouflage in different substrate complexities (sand and black and white gravel). More specifically, I investigated embryonic development time, hatching success (*i.e.*, survival rates), anxiety levels (namely time spent near a novel object and mobility) and ability to camouflage (latency, disruptiveness, background matching) under the different climate change-related treatments.

2. Materials and methods

2.1. Cuttlefish egg collection and husbandry

S. officinalis egg clutches were collected off Algés and Cascais, Portugal, by local fishermen, in May 2021 and transferred to the Guia Marine Laboratory (Cascais). The eggs were randomly placed in two recirculating aquaria systems, each comprising two water-baths (four water-baths in total) and a sump (**Figure 5**). Each system, corresponding to one treatment, contained four 9-liter plastic tanks (replicates), each connected to the bath through two small meshes, and receiving water directly from the sump. The eggs (248) were randomly distributed between treatments, acclimated during three days at control conditions (current annual mean Portuguese seawater conditions) and reared at:

a) control: 18ºC, pCO_2 = 420 µatm (20 eggs *per* replicate);

b) warming: 21ºC, pCO_2 = 420 µatm (20 eggs *per* replicate);

c) acidification: 18ºC, pCO_2 = 980 µatm (20 eggs *per* replicate);

d) warming and acidification combined: 21ºC, pCO_2 = ~ 980 µatm (22 eggs *per* replicate).

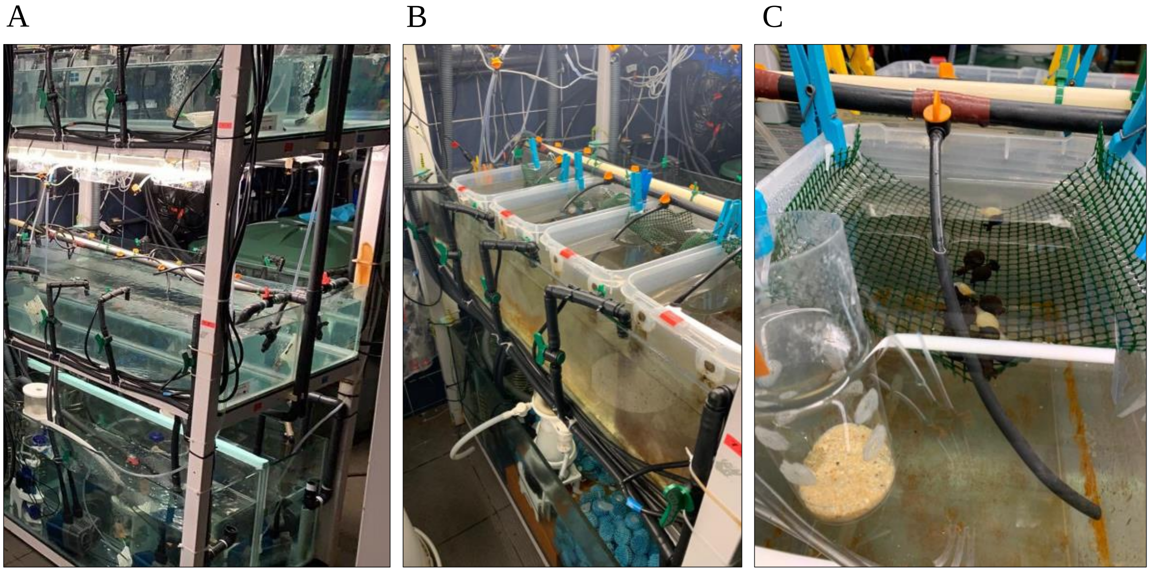

Figure 5 *– A) Recirculating system comprising two aquaria with two water-baths and a sump each. B) Four tanks in a bath (corresponding to a treatment). C) Cuttlefish eggs in a suspended net and cup containing a newborn cuttlefish*

Water was pumped directly from the sea and filtered through a 1-μm mesh and sterilized by a 12-W UV-sterilizer (Vecton 120 Nano, TMC-Iberia, Portugal). Water was continuously renewed with a water drip system in each bath. Each system was connected to a 270-L sump by 50-W pumps (TMC, V2 Power Pump, 3000 L h^{-1}), containing a protein skimmer (ReefSkimPro 400, TMC-Iberia, Portugal) and bioballs (ouriço®, Fernando Ribeiro Lda, Portugal). Additionally, one 35-W pump (TMC, V2 Power Pump, 2150 L h^{-1}) was connected to each bath, at 288-390 mL min^{-1} flow rate in each tank (renewal every ~ 30 min). The tanks were illuminated from above with LED, 8-W lights, in accordance with a photoperiod of 14 h light to 10 h dark. Water was kept oxygenated by means of eight air stones, two in each sump, and one in each bath connected to an air compressor (Medo Blower LA-120A, Nitto Kohki, Japan).

Temperature was kept through a temperature controller (XH-W3002, accuracy ± 0.1ºC, hysteresis 0.3ºC) connected to 150 W water heaters (Eheim GmbH & Co KG, Germany) and a water chiller (Hailea HC-150A). *Via* solenoid valves, pH was adjusted automatically, regulated by a Profilux controlling system (3N GHL, Kaiserslautern, Germany) connected to two pH probes (VWR double junction epoxy BNC, Germany, hysteresis 0.05). pH values were read every 2 s and downregulated by injection of a certified CO_2 gas mixture (Air Liquide, Portugal) through air stones, or upregulated by aerating the tanks with CO_2-filtered air, using soda lime (Sigma-Aldrich). Alkalinity was tested thrice a week with a digital titrator (Sulfuric Acid 0.1600N; Hach, USA) in order to adjust the pH corresponding to 980 µatm of pCO_2 through the CO2SYS Program 01.05 (Lewis & Wallace, 1998).

Salinity (Hanna refractometer, accuracy ± 1 PSU), oxygen levels and temperature (oximeter VWR DO220, accuracy ± 1.5%, ± 0.3ºC respectively), and pH (pHmeter VWR pHenomenal, accuracy ± 0.005) were monitored daily (**Table 1**). Ammonia/ammonium, nitrite and nitrate levels were be monitored every week through saltwater colorimetric tests (TropicMarin, Hünenberg, Switzerland) and maintained below 0.02 mg L^{-1} (nitrites and ammonia/ammonium; accuracies ± 0.02 mg L^{-1} and ± 0.03 mg L^{-1}, respectively) and 0.5 mg L^{-1} (nitrates; accuracy ± 0.5 mg L^{-1}).

***Table 1** – Seawater (SW) parameter values measured daily in each tank during exposure to treatments, shown as mean ± standard deviation. TA – Total Alkalinity, TCO_2 – Total CO_2, HCO_3 - Bicarbonate, ΩAr – Aragonite saturation state*

Parameters	Control	Acidification	Warming	Combined
Temperature	18.2 ± 0.13	17.8 ± 0.07	20.8 ± 0.07	20.8 ± 0.08
pH	8.152 ± 0.08	7.726 ± 0.08	8.095 ± 0.06	7.737 ± 0.04
Salinity	34.0 ± 0.47	34.2 ± 0.46	33.7 ± 0.53	33.9 ± 0.74
O2 (mg L^{-1})	8.09 ± 0.10	8.10 ± 0.08	7.75 ± 0.11	7.71 ± 0.14
O2 (%)	105.9 ± 1.3	105.1 ± 1.0	106.4 ± 1.5	105.7 ± 1.8
pCO_2 (µatm)	321 ± 91	973 ± 165	374 ± 88	953 ± 106
TA (µmol/kgSW)	2457 ± 224	2460 ± 232	2454 ± 214	2472 ± 235
TCO_2 (µmol /kgSW)	2139 ± 210	2351 ± 211	2151 ± 206	2344 ± 219
HCO_3 (µmol /kgSW)	1899 ± 199	2217 ± 195	1919 ± 195	2201 ± 202
ΩAr	3.57 ± 0.62	1.55 ± 0.35	3.47 ± 0.42	1.75 ± 0.28

Mantle length was inferred from novel object test videos in ImageJ 1.46r, compared to a two-cent coin. Hatching success and development duration were registered upon hatching. To discern individual specimens, newly-hatched cuttlefish were placed in plastic cups (labelled with cuttlefish number, treatment, replicate tank, and hatching date) with eight mesh-covered openings for water circulation, within their rearing tank.

2.2. Anxiety

From two to five days post-hatching, 180 cuttlefish (45 per treatment), were placed with a black spoon in a white circular arena (12 cm diameter), with a purple bottle cap in the centre (novel object), previously filled with 400 mL of water from the respective treatment. Black flaps were used to surround the arena and prevent cuttlefish from seeing the observer. Light was directed above and reflected throughout the chamber with white styrofoam, to diffuse it. A video camera (Canon, LEGRIA HF R56, 35 Mbps) recorded the arena from above at a *ca*. 90º angle for 20 minutes upon placing the cuttlefish in the arena (**Figure 6**).

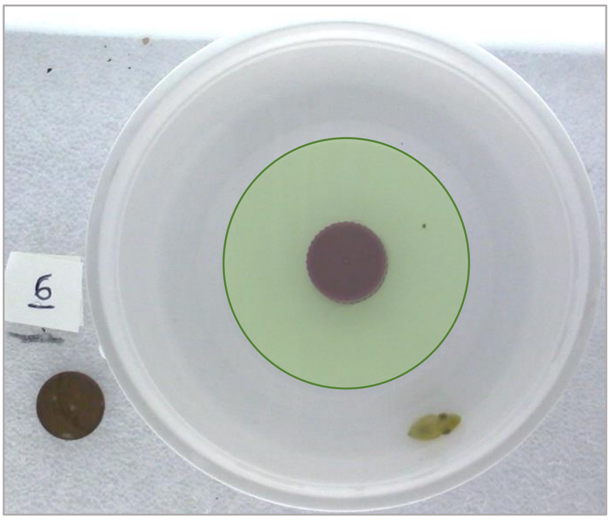

Figure 6 *– Photograph from a novel object test video frame. The purple cap is the novel object, and the green circle delineates the region of interest (in proximity to the object and slightly elevated)*

From video recordings, cuttlefish movements were tracked using the animal tracking software ToxTrac v2.61 (see Rodriguez *et al*., 2018), *via* the algorithm ToxId (Rodriguez *et al*., 2017). To assess anxiety, average acceleration, immobility rate and duration of staying near the object (time spent in the region of interest – a circle [7 cm diameter] that is slightly elevated, thus disincentivizing exploration behaviour), were extracted from videos. The detection rate (proportion of the video recording wherein the animal was detected by the software) was also registered. Video contrast was augmented beforehand by 130-150%. Results were considered exclusively when detection rates exceeded 90%.

2.3. Camouflage

From two to five days post-hatching, and at least five hours after the anxiety trial, 160 cuttlefish (40 per treatment) were placed, with the aid of a black spoon, in a white circular arena (12 cm diameter), with the bottom covered in either sand, to evoke a mottle pattern in the cuttlefish, or a gravel mixture of 60% black, 40% white (FishPlanet, Portugal), to evoke a disruptive pattern. Individual gravel area ranged from approximately 100% to 200% of the area of the cuttlefish's dorsal square – a light region expressed under the disruptive pattern in the centre of the mantle. The order of presentation of substrate patterns alternated between each trial, *i.e.*, if a cuttlefish underwent the camouflage trial in sand first, and gravel immediately after, the following cuttlefish was placed in gravel first, then sand. Cuttlefish were attributed a random number displayed in videos, in order to avoid observer bias in data analysis. The arenas were filled with water (400 mL) from the corresponding treatment and renewed between each trial.

A video camera (GoPro) recorded the arena for ten minutes after acclimation (considered when the cuttlefish remained more than five seconds stationary), to register attempts at burying in the substrate. Concomitantly, photographs (Canon PowerShot G7X Mark II, white balance-calibrated, shutter speed 1/15, F-stop f/11, ISO 250, 1080p, 60 fps) were taken remotely (Canon Connect application for mobile phones) at a ~ 90º angle (Zylinski *et al.*, 2011), whenever cuttlefish changed camouflage pattern or intensified the present pattern (**Figure 7**).

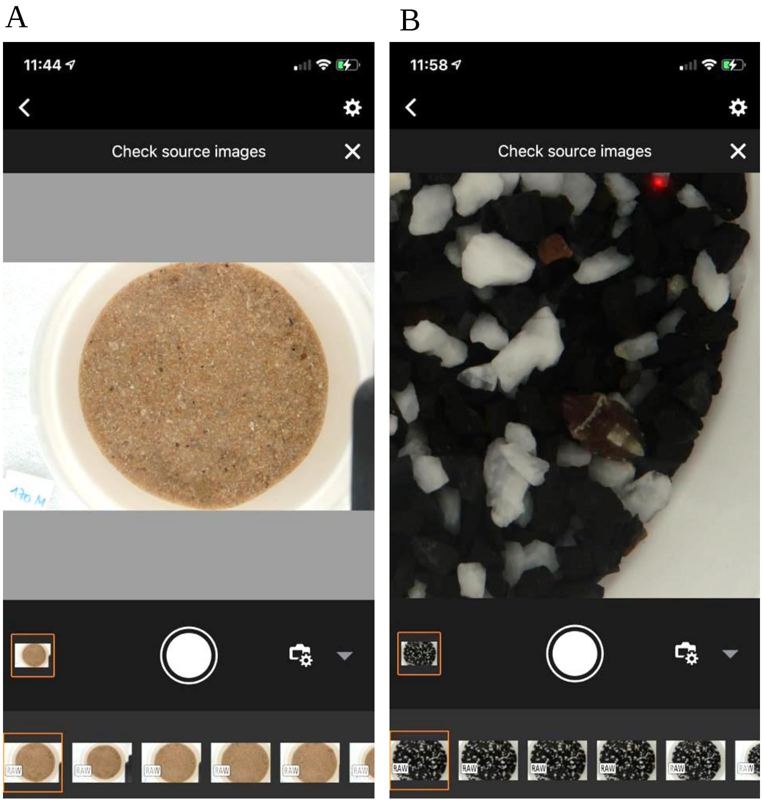

Figure 7 *– Photograph taken remotely from phone through the Canon Connect application in A) sand substrate and B) with zoom in gravel substrate*

To assess the ability to camouflage, latency to camouflage, camouflage scores, disruptivess and matching to the background were measured. Time past acclimation was registered, with the aid of a chronometer, upon taking each photograph. Camouflage latency was assumed as the time, following acclimation until the photo was taken - wherein cuttlefish camouflage was best suited to the environment (in sand, strong mottle; in gravel, dark uniform or strong disruptive pattern) (**Appendix A**). Due to latency data being highly zero-inflated, it was transformed to binary data (immediate – from 0 to 30 seconds – and delayed camouflage – upwards of 30 seconds). Further, difference in pixel values (grayscale) in the frontal and transversal body planes (**Figure 8**), and difference in pixel integrated densities (grayscale) between the cuttlefish's light region (dorsal square) and white substrate and dark region and black substrate (preference was given to gravel within the cuttlefish's field of vision) were extracted from photographs taken in gravel substrate through ImageJ (National Institute of Health, USA). Photographs were previously converted from CR2 to TIFF format. Finally, camouflage scores ranging 0 - 2 were attributed to cuttlefish in both substrates. In sand, score 0 represented animals in dark colours, which highly contrasted with the substrate. Score 1 was given to cuttlefish upon displaying uniform or weak mottle body patterns, distinguishing them from sand particles. Animals indistinguishable from the background were attributed score 2 (a strong mottle is required, with textured skin). In black and white gravel, score 0 corresponded to light colours in the entire body. Score 1 was given to weak disruptive body patterns, or dark uniform with a different colour from black gravel, that contrasted with the background. Score 2 was attributed to cuttlefish displaying a strong disruptive or dark uniform with similar colour to background gravel.

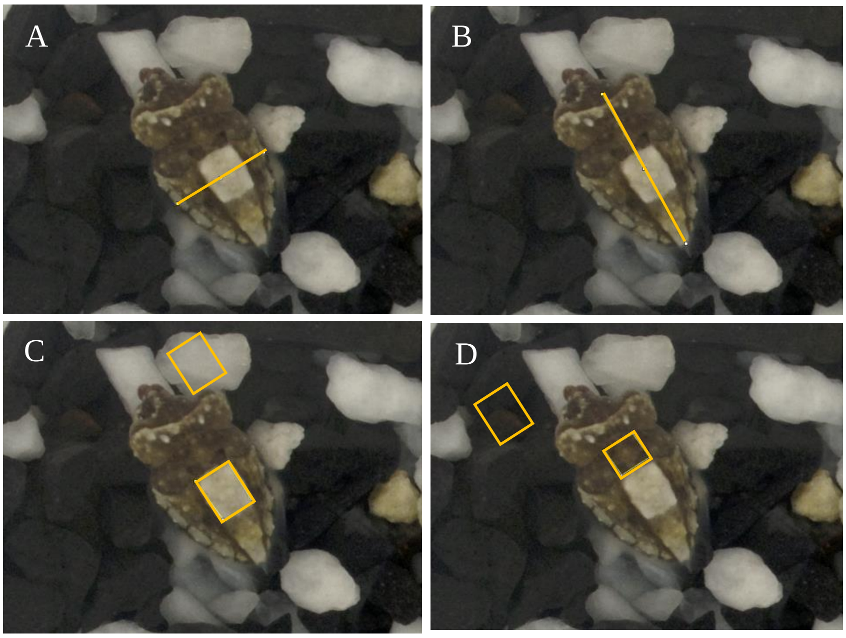

Figure 8 *– A) Cuttlefish transversal plane and B) frontal plane used to measure pixel intensities. Comparison of pixel integrated densities between C) white substrate and cuttlefish light region and D) black substrate and cuttlefish dark region in ImageJ software*

2.4. Data analysis

To test whether warming and acidification affect cuttlefish development time and size, linear models (LM) were fitted to development time and mantle length data, with treatment as four-level factor. Beta regressions (GLM, log link function; percentage of time in proximity of the object, immobility rate) and a LM (average acceleration) were fitted to anxiety data. The stepAIC (using the Akaike Information Criterion) function was used to determine whether the replicate, detection rate and mantle length influenced the response variables. LM were fitted to assess pixel value differences between treatments (in the cuttlefish's body planes and in comparison with the background). Generalized linear models (GLM) from the families Binomial (logit link function; latency to camouflage, time of acclimation, burial in sand) and Poisson (log link function, camouflage scores) were fitted. The influence of replicates, first substrate presented, cuttlefish mantle length and time elapsed between trials, was tested through the stepAIC function for each response variable.

All GLM assumptions (independence, normality and homoscedasticity of residuals) were tested. Type II Wald chi-squared tests were performed before each analysis to assess the influence of explanatory variables (treatment, first substrate presented, and detection rate). In order to avoid type I errors, p-values were adjusted through Tukey corrections. The admissible error was set at 0.05. Analyses were carried out in the RStudio, PBC, software.

3. Results

3.1. Development time and survival

Cuttlefish exhibited a prolonged embryogenesis under Acidification (A; ~ 50 days) relative to control conditions (~ 46 days; LM, z = 4.13, df = 203, p-value < 0.01). On the other hand, Warming (W) caused cuttlefish to hatch sooner (~ 35 days) (z = 8.40, p-value < 0.01). Such effect was reduced under Acidification and Warming combined (AW; ~37 days; z = -6.90, p-value < 0.01). Whilst W did not affect hatching success (98.8% in both control and warming conditions), A slightly lowered it (95%). Yet, the AW treatment dropped hatching success up to 72.7%. AW-exposed cuttlefish had reduced the mantle compared with W-exposed cuttlefish (LM, z = -3.33, df = 167, p-value < 0.01) (**Table 2**, **Appendix B**).

Table 2 *– Cuttlefish physical and physiological parameters after exposure to treatments. Mantle length was measured 2-5 days post-hatching. Values shown as mean ± standard deviation.*

	Control	**Acidification**	**Warming**	**Combined**
Sample size	35	26	36	39
Embryogenesis duration (days)	45.5 ± 3.7	50.2 ± 7.7	35.2 ± 7.8	37.2 ± 3.4
Hatching success (%)	98.8	95.0	98.8	72.7
Mantle length (mm)	7.3 ± 0.7	7.3 ± 0.7	7.7 ± 0.7	7.2 ± 0.6

3.2. Anxiety

No significant differences on average acceleration (Wald chi-squared test, df = 3, $\chi^2 = 1.35$, p > 0.05) and time spent immobile (Wald chi-squared test, df = 3, $\chi^2 = 3.85$, p > 0.05) were observed among treatments (**Figure 9**). The average acceleration was reduced by lower detection rates, which span 90-100% (Wald chi-squared test, df = 1, $\chi^2 = 31.13$, p < 0.01).

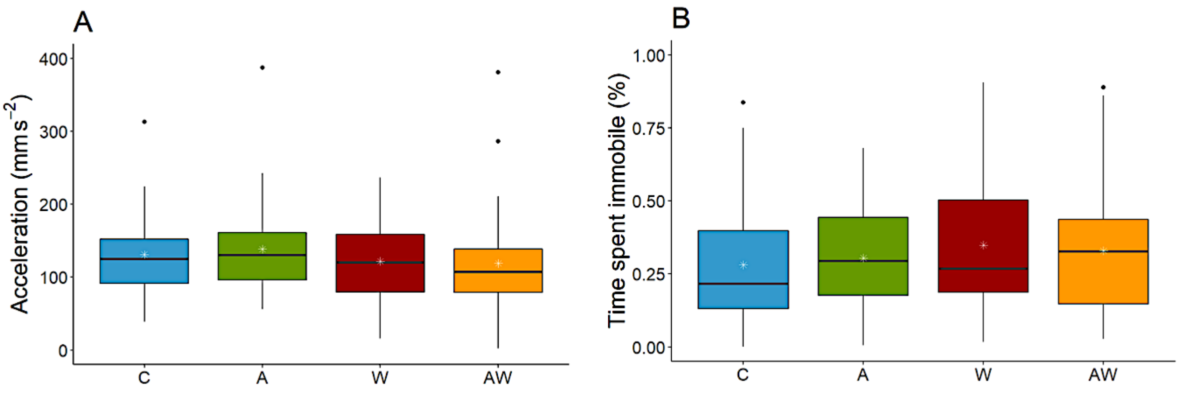

Figure 9 *– A) Average acceleration and B) percentage of time spent immobile of cuttlefish in the novel object test with relation to the different treatments: C (control), A (acidification), W (warming) and AW (acidification and warming combined). Whiskers represent the range from 25th to 75th percentiles, with the median as the inside bar. Outliers are shown as points, and the mean is represented by asterisks*

Moreover, the time spent near the object (region of interest) was also not affected by the different treatments (Wald chi-squared test, df = 3, $\chi^2 = 3.18$, $p > 0.05$) (**Figure 10**).

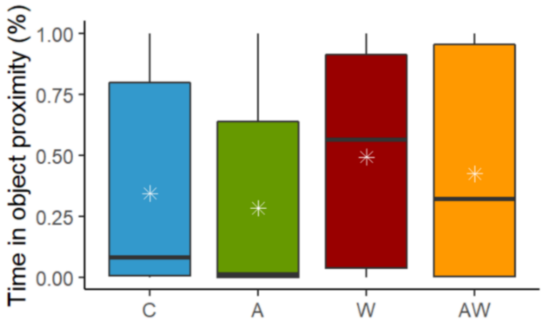

Figure 10 *– Percentage of time cuttlefish spent near the novel object relative to the treatments: C (control), A (acidification), W (warming) and AW (acidification and warming combined). Whiskers represent the range from 25th to 75th percentiles, with the median as the inside bar. The mean is represented by asterisks*

3.3. Camouflage

Time of acclimation did not vary significantly among treatments (Wald chi-squared test, df = 3; sand, $\chi^2 = 2.59$, $p > 0.05$; gravel, $\chi^2 = 1.03$, $p > 0.05$), nor did attempts at burying in the sand (Wald chi-squared test, df = 3, $\chi^2 = 1.91$, $p > 0.05$). Latency to maximum camouflage on the sand substrate also did not change among treatments (Wald chi-squared test, df = 3, $\chi^2 = 1.06$, $p > 0.05$; **Figure 11**). Yet, fewer cuttlefish camouflaged immediately upon acclimation in gravel under AW relative to W (binomial regression, z = 2.60, df = 167, p-value = 0.046). More cuttlefishes were able to camouflage immediately in sand (52%) than in gravel (40%). Camouflage in gravel was delayed when the gravel substrate was presented first (Wald chi-squared test, df = 1, $\chi^2 = 8.72$, $p < 0.01$) (**Appendix C**).

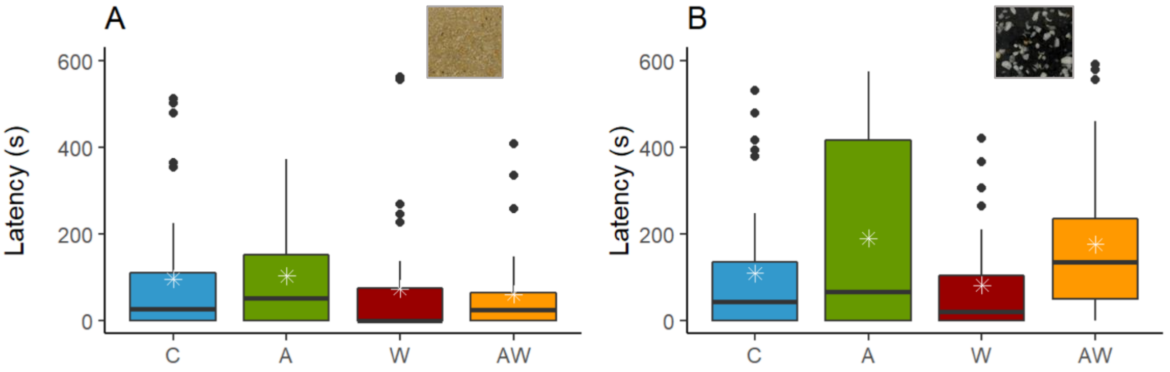

Figure 11 *– Latency to camouflage in A) sand and B) gravel substrates, with relation to different treatments: C (control), A (acidification), W (warming) and AW (acidification and warming combined). Whiskers represent the range from 25th to 75th percentiles, with the median as the inside bar. Outliers are shown as points, and the mean is represented by asterisks*

With respect to body planes pixel intensity differences in gravel, both W (LM, z = -3.48, df = 134, p-value < 0.01) and AW (LM, z = 2.73, df = 134, p-value = 0.032) treatments significantly enhanced body pattern contrast (**Figure 12**). Moreover, body contrast was reduced when gravel was presented first (Wald chi-squared test, df = 1, $\chi^2 = 8.72$, p = 0.003).

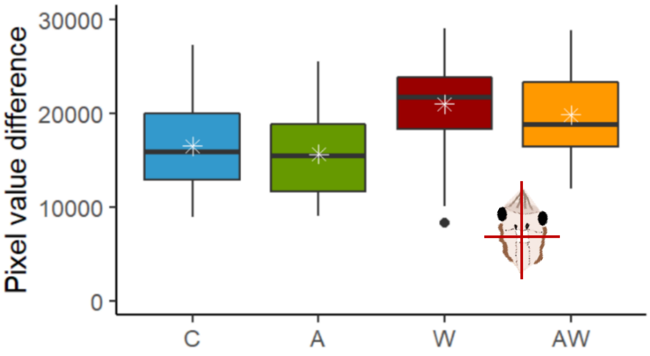

Figure 12 *– Body planes total pixel intensity difference (grayscale units) of cuttlefish exposed to treatments C (control), A (acidification), W (warming) and AW (acidification and warming combined. Whiskers represent the range from 25th to 75th percentiles, with the median as the inside bar. Outliers are shown as points, and the mean is represented by asterisks*

Treatments did not affect the difference between the cuttlefish's dark region and black gravel integrated pixel intensities (Wald chi-squared test, df = 3, $\chi^2 = 4.92$, p > 0.05) nor the difference between the cuttlefish's light region (dorsal square) and white gravel integrated pixel intensities (Wald chi-squared test, df = 3, $\chi^2 = 2.01$, p > 0.05) (**Figure 13**). Pixel integrated density of the dark region did not fall lower than that of black gravel.

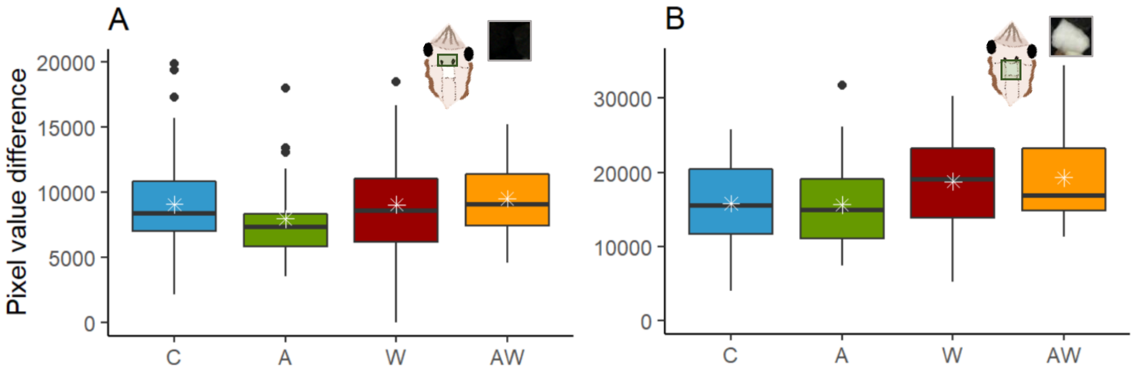

***Figure 13** – Difference of pixel values range (grayscale units) between A) black gravel and the cuttlefish's dark region and B) white gravel and the cuttlefish's light region (dorsal square) of cuttlefish, with relation to treatments: C (control), A (acidification), W (warming) and AW (acidification and warming combined). Whiskers represent the range from 25th to 75th percentiles, with the median as the inside bar. Outliers are shown as points, and the mean is represented by asterisks. Scales differ between plots*

Camouflage score did not change among treatments in sand (Wald chi-squared test, df = 3, $\chi^2 = 0.55$, $p > 0.05$) and in gravel (Wald chi-squared test, df = 3, $\chi^2 = 0.37$, $p > 0.05$). Cuttlefish presented a higher percentage of Score 2 camouflage and lower percentage of Score 0 in sand (81% and 0%, respectively) than in gravel (16%, 0.13%, respectively) (**Figure 14**). In sand, cuttlefish exposed to W and AW exhibited a slightly lower average and higher percentage of Score 1. However, in gravel, A resulted in a higher percentage of Score 0 cuttlefish camouflage.

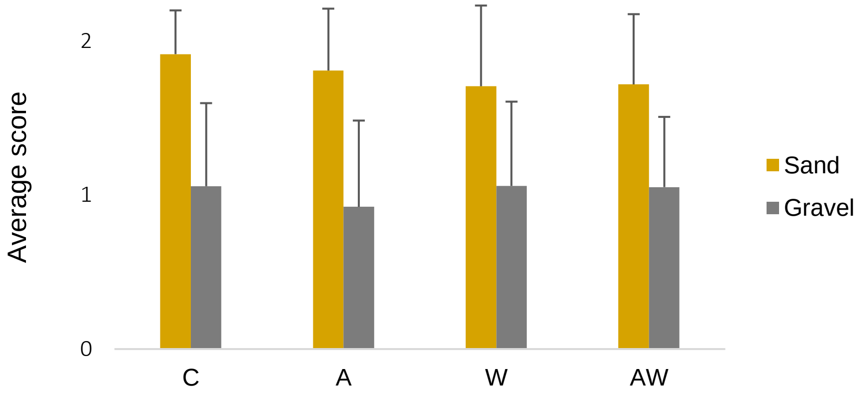

***Figure 14** – Average camouflage scores (0 – the animal is easily distinguishable from the background; 1 – the animal attempts to blend in to the background, but is still distinguishable to the naked eye; 2 – the animal is indistinguishable from the background) for each treatment – C (control), A (acidification), W (warming) and AW (acidification and warming combined) – attributed to cuttlefish in sand (yellow) and black and white gravel (grey)*

4. Discussion

4.1. Development time and survival

As previously reported (Dorey *et al.*, 2012; Rosa *et al.*, 2013; Moura *et al.*, 2019) for lower pH (Δ pH 0.4, 0.5, and 0.4, respectively), acidification had no relevant effect on hatching success in *S. officinalis* hatchlings. CO_2 partial pressures can be three times higher within the egg than surrounding SW pCO_2 (Dorey *et al.*, 2012; Rosa *et al.*, 2013), making them uniquely prepared for projected levels of OA. Moreover, cuttlefish are active swimmers, and consequently need an efficient ion transport system to maintain a stable blood pH during exercise, in order to cope with their own respiratory CO_2 (Gutowska *et al.*, 2008). Indeed, contrarily to most marine invertebrates, ectotherms with high metabolic rates, such as teleosts and cephalopods, are able to garner soft tissue mass and calcify under hypercapnia (Moura *et al.*, 2019). Additionally, as observed by Moura *et al.* (2019) exposure to A during embryogenesis was found to have no effect on the size of newly-hatched cuttlefish. Similarly to squid under Δ pH 0.4 (Rosa *et al.*, 2014) and cuttlefish under 980 µatm pCO_2 (Otjacques *et al.*, 2020), our findings indicate that acidification prolongs embryogenesis. Thus, A may pose a physiological burden on cuttlefish hatchlings, if paired with a downregulation of regulatory and metabolic genes (Hu *et al.*, 2011).

Warming, on the other hand, led to shortened development times, as a result of higher energy expenditures and turnover (Rosa *et al.*, 2012; Rosa *et al.*, 2013). Yet, acidification and warming were found to have an interacting effect on hatching success, reducing it substantially. This is corroborated by Rosa *et al.*, (2013), with more extreme conditions (+ 4ºC, Δ pH 0.5). However, Dorey *et al.* (2012) found no effect (+ 3ºC, Δ pH 0.4), which might suggest intraspecific differences in heat and hypercapnia tolerances associated with local adaptation. The combined treatment (AW) did not affect cuttlefish size (mantle length) at hatching, possibly due to the existence of an antagonistic effect between stressors, or because cuttlefish have developed mechanisms to temporarily cope with these stressors, such as improved systemic oxygen delivery through cellular and mitochondrial regulation (Oellermann *et al.*, 2012). Interestingly, AW-exposed cuttlefish displayed intermediate development durations between A and W, suggesting that acidification might reduce the negative effects caused by warming.

4.2. Anxiety

Average acceleration and immobility rate were not affected by climate change-related stressors (W, A and combined). Accordingly, Maneja *et al.* (2011) found that cuttlefish's ability to capture prey was only affected at 4000 µatm pCO_2, a very distant value from projected levels for the end of the century. Other pre-natal sources of stress, such as predator cues, were found to have no effect on locomotor activity (Lee *et al.*, 2020). Defensive behaviours, *e.g.*, approaching, retreating and inking, in the presence of a predator, were not influenced by embryonic exposure to predator cues in *S. pharaonis* and *S. officinalis* (Lee *et al.*, 2020). However, O'Brien *et al.* (2017) observed that embryonic stress (odour cues from predator and artificial lighting) increased attempts at capturing prey. According to these authors, locomotor activity levels did not change as a result of embryonic stress, but increased from maternal stress (daily removal of the reproducing mother from water). Arguably, in many species, activity levels can be considered a proxy for affective states, particularly anxiety. Notwithstanding, a shift in activity levels would likely impact survivorship. As per the threat-sensitive predator-avoidance hypothesis (Helfman, 1989), animals exhibit antipredator behaviour proportionately to the perceived threat from a predator. Indeed, antipredator behaviours are costly, as they reduce foraging and mating opportunities. Animals which can accurately assess predation risk, and adjust their behaviour accordingly, have therefore a better chance at survival.

Exploration-avoidance behaviour is the spatial exploration of novel situations. It can be reliably assessed through novel object tests (Takola *et al*., 2021). In the present case, time in proximity of the novel object was not affected by the treatments. This was also expected, as A did not affect shelter-seeking and hunting behaviours (Moura *et al*., 2019). Although not tested here, differences in behaviour among cuttlefish are probably attributable to temperament, *i.e*., individual behavioural differences which are repeatable over time and across situations (Réale *et al*., 2007), implying phenotypic plasticity. Whilst some cuttlefish spent most time immobile, on top of the novel object, others exhibited bursts of speed, high mobility and distancing from the object.

Cuttlefish hatchlings' exploration behaviours and mobility are not altered by A or W under the novel-object test. This suggests that cuttlefish are unlikely to feel higher levels of anxiety under these climate change-driven stressors. Such results could be explained by frequent exposure to extreme conditions, characteristic of coastal environments, paired with a broad phenotypic plasticity. However, the effects of prolonged exposure into adulthood to these stressors is unknown.

4.3. Camouflage

Cuttlefish is well adapted to sandy substrates, as it camouflaged more promptly in this substrate independently of the different climate change-related treatments. Moreover, hatchlings obtained higher camouflage scores in sand. In addition to chromatic patterns, texture patterns are equally necessary to match a sand background. Cuttlefish which did not exhibit skin texture, through the expression of major lateral papillae, *i.e*., protrusions borne of muscle expansions (Hanlon & Messenger, 2018), could not successfully blend in in sand. Furthermore, cuttlefish seem to perform more poorly if they are first presented with another substrate. Indeed, camouflage was delayed, body contrast in gravel was reduced, and cuttlefish displayed darker colours when gravel was the first substrate presented to cuttlefish. A possible reason for this is increased stress at being in a new environment where burial was less facilitated. Allen et *al.* (2010), for instance, found that *S. officinalis* show no preference for a particular substrate, save when they can bury themselves.

In white and black gravel, a more complex substrate, the combined effect of A and W delayed cuttlefish camouflage relative to W *per se*. The optic lobe undergoes rapid development during embryogenesis, specifically structural components of the cortex and radial column zone, which are responsible for the processing of visual information (Liu *et al*., 2017). Increased latency to maximum camouflage might indicate that this development was compromised, due to an exacerbation of extreme conditions inside the egg (high $p\text{CO}_2$, low O_2), especially in late embryogenesis. A possible ensuing metabolic depression, which can occur under hypercapnia, reduces protein synthesis and therefore growth (Rosa *et al*., 2013), at a time where cuttlefish are developing brain functions crucial for survival. Further, colour change is likely to entail energetic or metabolic costs related to the synthesis of pigments and cells, or changes in the state of chromatophore cells (Stuart-Fox & Moussalli, 2009). Therefore, these processes might be impeded by the existing higher energy expenditure caused by warming. For example, guppies (*Poecilia reticulata*) were found to increase food consumption following colour change, and avoid further colour changes (Rodgers *et al*., 2013). Nevertheless, the duration of cuttlefish acclimation did not differ between treatments. Higher acclimation times could lessen survivorship, considering that it is almost impossible to achieve crypsis during motion (Zylinski *et al*., 2009b).

Contrast in disruptive patterning is another indicator of camouflage performance, considering that cuttlefish adjust their contrast proportionately to the contrast of the background (Buresch *et al*., 2015). Interestingly, W, and, to a lesser extent, AW, enhanced body pattern contrast along the frontal and transversal body planes. Cuttlefish seemingly camouflage better when exposed to a stressor during embryonic development, or even when their progenitor is subjected to stress. Embryonic exposure to predator chemical cues led to strengthened disruptive patterns in *S. officinalis* (Lee *et al*., 2020). Furthermore, maternal stress (daily removal of the reproducing female from the water) was found to increase disruption in offspring (O'Brien *et al*., 2017). However, these cuttlefish did not show improved background matching. This could be due to cuttlefish resorting to dark uniform patterns instead of the expected disruptive pattern in black and white gravel. It is important to note that gravel size was not controlled (cuttlefish only produce disruptive patterns if white gravel measures around 40% to 120% of their dorsal square; Barbosa *et al*., 2007).

Given that camouflage in cuttlefish hatchlings is primarily a defense strategy, camouflage performance must be evaluated from the perspective of relevant predators (Merilaita *et al*., 2017). Putative cuttlefish predators (di- and tri-chromatic fish) have been shown to rely minimally on colour (Chiao *et al*., 2011), which is why grayscale is considered an adequate measure of camouflage efficiency. Yet, cuttlefish are well blended with the background when displaying a uniform dark colour in black and white gravel. To account for this, camouflage scores were attributed to cuttlefish, albeit from a human observer perspective. Camouflage scores consider luminance and colour, which are viewed from a human's perspective, and do not consider the environments cuttlefish are used to, greater turbidity, greater depths, distance from predators. Although there was no significant relationship, W- and AW-exposed cuttlefish exhibited a slightly lower score average than control in sand. Expectedly, this did not occur in gravel, as disruptiveness was increased in this substrate.

If climate change were to considerably hamper camouflage, cuttlefish would lose their primary defense strategy against predators. Although cuttlefish hatchlings take longer to achieve optimum camouflage under A and W combined, disruptiveness is enhanced under W. This denotes some capacity to withstand and adapt to change, similarly to adaptations to exposure to predators. *S. officinalis* juveniles have shown adaptability to artificial stressors (*e.g*., LED lighting), which have never been encountered by the species in its natural environment (O'Brien *et al*., 2017). Therefore, cuttlefish prospects of adapting to climate change stressors are promising.

4.4. Final considerations

Cuttlefish seem to be particularly resilient to acidification, a stressor that solely prolonged embryogenesis. However, warming had direct impacts on cuttlefish growth, leading to shortened development times and higher mortality. The combined effect of these stressors markedly reduced hatching success, denoting an interacting effect. No effect of acidification or warming on hatchlings' anxiety (exploration behaviour and activity level) was identified in this study. Conversely, disruptive camouflage was enhanced under warming, a finding also reflected in other type of stressors, such as predator cues (Lee *et al*., 2020) or maternal stress (O'Brien *et al*., 2017). Maximum disruptive camouflage, however, was delayed by acidification and warming combined in relation to warming alone, suggesting an underlying physiological or developmental burden of camouflage.

The present findings increase our understanding on the biological impacts of these climate change-related stressors on cephalopods. However, impacts of deoxygenation, hypoxia, and acute events such as marine heatwaves merit further investigation. Concurrently, as shown by O'Brien *et al*. (2017), maternal stressors have a larger impact on post-natal behaviours than direct embryonic stress. Thus, studying the impacts of combined climate change stressors on reproductive females and their offspring might provide valuable insights into an integrated climate change response. Furthermore, these impacts on gametes, zygotes and early development should also be understood, as these stages lack the specialized ion-regulatory epithelia that enables resilience to ocean acidification (Melzner *et al*., 2009).

Hilborn *et al.* (2020) suggest that fisheries lacking local management are at a greater risk of excessive harvesting and declining populations. Monitoring cuttlefish populations, *e.g*., through partnerships with fishermen, can therefore be crucial for the effective protection and fishing of these, and even of other marine animals. Moreover, the impacts of climate change on marine organisms should be taken into account in Marine Spatial Planning, in order to achieve anticipatory zoning capacity, adaptive management and dynamic ocean management (Frazão *et al.,* 2020)

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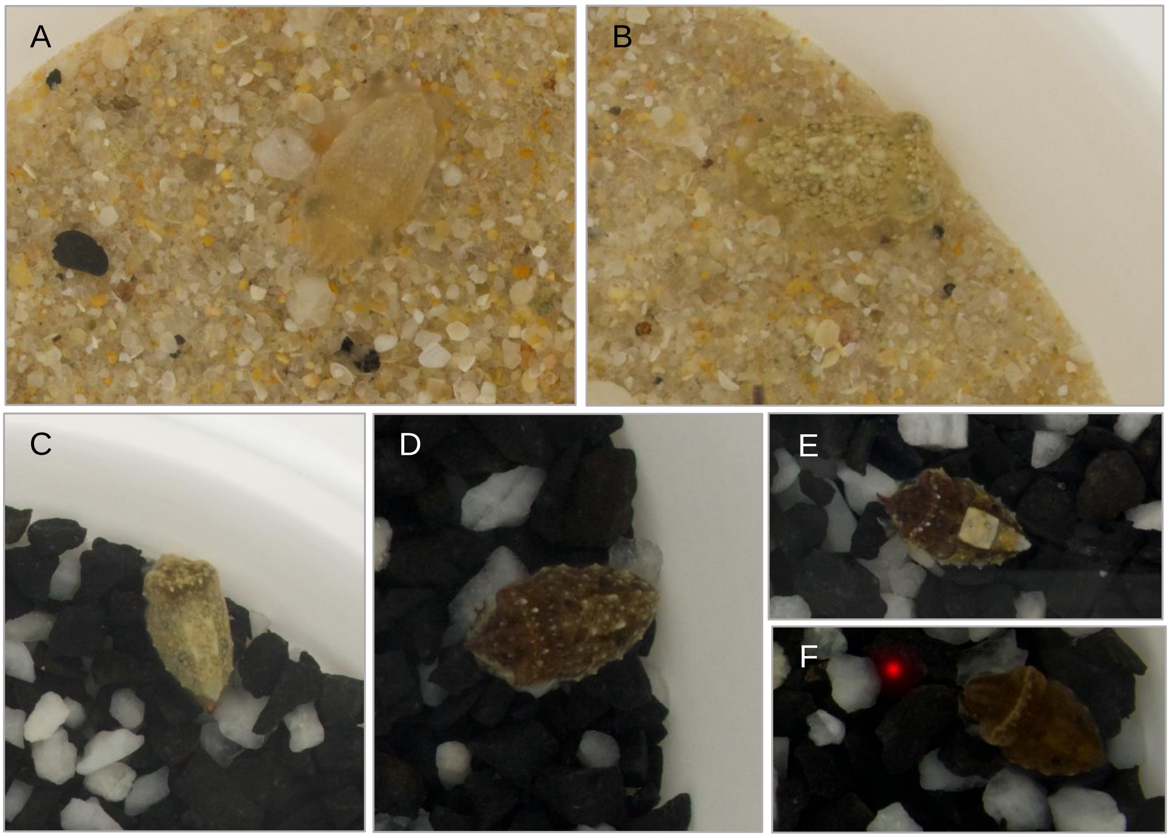
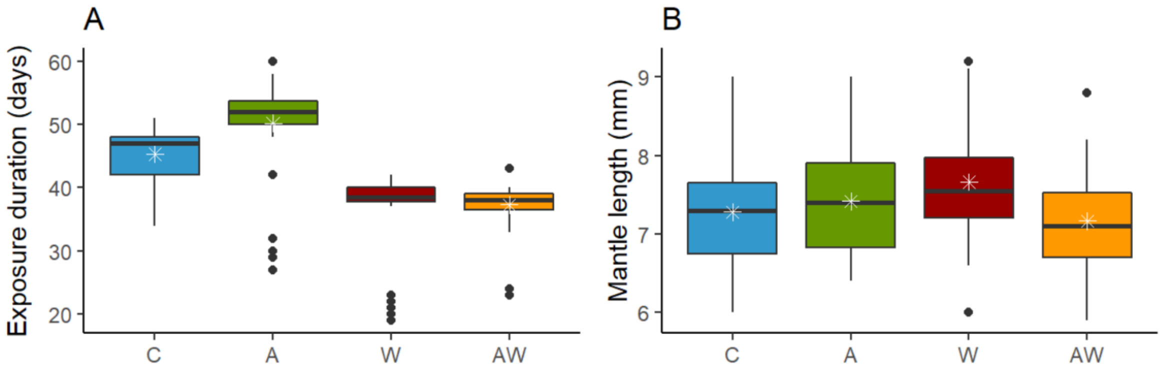
6. Appendices

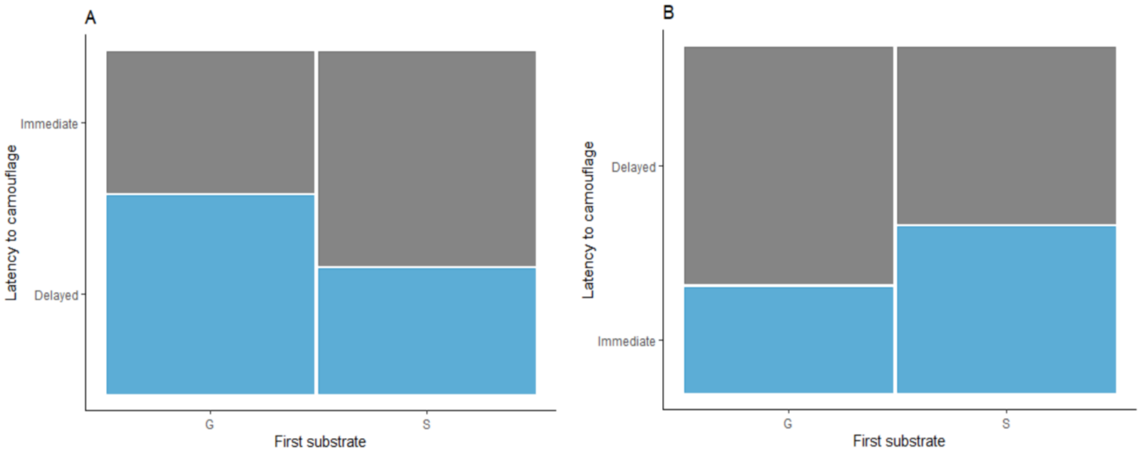
Appendix A – Examples of camouflage scores in sand: A) Score 1, B) Score 2; and in gravel: C) Score 0, D) Score 1, E) Score 2 (disruptive pattern), F) Score 2 (uniform pattern).



Appendix B – Influence of treatments – C (control), A (acidification), W (warming) and AW (acidification and warming combined) – on cuttlefish on A) embryogenesis duration and B) mantle length at 2-5 days old. Whiskers represent the range from 25th to 75th percentiles, with the median as the inside bar. Outliers are shown as points, and the mean is represented by asterisks.



Appendix C – Influence of substrate sequence (G – gravel, S – sand) presented to cuttlefish on A) latency to camouflage in sand, B) latency to camouflage in gravel (Immediate: 0-30 s, delayed: > 30 s).



Funding statement

This study had the support of Fundação para a Ciência e a Tecnologia (FCT) via UIDB/04292/2020, and by the Mar2020 Program through the Project VALPRAD (contract 16-01-04-FMP-0007).