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# Transgenerational responses of a gammarid amphipod to ocean acidification: effects on reproductive traits, mate detection and metabolism

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*"The sea, once it casts its spell, holds*
*one in its net of wonder forever."*

Jacques Yves Cousteau

*"Na prática, a teoria é outra..."*

**Agradecimentos**

Tal como em qualquer outro desafio na vida, o balanço entre o sucesso e o insucesso depende não só nas nossas próprias capacidades, mas também no apoio de todas aquelas pessoas que se unem a nós, em trabalho e em amizade, e que de alguma forma nos auxiliam a superar os obstáculos. São muitos, aqueles a quem sinto que devo agradecer por me terem ajudado a alcançar este novo e pequeno passo em direcção àquele que é o sonho de uma vida. Espero poder fazer a todos um pouco de justiça, e que revejam nestas palavras a gratidão que sinto, e que espero conseguir demonstrar um pouco no dia-a-dia.

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Não queria deixar de expressar, também, não um agradecimento, mas um pensamento de compaixão em particular. Em experimentação com animais, vemos passar dezenas, senão centenas de vidas – mais ou menos complexas – pelas nossas mãos. Aquilo que para muitos pode ser apenas trivial, mais um indivíduo amostrado no meio de muitos, é para os animais decisivo. Assim, a todos os pequenos seres que tornaram esta tese possível, espero ter-vos tratado a cada dia com o respeito merecido e não ter causado sofrimento desnecessário ou cruel. Nas palavras de Dalai Lama: «*Life is just as dear to a mute creature as it is to man. Just as one wants happiness and fears pain, just as one wants to live and not die, so do other creatures*».

**Abstract**

Ocean acidification (OA) poses a global threat to marine biodiversity. The rise in atmospheric carbon dioxide (CO2) concentration, resulting from anthropogenic activities, is responsible for the increase in the dissolved state of this gas in the oceans. The consequent changes in pH and seawater carbonate chemistry are responsible for the disruption of several biological processes (*e.g*. impairing survival and the maintenance of *fitness*-enhancing, physiological and behavioural, mechanisms) in certain marine groups. Disruption at the individual level, can originate negative cascading effects at higher levels of biological organization (*i.e*. populations and communities), which in turn can alter the underlying dynamics that control an ecosystem's structure and overall function. Current theories suggest that marine organisms might be able to maintain their performance in future OA conditions, either through acclimation or through evolutionary adaptation. Surprisingly, the effects of prolonged high-$CO_2$ exposure in crustaceans are still poorly known. The present dissertation investigates, for the first time, the transgenerational effects (*i.e*. over two generations) of ocean acidification in the physiology, behaviour (*e.g*. male mate-attraction) and reproductive traits (*e.g*. female investment, fecundity, mate-guarding and embryonic development) of the gammarid amphipod *Gammarus locusta*. Significant effects of ocean acidification were found for most reproductive traits. Although OA may initially stimulate female investment, transgenerational exposure led to an overall reduction in egg number and fecundity. The duration of mate-guarding behaviours was also diminished under high-$CO_2$ exposure. Individuals from the second generation (F1) exhibited metabolic depression (*i.e*. reduced oxygen consumption rates), and males also displayed a reduced ability to accurately identify and track the origin of female scent cues, thus hinting at a possible disruption of chemosensory abilities. Overall, negative transgenerational (*i.e*. parental) effects were observed for all reproductive traits, as well as survival, in the acidified lineage. The present findings suggest that exposure to a future ocean acidification scenario will likely lead to a reduction in the *fitness* of the natural populations of *G. locusta*.

**Keywords:** ocean acidification, transgenerational, evolution, behaviour, reproduction

**Resumo**

O ambiente marinho encontra-se sob ameaça das alterações climáticas. A acidificação dos oceanos, assim como o aumento da temperatura média das águas, constitui um risco global à biodiversidade marinha. O aumento significativo da concentração de dióxido de carbono ($CO_2$) na atmosfera, resultante das atividades humanas e experienciado desde o início da Revolução Industrial, tem vindo a provocar grandes alterações na química dos oceanos, num processo que pode ser designado por hipercapnia, *i.e.*, o aumento das concentrações de $CO_2$ nas águas, com o potencial para alterar significativamente muitos dos processos biológicos necessários à manutenção da saúde e equilíbrio dos ecossistemas. Ainda que inicialmente identificada como uma ameaça para organismos considerados mais suscetíveis (*e.g.* invertebrados calcificadores), a acidificação dos oceanos e, consequentemente, a hipercapnia, tem vindo a ser reconhecida como um potencial risco à sobrevivência e à manutenção dos mecanismos fisiológicos e comportamentais que são fulcrais à otimização do *fitness* dos organismos marinhos, e para o equilíbrio das populações naturais de inúmeros organismos marinhos. Tais efeitos irão levar, inevitavelmente, a repercussões tanto ao nível das comunidades, como à alteração da dinâmica que controla a estrutura e o equilíbrio dos ecossistemas naturais.

Quando o $CO_2$ entra no corpo dos indivíduos, por difusão, tende a equilibrar-se rapidamente em todos os compartimentos corporais, tendo um efeito predominantemente acidificante capaz de perturbar significativamente o equilíbrio ácido-base interno de um indivíduo. Desta forma, o $CO_2$ poderá levar a mudanças que irão afectar as taxas metabólicas e a alocação natural de energia entre os vários processos corporais do organismo. Embora os seres vivos marinhos possuam, no geral, mecanismos que permitem ajustar o organismo à hipercapnia, estes poderão não ser suficientes para exposições de longo prazo a determinados níveis de *stress* prolongado, e ter como consequência o comprometimento da sobrevivência, do metabolismo e do crescimento dos indivíduos, mas também da sua reprodução e do comportamento. Estima-se que as espécies iono- ou osmorreguladoras serão, em princípio, mais tolerantes aos níveis futuros de acidificação dos oceanos, uma vez que possuem os mecanismos compensatórios adequados que lhes permitem responder a perturbações externas ao equilíbrio ácido-base. Tipicamente, estas espécies habitam ambientes costeiros pouco profundos e sob influência de fontes de água doce, onde estão expostas a variações naturais e por vezes abruptas dos parâmetros abióticos – como o pH, salinidade, temperatura, *etc*.

Os crustáceos são considerados um grupo bastante resiliente e capaz de se adaptar às condições do oceano de amanhã. Durante a última década, tem-se verificado um aumento no interesse do estudo dos efeitos da acidificação nos crustáceos intertidais. Estes organismos desempenham um papel crucial nas cadeias tróficas, sendo não só consumidores primários e secundários de grande relevância, bem como servindo de alimento para níveis tróficos superiores – *e.g.* peixes, aves marinhas e limícolas, *etc*. São também caracterizados por uma elevada variedade de respostas a mudanças ambientais, desde regulação ativa face a alterações abióticas externas, a conformação às novas condições, *etc*. A maioria dos crustáceos são também espécies em contato direto com o meio envolvente, através de estruturas especializadas (como brânquias ou outras estruturas equivalentes), que são responsáveis por trocas de gás e de iões. Apesar de mais tolerantes à hipercapnia, o ajuste do equilíbrio ácido-base dos crustáceos deverá ter elevados custos metabólicos, pelo que a exposição prolongada a um ambiente ácido, dentro dos intervalos de projeção para o final do século, poderá afectar negativamente estas espécies.

Para a maioria dos organismos marinhos, e particularmente no grupo dos crustáceos, os efeitos de uma exposição prolongada e por várias gerações a concentrações elevadas de $CO_2$ no meio marinho são ainda relativamente desconhecidos. Os organismos poderão ser capazes de manter a sua performance nas condições futuras dos oceanos, quer através de aclimatação, quer através de adaptação evolutiva. A aclimatação supõe a existência de plasticidade fenotípica no que diz respeito às respostas fisiológicas,

morfológicas ou comportamentais, que auxiliam na manutenção do *fitness* dos indivíduos num novo ambiente. Por sua vez, a adaptação consiste na retenção nas populações por seleção natural dos genótipos favoráveis a um novo ambiente e que leva à alteração da estrutura natural das populações ao dirigir o equilíbrio fenotípico para um novo nível óptimo em relação às condições encontradas.

A aclimatação transgeracional ocorre quando um novo ambiente experienciado por uma geração paternal, influencia positivamente a performance da sua descendência para esse mesmo ambiente. Por vezes, é necessária uma exposição de várias gerações a um determinado factor de *stress* até que surjam diferenças significativas nas características de uma espécie entre dois ambientes diferentes. Assim, de modo a estudar a forma como a acidificação dos oceanos poderá ou não afectar os organismos no oceano de amanhã, são necessários estudos trans- e multigeracionais, que abordem esta questão em espécies com ciclos de vida curtos e de fácil manutenção.

O presente estudo visou aumentar o conhecimento existente acerca dos efeitos transgeracionais e da possível adaptação dos organismos marinhos à acidificação futura dos oceanos, no que diz respeito à fisiologia e aos processos reprodutivos de anfípodes gamarídeos. Neste sentido, foram estabelecidas duas populações laboratoriais de *Gammarus locusta*, que foram sujeitas por duas gerações consecutivas a um cenário de acidificação com valores estimados para o final do século (pH= 7.7 e $pCO_2$ = 900 μatm) e a um cenário controlo com valores de pH e pressão parcial de $CO_2$ actuais (pH = 8.1, $pCO_2$ = 400 μatm). Em ambas as gerações, foram recolhidos dados relativos a características específicas da história de vida desta espécie: i) investimento reprodutor das fêmeas (número médio de ovos no interior do marsúpio das fêmeas); ii) duração da parelha reprodutora (*mate-guarding*), iii) fecundidade média; iv) tempo médio de desenvolvimento embrionário; v) e apenas para a segunda geração, testes comportamentais focados na atração de machos por pistas olfativas de fêmeas recetivas, e do consumo médio de oxigénio (taxa metabólica de rotina). Os resultados do presente estudo mostraram efeitos significativos da acidificação dos oceanos na maioria das características reprodutivas desta espécie. Apesar de uma descida no pH poder, numa primeira instância e para a primeira geração exposta, estimular positivamente o investimento reprodutor das fêmeas, uma exposição transgeracional leva a uma redução significativa do número de ovos e da fecundidade. A duração da parelha reprodutora também foi significativamente afectada, tendo diminuído para os casais expostos ao ambiente acidificado, contudo não foram encontrados efeitos ao nível da duração do desenvolvimento embrionário. A sobrevivência aos 30 dias de idade foi também significativamente afectada, tendo diminuído em cerca de 19% em relação à população controlo e sugerindo a existência de efeitos metabólicos nefastos. Na segunda geração, foi identificada a presença de depressão metabólica em organismos de ambos os sexos, assim como uma perda da acuidade na localização da origem de pistas reprodutoras de fêmeas, aliada ao aumento do tempo de resposta (o estimular de comportamentos locomotores de busca) em machos sujeitos a condições de acidificação.

No geral, e para as características reprodutivas afectadas, assim como para a sobrevivência, foram encontrados efeitos transgeracionais (parentais) negativos na linhagem acidificada, que sugerem a possível diminuição do *fitness* das populações naturais de *G. locusta* num cenário de acidificação futura dos oceanos. Os efeitos da acidificação ao nível do metabolismo, aliados à depressão metabólica observada na segunda geração, ajudam a explicar a maioria dos resultados obtidos e sugerem a existência de efeitos nefastos da diminuição do pH (e aumento do $pCO_2$) numa espécie tipicamente considerada como resiliente.

**Palavras-chave:** acidificação, transgeracional, evolução, comportamento, reprodução.

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## List of abbreviations and symbols

| | |
|---|---|
| **OA** | ocean acidification |
| **$CO_2$** | Carbon dioxide |
| **$O_2$** | Oxygen |
| **ppm** | parts per million |
| ***p*$CO_2$** | partial pressure of Carbon dioxide |
| **$H_2CO_3$** | Carbonic acid |
| **$HCO_3^-$** | Bicarbonate |
| **$H^+$** | Hydrogen ion |
| **pH** | power of hydrogen |
| ***i.e.*** | 'that is' |
| ***etc.*** | '*etc*etera' |
| ***e.g.*** | 'for example' |
| **$pO_2$** | partial pressure of Oxygen |
| **MG** | mate-guarding |
| **TA** | total alkalinity |
| **WW** | wet weight |
| **RMR** | routine metabolic rate |
| **GLM** | generalized linear model |
| **GLMM** | generalized linear mixed model |
| **AIC** | Akaike information criterion |
| **CAC** | chemical alarm cues |
| **Hg** | Mercury |
| **Cu** | Copper |
| **gapdh** | Glyceraldehyde 3-phosphate dehydrogenase |
| **µatm** | micro atmospheres |
| **SCSS** | seawater carbonate system speciation |

# 1. Introduction

## 1.1. Ocean acidification

Since the beginning of the industrial revolution in the mid-eighteenth century, the world has witnessed geochemical changes that have not been experienced for the last 300 million years (Hönisch et al., 2012). The release of carbon dioxide ($CO_2$) into the atmosphere by agricultural, industrial, deforestation and transportation activities, is a serious concern. Current estimates set concentrations of this greenhouse gas higher than they have been for over 800 thousands years, while giving no hint to a possible decrease in the build-up rates (Lüthi et al., 2008). It is expected that the actual rate of atmospheric $CO_2$ build up will increase in the following years (IPCC, 2014), with concentrations displaying an escalating trend from 280 μatm (micro atmospheres, pre-industrial) to approximately 400 μatm (current levels) (Feely et al., 2009, Hönisch et al., 2012, IPCC, 2014).

$CO_2$ is one of the most relevant gases in our planet's atmosphere, being responsible for the control of both radiative heat balance of the Earth, and the calcium carbonate equilibrium in the oceans as well (Petit et al., 1999). $CO_2$ levels both in the air and in surface waters tend to equilibrate – in accordance with Henry's Law; as atmospheric partial pressures for this gas increase, leading to the equivalent dissolution of $CO_2$ (Pörtner et al., 2004). The ocean's carbonate chemistry is governed by a series of abiotic and biologically mediated chemical reactions (see Figure 1.1.1a), which depend on the stability of abiotic parameters such as ocean temperature and alkalinity. It is the flux of carbon dioxide between the atmosphere and the ocean - which is a function of surface mixing and differences in concentration between surface waters and the air – that keeps the ocean's carbon chemistry in check, and maintains a positive environment for current ecological processes, as well as the survival and maintenance of *fitness*-enhancing, physiological and behavioural mechanisms in marine organisms (Feely et al., 2009, Munday et al., 2013, Sunday et al., 2014).

Ocean acidification results from the abnormal increase in partial pressure of carbon dioxide ($pCO_2$) in surface waters, which in turn leads to the reduction in mean surface water pH levels (Sunday et al., 2014). The increase in $pCO_2$ is associated with the increased uptake of fossil fuel-derived $CO_2$ from the atmosphere (Orr et al., 2005, Feely et al., 2009, Sunday et al., 2014), which in turn leads to the unbalancing of normal seawater carbonate chemistry. As $pCO_2$ increases in seawater, there is a subsequent increase in the rate of the reactions that form carbonic acid ($H_2CO_3$). Most of this acid naturally dissociates to form $HCO_3^-$ and $H^+$, and the latter reacts in turn with carbonate ions to produce additional bicarbonate ions (see Figure 1.1.1b). This shift in the carbon chemistry, by "injecting" increased quantities of carbon dioxide into the ocean, contributes to the progressive acidification of seawater, with an increase in hydrogen ion concentration, the associated decline in pH levels, and the reduction in the concentration of free carbonate ions (Feely et al., 2009), as well as the saturation state of calcium carbonate minerals.

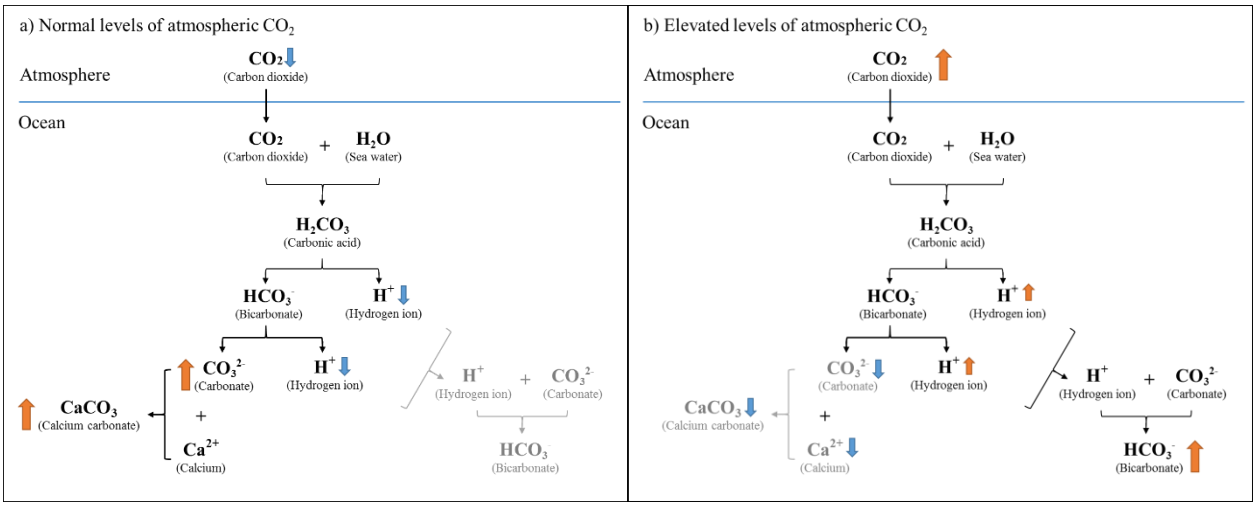



**Figure 1.1.1.** Ocean-atmosphere carbon dioxide exchange: the ocean's carbon chemistry in (a) normal atmospheric levels of carbon dioxide, and (b) elevated levels of atmospheric carbon dioxide. Coloured arrows signify high (orange) or low (blue) levels of a chemical compound. Sections in black point out the primary chemical reactions which occur in each scenario.

This increase in the ocean's acidity has gradually occurring, translated in a decrease of approximately 0.1 pH units since the beginning of the industrial era (Caldeira and Wickett, 2003, Orr et al., 2005, Doney et al., 2009). The majority of OA projections place a future increase of $pCO_2$ from current levels (~400 µatm), to approximately 880-990 ppm by the year 2100 (see Figure 1.1.2). This would mean an additional decrease of 0.3 pH units coupled with an increase in the ocean's hydrogen ion concentration of approximately 2.5 times, relative to pre-industrial levels (Caldeira and Wickett, 2003, Friedlingstein and Solomon, 2005, IPCC, 2014), which could pose a serious risk for marine organisms in the near future, as will be discussed in the next section.

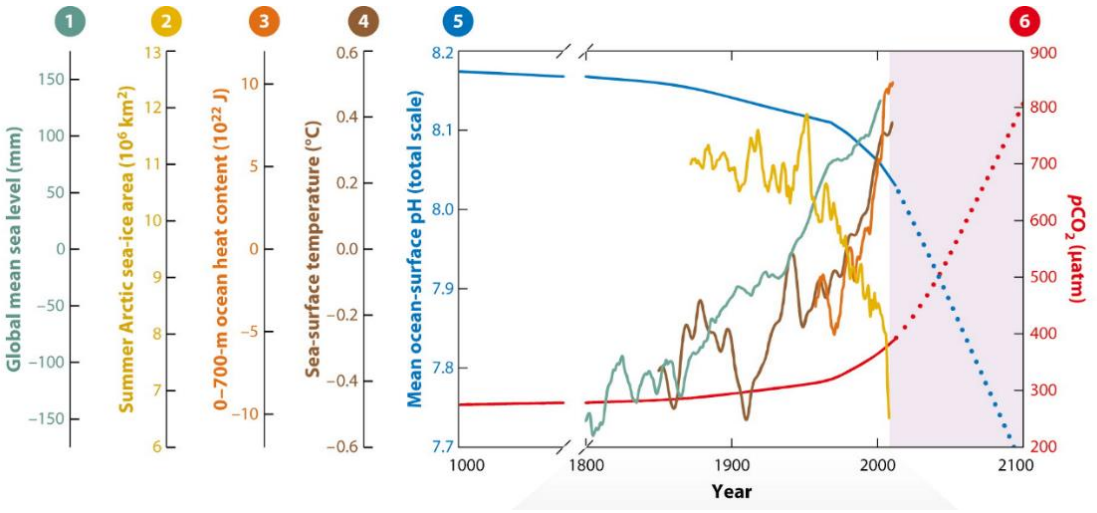



**Figure 1.1.2.** Changes in (1) global mean sea level (teal line); (2) summer Arctic sea-ice area (yellow line); (3) 0–700 m ocean heat content (orange line); (4) sea-surface temperature (brown line); (5) mean ocean-surface pH (blue line); and (6) atmosphere $pCO_2$ (red line). Light purple shaded region denotes projected changes in pH and $pCO_2$ consistent with the Intergovernmental Panel on Climate Change's twenty-first-century A2 emissions scenario with rapid population growth (Doney et al., 2012)

### *1.1.1 Effects of OA on crustaceans and other marine organisms*

General concern about the severe impacts of OA in marine organisms has increased over the past decades (Orr et al., 2005, Hoegh-Guldberg, 2007, Kroeker et al., 2010, Sunday et al., 2014). Changes in ocean chemistry associated with OA will elicit both direct and indirect effects in the physiological functioning, behaviour and demographic traits of most marine organisms. These effects will translate into populational and community level changes - which can ultimately alter the underlying dynamics that control an ecosystem's structure and overall function (Doney et al., 2012) – and acidification could, therefore, have major impacts on food webs and global geochemical cycles (Orr et al., 2005, Doney et al., 2009, Feely et al., 2009, Doney et al., 2012).

It is generally accepted that the degree to which a particular species will be affected by projected OA will ultimately depend on its physiological performance. When faced with a stressor, organisms initially respond with physiological and behavioural adaptations, which are conditioned by their evolutionary history (Doney et al., 2012, Somero, 2012). If conditions are physiologically tolerable, individuals of said species can acclimate or adjust their physiology to cope with the new stressor. As acclimation at an individual level occurs, then a species may also (but not necessarily) adapt to the new conditions on the long-term - by increasing both the abundance and the reproductive success of those genotypes that are most tolerant to the new conditions - over subsequent generations (Doney et al., 2012, Munday et al., 2013). If, on the contrary, these conditions prove to be physiologically unfavourable, individuals and populations will either promote migration into areas where conditions are more suitable, induce changes in phenology and advance or delay certain life-history events, or even face local extinction events (Parmesan, 2006).

Depending on species ability to acclimate or adapt to OA, some taxa have been classified as being potentially more vulnerable than others (Whiteley, 2011, Doney et al., 2012). Invertebrates such as cnidarians, molluscs and echinoderms have been extensively studied for their well-known vulnerability to changes in the ocean's chemistry – for being poor iono-regulators and having a reduced ability to buffer the acidifying effects of high-$CO_2$ concentrations in their body compartments (Fabry et al., 2008, Doney et al., 2009, Dupont et al., 2010). For these and other groups, OA is known to affect many physiological processes and life-history patterns: in calcifying organisms, OA is thought to increase the energy costs of calcification (Pörtner et al., 2004, Hofmann et al., 2010, Doney et al., 2012), and some species have been known to undergo dissolution of their calcified skeletons or suffer significant reductions in their calcification rates, when exposed to acidic environments (Fabry et al., 2008, Whiteley, 2011). Reproduction and metabolism can also be affected, and some species have shown reduced fertilization success, shorter developmental rates and larval sizes (Kurihara, 2008), and lower oxygen-binding capacity ($O_2$) of respiratory proteins - thus impairing an individual's aerobic scope (Pörtner et al., 2004).

The effects of OA in marine organisms result from the entry of $CO_2$ by diffusion in the individual body, rapidly achieving equilibrium in all body compartments, which then has a predominantly acidifying effect on an individual's internal acid-base balance (Pörtner et al., 2004). By causing changes in one or in a combination of the body compartment's acid-base parameters, $CO_2$ may affect overall metabolic rates, as well as the partitioning of energy between individual metabolic processes (Pörtner et al., 2004). Disturbances in the acid-base balance can frequently be compensated in order to re-

establish the original or a new equilibrium in the body fluids, and for this purpose, water breathers rely almost exclusively on ion exchange mechanisms with the exterior.

Changes in the ocean's and the individual's carbonate chemistry compromise the excretion of $CO_2$ from the haemolymph, leading to increases in its concentration in the extracellular compartment and to variations in the haemolymph's pH levels (Whiteley, 2011). The resulting variation of pH levels in haemolymph can be buffered by adjusting the body's acid-base equilibria, maintaining an environment favourable with most protein activity. Rises in intracellular $H^+$ can, however, disrupt protein synthesis, iono-regulation, cell volume and overall metabolism. This interferes with organism's capacity to cope with pH disruptions in the haemolymph, as they can only be tolerated to some extent, before causing medium to long term disturbances (Whiteley, 2011). Acid-base balance disruptions both in extra- and intracellular environments can have far-reaching consequences, compromising survival and disrupting relevant biological processes such as metabolism and individual growth. Species that are strong iono- and osmoregulators, are likely to be most tolerant to OA, because they own the compensatory mechanisms that enable them to respond to acid-base disruptions. These species typically inhabit shallow coastal environments, under freshwater influence, where they experience natural variations in abiotic parameters – *i.e*. seawater $pCO_2$, $pO_2$, salinity and temperature (Pörtner et al., 2004, Whiteley, 2011, Kelly and Hofmann, 2013).

Crustacea have thus been considered a fairly resilient group of species, capable of adjusting to future OA conditions. However, in the past decade, a growing interest on how OA will directly affect these intertidal dwellers has shed some light on the subject. Crustaceans are primary and secondary consumers, and constitute an important food source for higher trophic levels. As abundant and frequent living organisms of intertidal and estuarine areas, which are known to display broad and rapidly changing fluctuations of physical factors (Whiteley, 2011), crustaceans also show a wide variety of responses to abiotic change – from active regulation against external changes, to conformation to new conditions.

The majority of crustacean species are committed water-breathers, in which there is close contact with the external environment through specialized structures – *i.e*. gills or others equivalent – that are responsible for respiratory gas and ion exchange (Taylor and Taylor, 1992). Despite being more tolerant to hypercapnia - because they maintain higher $HCO_3^-$ concentration in the haemolymph - acid-base adjustments made by crustaceans are likely to be metabolically expensive if exposure to acidified conditions is prolonged over weeks or months, due to the dependence on bicarbonate ($HCO_3^-$) ion uptake from the seawater (Whiteley, 2011). If these compensation costs are indeed significant, then it is possible to infer that crustaceans could be adversely affected by OA, and that the costs will either be limiting and restrict homeostatic processes, or energy will be diverted away from other processes, with individual performance being affected in both scenarios (Pörtner et al., 2004, Whiteley, 2011).

There is indeed growing evidence that OA may affect crustacean populations by influencing growth and / or reproductive performance in adults, and in determining life-history traits such as larval development and settling (Whiteley, 2011). Some described effects of short-term exposure to high-$CO_2$ in crustaceans include changes in egg production - *e.g*. *Palaemon pacificus* (Kurihara, 2008) - increased embryonic development duration characterized by a delayed time to hatching – *e.g*. *Semibalanus balanoides* (Findlay et al., 2009) – reduced survival and growth in amphipods – *e.g*. *Peramphithoe parmerong* (Poore et al., 2013) and *Gammarus pulex* (Taylor et al., 1994) – changes in palatability to food sources – *e.g*. *Peramphithoe parmerong* (Poore et al., 2013) –and changes in the expression of metabolically relevant enzymes in the amphipod *Gammarus locusta* (Hauton et al., 2009), *etc*.

OA is also known to effectively disrupt chemically-mediated, *fitness* enhancing behaviours in both fish and crustacean groups – *e.g*. *Pagurus bernhardus* (de la Haye et al., 2012), among others (Briffa et al., 2012). Most results indicate that exposures to $CO_2$ enriched water affect the olfactory system on some level that is not directly linked to the chemical modification of odour cues (Leduc et al., 2013). Therefore, exposure to high-$CO_2$ (and the resulting hypercapnia) is responsible for the majority of observed disruptions in the physiology and behaviour of marine animals, particularly in crustaceans, which behaviour can be directly affected by direct interference in acid-base balance maintenance (Pörtner et al., 2004, Briffa et al., 2012). Hypercapnia could lead to behavioural changes through a series of processes: i) as a consequence of systemic physiological changes that reduce the energy available to the individual or alter its metabolic processes (Pörtner et al., 2004); ii) changes in neural mechanisms required for processing information (Nilsson et al., 2012); iii) or the disruption of the signal reception – through physical impairment of receptive organs, alteration of the receptors or in some situations, changes in the signalling molecules (Briffa et al., 2012).

The impacts resulting from long-term high-$CO_2$ exposure in crustaceans, and many other marine groups, remain relatively unknown (Whiteley, 2011). While some species show negative effects to OA exposure, others show no measurable effects or are even positively affected by exposure to mid- to high-$CO_2$ conditions (Whiteley, 2011). Thus, it is highly important to include crustacean species as models in trans- and multigenerational experiments, to better understand the subtle effects of ocean acidification that may be concealed by resilience to short-term exposures.

### *1.1.2 Trans-/multigenerational approaches on OA*

When studying the effects of ocean acidification or other environmental stressors on marine organisms, it is important to consider the potential long-term, evolutionary effects. For some time, studies focused on the impact of high-$CO_2$ exposure stress on either 'adult individuals' or 'larvae' (Parker et al., 2012) and ignored the potential link that exists between different life-history stages and different generations, and possible effects that might be passed down from adults to their offspring – parental effects (see Figure 1.1.3) (Dupont et al., 2010, Kroeker et al., 2010, Parker et al., 2012).

Marine organisms might be able to maintain their performance in future OA conditions either through acclimation or through evolutionary adaptation (Munday, 2014, Sunday et al., 2014, Calosi et al., 2016). Acclimation involves phenotypically plastic responses at the physiological, morphological or behavioural level, which can help maintain individual *fitness* in a new environment (Sunday et al., 2014). Phenotypic plasticity is the capacity of one genotype to produce a range of phenotypes under different environmental conditions (Fordyce, 2006), which plays a key role in determining an organism's *fitness* and subsequent ecological performance when challenged with highly selective pressures (Gibbin et al., 2017).

Adaptation, involves the selection on genetic variation, by retaining favourable genotypes in a population, which in turn alters its structure by shifting the average phenotype towards a new, optimal *fitness* peak for the conditions encountered (Sunday et al., 2014). Acclimation, in turn is a short-term response which can either be reversible, developmental or transgenerational. Reversible acclimation occurs in hours to days or even months, and allows an individual to temporarily adjust its physiology and behaviour to survive the stress imposed by a sudden change. Developmental acclimation can occur when exposure to a novel environment happens during early-life phases of an individual, enhancing its ability to survive to that same environment later in life. Finally, transgenerational acclimation occurs when the environment experienced by the parent positively influences the performance of the offspring exposed to that same environment (See Figure 1.1.3). Sometimes, a phenomenon called "phenotypic

buffering" can lead to the apparent absence of differential responses in certain traits, between different environments, but a longer, multigenerational exposure might be enough to elicit clear positive or negative responses (Munday, 2014, Sunday et al., 2014).

There is growing consensus that on short ecological time-scales, evolution can occur (Carroll et al., 2007) - through standing variation or from the appearance of new mutations (Kelly and Hofmann, 2013) - and that adaptation could reduce or compensate the risk of extinction during periods of rapid environmental changes (Bell and Gonzalez, 2009). Both genetic adaptation and acclimation can help organisms persist in the face of environmental change, and understanding the links between these processes will be critical for predicting the evolutionary responses of marine organisms to OA (Sunday et al., 2014).

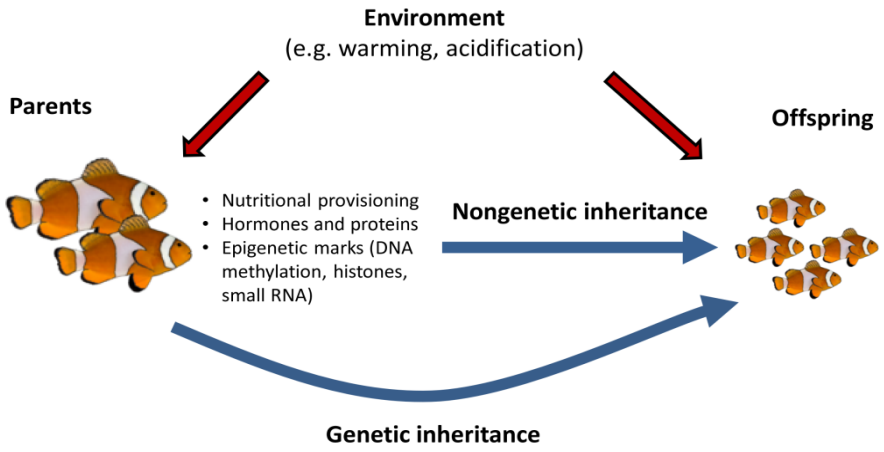



**Figure 1.1.3.** Parents influence the phenotype of their offspring through both genetic and non-genetic pathways. The environment experienced by parents can influence the phenotype of their offspring through a variety of non-genetic mechanisms. In fishes, for instance, these mechanisms include nutritional provisioning of eggs, transfer of hormones or proteins to eggs, or epigenetic marks from either the mother or the father. Transgenerational acclimation occurs when the performance of offspring in a particular environment is improved when parents have experienced the same environment (Munday, 2014).

A possible approach to measure the scope for adaptation is experimental evolution (Kelly and Hofmann, 2013). In this approach, populations of (typically) short-lived organisms of easy maintenance in laboratory, are exposed to simulated future conditions over a set of generations, and then are compared to control populations for the evaluation of possible signs of adaptation. Multi-generational experiments are needed to anticipate the adaptive capacity of marine organisms over the next century given the current rate of increase of atmospheric $CO_2$ (The Royal Society, 2005).

Some of the most recent multigenerational approaches for OA in marine animal groups are summarized in Table 1.1.1. Most negative effects that occur at a transgenerational level seem to affect fecundity, development metabolism and behaviour. Despite the existence of examples of parental effects or adaptive potential that could alleviate some of these impacts, there are also species that show increasingly negative consequences of OA exposure with each subsequent generation. It is still unknown how most marine groups will fare in the future, due to intra-group responses being so variable. It is important to conduct broader, longer and more holistic multigenerational experiments, in order to ensure the understanding of how marine organisms will change under the selective pressure of future global change drivers, and better predict population, community and ecosystem level responses (Calosi et al., 2016).

**Table 1.1.1**. Examples of trans-/multigenerational studies with ocean acidification (OA) as a main stressor (sometimes with complementary stressors) in crustaceans, teleost fishes and molluscs.

| Group | Type | Species | Stressor | $pCO_2$ (µatm) pH | Generations | Outputs / Objectives | O.A effects | Reference |
|---|---|---|---|---|---|---|---|---|
| Mollusc | Trans | *Saccostrea glomerata* | OA | 380, 856 | F0 - F1 | Larvae development;<br>Growth;<br>Survival response;<br>Standard Metabolic Rate (SMR). | Direct exposure on larvae:<br>↓ development rate and growth;<br>↓ survival;<br>Carry-over effects on larvae from adults reared on high $pCO_2$:<br>↑ size and development rates;<br>Similar survival with control group | Parker et al. (2012) |
| Polychaeta | Multi | *Ophyrotrocha labronica* | OA<br>Warming | pH 8 ; 7.6 | F1-F6 | Juvenile developmental rate;<br>Survival to sexual maturity;<br>Average reproductive body size;<br>Fecundity;<br>Cellular reactive $O_2$ content;<br>Mitochondrial density;<br>Mitochondrial capacity. | Minor effects on juvenile developmental rate;<br>OA + warming:<br>- Negative effects on mitochondrial capacity. | Gibbin et al., (2017) |
| Crustacea | Trans | *Tisbe battagliai* | OA<br>Cu | 188 - 616 | F0 - F1 | Naupliar production per brood and per generation;<br>Naupliar growth;<br>Cuticle composition;<br>Copepod Cu uptake with decreasing pH. | ↓ naupliar production;<br>↑ growth;<br>↓ sulphur, phosphorus and calcium | Fitzer et al. (2013) |
| | Multi | *Calanus finmarchicus* | OA<br>Food limitation | ambient, 1080, 2080, 3080 | F0 - F2 | Energy budget;<br>Growth;<br>Development rate;<br>Fecundity;<br>Fertility. | Negative effects on ontogenetic development, somatic growth, fecundity;<br>Adaptive potential in the development rate between F0 and F1 | Pedersen et al. (2014) |
| | Multi | *Pseudocalanus acuspes* | OA | 400, 900, 1550 | F0-F2 | Egg production rates;<br>Egg clutch sizes;<br>Respiration rates. | ↓ fecundity at 900 µatm in 2nd generation adults<br>↑ metabolic rate;<br>These effects were reversible;<br>Transgenerational effects partly reduced O.A effects at 1550 µatm ; | Thor & Dupont (2015) |

**Table 1.1.1**. (continued) Examples of trans-/multigenerational studies with ocean acidification (OA) as a main stressor (sometimes with complementary stressors) in crustaceans, teleost fishes and molluscs.

| Group | Type | Species | Stressor | $pCO_2$ (µatm) pH | Generations | Outputs / Objectives | O.A effects | Reference |
|---|---|---|---|---|---|---|---|---|
| Crustacea | Trans | *Acartia bifilosa* | OA | 356 - 1231 | F0 - F1 | Egg production rate;<br>Hatching success;<br>Female size;<br>Female antioxidant capacity. | ↓ adult female size;<br>↓ egg hatching success;<br>Treshold (~1000µatm): maternal effects stop alleviating negative effects on egg hatching and nauplii development. | Vehmaa et al. (2016) |
| | Multi | *Tigriopus japonicus* | OA<br>Hg | 400, 1000 | F0-F3 | Metal accumulation;<br>Survival and sex ratio;<br>Developmental time;<br>Fecundity | Significant interaction between Hg and high $pCO_2$:<br>↓ Hg accumulation at each generation | Yan Li et al (2017) |
| Teleost fish | Trans | *Gasterosteus aculeatus* | OA | 400, 1000 | F0-F1 | Survival 30, 60, 90d post-hatch;<br>Growth rates;<br>Maternal $CO_2$ on fecundity;<br>Egg number, weight and diameter;<br>Otolith size, area and weight. | ↑egg production;<br>No trade-off between fecundity and egg quality;<br>↑survival 90 days pot-hatch;<br>Significant paternal effects: ↓growth;<br>Paternal transgenerational effects on survival and growth only in early life stages;<br>Strong paternal effects on otolith size 100 days post-hatch:<br>↑ otolith size in the offspring from fathers acclimated to high-$CO_2$; ↑ otolith area in the offspring of both maternal and paternal high-$CO_2$ | Schade & Clemmesen (2014) |
| | Trans | *Amphiprion melanopus* | OA | 446, 656, 912 | F0 - F1 | Olfactory preference trials;<br>Behavioural lateralization | ↓ avoidance response to chemical alarm cues (CAC)<br>↑ attraction to chemical alarm cues in juveniles reared at high-$CO_2$<br>Loss of innate avoidance for CAC in juveniles from parents maintained at mid-$CO_2$ and high-$CO_2$;<br>Disruption of behavioural lateralization on juveniles reared in high-$CO_2$; | Welch et al. (2014) |

## 1.2. Study species: the amphipod *Gammarus locusta*

### *1.2.1 Distribution and ecology*

*Gammarus locusta* (Linnaeus, 1758) (Figure 1.2.1) is a relatively known and frequent species in both coastal and estuarine waters along the North-eastern Atlantic Ocean (Figure 1.2.2), namely from the coasts of Norway (Lincoln, 1979a), Iceland (Ingólfsson, 1977) and the British Isles (Lincoln, 1979b) – to the Baltic and North seas, the Azores Islands, and the Atlantic coasts of France, Spain and Portugal (Maren, 1975, Costa and Costa, 1999, Costa, 2000, Costello, 2010). In Portugal, its occurrence has been reported in numerous locations along the coast, such as the Mondego, Sado, Mira, Aveiro and Alvor estuaries, among others (Costa and Costa, 1999, Neuparth et al., 2002).

**Figure 1.2.1.** *Gammarus locusta* (Linnaeus, 1758)

*G. locusta* is described as a marine, euryhaline species (Remane and Schlieper, 1958), and has won the connotation of being «*the most common, purely marine form in the temperate eastern atlantic ocean*» (Stock, 1967), which even though it is not accurate, this conotation emphazyses the high abundance of this amphipod. Records on this species' occurrence indicate that it prefers conditions that are closer to the open sea (Costa, 2000), but this amphipod still penetrates brackish waters to some extent and, according to some authors, tolerates considerable reductions in salinity - given that an adequate acclimation period is provided (Costa, 2000). In fact, individuals are frequently found in the infra- and intertidal zones, and can endure a wide range of salinities – from as low as 5 and up to 40 (Costa and Costa, 1999). They are also able to adapt to very different types of habitats - which include rocky coastlines and sandy or muddy substrates, as well as *Zostera* sp. beds – as long as these are associated with a high coverage of macroalgae (Oliveira Costa et al., 1996, Costa and Costa, 1999, Ruppert, 2004). The macroalgae's structures, and their filamentous and branded epiphytes create a complex environment, which acts as the perfect substrate for shelter and refuge from predators, as well as a food source for this gammarid.

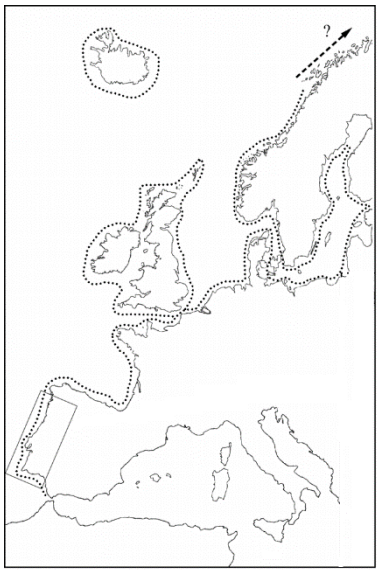


**Figure 1.2.2.** Distribution of *Gammarus locusta* in the NE Atlantic Ocean – adapted (Costa et al., 2004).

### *1.2.2 Trophic relationships*

*G. locusta* is an omnivorous, opportunistic, deposit-feeder species (Costa and Costa, 2000), whose diet includes a variety of different seaweeds and the occasional predation of smaller organisms or even conspecifics. This species has been shown to consume a variety of food items, and maintains relationships of herbivory, detritivory, and cannibalism, both in the wild and in laboratory conditions (Costa and Costa, 1999, Costa and Costa, 2000, Costello, 2010). Studies have shown that the preferred food source for this small amphipod are macroalgae – such as *Ulva* spp. and *Enteromorpha* spp – which represents the major component of its natural diet, followed by detritus. This preference for algae makes this amphipod as an important mesograzer, helping in the suppression of overgrown ephemeral algae, *e.g.* in seagrass beds (Andersson et al., 2009).

This amphipod species is also crucial for higher trophic levels in coastal and estuarine food webs, being preyed upon by fish (Kuhlmann et al., 1982), shorebirds, and many other groups like nemertenians and larger crustaceans (Costa and Costa, 1999, Macneil et al., 1999). Its role as a consumer and a prey, alongside its wide latitudinal distribution (Figure 1.2.2), range and abundance, contribute to its high ecological relevance in numerous European coastal systems (Costa and Costa, 2000).

### *1.2.3 Life cycle and reproduction*

Amphipods display a great diversity of life-history patterns (Sundelin et al., 2008) – *e.g.* semelparity, iteroparity, semi-annual, annual, biannual or perennial life cycles, presence or absence of pre-copulatory mate guarding (MG) behaviours, *etc*. - that are directly influenced by latitude, depth and salinity (Appadoo and Myers, 2004). Overall, the general life cycle of a gammarid amphipod can be summed according to Figure 1.2.3.

In southern areas of its distribution, such as at the Sado estuary, *G. locusta* exhibits a multivoltine life cycle. This means that over a year, a female will produce more than two broods, and that reproductive activity is more or less continuous throughout most of this period (Costa and Costa, 1999). The cycle is generally completed in 40 to 50 days, at 15 ºC (Neuparth et al., 2002), when individuals have achieved sexual maturity, mated, and given birth to a new generation. Increases in temperature have been linked to an average four-week reduction in the life-span of *G. locusta*, as well as an acceleration and condensation of their life cycle – which includes the anticipation of the age at maturity, and higher population growth rates (Neuparth et al., 2002).

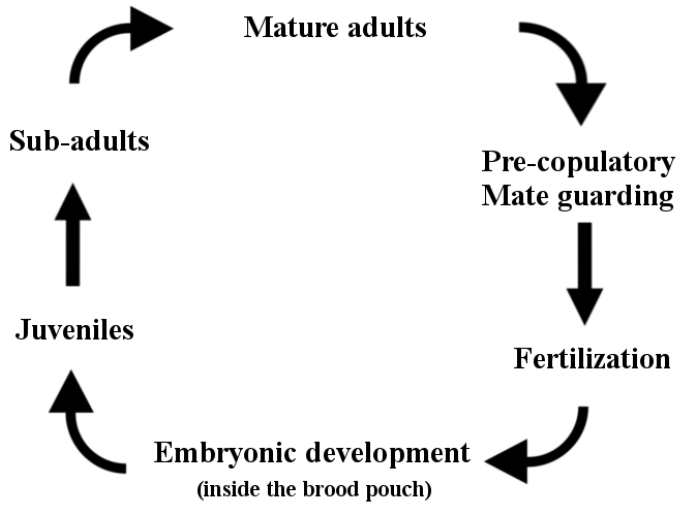



**Figure 1.2.3.** Life cycle of a Gammarid amphipod.

The gender of adult *G. locusta* is clearly identifiable, since males and females exhibit some degree of sexual dimorphism. Males are characterized by the existence of a genital papillae on the ventral surface of the 7$^{th}$ thoracic segment, and can be easily differentiated by their enlarged 2$^{nd}$ pair of gnathopods. Females, in turn, can be distinguished by the presence of brood plates (*i.e*. oostegites), which hold the eggs and developing embryos in the ventral area and thus create a brood pouch or marsupium. In females, the visual indicator of sexual maturity is the development of setae on the surface of the brood plates (Sundelin et al., 2008).

In the Sado estuary, females reach maturity at approximately 28 days of age (or 7 mm in length), depending on habitat conditions (Costa and Costa, 1999, Hauton et al., 2009), and a female's investment in her progeny begins right at oogenesis. In crustaceans, large quantities of lipids are deposited into developing oocytes – mainly monounsaturated fatty acids – which are used during embryo development (Morais et al., 2002, Rosa et al., 2005). The number of eggs produced by a female varies considerably between different species (Sundelin et al., 2008). For example, *G. minus*, produce an average of 4 to 9 eggs per female (Glazier et al., 1992), while *Gammarus zaddachi* is known to produce over one hundred eggs per brood (Cheng, 1942).

Shortly before mating, and following the parturial moult – *i.e*. the moult that signals a female's maturity and reproductive availability, with the added development of setae on the brood pouches – the female's oocytes are sorted into eggs that will pass down through the oviduct wall and exit into the female's marsupium (Sutcliffe, 2010b). The passage of the eggs into the brood pouch can only happen during a short-time period, after the moult has occurred and the new exoskeleton is still soft, because this is the time when the oviduct wall is still malleable enough to conform to the egg's passage.

This reduced window of time created a challenge for amphipod reproduction since, despite the fact that the female's moult, and thus fertility, is predictable, males have few opportunities to find and mate with fertile females unless they develop a strategy to maximize their probabilities. It is believed that this is the reason behind the evolutionary appearance of a precopulatory stage called *mate-guarding* (MG), in which males will guard and effectively carry a pre-moulting female to ensure their place as the fertilizers of the eggs – males will use their enlarged pair of gnathopods to clasp and hold the smaller females until they moult (Steele and Steele, 1986). Precopulatory MG can last up to 8 – 10 days, and is dependent on the female's moult and external abiotic conditions (Sutcliffe, 2010b)

When body contact occurs between a mature male and a receptive female, the male will catch and clasp the female by an appendage and place her lengthwise beneath him, in the position of precopulatory amplexus. The male is able to swim whilst carrying the female, which can either remain in a curled position or straighten her body and contribute to the locomotor activity, by beating her pleopods (Borowsky, 1984, Ward, 1985, Sutcliffe, 2010a). After the eggs have passed through into the marsupium, the male rotates the female underneath him so that he can deposit his spermatophores and hence fertilize the eggs (Sutcliffe, 2010a).

These processes of male attraction and MG rely on chemosensory information that is transmitted by means of waterborne chemical signals produced by the females. It has been suggested that secretion of these hormones happens in two stages: the secretion of a "*primary ovarian hormone*" by the ovary's primary follicular cells, which controls the development of the oostegites (Sutcliffe, 2010a); and the secretion of a "*temporary ovarian hormone*", at the secondary follicular cells, which controls the development of temporary sexual features such as the long ovigerous setae that cover the oostegites during vitellogenesis (Sutcliffe, 2010a).

Males use their aesthetascs to detect female pheromones, thus enabling the search for a cue releasing female and the initiation of precopulatory behaviours (Gleeson, 1982, Hallberg et al., 1997). In some species – *e.g. G. pulex* and *G. duebeni* – the urine of female's acts as an attractant solution for males in the water column (Dunham, 1978, Borowsky, 1985). The presence of a specific pheromone, probably ecdysone or ecdysterone - which control the impending moult in the female and hence signals the short fertility period (Ducruet, 1975) – or a mixture of substances produced at the same time, is suggested to be the vehicle of this attraction (Hammoud et al., 1975).

In *Gammarus*, attraction is present in two different levels: on long distances – by means of a long distance pheromone, probably present in the female's urine; and on short distances – by means of a body contact pheromone, secreted in the female's exoskeleton. While the first is able to operate at a distance to attract nearby males (Dahl et al., 1970, Borowsky, 1985, Borowsky and Borowsky, 1987), the second is released in the female's external body cuticle and elicits courtship and MG behaviour in males (Sutcliffe, 2010a). In 1985, Borowsky demonstrated the existence of long-distance sexual attraction in *Gammarus palustris*, showing that when males were exposed to cues from conspecifics and congenerics in different maturity stages, in binary choice Y-mazes, they were «*more often attracted to receptive females*», compared to non-receptive females and other males (Borowsky, 1985). The effect of other cues (*e.g.* from fish, invertebrates, algae and from the environment) as attractants and repellents on gammarid amphipods has also been described (Williams and Moore, 1985).

At ambient conditions, embryonic development – spanning from egg incubation to post-hatch juveniles – has been described to last from 15 to 18 days (Neuparth et al., 2002, Maranhão and Marques, 2003, Sutcliffe, 2010a). For a couple of days, post-hatch juveniles are able to move freely in and out of the female's marsupium, and begin taking chances in feeding outside the brood pouch. Juvenile development until adulthood takes 6 to 7 weeks at 15 ºC, and it is estimated that *G. locusta* life span can reach from 6 months (Neuparth et al., 2002) to a full year (Ruppert, 2004).

# 2. Objectives

The objective of the present dissertation was to investigate for the first time the transgenerational effects of OA exposure (pCO2 = 800-900 μatm) on the physiology, behaviour and reproductive processes of a gammarid amphipod – *G. locusta*. Understanding how reproductive traits and metabolism, may or may not be impacted by environmental change, and whether these effects are carried throughout subsequent generations, will allow us to infer about the sustainability of amphipod populations in an acidified ocean. Within this context, I hypothesised that this scenario could lead to:

1. Reduction in MG behaviour duration due to metabolic stress;

2. Decrease in female investment in reproduction (number of eggs per female) as a result of energy reallocation;

3. Negative effects on fecundity (number of surviving juveniles per female);

4. Decreased survival into adulthood;

5. Disruption of male chemosensory reception / attraction to female waterborne pheromones;

6. Negative effects on the amphipod's aerobic metabolic rates induced by physiological stress.

The transgenerational analysis between two successive generations (parental F0 and F1) allowed to test hypothesis 1 through 4. Chemosensory disruption and the possibility of metabolic alteration (hypothesis 5 and 6) were tested only on the F1 generation, due to constraints on setup availability and optimization.

# 3. Materials and Methods

## 3.1. Amphipod collection and stock maintenance

A stock culture of *G. locusta* was established using a mix of individuals collected in the wild from a clean site in the South margin of the Sado Estuary (38°27′N,08°43′W), Portugal, and others from a pre-existing culture, originally collected in the same estuary, and kindly provided by the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR). The sampling site has been previously used by other authors for amphipod sampling and collection (see Figure 3.1.1), and has been referenced as a relatively non-contaminated, clean site, with high biological diversity (Costa and Costa, 1999, Neuparth et al., 2002, Martinez-Haro et al., 2016).

The collection process included the gathering of large macroalgae clusters, and the scrapping of the first layers of sandy sediment in the intertidal area at low tide, to ensure recovery for the maximum possible number of individuals. The algae and sand were thoroughly washed and stored in coolers for transportation with enough water and aeration. Upon arrival at the Laboratório Marítimo da Guia (LMG), in Cascais, these were washed and sorted, and all material processed to ensure collection of the maximum number of live individuals of the target amphipod species, for stock reinforcement.

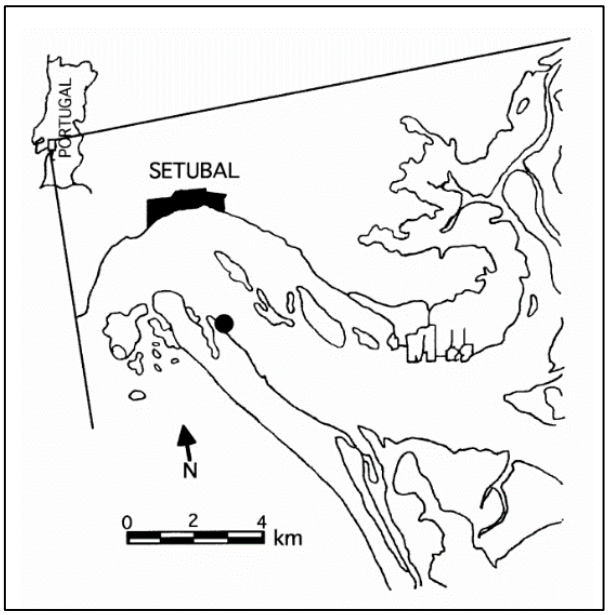



**Figure 3.1.1.** Geographic location of the Sado estuary and the sampling site, indicated by a black circle (Costa and Costa, 1999)

All stocks were kept under stable conditions similar to those found in their natural environment, and the same physico-chemical conditions were applied in the control treatments during the experimental phase (See Table 3.1.1). Individuals were left to acclimate to laboratorial conditions for at least two generations (approximately 60-70) days.

**Table 3.1.1**. Abiotic conditions chosen for stock maintenance.

| Parameter | Conditions |
|---|---|
| Temperature | 18 ± 0.5 ºC |
| pH \| $pCO_2$ | 8.0 ± 0.5 \| ~400 µatm |
| Photoperiod | 12h light : 12h dark |
| Salinity | 35 ± 0.5 |

A continuous, fresh supply of green algae (*Ulva* sp.) was maintained and fish flakes were added once a week as nutritional enrichment, following Costa (1996), who showed that the use of this supplement stimulated individual growth rates and reduced cannibalism in *ex-situ* populations (Costa and Costa, 2000). Stocks were filled with a 2 cm layer of sandy substrate and rocks and small PVC pipes to simulate natural shelter. Water was continuously aerated and was fully replaced at least two times a week.

## 3.2. OA exposure

OA experimental design followed the guidelines of experimental design for acidification research (Cornwall and Hurd, 2016). The flow-through aquatic systems were set in order to maintain total alkalinity, dissolved inorganic carbon speciation due to bacterial activity and acidification of treatments, and the setup consisted in two 20 L tank setups (5 independent replicate tanks per treatment). F0 gammarids were exposed to the following experimental conditions: i) control scenario (18 °C, pH 8.0, 300-400 µatm); and ii) hypercapnic scenario (18 °C, pH 7.7, 800-900 µatm). The values chosen for pH and $pCO_2$ levels for the control and acidified groups were defined according to relevant climate change projections (Doney et al., 2012, IPCC, 2014), and temperature was kept constant at mean annual values for the individual's area of origin in the Sado estuary Table 3.2.1. pH levels were adjusted automatically, via solenoid valves, using the Profilux controlling system (Profilux 3.1, GHL, Germanry) connected to individual pH probes, which monitored values every 2 seconds and lowered the pH by injection of a certified CO2 gas mixture (Air Liquide, Portugal) via air stones, or upregulated by aerating the tanks.

**Table 3.2.1**. Seawater physiochemical parameters in all experimental setups. Temperature, salinity and pH were measured daily and averaged per replicate and per treatment. Total alkalinity (TA) and $pCO_2$ were quantified using the $CO_2$SYS software. *Values* represent mean ± SD.

| Generation | Treatment | Temperature (º C) | Salinity | pH | TA (µmol/kgSW)* | $pCO_2$ (µatm)* |
|---|---|---|---|---|---|---|
| F0 | C | 18,3 ± 1,3 | 35 | 8,09 ± 0,07 | 1932,24 ± 109,83 | 375,86 ± 67,73 |
| | A | 18,4 ± 1,4 | 35 | 7,74 ± 0,12 | 1971,54 ± 64,26 | 827,52 ± 73,19 |
| F1 | CC | 18,8 ± 0,8 | 35 | 8,01 ± 0,10 | 2126,45 ± 112,34 | 354,15 ± 28,68 |
| | AA | 18,8 ± 0,6 | 35 | 7,68 ± 0,09 | 2044,07 ± 140,38 | 825,48 ± 71,47 |
| | AC | 18,7 ± 0,6 | 35 | 8.02 ± 0.06 | 2105,65 ± 108,93 | 366,78 ± 20,54 |
| | CA | 18,8 ± 0,6 | 35 | 7,69 ± 0,12 | 1943,55 ± 88,44 | 803,18 ± 27,82 |

Recording of temperature, salinity and pH was performed daily. The quantification of pH in the replicate tanks was determined using a pH meter (826 pH mobile, Metrohm, Germany) connected to a glass electrode (Schott IoLine, SI analytics), calibrated with TRIS-HCI (TRIS) and 2-aminopyridine-HCl (AMP) (Mare, Belgium) seawater buffers. Seawater carbonate system speciation (SCSS) was quantified following the same method described in Repolho et al. (2017). SCSS was calculated from TA (total alkalinity, measured by spectrophotometry at 595 nm) and pH measurements. Values of bicarbonate and pCO2 were calculated using the $CO_2$SYS software (Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, USA), with dissociation constant, as refitted.

Temperature inside the tanks was kept constant by partial immersion in a large bath, supplied with water that was conditioned by a chiller and thermostats to the desired levels. The tanks were illuminated from above with white fluorescent lamps, with a fixed photoperiod of 12 h light and 12 h dark). Daily water renewal for each replicate was calculated to be approximately 10 times its volume.

## 3.3. Transgenerational design

The transgenerational experimental design is summarized in Figure 3.3.1.

Thirty MG couples were randomly selected from the stock tanks at the beginning of the experimental phase. These individuals were maintained in two 60-L tanks (15 couples for each treatment) and gradually acclimated to the desired treatment conditions (*i.e*. pH was maintained at 8.0 for the controls and decreased gradually by 0.1 units per day in the acidified treatments, until reaching the target pH). This procedure guaranteed that the first generation (F0 or paternal generation) was conceived and developed already under the respective treatment conditions and, as such, exposure to each pH level was assured since shortly before the beginning of the embryonic development.

Following acclimation, normal gestation was allowed to continue with removal of the males from the pre-F0 population after the end of the MG behaviour. Upon birth and exit from the mother's brood pouch, new-borns were randomly sorted into the 4-L replicate tanks (Figure 3.3.2). A total of 25 new-borns were randomly placed into each treatment's 5 replicates.

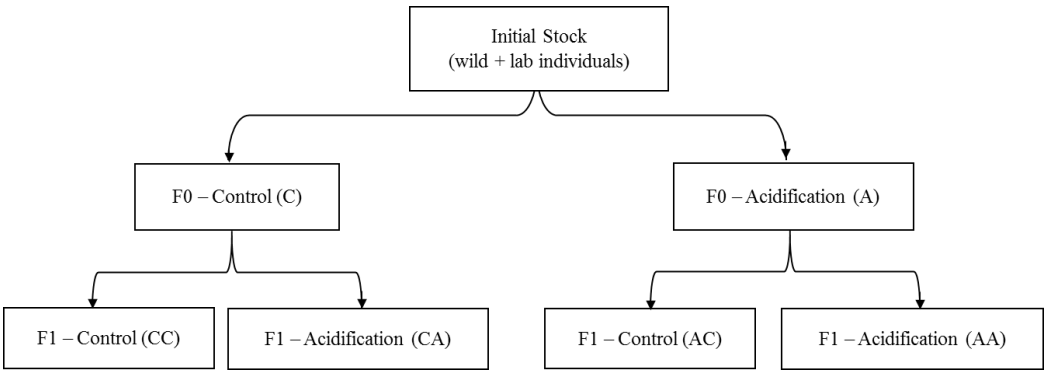



**Figure 3.3.1.** Global experimental design: after ensuring 60 to 70 days of stock acclimation, the last new-born individuals were left to develop and reach maturity. These individuals would be separated into two groups: one control and one acidified, which would then constitute the parental generation (F0). The progeny resulting of the intra-group reproduction would be separated, with half being subjected to the same treatment conditions as their progenitors, and the other subjected to conditions of the opposing treatment.

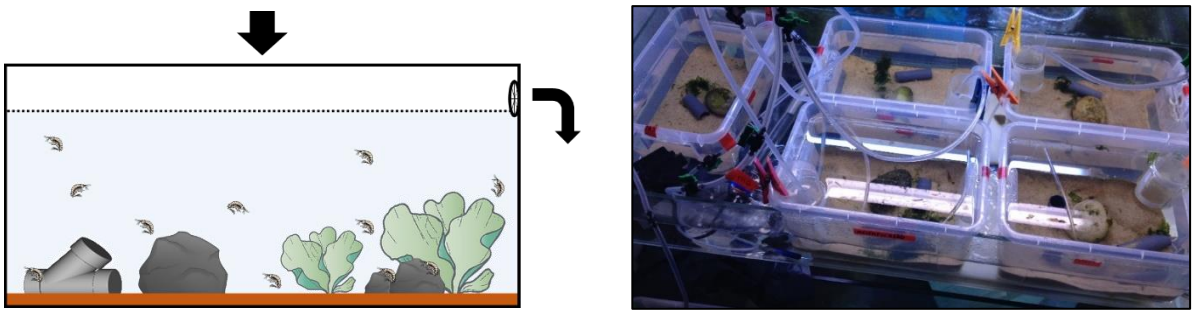


**Figure 3.3.2.** Schematic (left) and photograph (right) of the 4 L replicate tanks used in the setup. In the photograph, the tubes which conducted water from the mixture tanks by gravity can be observed, as well as the stones and PVC pipes placed above the substrate for shelter and the small clusters of *Ulva* sp. supplied for both shelter and nutrition.

Each experimental generation was reared from juvenile to sexual maturity (approximately 30 days of age). After this period, sampling of reproductive, behavioural and metabolic outputs (*i.e.* the latter only for the F1 generation, as previously mentioned) took place, with the collection of new-borns to ensure the establishment of the following generation. Population size depended on youngling recruitment, and replicates were populated with 25 new-born F1 individuals.

## 3.4. Reproductive traits

### *3.4.1 Reproductive traits*

Reproductive outputs were measured continuously upon each generation's pre-reproductive phase. Starting at approximately 20 days of exposure, when overall size, behaviour and sexual dimorphism indicated that individuals would soon reach maturity, tanks were checked twice a day for recently formed pre-copulatory MG couples. When identified, these couples were separated from the group and placed into transparent plastic cups (220-mL) filled with their respective replicate's water. This allowed for an accurate measurement of the desired outputs and also helped to minimize the risk of cannibalism of females by other extra-couple individuals or aggressiveness interfering with mating and embryonic development. After couple separation, males were quickly removed from the cups and reintroduced into their original population. The time (in days) from MG initiation until separation was registered. Afterwards, approximately half of the ovigerous females were sacrificed for determination of female investment, while the other half was allowed to complete gestation, with measurement of embryonic development time and fecundity. A total of 146 females (50 ♀ in F0 and 96 ♀ in F1) and 61 males (for the MG duration output) were sampled.

### *3.4.2 Female investment*

Given the difficulty of isolating a female prior to reaching sexual maturity, without resorting to invasive and time consuming morphological analysis (due to the apparent similarities between immature females and young females, and to the lack of synchronization in female's reproductive cycles), only ovigerous females possessing fertilized eggs were sampled. Investment was then accounted as the total number of eggs present in the marsupium of a gravid female, after MG behaviours had ceased and the couple had separated.

Previous trials demonstrated that females could be anesthetized either via short time exposure to low temperatures or carbonated water (Sundelin et al., 2008), followed by lateral and ventral photography and consequent egg count with ImageJ. This allowed females to be returned to their respective population, preventing their sacrifice, and even allow for a direct comparison between a female's investment and the total number of younglings that reached the end of their embryonic development. However, comparisons between photographic data and data from the sacrifice of a number of females showed a disparity of over 60% in the total number of eggs. As such, in order to ensure a correct measurement of the number of eggs inside the brood pouch, females were sacrificed by freezing (-80 ºC) shortly after the parting of the male.

After sacrifice, a female's marsupium would be gently scrapped using a blunt laboratory tweezer, in the anterior-posterior direction. This allowed for efficient egg removal and minimized potential egg damage or loss (Figure 3.4.2)

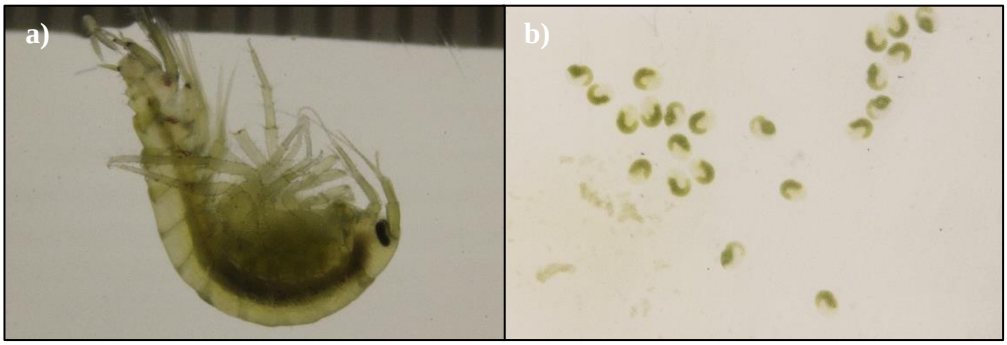



**Figure 3.4.2. (**a) Recently sacrificed female with marsupium filled with eggs. (b) Some of the sacrificed female's eggs following the marsupium's scrapping, and with embryonic features already visible.

### *3.4.3 Embryonic development and Fecundity*

To estimate the average duration of embryonic development, the amount of time (in days) spanning from oviposition and male fertilization, to the exit of all juveniles in the female's brood pouch was registered. Females with recently fertilized eggs were kept separated from the rest of the population, and maintained in plastic cups until eventual youngling departure. Small pieces of algae were supplied to ensure enough food was available for the female and her progeny, thus minimizing losses by maternal cannibalism.

When the marsupium appeared empty, water from the cups was processed and juveniles separated from the females. Female fecundity was accounted as the number of juveniles released per female in each replicate (Maranhão and Marques, 2003). Following separation from their mother, juveniles were counted and pooled in small tanks per replicate, in preparation for the next experimental generation.

## 3.5 Behavioural trials

In order to investigate the possible disruption of a male's chemosensory reception to female scent cues in the water column, a binary-choice experiment was designed. The chosen experimental setup (Figure 3.5.1) was based on a system that was firstly described in 1913, and developed to study the response of fishes to different levels of dissolved $O_2$ and $CO_2$ (Shelford and Allee, 1913). Since then, this design has been used and adapted by several authors to test chemical preference and avoidance in marine organisms (Hall et al., 1982, Hall et al., 1984, Pinkney et al., 1985, Dempsey, 2009)

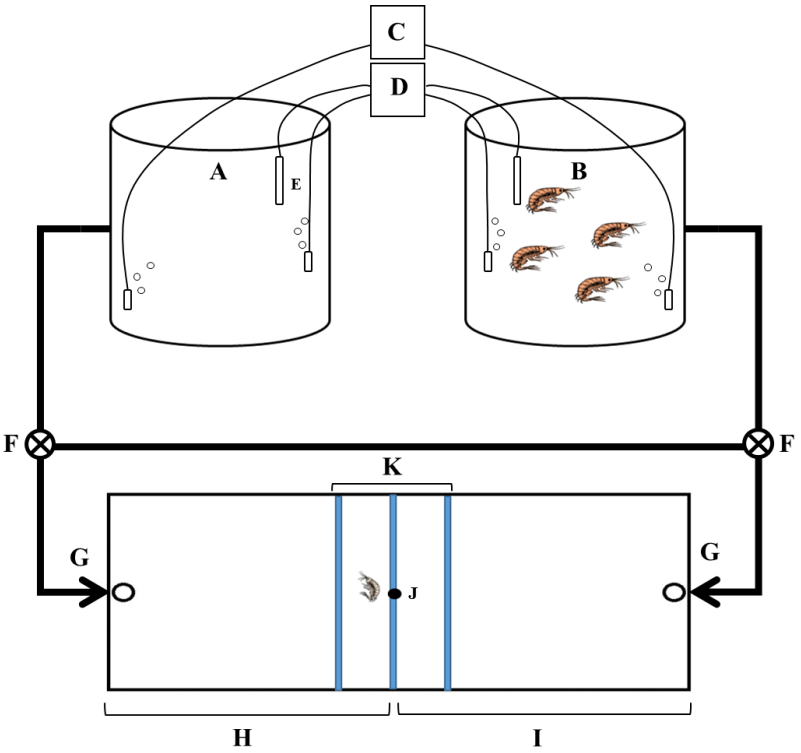



**Figure 3.5.1.** Binary-choice setup used in the behavioural trials to test for disruption on male-mate attraction in *G. locusta*. A – Control cue tank; B – Test cue tank with females; C – Air compressor; D – $CO_2$ injection system; E – pH probe; F – Easy-switch cue side system; G – cue input; H – Association zone A; I – Association zone B; J – centre drain; K – Starting zone.

A 60 x 20 cm rectangular arena, with a water depth of 3 cm, was used (Figure 3.5.2). A single individual was placed in the choice arena, where it would be able to swim freely between two opposing water masses (*i.e.* one containing female scent cues to incite a males' response, and other corresponding to clean filtered seawater). The water masses were continuously supplied, in a flow through manner, by two 20 L header tanks, in which cues had been prepared for at least 48 hours prior to trials, by leaving the highest possible number of adult females in a tank to release odours (*e.g.* urine and pheromones) capable of attracting males. A drain, placed at the centre of the starting zone, allowed a constant water height while ensuring that both cue flows continuously drained away, hence creating two opposing water currents. As mentioned, one header tank was filled with normal filtered seawater, while the other was filled with water from the same source and populated with 30 to 35 stock adult females. Both header tanks were continuously aerated. Amphipod males were tested in the respective treatment-conditioned water. The possibility that acidified water would damage or degrade the female cues involved in male attraction was accounted for.

**Figure 3.5.2** Overhead view of the binary-choice arenas.

Preliminary testing was performed for setup optimization, in order to establish optimum flow rates to prevent mixture between the two water masses (See Figure 3.5.3 and Video 1 in supplemental). These tests consisted on the definition of a specific flow and by visual analysis using a blue dye (food colouring).

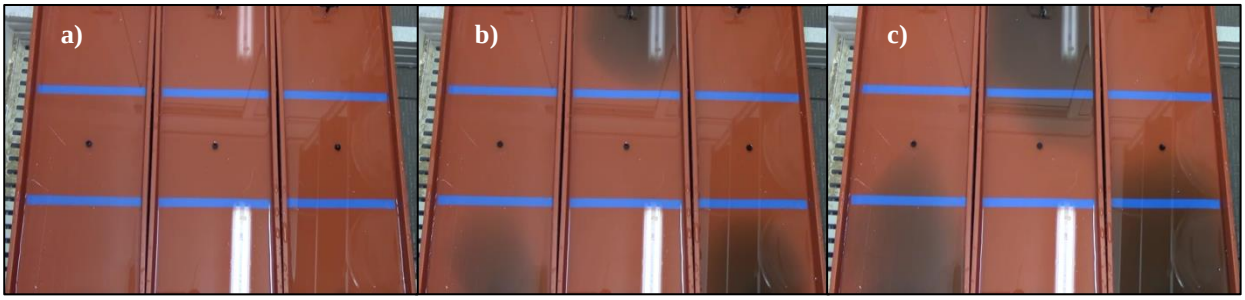



**Figure 3.5.3.** Blue dye test for setup optimization. (a) 0 sec; (b) 600 sec; (c) 1200 sec. During trials, and as demonstrated in the video in supplemental, after 20 minutes of cue input there was no mixture of the water masses in either association zone. Larger version included in appendix 9.1.

Following setup optimization, an amphipod's position after being released from the starting compartment could be accurately determined – association zone A or B – with minimum error. The starting zone was used to randomly assign the position of the starting compartment in each arena, and to minimize possible biases and uncontrolled variables that could interfere with the water flow and hence prevent a confident analysis of the first seconds of trial run following release. Each trial consisted of a total run time of 20 minutes: 10 minutes acclimation and initial exposure, and 10 minutes of free movement inside the arena. Amphipods were placed in small perforated holding containers inside the starting zone, to acclimate to the water flow and overall surroundings. Acclimation duration was decided after preliminary trials, as it ensured a minimum exposure to the cues to allow for initial stimulation. After 10 minutes, the containers were lifted one by one, and the amphipod's movements were recorded with a video camera for posterior analysis. A total of 24 adult males from the F1 generation (12 per main treatment, 2 to 3 per treatment) were randomly selected from the replicate tanks and tested two times, with cue input reversal. The response time of an amphipod – the time required for it to start moving – as well as first choice of direction and cumulative time (*i.e.* the mean proportion of time spent in the presence of the female cue, compared to the total amount of time spent in both association zones) were

registered. Out of the 24 males used in these trials only those that met the following requirements were included for the statistical analysis: i) an individual's initial response time was less than one minute (60s); ii) a male's first choice for direction of movement was clearly defined (*i.e*. the male did not move to the centre drain, and instead chose one of the association zones); iii) an individual spent at least 7 minutes (70% of the total trial time) exhibiting active searching or exploratory behaviours.

## 3.6 Routine Metabolic Rate (RMR) – Oxygen consumption

Measurements of routine metabolic rate ($O_2$ consumption rate) were used to address possible effects of OA on amphipod's metabolic *fitness*. RMR is an ecologically relevant measure of metabolism, that is applied in situations when an animal's locomotor activity cannot be completely controlled (*i.e*. the animal is neither completely still, nor completely active during measures) (Ikeda, 2016). The design consisted of a static, intermittent flow-through respirometry system (Clark et al., 2013) and is represented in Figure 3.6.1. The dimensions and volume of the chamber ($V_{chamber + tubbing}$ = 90 mL) were defined according to the average individual size of *G. locusta*, so that a large individual could easily be accommodated in a static position. At the same time, the combined volume of chamber plus tubbing was small enough to allow a correct measurement of individual amphipod's $O_2$ consumption rate, without it mixing with background respiration noise – the possible consumption by microorganisms in the water column.

Oxygen measurements were achieved by attaching a small sensor spot (thin sheet of material coated in relevant $O_2$ sensing chemicals), on the inside of one of the transparent faces of the cylindrical chambers. A fibre-optic sensor was attached in the same area on the outside of the chamber, which measured small changes in the sensor spot material's colour, which reacted to the amount of dissolved oxygen in the water volume. Recirculation was needed in order to prevent water stratification that could potentially bias measurements: this was achieved by using a peristaltic pump (Masterflex L/S® series), which ensured a continuous flow of approximately 40 ml $min^{-1}$. The entire system was submerged in a bath of oxygenated and previously conditioned water to the desired pH and temperature levels.

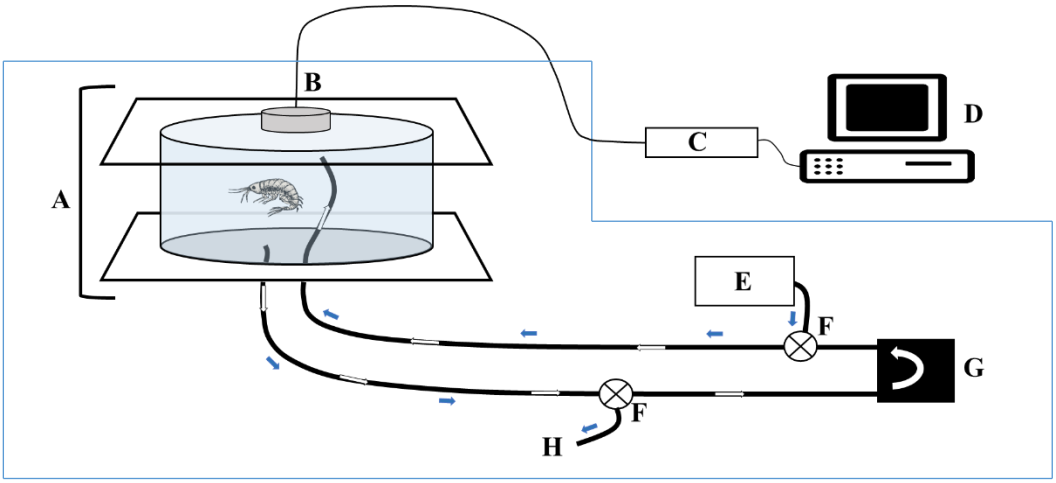



**Figure 3.6.1.** Schematics of the static, intermittent flow respirometry system used in for the assessment of oxygen consumption rates in *G. locusta*. A) sealed acrylic chamber; B) Fibre-optic oxygen sensors (optoads); C) "Firesting" – fibre-optic oxygen meter; D) Computer with Pyro Oxygen Logger Software (PyroScience™); E) Flush pump; F) Flush input / output valve; G) Peristaltic pump; H) Treatment conditioned bath (Temperature and pH controlled). White arrows represent permanent water circulation ensured by the peristaltic pump; Blue arrows represent intermittent path of flush of oxygenated water.

Each amphipod was gently introduced into one sealed acrylic chamber, following sensor calibration in $O_2$ saturated water, and then left for 40 minutes to acclimate to the chamber with recirculating water. Following acclimation, a 5-minute flush with oxygenated water from the bath signalled the start of a 25-minute measurement period, and was performed by means of a flush pump. Each flush ensured that $O_2$ levels inside the chamber returned to start values, and the run-time of 25 minutes ensured that they never fell below 80% air saturation levels (Hughes, 1973, Clark et al., 2013). A total of 3 flushes (three replicate measurements) were made for each individual and no individual was used twice. Both acclimation and measurement periods were based on Ramus & Forward (2011), who measured oxygen uptake rates in the amphipod *Talorchestia longicornis*. In order to ensure proper acclimation to the chamber and to the respirometry system, an acclimation time of 40 minutes was considered for *G. locusta*. A total of 28 individuals (14 per treatment) were sampled using this methodology. Efforts were made to ensure that amphipods remained not stressed for the majority of run time, to minimize the possible disturbance of $O_2$ gradients, or peaks of activity (Clark et al., 2013): tests were conducted under reduced lighting and the experimenter's movements next to the respirometry system were minimized and concealed from the animal's view, in order to reduce possible stress-induced movements.

Oxygen consumption during each 25-minute run was logged in Pyro Oxygen Logger Software®. After each test, the individual amphipod's wet weight (WW) was determined to the nearest $1e^{-5}$ g, by removing excess water with paper towelling for 5 to 10 seconds, and then transferring them to a tared petri-dish (Halcrow and Boyd, 1967). Sex as well as a qualitative measure of the animal's activity during trials were registered. Data from animals that were apparently stressed was not taken into consideration. Determination of mass-specific RMR (µmol $O_2$ $g^{-1}$ $h^{-1}$) is widely common in fish and can also be applied to molluscs and crustaceans (Clark et al., 2013), according to the formula presented below.

**3.6.1.** Routine Metabolic Rate $\left(\mu\text{mol O2 g}^{-1}\ \text{H}^{-1}\right) = \frac{[(Vr-Vf)*\Delta O2]}{WW\ (g)*\ \Delta t\ (s)}$

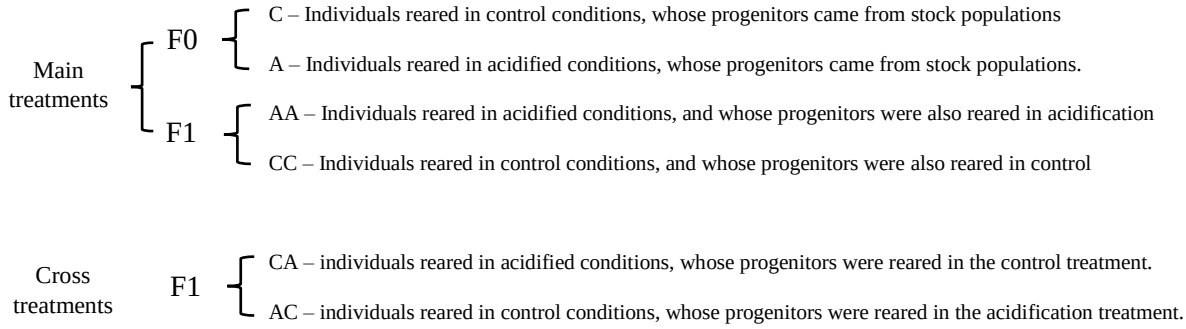
The slope of oxygen consumption for each 25-minute registry period was calculated by applying a linear regression to the continuous raw trace ($\Delta_{O2}/\Delta_t$), and only slope regressions with coefficients ($R_2$) greater than 0.90 were used. Given that an individual's volume ($V_f$) did not constitute over 5% the total volume of the chamber + tubbing ($V_r$), the individual volume was discarded for all calculations.

# 4. Data analysis

For all reproductive outputs, intra-generation and intra-treatment comparisons, as well as cross-treatment comparisons were performed to evaluate transgenerational effects of ocean acidification, and possible carry-over effects.

In behavioural and metabolic outputs, results concern only the main acidified (AA) and main control treatments (CC) from the F1 generation and, thus, no transgenerational analysis is performed.

Important terminology used for the analysis, and which will be used in the results and discussion sections follows:




Analysis of reproductive outputs was performed via generalized linear models (GLM) using the Poisson family of distribution. When Poisson over dispersion was different than 1, the family of distribution was changed to Negative Binomial (dispersion higher than 1) or to Quasipoisson (dispersion smaller than 1), and model definitions adjusted accordingly. Behavioural outputs were also analysed with resort to GLM, with the appropriate families of distribution: Gaussian (response time), Gamma (cumulative time) and Binomial (first choice). Oxygen consumption rates were analysed via GLMs for continuous variables (Gaussian).

For all models, «Generation», «Treatment», and «Replicate» and, in the behavioural trials, «Test» and «Individual» were included as factors, to account for intrinsic variability in random effects analysis. The best model for each output was ultimately chosen with resource to the calculation of the Akaike Information Criterion (AIC) – as a rule, the best model was the one which featured the smallest AIC. All data analysis was performed in RStudio Software (Version 1.0.143– © 2009-2016 RStudio, Inc.)

# 5. Results

## 5.1. Survival

Results for the survival (%) analysis are presented in figure 5.1.1 and in table 5.1.1. Survival of individuals was significantly affected by high-$CO_2$ exposure for the first generation (GLMM, z: -1.97, $p < 0.05$), with a mean value of approximately 65%, below survival of F0 amphipods exposed to control conditions (75%). In F1, survival in control (CC) and acidified (AA) treatments remained stable compared to the F0 equivalents while cross treatments AC and CA exhibited lower survival rates (CC x AC – GLMM, z: 2.90, $p < 0.001$; CC x CA – GLMM, z: 3.41, $p < 0.001$). Individuals that were born and raised under acidified conditions as their parents, named AA in the figure 5.1.1b, showed a wide range of survival values, from approximately 30 to 90%. No significant differences were found between the main acidified and control groups (GLMM, z: -1.61, $p > 0.05$). Likewise, cross-treatments AC and CA showed no differences between them (GLMM, z: 0.57, $p > 0.05$) nor between the main F1 acidified treatment (AA x AC – GLMM, z: 1.36, $p > 0.05$; AA x CA – GLMM, z: 1.92, $p > 0.05$).

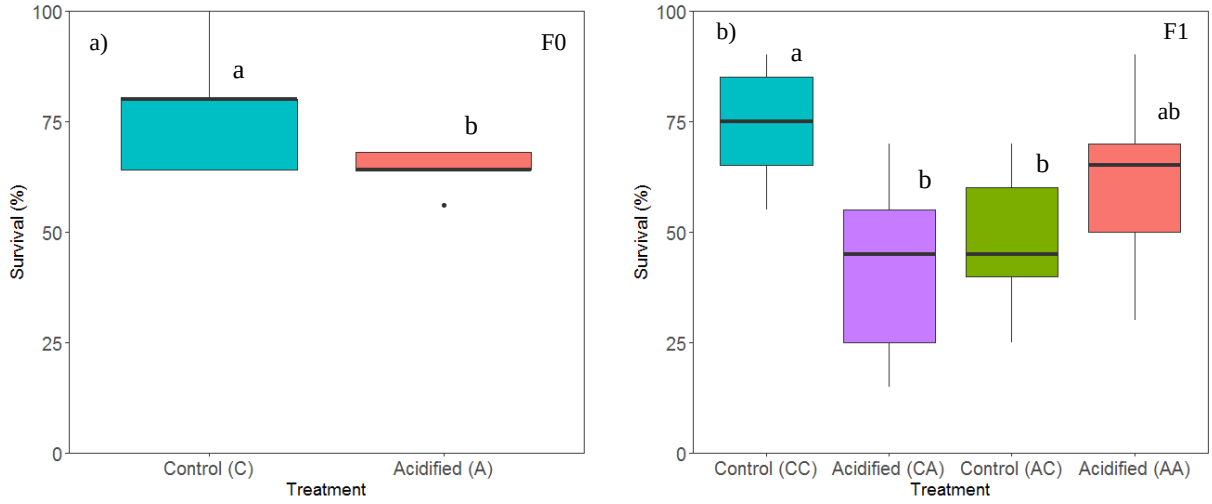



**Figure 5.1.1.** Survival ratio (%) by treatment for F0 (a) – Control (C) and Acidified (A); and F1 (b) – Control (CC), Acidified (AA) and cross treatments Control (AC) and Acidified (CA). a) $n_{CC} = 25$, $n_{AA} = 25$; b) $n_{CC} = 25$, $n_{CA} = 25$, $n_{AC} = 25$, $n_{AA} = 25$ Values represent mean percentages ± SD. Statistical differences (p-value < α, α = 0.05) are represented by different superscript letters over each plot.

Both control and acidified treatments showed no significant differences from F0 to F1 (Acidification – GLM, z: -0.641, $p > 0.05$; Control – GLM, z: -0.194, $p > 0.05$). Regarding cross-treatment comparisons, only the parental cross F1 CA x F0 CC revealed significant differences in mean replicate survival (see Table 5.1.1).

**Table 5.1.1**. Results of the GLMM models (Poisson with replicate as random effect) applied on the cross-treatment comparisons for analysis of carry-over effects in the Cumulative time analysis. Statistical differences at p-value < α, α = 0.05.

| Cross comparison | z value | p-value |
|---|---|---|
| Parental cross: F1 CA x F0 CC | -3.170 | p < 0.001 * |
| Parental cross: F1 AC x F0 AA | -0.711 | 0.4769 |
| Acidification cross: F1 CA x F0 AA | -1.280 | 0.2007 |

## 5.2. Reproductive Traits

### *5.2.1 Mate-guarding duration*

The results of the mate-guarding duration analysis are presented in Figures 5.2.1 and table 5.2.1. Data revealed that mate-guarding duration lasted, in average, up to five days. In the parental generation (F0), MG duration was significantly reduced in couples subjected to high-$CO_2$, compared to the control group (GLM, t: 2.995, $p < 0.05$). In the F1 generation, both the main acidified (AA) and control treatments (CC) showed no significant differences (GLM, t: 1, $p > 0.05$. Regarding cross-treatments in F1 comparisons, significant differences were found between the CC and CA treatments (GLM, t: -4.363, $p < 0.05$), but no significant differences between AC and AA (GLM, t: -1.560, $p > 0.05$).

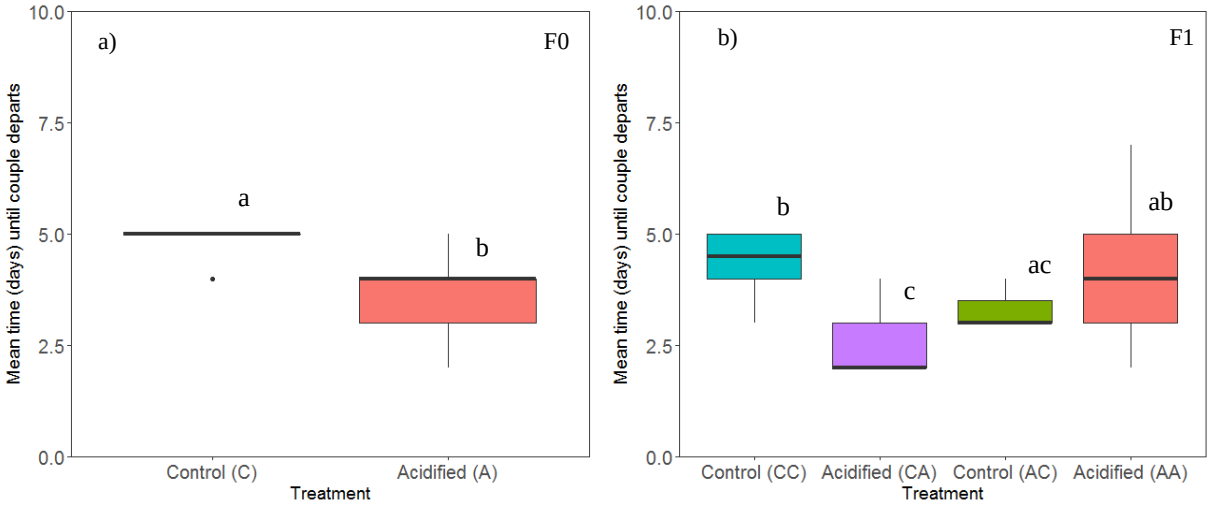



**Figure 5.2.1** Mate-guarding duration: mean number of days until a mate-guarding couple parted for F0 (a) – Control (C) and Acidified (A); and F1 (b) – Control (CC), Acidified (AA) and cross treatments Control (AC) and Acidified (CA). Values represent mean ± SD. a) $n_{CC} = 8$, $n_{AA} = 10$; b) $n_{CC} = 12$, $n_{CA} = 8$, $n_{AC} = 7$, $n_{AA} = 12$. Statistical differences (p-value < α, α = 0.05) are represented by different superscript letters above each plot.

No significant inter-generation differences (F0 x F1) were found in either of the main treatments (Acidification – GLM, t: 0.738, $p > 0.05$; Control – GLM, t: -1.746, $p > 0.05$).

**Table 5.2.1**. Results of the GLM (Quasipoisson) models applied on the cross-treatment comparisons (Parental and acidification crosses) for analysis of carry-over effects in mate-guarding duration. Statistical differences at p-value < α, α = 0.05.

| Cross comparison | t-value | p-value |
|---|---|---|
| Parental cross: F1 CA x F0 CC | 6.811 | p < 0.001 * |
| Parental cross: F1 AC x F0 AA | -0.727 | 0.478 |
| Acidification cross: F1 CA x F0 AA | -2.468 | 0.025 * |

### *5.2.2 Female investment*

The results regarding the female investment, expressed as the mean number of eggs carried, are shown in Figure 5.2.2.

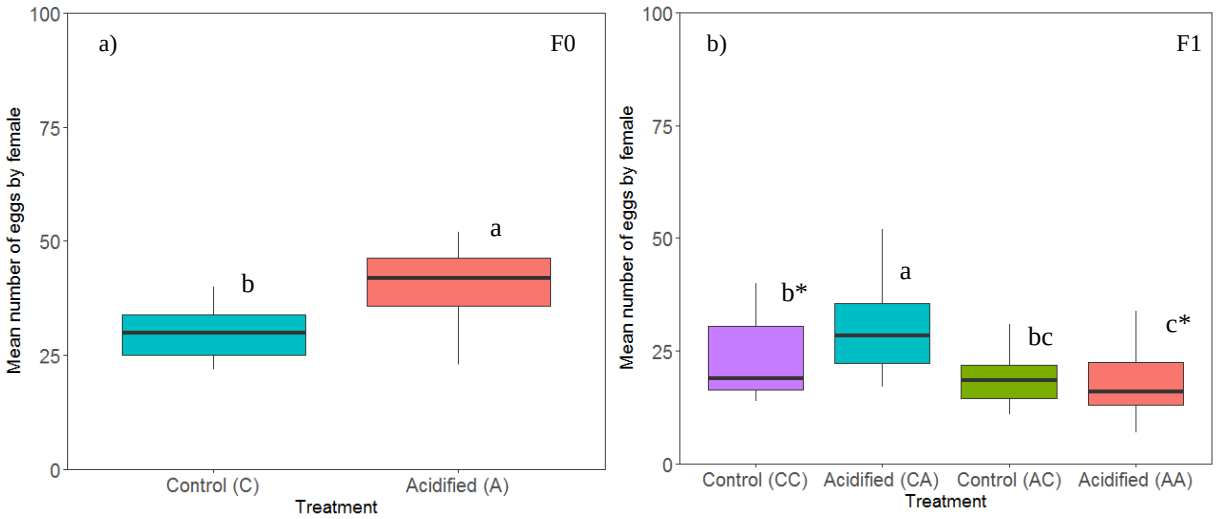



**Figure 5.2.2.** Female investment: mean number of eggs by female for F0 (a) – Control (C) and Acidified (A); and F1 (b) - Control (CC), Acidified (AA) and cross treatments Control (AC) and Acidified (CA). Values represent mean ± SD. a) $n_{CC}$ = 12, $n_{AA}$ = 13; b) $n_{CC}$ = 15, $n_{CA}$ = 10, $n_{AC}$ = 14, $n_{CA}$ = 15. Statistical differences (p-value < α, α = 0.05) are represented by different superscript letters above each plot. Asterisks represent significant differences between generations (F0 x F1) (p-value < α, α = 0.05).

Females from the first generation that were reared in a high-$CO_2$ environment produced significantly more eggs than their control counterparts (GLM, z: -3.269, $p < 0.0001$), but this tendency was reversed in F1 (GLM, z: 2.185, $p < 0.05$). Also in F1, the cross-treatment CA showed an egg count per female significantly higher than the other treatments, and - just as in F0 - this treatment produced significantly more eggs than the control group of the same generation (GLM, z: 1.997, $p < 0.05$).

Regarding inter-generation comparisons (F0 x F1), a significant reduction in the number of eggs per female was observed in both the acidified (GLM, z: -6.784, $p < 0.0001$) and the control treatments (GLM, z: -2.396, $p < 0.05$), but from F0 to F1 differences are more pronounced for the high-$CO_2$ group (see Figure 5.2.2). Cross comparisons for analysis of carry-over effects and first exposure equivalent are present in Table 5.2.2.

**Table 5.2.2**. Results of the GLM models (Negative Binomial) applied on the cross-treatment comparisons for analysis of carry-over effects in Female investment. Statistical differences at p-value < α, α = 0.05.

| Cross comparison | z-value | p-value |
|---|---|---|
| Parental cross: F1 CA x F0 CC | 0.959 | 0.338 |
| Parental cross: F1 AC x F0 AA | -7.116 | p < 0.001 * |
| Acidification cross: F1 CA x F0 AA | -2.886 | p < 0.001 * |

### *5.2.3 Duration of embryonic development*

Results of the analysis of duration of embryonic development are presented in Figure 5.2.3. No significant effects of high-CO2 exposure on embryonic development duration were revealed by the statistical analysis for the F0 (A x C - GLM, z: 0.152, p > 0.05) or F1 generations (A x C – GLM, z: 0.228, p > 0.05). Cross-treatments also showed similar mean durations as the other F1 groups (on average 10 days ± SD until all new-borns had left the marsupium).

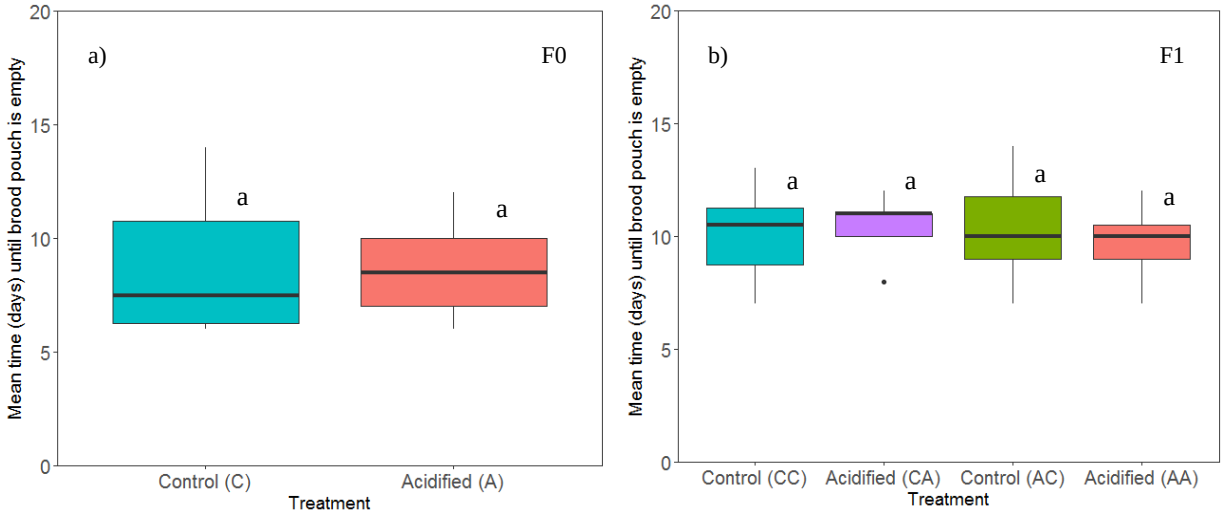



**Figure5.2.3.** Embryonic development: mean number of days until a female's brood pouch was empty of new-borns for F0 (a) – Control (C) and Acidified (A); and F1 (b) – Control (CC), Acidified (AA) and cross treatments Control (AC) and Acidified (CA). Values represent mean ± SD. a) $n_{CC}$ = 10, $n_{AA}$ = 10; b) $n_{CC}$ = 12, $n_{CA}$ = 8, $n_{AC}$ = 9, $n_{AA}$ = 12. Statistical differences (p-value < α, α = 0.05) are represented by different superscript letters above each plot.

Duration of embryonic development remained stable for both main treatments across generations, with no significant differences being revealed between F0 and F1 (Acidification – GLM, t: 1.762, p > 0.05; Control – GLM, t: 1.314, p > 0.05).

In cross-treatment comparisons (see Table 5.2.3), no significant differences were revealed in the parental cross comparisons (F1 CA x F0 CC; F1 AC x F0 AA), and however, the CA treatment registered a significantly higher duration of embryonic development (approximately 11 days ± 1) compared with the first acidified treatment in F0 (approximately 9 days ± 2).

**Table 5.2.3**. Results of the GLM (Quasipoisson) models applied on the cross-treatment comparisons for analysis of carry-over effects in embryonic development duration. Statistical differences at p-value < α, α = 0.05.

| Cross comparison | t-value | p-value |
|---|---|---|
| Parental cross: F1 CA x F0 CC | -1.499 | 0.152 |
| Parental cross: F1 AC x F0 AA | 1.836 | 0.085 |
| Acidification cross: F1 CA x F0 AA | 2.136 | 0.048 * |

*5.2.4 Fecundity*

The fecundity analysis results are presented in Figure 5.2.4 and Table 5.2.4.

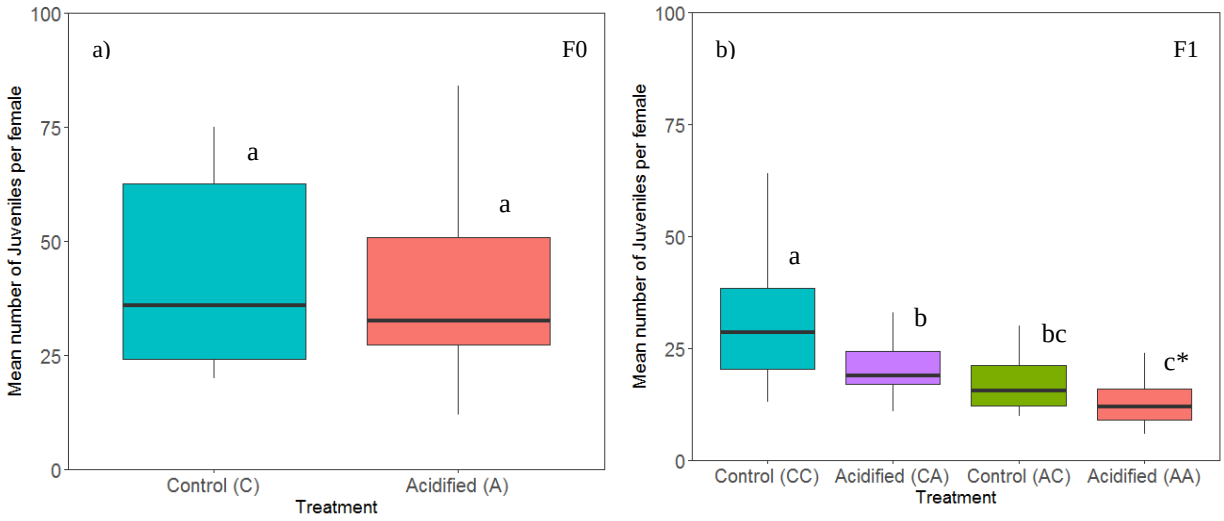



**Figure 5.2.4.** Fecundity: mean number of juveniles that exited a female's brood pouch for F0 (a) – Control (C) and Acidified (A); and F1 (b) – Control (CC), Acidified (AA) and cross treatments Control (AC) and Acidified (CA). a) $n_{CC}$ = 14, $n_{AA}$ = 14; b) $n_{CC}$ = 10, $n_{CA}$ = 8, $n_{AC}$ = 8, $n_{AA}$ = 7. Statistical differences (p-value < α, α = 0.05) are represented by different superscript letters above each plot. Asterisks represent significant differences between generations (F0 x F1) (p-value < α, α = 0.05).

No significant differences were found on fecundity for the F0 generation (GLM, z: 1.356, p > 0.05), between acidified and control treatments. However, fecundity declined significantly from F0 to F1 in the acidified group (Acidification – GLM, z: -4.684, p < 0.001), whilst the F1 main control group remained unaltered (Control – GLM, z: 1.356, p > 0.05). Fecundity for F1 AA was also significantly lower than F1 CC (GLM, z: 4.286, p < 0.001). Cross-treatments CA and AC were statistically similar (GLM, z: 0.786, p > 0.05), and with fecundity values significantly lower than control (CC x CA – GLM, z: -3.175, p < 0.05; CC x AC – GLM, z: -2.363, p < 0.05). All cross comparisons featured significant differences (see Table 5.2.4).

**Table 5.2.4**. Results of the GLM models (Neg Binomial) applied on the cross-treatment comparisons for analysis of carry-over effects in the number of juveniles per female. Statistical differences at p-value < α, α = 0.05.

| Cross comparison | Z value | p-value |
|---|---|---|
| Parental cross: F1 CA x F0 CC | 3.689 | p < 0.001 * |
| Parental cross: F1 AC x F0 AA | -3.21 | p < 0.001 * |
| Acidification cross: F1 CA x F0 AA | -3.852 | p < 0.001 * |

## 5.3. Behavioural outputs

Results for the behavioural trials performed at the F1 generation are presented in the next section.

### *5.3.1 Response time*

Results for the response time analysis are presented in Figure 5.3.1. Males reared in the high-$CO_2$ treatment showed significantly longer response times upon release, compared to the control group (LMM, t: -2.455, $p < 0.05$).

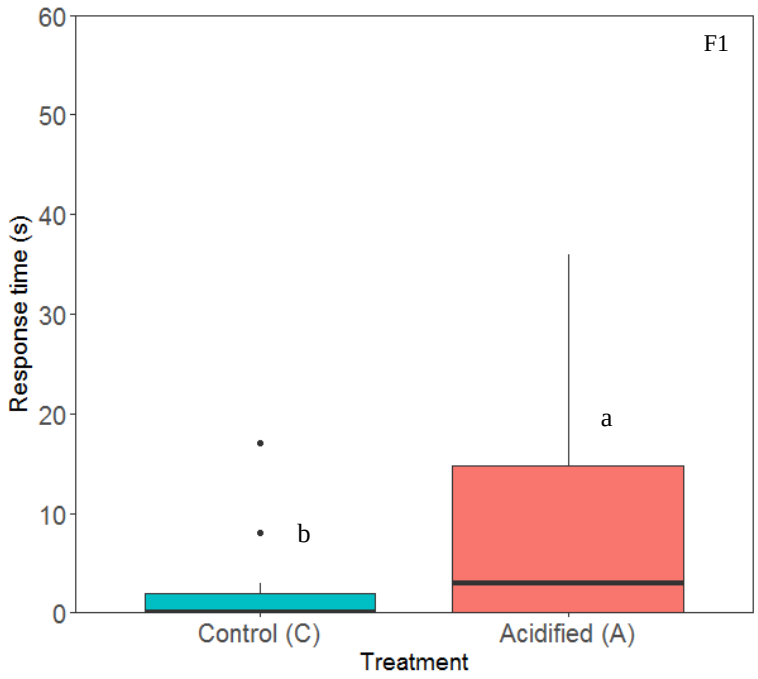



**Figure 5.3.1.** Mean response time (seconds) until an individual started movement towards one of the association zones, after release from the starting compartment. Comparison between treatments - control (C) and acidified (A); for the F1 generation. $n_{CC}$: 9, $n_{AA}$: 11. Statistical differences (p-value < α, α = 0.05) are represented by different superscript letters above each plot.

### *5.3.2 First choice*

Results of the first choice analysis are presented in Figure 5.3.2. Regarding the frequency of correct choice (female cue present) in the total number of trials, no significant differences were found between the acidified and the control groups (GLMM, z: 1.50, p > 0.05).

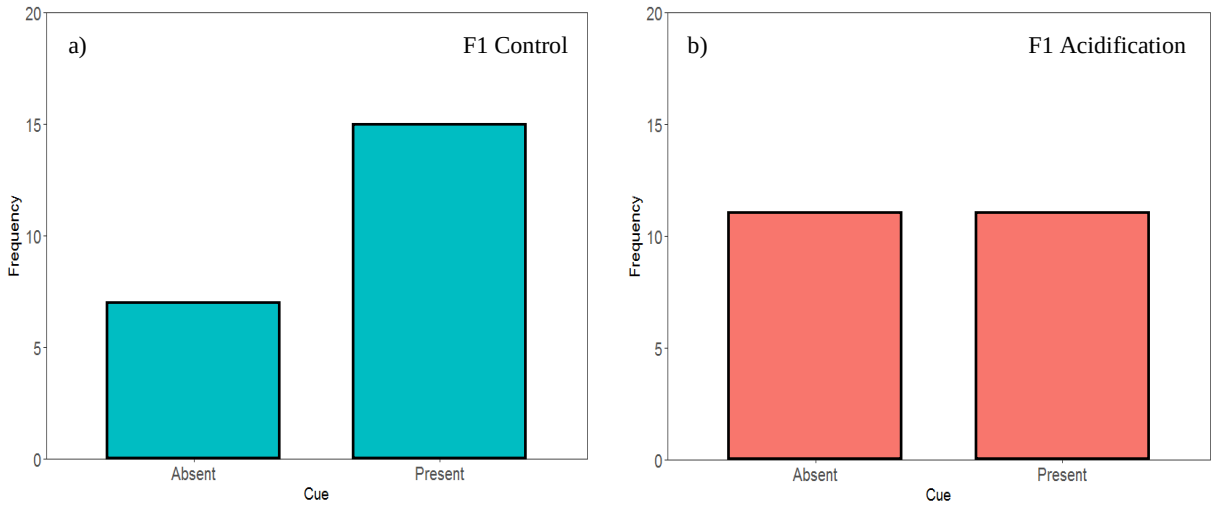



**Figure 5.3.2.** First Choice: bars represent absolute frequencies for the first choice of the direction of movement upon release from the starting compartment: cue present vs cue absent, in the control (a) and acidified (b) treatments. $n_{CC}$: 11, $n_{AA}$: 11.

### *5.3.3 Cumulative time*

Regarding cumulative time, males from the acidified treatment spent significantly less time (in proportion) than control males in the association zone with the female cue (GLMM, t: 2.292, p > 0.05) (Figure 5.3.3).

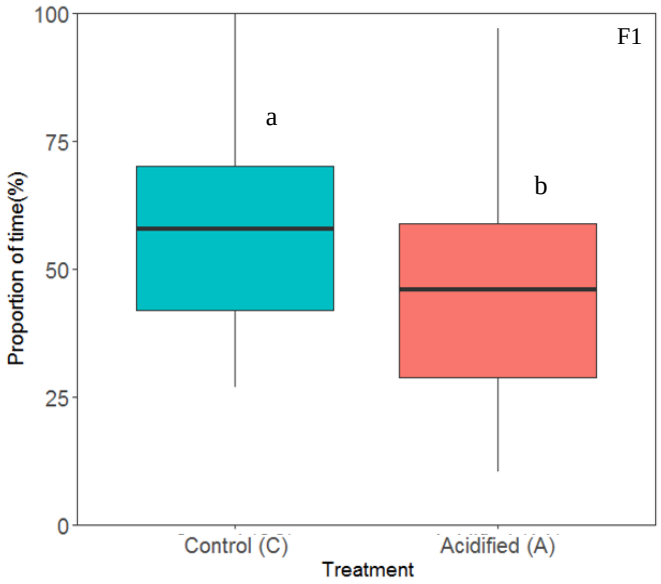



**Figure 5.3.3.** Cumulative time: mean proportion of time spent in the presence of the test (female scent) cue, for each treatment in the F1 generation. $n_{CC}$: 11, $n_{AA}$: 11. Total trial time was 10 minutes. Statistical differences (p-value < α, α = 0.05) are represented by different superscript letters above each plot.

## 5.4. Oxygen consumption rates

Results of the metabolic trials performed at the F1 generation are featured in Figures 5.4.1 and 5.4.2. Oxygen consumption rates ($VO_2$ µmol $g^{-1}$ $h^{-1}$) were significantly lower for individuals exposed to acidified conditions, compared to control individuals (LMM, t: 2.865, $p < 0.05$). Gender comparisons for each treatment, however, revealed no significant differences in consumption rates between females and males for either treatment group (Acidified – LMM, t:-0.418, $p > 0.05$; Control – LMM, t: -2.062, $p > 0.05$).

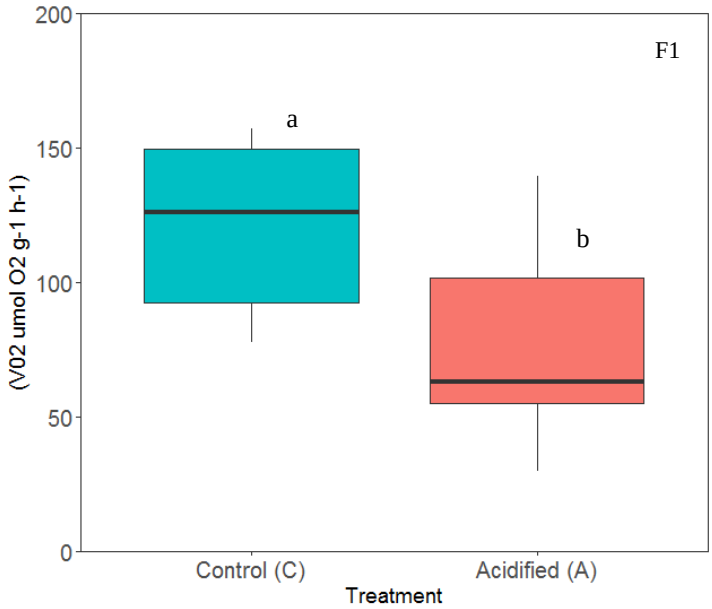



**Figure 5.4.1.** Mean oxygen consumption rates over three 25-minute measurement periods for the control (C) and acidified (A) treatments. Values represent mean values ± SD. $n_{CC}$: 14, $n_{AA}$: 14. Statistical differences (p-value < α, α = 0.05) are represented by different superscript letters above each plot.

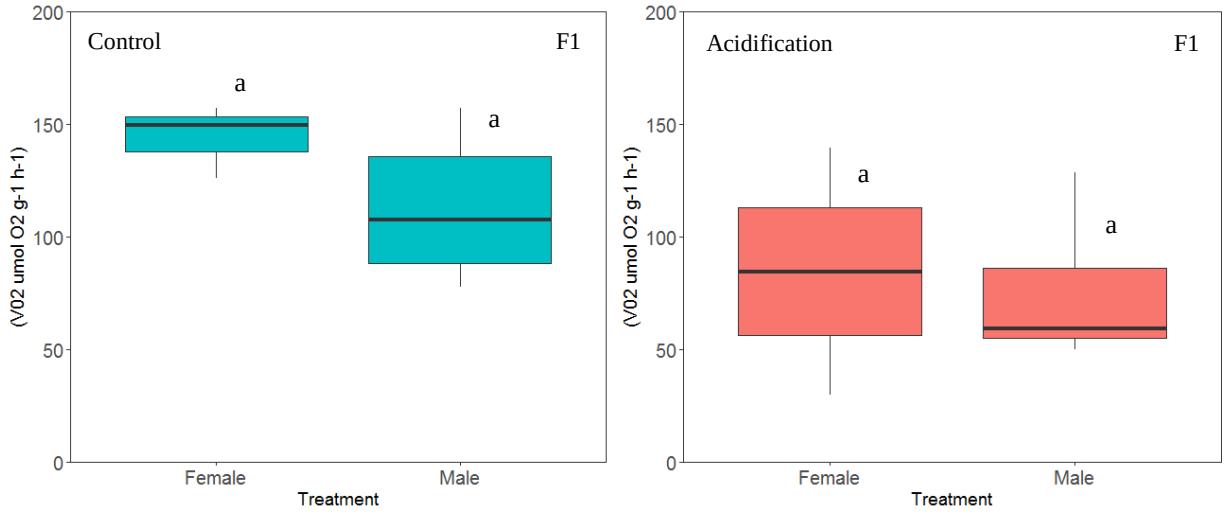



**Figure 5.4.2.** Mean oxygen consumption rates, by gender, over three 25-minute measurement periods for the control (a) and acidified (b) treatments. Values represent mean values ± SD. $n_{AA}$: 14, $n_{CC}$: 14. Statistical differences (p-value < α, α = 0.05) are represented by different superscript letters over each plot.

# 6. Discussion

## 6.1. Survival

High-$CO_2$ was responsible for causing a significant decline in the survival of *G. locusta* after 30 days under experimental conditions. In the parental generation, survival decreased from approximately 75-80% in the control group, to approximately 60-65% in the acidified treatment. For the same species, Hauton et al., (2009) reported similar survival rates (~65%) after a 25-day exposure to a 7.8 pH. The fact that survival in F1-CA was similar to the acidified treatment in F0 and was significantly lower compared to both F1-CC and their parent generation treatment F0-CC, reinforce evidence that within-generation decrease in pH levels (acclimation exposure) significantly reduces survival for this amphipod. There was also evidence for the existence of parental effects in the cross from the acidified into the control conditions in F1 (F1-AC). Survival for this group was significantly lower than both the F1-AA and F1-CC treatments, and was similar to F1-CA. Although on F1 comparisons, the F1-AA treatment did not exhibit differences regarding the control group, this did not hint at a possible transgenerational acclimation by prolonged exposure. The absence of differences under OA between both generations, suggests that survival could be significantly reduced on a transgenerational level.

Decreases in survival could be related to impacts of OA on calcification and metabolism (Kroeker et al., 2010), which could result in altered energy allocation and consequent *fitness* reduction. Previous multigenerational studies have shown varied survival responses to OA in invertebrates. In the calanoid copepod, *Acartia tsuensis*, exposure to OA for two generations revealed no clear impact on survival (Kurihara, 2008). In the oyster *Saccostrea glomerata*, despite short-term transgenerational exposure to OA led to decreases in survival (Parker et al., 2012), prolonged exposure revealed the existence of positive carry-over effects, with larvae and juveniles exhibiting increased resilience (Parker et al., 2015). It is possible that a multigenerational exposure to OA could reveal the existence of positive carry-over effects regarding survival.

## 6.2. Reproductive traits

### *6.2.1 Mate-guarding*

The significant reduction of the duration of precopulatory MG in the first acidified generation (both in F0-AA; and in F1-CA), suggests that first-generation acclimation to high-$CO_2$ conditions could affect precopulatory behaviour and reproductive success in *G. locusta*.

The comparison of F1-CA with its parent group, as mentioned above, emphasises the short-term effects of acidification that significantly reduced the duration of MG behaviours. The other parental cross, F1-AC, showed that despite being reared in control conditions, the progeny of acidified individuals still exhibited shorter-duration MG periods. This hints at the presence of parental effects that are not alleviated nor worsened by a return to normocapnic conditions. Also, the significant differences between both first-generation exposure to high-$CO_2$ treatments (F0-AA and F1-CA), although initially unexpected, don't go against these assumptions, since MG duration was shorter in F1-CA.

Shorter MG duration could be related with increased metabolic costs under OA. There is evidence that in *Gammarus* sp., males that are in poor energetic condition may be less able to pay the energetic costs associated with precopulatory mate-guarding (Plaistow et al., 2003). Hence, acidified males could be investing in shorter-duration MG behaviour, with the possible trade-off of increasing the probability of losing the role as the fertilizer of the eggs (*i.e.* if a male leaves the female before fertilization occurs), as a response to metabolic stress. Another possibility could be the reduction of parturial moult duration in pre-mature females - thus allowing shorter but still effective MG by males. However, sustained decreases in ocean pH of at least 0.5 units have already been proved to have no significant impacts in the moulting cycle of *G. locusta* (Hauton et al., 2009), suggesting the former hypothesis is the most probable one.

### *6.2.2 Female investment and fecundity*

First-generational exposure to high-$CO_2$ resulted in a significantly larger investment by females in the mean number of eggs of the first brood (for both F0-AA and F1-CA). Positive stimulation of egg production in response to high-$CO_2$ induced stress is known to occur in crustaceans (Schade et al., 2014), although this stressor frequently induces either an effective decrease in female investment per brood, or no effects at all (Kroeker et al., 2010). Transgenerational exposure to high-$CO_2$, however, revealed a pronounced decrease in egg production in F1-AA, compared to its parent treatment and to the F1-CC. This could imply that the physiological stress felt since birth or transmitted through the maternal line (possibly energetic trade-offs) not only led to a decrease in egg production regarding their parental treatment, but also resulted in an overall reduction in the number of eggs compared to the control group in F1.

The F1-AC treatment produced a mean number of eggs per brood similar to both F1 acidified and control groups. This suggests that negative effects of acidification have been introduced into the F1 generation during either egg or embryo development (early in life, and possibly through maternal effects) and were not necessarily alleviated by transfer into control conditions. It is possible that some maternal effects are being transferred from F0 to F1. The fact that F1-AC and F1-AA had similarly reduced female investment suggests that their individual *fitness* was affected on some level, either during egg formation (in egg quality) or during embryonic development.

Egg number dropped from F0 to F1 for both the acidified and control groups, for reasons that are unknown. This overall drop in egg count helps to explain why no significant differences were found between F1-CA and F0-CC, as was expected, despite the former clearly exhibited a higher egg production regarding F1-CC. The magnitude of decrease between F0 and F1 for both main treatments was, however, different. In the acidified group, the mean egg number was reduced by approximately 20%, while in the control treatment, the decrease was only of 10%.

Concerning fecundity, OA did not produce immediate effects (positive or negative) to F0-AA, but, in the second generation, a significant decrease was observed for the main acidified treatment. This is consistent with a multigenerational study on the copepod *Acartia* sp., which exhibited a consistent decrease on egg hatching success over subsequent generations exposed to high-$CO_2$ (Vehmaa et al., 2016). Another study, also on a copepod species (*i.e. Pseudocalanus acuspes*), found that second generation adults exhibited a 29% decrease in fecundity at 900 μatm, compared to control conditions (Thor and Dupont, 2015).

The absence of effects in F0-AA for this reproductive trait suggests that the increased egg production was enough to ensure that mean fecundity remained at "normal" or control levels. However, increased investment did not result in increased fecundity. In the F1 generation, mean fecundity suffered a significant drop, concomitant with the decline in egg investment for the same treatment. It is possible that the metabolic costs associated with the accumulated effects of two-generation exposure could have led to a temporary shift in the allocation of energy resources that would normally be used for reproduction, *i.e*., the female investment in the number of mature oocytes that are deposited as eggs in the brood pouch and, possibly, egg quality (Neuparth et al., 2002).

Similar fecundity levels were expected between F0-CC and F1-CA, following the trend established by the high-$CO_2$ group in F0 (F0-AA), but this was not the case. Nonetheless, mean fecundity was still significantly higher than the same generation's main acidified treatment (F1 AA), as expected, despite being significantly smaller than both CC (F0 and F1) and AA (F0) groups. Maternal cannibalism (*i.e*. during development, when new-born individuals are already capable to enter and exit the brood pouch at will) could help explain this apparent disparity. Decreases in pH levels have already been linked to an increase in the number of cannibalistic crustacean species in acutely acidified lakes (Havens, 1991), and cannibalism can also account for a significant portion of mortality rates in *G. locusta* (Christie and Kraufvelin, 2004, Costa et al., 2005)

The results also suggest the existence of negative maternal effects regarding fecundity that were present in both F1-AA and F1-AC groups. Mean fecundity for F1-AC was significantly reduced when compared to this treatment's parent generation, and just as reduced as F1-AA, and followed the same tendency as female investment for the same treatment. Vehmaa (2016), also showed the existence of significant negative maternal effects of pH on egg hatching success in *Acartia* sp.

The (~) 10% reduction in the mean number of eggs in the control group from F0 to F1 was not followed by a significant reduction in the number of younglings that successfully hatched and reached the age of brood-exit. This could be explained by normal female variability in this trait – since females that were sampled for egg count were not the same that were sampled for fecundity, as discussed in the materials and methods section – and due to the high deviation ranges experienced for both these outputs in control.

### *6.2.3 Embryonic development*

The duration of embryonic development was unaffected by acidification. There is a lack of literary information regarding this particular trait for amphipods, however, in *Echinogammarus marinus* (Leach, 1815), exposure to pH 7.5 resulted in delayed embryonic development at low salinities (22), but this effect was absent at 35 (Egilsdottir et al., 2009). Also, similar reductions in pH had no effect on the growth rate of juveniles (from adolescence to sexual maturity) in the same species as ours (Hauton et al., 2009). It seems plausible that transgenerational exposure to ocean acidification by itself and in a reduced number of generations will not produce changes in the development rate of this marine amphipod, as it was reported for other invertebrate species - *e.g*. *Saccostrea glomerata* (Parker et al., 2012). It is possible that multigenerational exposure could, however, produce significant effects in the duration of embryonic development that have not been triggered by the current experiment duration.

## 6.3. Behavioural traits

The behavioural trials showed, overall, that OA can significantly affect the ability of gammarid males to successfully locate and track the origin of female waterborne cues. Males from F1-AA (who were 2nd generation-exposed to high-$CO_2$), spent significantly less time (in proportion regarding total test time) in the association areas in which the female scent cue was present. On the other hand, males from the control condition spent the majority of trial time, on average, in the correct association area and hence, exhibiting the normal levels of attraction and cue tracking that would be characterized by normal chemosensory reception.

The decreased proportion of time spent in the presence of female cues could indicate a decreased ability to accurately track and locate the origin of a cue trail in the water column, as has already been described for *Eurytemora affinis* and *Temora longicornis* (Seuront, 2010). Acidified males did exhibit a mean proportion of 50% of the time spent in the correct association area, despite the increased deviation range for both treatments. This supports the possibility that males could be unable to detect the female pheromones, since this proportion is neither indicative of attraction nor avoidance. The fact that there were no statistical differences between F1-AA and F1-CC regarding first-choice means that, on average, acidified males can still detect and be positively stimulated by exposure to a female pheromone trail. This would suggest that although males exposed to OA are not able to correctly identify and track the origin of this cue, they are still being positively stimulated by it to some extent. Also, the response time at trial start exhibited by males was also significantly higher under OA. This could suggest that such males experienced, in the F1 generation at least, a reduction in stimuli response time linked to physiological-induced locomotor impairments.

The possibility that using treatment-conditioned water for the behavioural tests would damage or degrade the female cues involved in the attraction of males was dismissed. Although the effect of pH on signalling molecules in the context of OA has yet to be sufficiently explored on a molecular level (Roggatz et al., 2016), so far only in one freshwater system has molecular evidence for cue degradation been described, which directly affected individual response to alarm cues – the cue in question being a naturally occurring purine derivative, hypoxanthine (Brown et al., 2002). Marine studies involving $CO_2$-induced acidification and the testing of behavioural responses to olfactory stimuli in fish, showed that, in overall, the observed changes were not caused by chemical modification of the odour cues, at least for a change in pH levels of approximately 0.5 units (Leduc et al., 2013). Also, aquatic sex pheromones that are known to be potentially affected by pH include peptides, nucleosides, thiols and organic acids (Hardege et al., 2011), while hormones that are most likely involved in acting as male attractants in long distances for gammarid amphipods (like the active moulting hormone 20-OH-ecdysone) belong to the steroid compound class (Thummel and Chory, 2002), that are not included in the mentioned hormone categories.

The present study showed, for the first time, sexually-related chemo-sensory disruption in marine amphipods. In marine fish, OA was also responsible for the loss of avoidance behaviour in larvae of the anemonefish *Amphiprion melanopus* to chemical alarm cues, as well as a reversal to the elicited response with attraction to this cue (Welch et al., 2014). Yet, such study showed that transgenerational acclimation alone was not able to reverse these effects. Further testing on subsequent generations of *G. locusta* is, therefore, important in order to address possible transgenerational effects on this particular trait.

### 6.4. Metabolism

Results from the mass-specific RMR analysis showed that mean routine $O_2$ consumption rates were significantly reduced in amphipods that had been exposed to high-$CO_2$ for two generations (F1-AA). This effect can be the direct result of respiratory-stress induced by hypercapnia – abnormally high concentrations of dissolved $CO_2$ in the haemolymph (Pörtner et al., 2004). The decrease in extracellular pH unbalances the regulation of the intracellular acid-base status in the organism (by slowing down the rate of $H^+$ equivalent ion exchange in $Na^+/H^+$- and $Na^+$- dependent $Cl^-/HCO_3^-$ transporters), and when uncompensated, this decrease leads to acidosis-induced metabolic depression (Pörtner et al., 2004). Metabolic depression is considered as a time-limited adaptation strategy to survive unfavourable conditions, such as hypercapnia (Guppy and Withers, 1999), since it reduces the energy requirements of acid-base regulation (Pörtner et al., 2004). In many calcifying and non-calcifying marine invertebrates tolerant to $CO_2$ oscillations, prolonged high-$CO_2$ exposure has been known to cause drops in metabolic rates and even, sometimes a phenomenon similar to anaesthesia (Reipschläger and Pörtner, 1996, Pörtner et al., 2004, Michaelidis et al., 2005).

By comparing female and male RMR, no significant differences were found which could indicate metabolic depression in either sex for each treatment, despite slightly higher values in female consumption rates. This is a solid indicator that both males and females are equally subjected to metabolic depression as a possible compensation for extracellular acidosis and, thus, are equally metabolically affected by pH reductions in the environment. These results help support some of the previously stated hypothesis, in which disruption of normal metabolic processes by OA might be playing a role in modulating other life-history traits or behaviours.

# 7. Conclusions

Ocean acidification is a pervasive stressor that will undoubtedly pose risks for marine biodiversity in the future. Some marine species will have the ability to adapt to future global change, through transgenerational plasticity and epigenetics, but this adaptive potential can be relatively finite, due to the presence of evolutionary trade-offs and reduction of extant genetic diversity (Calosi et al., 2016). Our results highlight the hypothesis that gammarid amphipods, which are commonly considered more tolerant than other species to future climate change, are also prone to negative effects of high-$CO_2$. A summary of the results of all reproductive, behavioural and metabolic analysis is featured in Table 7.1.

First-exposure individuals showed an increase in female reproductive investment, a reduction in MG duration and decreased survival, despite exhibiting normal fecundity levels. The adoption of a different reproductive strategy by females, could pose an active response regarding prolonged physiological stress, which could aim to maximize genetic variability and thus, the chances that new, more adapted phenotypes, could arise. Short-term survival (at 30 days) was also affected, leading to survival ratios approximately 19% lower than in control.

In marine invertebrates, the maintenance of acid-base balance is an energetically costly process that does not occur spontaneously (Pörtner et al., 2004). Previous exposure to reduced pH during growth (from juvenile to maturity) for *G. locusta* has shown an increased and sustained expression of the *gapdh*

gene (glyceraldehyde 3-phosphate dehydrogenase), which suggests that subtle changes in the organism's physiology do take place on OA scenarios (Hauton et al., 2009). This supports the possibility that OA, as the organism's physiology attempts to compensate acid-base balance, is causing some important and negative effects on the metabolism of *G. locusta*, evidenced by changes in mean survival and in reproductive traits. In fact, in the second generation, both male and female amphipods suffered some degree of metabolic depression, that is thought to constitute a survival enhancing strategy under stressful conditions, as it allows an active defence against disturbances in metabolism and tissue functioning induced by hypercapnia (Lannig et al., 2010). This result helps to explain the significant increase in response time observed in the behavioural trials for high-$CO_2$ exposed males, since metabolic depression can be behaviourally expressed as a reduction in activity (anaesthesia). Not only did males take more time to react to the female cues, but their preference responses were less clear than males in the control group, suggesting that OA is potentially acting as a chemosensory disruptor. Decreased metabolic rates could also explain the reduction in MG duration in the first generation, although no metabolic data was collected for the parental generation. Smaller amounts of energy could be available for processes other than maintaining metabolism and acid-base balance, which could lead to males investing less in the active guarding of females, and potentially reducing the number of successful sexual occurrences between mates. Females in F1 also experienced a drop in investment by number of eggs, which culminated in reduced progeny.

The presence of parental effects was also identified. In female investment, MG, survival and even in fecundity, individuals reared in control conditions whose parents came from the acidified parental group always exhibited negative tendencies of traits, that either followed the negative trend of their parental generation (MG duration and survival) or the trend exhibited by the acidified population of the second generation (female investment and fecundity). Although two particular results seemed to hint to a possible transgenerational acclimation in F1-AA (MG and survival, specifically), these are most likely due to the high deviance range of the data for these outputs in this particular treatment. It could be, still, that prolonged transgenerational exposure is indeed acting towards acclimation of the population - with the potential genetic variability induced by high female investment in F0-AA – and that the high deviation of the data is indeed a sign of this variability.

**Figure 7.1.** Summary table of the results for the reproductive traits, behaviour and metabolism analysis. Arrows signify increase (↑) or decrease (↓) for a determined measurement, for the acidified treatments of first exposure (F0-A; F1-CA) and second generation (F1-AA).

| Trait | Acidified population's response regarding control | | |
|---|---|---|---|
| | 1st exposure (F0-A; F1-CA) | F1 (AA) | Parental effects (F0 -> F1) |
| **Survival at 30 days (%)** | ↓ | no differences | negative effects |
| **Reproduction** | | | |
| *Duration of mate-guarding (days)* | ↓ | no differences | negative effects |
| *Number of eggs per female* | ↑ | ↓ | negative effects |
| *Duration of embryonic development (days)* | no differences | no differences | absent |
| *Number of juveniles per female* | no differences | ↓ | negative effects |
| **Behaviour** | | | |
| *Response time (sec)* | - | ↑ | - |
| *First-choice* | - | no differences | - |
| *Cumulative time (%)* | - | ↓ | - |
| **Metabolism** | | | |
| *Routine metabolic rate (RMR)* | - | ↓ | - |

Further experimental tests are necessary to improve scientific knowledge towards understanding the impacts of OA in amphipod's biological traits. Not only increasing the number of experimental generations, but also complementing these outputs with other useful data that could help shed some light on the exact mechanisms that might be at play. First, regarding reproductive trials, it would be important to complement female investment (in number) with data on egg quality, size and shape, and following embryonic development to address the existence of abnormalities of development in younglings. Mate-guarding duration should be complemented by a quantitative analysis of successful mate encounters – which should be reduced, since high-$CO_2$ negatively affected the ability of males to identify the origin or the presence of female cues in the water current. Since males rely on both long and short distance chemical cues to track females and initiate MG behaviour, specific tests should be designed to assess possible disruption in the detection of contact-pheromones, and in possible modification of the success of MG initiation. It is also important to test for chemosensory disruption of other *fitness*-enhancing chemically-based behaviours: i) does OA only affect the detection of sexual cues, or does it disrupt all of the amphipod's chemoreception mechanisms and equally decreases individual's response to other conspecific and heterospecific, or even environmental cues? ii) does OA impair the conspecific recognition mechanisms that elicit gregarious behaviours in this species, and, does it decrease the ability to detect predator and injured conspecific cues in water-currents - thus increasing mortality by intra- and interspecific predation?

It is also important to assess whether metabolic depression carries on in subsequent generations, and if individual survival is indeed maximized, and amphipods eventually begin to show a positive comeback from the apparent negative effect of OA, thus hinting at transgenerational acclimation.

Trans- and multigenerational approaches to OA and other stressors, as well as possible synergies, in climate-change ecology are fundamental in order to help managers and policy-makers in the development of measures towards the protection of global environments. It is only through long-term experimental evolutionary studies that scientists will be able to accurately understand the expected effects of these stressors on different levels of biological organization (*i.e*. from molecules and cells, to communities and ecosystems).

## 8. References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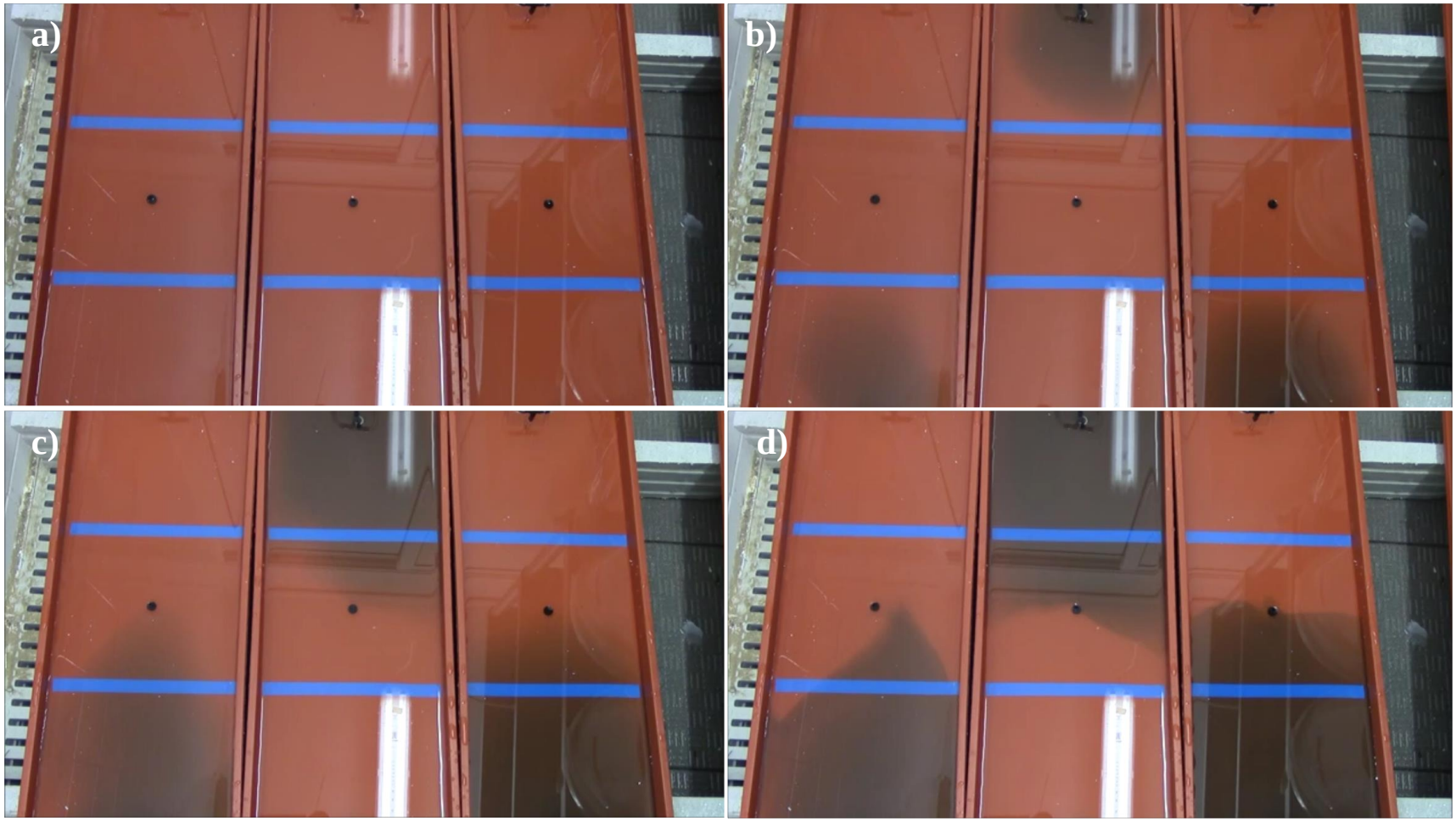
# 9. Appendices (supplemental material)

## 9.1. Blue dye test for behavioural setup optimization




**Supplemental Figure 9.1.** Blue-dye test for behavioural setup optimization: (a) 0 sec; (b) 600 sec; (c) 1200 sec (d) 3000 sec. During trials, and as can be seen in the video 1 in supplemental, after 20 minutes of cue input there was no mixture of the water masses in either association zone.