Combined effects of warming, acidification and the synthetic progestin levonorgestrel on the fitness of the marine amphipod *Gammarus locusta* (Crustacea)

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To my beloved family who’s always consistently reminding me that no matter the type and/or magnitude of a challenge, there’s a light at the end of the tunnel.
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ABSTRACT

On the facet of global warming, marine invertebrates are harshly challenged to adapt in a changing ocean. Global warming and increased atmospheric CO$_2$ are causing the oceans to become warmer and more acidic. According to the 5th assessment report of the Intergovernmental Panel on Climate Change (IPCC), global mean sea surface temperature is expected to increase up to 4°C and ocean pH is expected to decrease by Δ 0.5 pH units until 2100. The stimulatory effect of ocean warming coupled with the sedative effect of low pH is lethal for marine invertebrates physiological processes. Moreover, adapting to these climate variables may become more difficult due to their possible interaction with contaminants, such as Endocrine Disrupting Chemicals (EDCs).

To our knowledge there is almost no information in the literature regarding the impact of multiple climate drivers and EDCs on the aquatic organisms. Thus, in this study a combined effect of the near future ocean warming (+ 4°C) and acidification (Δ 0.5pH units) with the synthetic steroid, levonorgestrel was evaluated on the fitness of the marine amphipod Gammarus locusta. The experiment employed a full factorial design manipulating temperature [ambient (18°C) and warming (+ 4°C)], CO$_2$ [normocapnia (pH 8.1) and hypercapnia (pH 7.6)] and the progestin levonorgestrel (LNG: L1 – 10 ng L$^{-1}$ and L2 – 1000 ng L$^{-1}$, control – no progestin and solvent control – vehicle ethanol (0.01%)). The study was divided in two phases: 1) pre-reproduction phase, which lasted for 21 days and 2) post-reproduction phase that continued for more 7 (± 2) days. The responses of G. locusta were evaluated through multiple outputs like survival, growth, consumption rate, fitness condition and reproductive traits (fecundity and embryonic development).

Warming condition (22°C, pH 8.1) revealed to be the most negative scenario for the G. locusta survival (30% - 50%) compared to the ambient condition (70% - 90%). However, under the combined effect of warming and acidification the survival was higher (60% - 80%). Additionally, elevated temperature reduced fitness condition and interacted with LNG, suppresing the consumption rate. In addition, elevated temperature seems to slightly suppress the growth rate, however, the acidification and LNG concentrations had a more negative effect on growth rate. On the other hand, the G. locusta fecundity was significantly reduced under higher temperature and a synergistic effect with higher LNG concentrations was detected. For the embryonic development time, a significant reduction was observed with the increase of temperature (4 - 6 days) compared to ambient temperature (6 – 12 days). On the other hand, hypercapnia under ambient temperature prolonged embryonic development.
Our findings suggest that the future warming scenario may exceed *G. locusta* lethal thermal limit affecting all levels of physiological performance. On the other hand, acidification *per se* seems to have had some negative effects on metabolism and development of the organisms, however the impact was not so drastic like the warming condition alone. The interaction between LNG and warming revealed to be more relevant at the reproduction level, meaning that the higher the temperature and LNG concentration, the lower the fecundity recorded for this invertebrate species. Concluding, that the novelty of this research may have a strong impact on the management and conservation of coastal ecosystems on the facet of climate change.
RESUMO

No âmbito das alterações climáticas, os invertebrados marinhos são fortemente desafiados a adaptarem-se a um ambiente de mudança. Devido ao aquecimento global e aumento do CO₂ os oceanos têm vindo a sofrer um aumento progressivo de temperatura e acidez. De acordo com o 5º relatório do IPCC, a temperatura média superficial do oceano poderá aumentar até 4º C e o pH diminuir até 0.5 unidades até ao final do século XXI. O efeito estimulador do aumento de temperatura associado a um efeito mais inibitório por parte do pH mais baixo pode ser letal para a maioria dos processos fisiológicos que ocorrem nestes invertebrados. Por outro lado, a adaptação a estes fatores climáticos pode ser dificultada pela interação com contaminantes, nomeadamente os compostos disruptores endócrinos.

De acordo com o nosso conhecimento, existe uma grande lacuna na literatura relativamente aos efeitos de múltiplos fatores climáticos associados a estes compostos endócrinos ao nível dos organismos aquáticos. Assim, este estudo teve como principal objetivo avaliar os efeitos combinados do aumento de temperatura (+ 4ºC), acidificação (Δ 0.5pH) e a presença da hormona sintética levonorgestrel (LNG) na história de vida do anfípode marinho, Gammarus locusta. Desta forma, foi desenvolvida uma experiência multifatorial em que foram manipulados 3 fatores, temperatura (ambiente e aquecimento (+ 4ºC), CO₂ (normocapnia e hipercapnia) e a hormona levonorgestrel (L1 – 10 ng L⁻¹ e L2 -1000 ng L⁻¹). O estudo foi dividido em 2 fases: 1) pre-reprodução (21 dias) e 2) pós-reprodução (7 ± 2) dias. As respostas do anfípode foram avaliadas através de diferentes parâmetros: sobrevivência, estado de condição, taxas de consumo e crescimento e ainda, reprodução (fecundidade e tempo de desenvolvimento embrionário).

Verificou-se que o cenário de aquecimento (22ºC, pH 8.1) foi o que desencadeou efeitos mais negativos ao nível da sobrevivência da espécie (30-50%), comparativamente à condição ambiental (70-90%). Contudo, num cenário combinado de aquecimento e acidificação os valores de sobrevivência foram superiores (60-80%). Por outro lado, a temperatura mais elevada foi responsável por uma diminuição do estado de condição dos organismos e em interação com a hormona LNG verificou-se um efeito negativo ao nível das taxas de consumo. Também se observou uma diminuição das taxas de crescimento sob o efeito da temperatura mais elevada, no entanto, o efeito da acidificação foi mais significativo. Por outro lado, a fecundidade da espécie foi significativamente reduzida sob o efeito da temperatura mais elevada e também se observou um efeito sinérgico com a presença de concentrações mais elevadas de LNG. Relativamente ao tempo de desenvolvimento embrionário, observou-se uma redução significativa do mesmo sob o efeito da temperatura mais elevada (4-6 dias) comparativamente à temperatura ambiente.
(6-12 dias). Por outro lado, verificou-se que a acidificação foi responsável pelo prolongamento do tempo de desenvolvimento embrionário.

Assim, os nossos resultados sugerem que num cenário futuro de aquecimento global (+ 4°C), este pode exceder os limites térmicos letais para a espécie, afetando toda a sua fisiologia. Por outro lado, a acidificação por si só, apesar de ter condicionado o desenvolvimento dos indivíduos, parece não ter um efeito tão negativo como o aumento da temperatura. Por sua vez, a interação entre a hormona LNG e fatores climáticos, especialmente a temperatura, parece ser mais relevante ao nível da reprodução da espécie. Concluindo, estes resultados, devido a sua novidade, poderão ter um forte impacto ao nível da gestão e conservação dos ecossistemas costeiros face a um cenário de alterações globais.
INDEX

1 INTRODUCTION.................................................................................................................. 1
1.1 CLIMATE CHANGE AND ITS EFFECTS ON MARINE ORGANISMS .............................. 1
1.2 ENDOCRINE DISRUPTING COMPOUNDS’ (EDC’s) ......................................................... 4
1.3 PROGESTINS .................................................................................................................... 4
1.4 CLIMATE CHANGE AND AQUATIC CONTAMINANTS..................................................... 6
1.5 GAMMARUS LOCUSTA .................................................................................................. 7
1.6 OBJECTIVES ................................................................................................................... 8

2 MATERIALS AND METHODS .......................................................................................... 11
2.1 AMPHIPOD COLLECTION ............................................................................................... 11
2.2 EXPERIMENTAL DESIGN ............................................................................................... 11
2.3 PHARMACEUTICAL......................................................................................................... 15
2.4 AMPHIPOD LENGTH AND WEIGHT ............................................................................. 15
2.5 AMPHIPOD CONDITION INDEX .................................................................................. 15
2.6 CONSUMPTION RATE .................................................................................................... 16
2.7 REPRODUCTIVE TRAITS............................................................................................... 16
2.8 QUANTIFICATION OF LEVONORGESTREL (HPLC-DAD) ........................................... 16
2.9 DATA ANALYSIS .......................................................................................................... 18

3 RESULTS .......................................................................................................................... 19
3.1 LEVONORGESTREL QUANTIFICATION ........................................................................ 19
3.2 AMPHIPOD SURVIVAL ................................................................................................... 20
3.3 FULTON CONDITION INDEX ....................................................................................... 21
3.4 AMPHIPOD GROWTH RATE .......................................................................................... 22
3.5 RELATIVE CONSUMPTION RATE (RCR) ...................................................................... 23
3.6 G. LOCUSTA FECUNDITY AND EMBRYONIC DEVELOPMENT ...................................... 24

4 DISCUSSION ..................................................................................................................... 27
4.1 SURVIVAL AND FITNESS CONDITION AND GROWTH ............................................. 27
4.2 RELATIVE CONSUMPTION RATE (RCR) ...................................................................... 30
4.3 FECUNDITY AND EMBRYONIC DEVELOPMENT ......................................................... 31
4.4 CONCLUSION ............................................................................................................... 33
List of Figures and Tables

**Figure 1:** General scheme on the life cycle of *Gammarus locusta* under ambient conditions (Page - 8)

**Figure 2:** A detailed scheme illustrating a sub-set of the experimental system. Abbreviations, F- female, M- male, C- control, SC- solvent control, L1- LNG low concentration and L2- LNG high concentration. (Page - 14)

**Figure 3:** Basic scheme on the analytical method for the pre-concentration and quantification of the progestin LNG. *Adopted from (Ribeiro et al., 2007)*; (Page - 17)

**Figure 4:** Survival (%) of *G. locusta* individuals exposed to different combinations of temperature, pH and LNG concentrations (n = 12). Abbreviations, C- control, SC- solvent control, L1- 10ngL$^{-1}$ LNG and L2- 1000ngL$^{-1}$ LNG. (Page - 21)

**Figure 5:** Fulton condition index ($K$) for *G. locusta* exposed to different combinations of temperature, pH and LNG concentrations at day 21. Values represent mean ± SD. Abbreviations, C- control, SC- solvent control, L1- 10ngL$^{-1}$ LNG and L2- 1000ngL$^{-1}$ LNG. (Page - 22)

**Figure 6:** Growth rates of *G. locusta* individuals exposed to different combinations of temperature, pH and LNG concentrations. Values represent mean ± SD. (Page - 23)

**Figure 7:** RCR of *G. locusta* individuals at the (A) 1$^{st}$ week of exposure and (B) 3$^{rd}$ week of exposure. Values represent mean ± SD. Different letters indicate significant differences among treatments (3-Way ANOVA, Post-Hoc analysis $p < 0.05$). (Page - 24)

**Figure 8:** Total number of newborns released by female *G locusta* during the post-reproduction phase exposed to the different treatments. Values represent mean ± SD. (Page - 25)

**Figure 9:** Figure 8: Embryonic development time of *G locusta* exposed to the different treatments. Values represent mean ± SD. (Page - 26)

**Table 1:** Description of the different treatments to which *G. locusta* were exposed. C – control, SC – solvent control, L1 - LNG (10 ng L$^{-1}$) and L2 - LNG (1000 ng L$^{-1}$). (Page - 13)

**Table 2:** Levonorgestrel concentrations (ng L$^{-1}$) in waters during 21 days exposure. The water was sampled 30 minutes after the first injection ($T_0$) and 90 minutes later ($T_{90}$). (Page - 19)
1 INTRODUCTION

1.1 Climate Change and its Effects on Marine Organisms

Human influence on the climate system is apparent and inevitable. According to the Intergovernmental Panel on Climate Change (IPCC), the increase in global population and consequently on economic growth have been the major drivers for the increase of greenhouse gases (GHG) in the last three decades (IPCC, 2014). Anthropogenic GHG emissions have led to the rise in the concentration of carbon dioxide (CO$_2$) in the atmosphere, which remains as the most important GHG. Thus current high CO$_2$ levels have been the prominent cause of observed global warming and ocean acidification (OA) since the mid 20$^{th}$ century (Fabry et al., 2008, Feely et al., 2009, Arenas and Vaz-Pinto, 2014, IPCC, 2014).

As the ocean is getting warmer it also acts as a reservoir absorbing about 40% of emitted anthropogenic CO$_2$ gas every year (Feely et al., 2009, Arenas and Vaz-Pinto, 2014). Seawater carbonate chemistry is a product of two main processes; (1) abiotic chemical reactions (CO$_2$ dissolution, acid-base chemistry) and (2) biologically mediated reactions (photosynthesis and respiration/decomposition) (Feely et al., 2009). As the ocean absorbs CO$_2$ continuously, carbonate chemistry is modified, decreasing carbonate ion (CO$_3^{2-}$) concentration and saturation state of CaCO$_3$. As a consequence, oceans become more hypercapnic having deleterious impacts on marine invertebrates, particularly on calcifying species (Kurihara et al., 2007, Byrne, 2011, Byrne et al., 2013b). Therefore, aquatic organisms are currently challenged by these two climate variables (i.e. warming and ocean acidification) acting together and altering ecotypes at a wide geographical range (Feely et al., 2009, Arnberg et al., 2013). The distribution, performance, population abundance and behavior of marine invertebrates are governed
by vital seawater parameters like partial pressure of CO$_2$ ($p$CO$_2$), calcium carbonate (CaCO$_3$) saturation and power of hydrogen ions (pH) (Byrne, 2011, Byrne and Przeslawski, 2013).

Oceanic pH is predicted to decrease by 0.3 – 0.5 pH units in the mid 21st century (IPCC, 2014). As oceanic pH decreases due to increasing $p$CO$_2$, this condition threatens the physiological development, reproduction and calcification of many marine invertebrates like crustaceans, echinoderms and molluscs (Doney et al., 2009, Byrne, 2011, Ross et al., 2011). Previous studies have already shown unparalleled implications of the near future scenario of OA on a variety of marine animals (Kurihara et al., 2004, Byrne and Przeslawski, 2013, Carter et al., 2013, Kurihara et al., 2013). Even more challenging, idiosyncratic effects are abundant in literature with different invertebrate species responding differently to ocean acidification. Some species can be negatively affected by more acidic waters, like the shrimp *Palaeomon pacificus*, that suffered a reduction in growth, survival, molting frequency and egg production when exposed to lower pH (7.89 and 7.64) (Kurihara et al., 2008). While, for most copepod species tested in previous studies, the negative impact of acidification on the survival and reproduction of individuals was only observed in more extreme condition of 6.8pH units (Fitzer et al., 2012, Weydmann et al., 2012).

In addition, invertebrate sensitivity towards acidic conditions varies within life stages. Adult individuals can be less sensitive to acid conditions compared to their early life stages. Larval stages have been reported to be the most sensitive in acidic conditions, often inflicting negative impacts on survival, growth, calcification, metamorphosis and physiology (Dupont and Thorndyke, 2009, Ross et al., 2011). Additionally, it has been observed that cleavage rate and skeletogenesis are greatly affected at lower pH levels (Kurihara and Shirayama, 2004). This has been observed in a variety of invertebrate taxa from echinoderms, crustaceans, molluscs, polychaetes and cnidarians (Dupont and Thorndyke, 2009). The alarming concern herein is the dramatic impact of acidic conditions on early life stages of invertebrates, which can ultimately have a profound effect on population dynamics.

Hence inferences on the impacts of OA on invertebrates require further research as species and life stages within species may respond differently to acidic conditions. Also, Dupont and Thorndyke (2009) did acknowledge the need for medium and long-term exposure assays, as most recent studies have been a result of short-term acute assays. Additionally, It is extremely important to evaluate the effects of multiple climate drivers (e.g. warming and acidification), instead of single stressor experiments on marine species. Ocean warming (OW) due to increasing GHG in the atmosphere is the other global climate issue. Elevated temperature has been observed detrimental on marine
Introduction

Invertebrates, survival, growth, physiological functions, metabolism and trophic interactions (Somero, 2002, Edwards and Richardson, 2004). OW is known to speed up organism metabolism and development. Predicted increase in OW (2° - 4°C; mid 21st century) by the IPCC (2014) climate model has drawn researchers to investigate warming influence on invertebrate development. The impacts of ocean warming have proven to stimulate embryonic and larval development causing organisms to appear earlier than normal in their environment (Maranhão and Marques, 2003, Arnberg et al., 2013). However, global ocean warming also reduces survival (Arnberg et al., 2013), induces abnormal larval development and leads to lower fecundity rates with a high mortality rate (Nguyen et al., 2012). More important, in the context of speciation, ocean warming imposes the threat of ‘phenology mismatch’. This mismatch recognizes that organisms in every trophic level respond differently to ocean warming; hence the stimulatory effect of temperature on an organism’s developmental process can inflict mismatch across functional groups within and between trophic levels (Edwards and Richardson, 2004).

Besides climate change, over the last two decades a great attention has been given to the environmental health risks posed by endocrine disrupting chemicals (EDCs). These substances are widespread throughout the aquatic ecosystems; however, the risks they pose on aquatic organisms are largely unknown. And, to our knowledge there is a lack of information regarding the combined effects of climate stressors and EDCs on coastal organisms. According to recent investigation it is expectable that global warming could strengthen the efficacy of EDC’s (Schiedek et al., 2007, Noyes et al., 2009, Nikinmaa, 2013). Recent research on this hypothesis speculates that increasing temperature can enhance chemical uptake across membranes and coupled with decreasing pH, fundamental enzyme and protein function could also be distorted due to its narcotic effect. Chemical uptake in the gills of the rainbow trout Oncorhynchus mykiss was enhanced under low pH (6.4pH units – 8.4pH units) compared to an alkaline pH condition (9.2pH units). Nine weakly acidic chlorophenol was investigated in this experiment and regardless of their different ionization constants, the gills of O. mykiss still exhibited greater uptake in a pH ranging from 6.4pH units – 8.4pH units. The author also added that these findings indicate considerable bioavailability of ionized forms that varies with pH (Erickson et al., 2006a, Erickson et al., 2006b). Additionally, temperature is known to increase the toxicity of chemicals, which could be detrimental in aquatic organisms. The crustacean Carcinus maenas exposed to a combination of extreme temperatures and copper altered cardiac performance (Camus et al., 2004). Particularly at higher temperature (25°C) the author hypothesized that enhanced uptake of copper by C. maenas caused erratic cardiac performance, which reflected enhanced copper toxicity. However, there’s still a high
uncertainty about these effects, especially in combination with other stressors (e.g. ocean warming and acidification).

1.2 **Endocrine Disrupting Compounds’ (EDC’s)**

According to Segner *et al.* (2003), EDCs are natural and manmade substances that can potentially interfere with the endocrine system of organisms. These compounds have the ability to disrupt the synthesis, secretion, transport, binding action and/or the elimination of endogenous hormones in the body. Disturbances in these processes entails a negative effect on an organisms’ homeostasis, development, reproduction and behavior (Segner *et al.*, 2003). Studies have revealed that chronic exposure of crustaceans and other invertebrates to anthropogenic EDCs as low as nanograms per liter (ng/L) and micrograms per liter (µg/L) is harmful (Watts *et al.*, 2002, Matozzo *et al.*, 2008). De Lange *et al.* (2006) reported that fluoxetine and ibuprofen at low concentration (10ngL\(^{-1}\) – 100ngL\(^{-1}\)) decreased the activity of *Gammarus pulex*, In the same study, the cationic surfactant, cetyltrimethylammonium bromide also exhibited similar response in an increasing concentration (De Lange *et al.*, 2006). Additionally, Watts *et al.* (2003) exposed the larvae of *Chironomus riparius* to low concentrations (10ngL\(^{-1}\) – 1000ngL\(^{-1}\)) of 17\(\alpha\)-ethinylestradiol and bisphenol-A. Molting was delayed and individual wet weight was significantly reduced at 1000ngL\(^{-1}\). Additionally, at the lowest concentration deformities were observed in the mouthpart of the organisms. It has also been observed that these EDCs have the potential to alter sex ratio in aquatic invertebrates (Ford *et al.*, 2004, Ford *et al.*, 2006). Still, in the course of species distribution and abundance the unparalleled impact of EDCs on the reproductive output or aquatic invertebrates is of high concern (Watts *et al.*, 2001, Cold and Forbes, 2004, Vogt, 2007, Yang *et al.*, 2008, de los Santos *et al.*, 2015).

Among these EDCs are the synthetically produced progestin’s (also known as gestagens), which are widely produced for various medicinal purposes. Therefore, recent efforts have been focused on analyzing whether this synthetic progestin’s can inflict behavioral transformation on invertebrates, as it has been observed and documented extensively with teleost species (Fent, 2015, Zhao *et al.*, 2015).

1.3 **Progestins**

In the last decade progestins discharged in the aquatic systems have gradually been receiving an increasing attention (Orlando and Ellestad, 2014, Fent, 2015). Progestin’s belong to a group of steroid hormones recently known to interact with endogenous steroid receptors, causing behavioral and physiological changes, particularly in fish species (Kumar *et al.*, 2015). According to Fent (2015), there are 20 progestin’s (1 natural and 19 synthetic), which either derives from Progesterone (P4) or the closely
related androgenic steroid hormone, testosterone. These progestins have been widely produced primarily for contraceptive pharmaceuticals, which can be taken individually or in combination with other synthetic steroids. Also, other uses of these progestins include hormone replacement therapy, prevention of endometrial cancer, treatment of dysfunctional uterine bleeding, palliative appetite stimulation and growth promoters in animal farming (Fent, 2015, Kumar et al., 2015). These compounds seep in aquatic systems through wastewater treatment plants and runoff waste from livestock farms (Fent, 2015). Some recent studies have already demonstrated the negative effects of these substances on fish species, particularly on gene expression and sex differentiation (Zucchi et al., 2013, Liang et al., 2015), physiological development (Runnalls et al., 2013), circadian rhythm response (Zucchi et al., 2013) and most crucial, interfering with fish reproductive processes (Zeilinger et al., 2009, Paulos et al., 2010, Runnalls et al., 2013, Runnalls et al., 2015). However, to our knowledge there is a huge lack of information regarding the effects of these progestin’s on aquatic invertebrates.

Levonorgestrel (LNG) a synthetic steroid used as a contraceptive as well as a post-coital contraception modality (“the morning after pill”) and is grouped as one of the 2nd generation progestin compound (Contardo-Jara et al., 2011, Kumar et al., 2015). Being one of the oldest progestin, LNG is considered relatively nonspecific showing cross-reactivity with human androgen receptors (Fent, 2015). LNG has been detected in surface waters at low concentrations (ngL⁻¹), however it has triggered abnormal functional properties on fish species (Kumar et al., 2015). This synthetic steroid interacts with nuclear receptors such as androgen receptor (AR), estrogen receptor (ER), glucocorticoid receptor (GR) and mineralocorticoid receptor, acting as ligand transcription factors (Fent, 2015). Studies have revealed that administration of LNG on certain fish species disturbed spawning ability (Runnalls et al., 2013), altered female physiology (masculinization) and female fecundity (Hua et al., 2015, Frankel et al., 2016), harm egg production (Runnalls et al., 2015, Frankel et al., 2016) and impaired reproduction (Zeilinger et al., 2009, Runnalls et al., 2015). In this context, it is evident that due to the ability of LNG to interact with natural hormone receptors often jeopardizing fish reproduction, its ecological impact should not be undervalued. Bearing this in mind, crustacean endocrinology studies have revealed the presence of steroid related hormones that are structurally similar to vertebrate counterparts that are endogenously produced (Teshima and Kanazawa, 1971, Warrier et al., 2001, Ye et al., 2008, Lewis et al., 2012, Lewis et al., 2015). Conversely, while research on LNG has been focused solely on fish species, its potential impact on invertebrate like crustaceans, has received biased attention.
Given the background on the potential ecological risks of LNG, model crustacean species certainly deserve equal research consideration. Despite recent literature on the presence of steroid hormone and their receptors for crustaceans (Köhler et al., 2007, Lewis et al., 2012, Lewis et al., 2015), LNG research has been solely focusing on fish species. Nonetheless, some previous studies have already reported the effects of these progestins on other invertebrate species, like the bivalves, *Mytilus galloprovincialis* and *Dreissena polymorpha* (Contardo-Jara et al., 2011, Dimastrogiovanni et al., 2015). Both studies reinforced the ability of species, *M. galloprovincialis* and *D. polymorpha* to metabolize and eliminate the progestin employed. However, the latter species in particular, being administered with LNG reported relevant extent of protein damage (Contardo-Jara et al., 2011).

Due to the lack of information about the effects of progestin’s on invertebrate species, and considering their relevance on several trophic levels, it is important to extend the research to different taxonomic groups (like e.g. crustaceans).

### 1.4 Climate Change and Aquatic Contaminants

According to the literature there is an indication that environmental variables can influence the efficacy of contaminants (Noyes et al., 2009, Hooper et al., 2013). Higher temperature is known to increase aquatic contaminant uptake via an increased metabolic rate and decrease in oxygen solubility (Schiedek et al., 2007). A study conducted by Patra et al. (2007), investigated the impact of endosulfan, chlorpyrifos, and phenol on the thermal tolerance of four species of fish, *Bidyanus bidyanus*, *Melanotaenia duboulayi*, *Hypseleotris klunzingeri*, and *Oncorhynchus mykiss*. The result showed that the three contaminant decreased thermal tolerance in all four species (Patra et al., 2007). Additionally, Camus et al. (2004) reported that the crustacean *Carcinus maenas* exposed to sub-lethal concentration of copper observed a change in heart rate only at extreme temperatures. The authors concluded, that cardiac arrest at the lowest temperature (5°C) might be a physiological response to limited copper uptake. On the contrary, erratic heart rate at higher temperature (25°C) reflects increased copper toxicity (Camus et al., 2004).

These two examples highlight the two main philosophies oriented around climate variable - toxicant interaction, 1) climate induced toxicant susceptibility (CITS) and 2) toxicant induced climate susceptibility (TICS). The later reflects the scenario where climate variables enhance the toxicity of chemicals and could potentially affect aquatic organisms residing at the edge of their physiological tolerance range. TICS on the other hand involves the detrimental impact of toxicants in the ability of aquatic organisms to acclimate to a stress inflicted by extreme climate variables (Hooper et al., 2013). Still, Hooper et al. (2013) in a comprehensive mechanistic approach projected possible impact
of this interaction which includes changes in metabolic process (hyperphagia, shivering, estivation, and hibernation), structural complexity (metamorphosis, tissue resorption and growth), homeostasis (water retention, ion balance, nutrient absorption, reproductive processes) and can trigger associated behavioral changes such as foraging, feeding, mating, reproduction, and even migration. Further, Noyes et al (2009) confirmed that, the increase in water temperature would have a substantial effect on the biotransformation of contaminants to bioactive metabolites and hence impairing homeostasis (Noyes et al., 2009). Attending to this, the hypothesis on the effectiveness of LNG as a contaminant being enhanced by ocean acidification and warming, gains some value undoubtedly.

1.5 Gammarus locusta

The amphipod Gammarus locusta (Linnaeus, 1758) is an epibentic marine crustacean that inhabits the littoral and infralittoral zones and even penetrates through the brackish waters (Costa and Costa, 1999). G. locusta has a wide biogeography along the North East Atlantic and particularly in Portugal, its occurrence has been reported in a number of sites along the Portuguese coast namely, Mondego, Sado, Mira, Aveiro, Alvor, Óbidos and Quateira beach (Costa and Costa, 1999, Neuparth et al., 2002). In the wild, animals inhabit a variety of substrate yet it is more commonly associated with sand coupled with pebbles and macroalgae (Costa and Costa, 1999). In terms of dietary preferences, G. locusta is an omnivorous species. It is a multivoltine organism reproducing throughout the year. Sexual dimorphism is observed in this species, where males are larger in size than females and possessing a profound gnathopod on the second thoracic segment, which is vital for precopulatory behavior (Costa et al., 1998, Costa and Costa, 1999). G. locusta along with other species from the same genus are well recognized as models for EDC assays and also to investigate the impacts of extreme climate variables (Neuparth et al., 2002, Kunz et al., 2010).

Most of the species of the genus Gammarus spp are characterized by a short life cycle, which follows the general scheme illustrated in figure 1. Briefly, adult male with its 1st and 2nd pair of gnathopod catches and holds females ventrally in a lengthwise position. Then male guards the female until it molts, where it finally turns the female ventral side up and repeatedly places sperm onto her ventral surface closer to the oviduct. This precopulatory period can last up to 8 - 10 days and is dependent on female molting and external conditions like temperature (Sutcliffe, 2010). Gamete fertilization occurs in the marsupium of females where fusion of the gametes occurs within minutes of deposition. At ambient conditions, G. locusta embryonic development from egg incubation to post-hatch juveniles can range from 15 – 18 days (Neuparth et al., 2002, Maranhão and Marques, 2003, Sutcliffe, 2010). Pot-hatch period, where juveniles can move freely in and
out of the female's marsupium can take another 1 – 2 days. Under ambient conditions, development from juveniles to sub-adults ranges from 3 – 4 weeks and maturity is reached 6 – 7 weeks (Adults) after the post-hatch period (Neuparth et al., 2002, Sutcliffe, 2010). Finally, its estimated that the life span of G. locusta ranges from 5 – 6 months (Neuparth et al., 2002).

![Diagram of life cycle of Gammarus locusta](image)

Figure 1: General scheme on the life cycle of *Gammarus locusta* under ambient conditions.

*Gammarus locusta* is sensitive to a wide array of contaminants. Hence, it has been employed extensively in the Northern hemisphere as a model for water quality assessment and ecotoxicological assays (Gerhardt, 2011). It has been used as a model to test pharmaceutical contaminants (Neuparth et al., 2014), sediment toxicity (Costa et al., 1998, de los Santos et al., 2015), ecological impacts of climate change (Eklöf et al., 2012) and other EDC's potentially detrimental to their life history and population dynamic (Gerhardt, 2011). *G. locusta* can be considered as an excellent model for ecological and ecotoxicological studies, since it is easily accessible, tolerant to a wide range of parameters, easy to maintain and reproduce in laboratory and most important, it has a short life cycle (Neuparth et al., 2002). This later characteristic is of great importance as it can allow us to investigate environmental stress along different stages of its life cycle.

### 1.6 Objectives

The main goal of this study was to evaluate the effects of combined multiple stressors (warming, acidification and different concentrations of levonorgestrel) on distinct life stages of the amphipod *Gammarus locusta*. Thus, the main objectives of this work
were to evaluate the effects of those stressors on several endpoints of *G. locusta*, such as survival, growth, consumption rate and reproductive traits (embryonic development and fecundity).
CHAPTER TWO

2 MATERIALS AND METHODS

2.1 Amphipod Collection

Amphipods were obtained from a permanent laboratory culture system at CIIMAR facilities (Portugal) in which the original specimens were collected from the south margin of Sado estuary, Portugal (Neuparth et al., 2002).

_G. locusta_ individuals were separated in different size classes from which sub-adult (with approximately 3-4 weeks) were selected to be used in the experiment. During acclimation period (4 days), animals were fed with _Ulva sp_ on an _ad-libitum_ basis and maintained in a semi static system whereby 100% of the water was changed twice a week. Tanks were filled with a sand layer (1cm) and pebbles in order to mimic the natural environment. Photoperiod was set to 18h light: 6h dark to simulate summer conditions. The organisms were acclimated under the present day temperature and normocapnia (18°C, pH 8.1) and salinity 33-35. These conditions corresponded to the mean sea surface temperature (SST) and pH at the Sado estuary, which is the original site of the _G. locusta_ population.

2.2 Experimental Design

The experimental set-up followed a full factorial design manipulating temperature [present-day temperature and warming (+ 4°C)], CO₂ [normocapnia and hypercapnia (Δ pH 0.5 units)] and the progestin levonorgestrel (LNG: L1 – 10 ngL⁻¹ and L2 – 1000 ngL⁻¹, control – no progestin and solvent control – vehicle ethanol (0.01%)).

In order to avoid the interdependence or non-randomly interspersed treatment replicates that is frequently common in ocean acidification studies, we have implemented
one of the experimental models suggested by Cornwall and Hurd (2015). Therefore, a model with different tank types (i.e. storage tank, mixing tank, experimental tank) was applied. For a review of these type of experiments and the challenges they pose see Cornwall and Hurd (2015). The continuous flow-through system was divided in four levels. The 1st level corresponded to the reservoir tank (i.e. seawater tank), the 2nd fitted to the CO$_2$ mixing tanks (i.e. acidic and non-acidic), the 3rd corresponded to the LNG mixing flasks and finally the 4th level corresponded to the experimental units level (figure 2).

Water flow through the different tanks was maintained by gravity whereby water level in each tank was controlled by a float valve that closed once water level reached the required volume. The same mechanism was employed to control water in CO$_2$ mixing tanks and contaminant mixing flasks in the 2nd and 3rd levels, respectively. The seawater reservoir tank (350L) was connected directly to the main seawater source through a 10µm filter. Seawater from this reservoir was then channeled to the 2nd level, which comprised the CO$_2$ mixing tanks (50L). CO$_2^-$ chemistry in the acidic tanks was manipulated by diffusing pure CO$_2$ from a gas tank. CO$_2$ gas flow was regulated through a pH stat system (Aqua Medic® AT Control-SW, version 9.0). This was made possible as the AT Control automatically opened or closed a solenoid valve when pH readings in the acidic tanks deviated from the predetermined set points by 0.1pH units. pH readings were recorded through pH electrode sensors. CO$_2$ mixing tanks were maintained with aeration to facilitate CO$_2$ diffusion.

Following acidification, seawater from both acidic (Ac) and non-acidic (NAc) tanks was directed to the contaminant mixing flasks. Each Ac and NAc tank continuously filled three contaminant glass flasks (5L each), where colored codes were used to distinguish treatments into LNG contaminant flaks, solvent control flasks and control flasks.

The flow rate from the contaminant mixing flasks (3rd level) to the respective experimental units was ensured by a valve mechanism and maintained at 0.009 Lmin$^{-1}$. Experimental units assigned in different treatments were maintained at different temperatures in water baths (30L), in order to keep the temperatures more stable during the experiment. Temperature within the water baths were also regulated by the AT Control, which automatically heated the tanks whenever temperatures deviated from predetermined set points by 0.1°C. Experimental units were illuminated with artificial light apparatus suitable for marine set ups. Photosynthetic Active Radiation (PAR) was measured with a universal light meter (ULM – 500; WALZ) and maintained at 14µmol photons m$^{-2}$ s$^{-1}$.

Each experimental unit was continuously aerated and concurrently, water level in each unit was maintained at 0.55L through a dewatering tube connected to an external...
Materials and Methods

E. Loganimoce  
Master Degree

flask (0.80L), which was working on the principle of communicating vessels. From these external flasks, contaminated water was directed to a contamination tank (20L) and with a submersible pump (3000L h⁻¹) water was pumped continuously to the decontamination chamber (120L). Wastewater was disposed through an activated-charcoal filter before being totally eliminated.

The experiment was divided in two phases: 1) pre-reproduction phase, which lasted for 21 days, and 2) post-reproduction phase that last more 7 (±2) days according to the embryonic development time for the different treatments. Amphipods were exposed to 16 different treatments indicated in the table 1.

Table 1: Description of the different treatments to which G. locusta were exposed. C – control, SC – solvent control, L1 - LNG (10 ng L⁻¹) and L2 - LNG (1000 ng L⁻¹).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>18°C, pH 8.1, C</td>
</tr>
<tr>
<td>T2</td>
<td>18°C, pH 8.1, SC</td>
</tr>
<tr>
<td>T3</td>
<td>18°C, pH 8.1, L1</td>
</tr>
<tr>
<td>T4</td>
<td>18°C, pH 8.1, L2</td>
</tr>
<tr>
<td>T5</td>
<td>18°C, pH 7.6, C</td>
</tr>
<tr>
<td>T6</td>
<td>18°C, pH 7.6, SC</td>
</tr>
<tr>
<td>T7</td>
<td>18°C, pH 7.6, L1</td>
</tr>
<tr>
<td>T8</td>
<td>18°C, pH 7.6, L2</td>
</tr>
<tr>
<td>T9</td>
<td>22°C, pH 8.1, C</td>
</tr>
<tr>
<td>T10</td>
<td>22°C, pH 8.1, SC</td>
</tr>
<tr>
<td>T11</td>
<td>22°C, pH 8.1, L1</td>
</tr>
<tr>
<td>T12</td>
<td>22°C, pH 8.1, L2</td>
</tr>
<tr>
<td>T13</td>
<td>22°C, pH 7.6, C</td>
</tr>
<tr>
<td>T14</td>
<td>22°C, pH 7.6, SC</td>
</tr>
<tr>
<td>T15</td>
<td>22°C, pH 7.6, L1</td>
</tr>
<tr>
<td>T16</td>
<td>22°C, pH 7.6, L2</td>
</tr>
</tbody>
</table>

Each treatment included 6 replicates (glass cups of 650ml each) and within each one were added 12 individuals of G. locusta. 2 of these amphipods were isolated in small plastic perforated flasks (5ml) in order to evaluate individual survival, growth and
consumption rates. The other 10 individuals were maintained free in the cups, for the post-reproduction phase. The later were separated by sex and half of the replicates were maintained with males and the other half with females in order not to compromise the final reproduction (figure 2).

Glass cups and the small microcosm flasks simulated natural environment with a thin layer of sand and Ulva sp (2 discs; 0.0708g w/w, ±0.102 per individual). The latter was replaced thrice a week. During the pre-reproduction phase, survival was checked at days 7, 15 and 21 and the organisms sampled at day 21 were examined for growth and condition estimates. In the post-reproduction phase, administration of LNG was stopped since the objective was to observe effects only on the progenitors and not on the offspring.

During the experimental period, water temperature and pH were controlled and varied in the range of; ambient temperature - 18.230°C ± 0.447, warming temperature - 21.791°C ±0.587, normocapnia - 8.057pH ±0.0562 and hypercapnia - 7.520pH ±0.255.

Figure 2: A detailed scheme illustrating a sub-set of the experimental system. Abbreviations, F- female, M- male, C- control, SC- solvent control, L1- LNG low concentration and L2- LNG high
Materials and Methods

concentration. (Image designer, João Mario Oliveira)

2.3 Pharmaceutical

The standard levonorgestrel (LNG) was purchased from Sigma-Aldrich (Steinheim, Germany). Stock solutions were prepared with analytical ethanol supplied by Merck, and stored in dark at -20°C. The two solutions, L1- 10 ngL⁻¹ and L2- 1000ngL⁻¹ LNG were administered 3 times daily in their respective mixing flasks, in order to simulate episodic discharges from a contamination source.

2.4 Amphipod Length and Weight

All isolated amphipods were measured and weighted at day 1 and then at day 21. Amphipods were previously photographed individually using the Olympus cell^B (analySIS image processor version 5.1) program.

The program, ImageJ (version 1.50i) (Rasband, 2016) was then used to determine the individual Metasomatic Length (ML) to the nearest 0.1mm, following the same procedures as Neuparth et al. (2002). ImageJ has also been employed to determine length of microorganisms (Mörck and Pilon, 2006) and even on animal from the same order as those used in this experiment (Foley et al., 2010). ML is defined as the distance between the anterior end of the rostrum and the posterior end of the last metasomatic segment (Neuparth et al., 2002). Since it is difficult to determine TL of animal’s due to its lateral resting position, alternatively ML was measured (Costa and Costa, 1999). The relationship between TL and ML had been previously determined by Costa and Costa (1999) and is represented by the following linear regression equation:

\[ TL = -0.153 + 1.218 \times (ML) \]

Hence, TL of individuals along the exposure phase was determined using the above linear regression.

2.5 Amphipod Condition Index

Amphipods length and wet weight data was used to determine the fitness condition of test subjects along the exposure period. In this case the Fulton Condition Index (K) was applied and is, expressed by the following:

\[ K = \frac{W}{TL^3} \times 100 \]

Where: W = mean weight and TL³ = mean total length.
This index is typically used for fishes (Rosa et al., 2014) but it has also been applied for crustaceans (Enin, 1994, Moreira et al., 2015).

2.6 Consumption Rate

The estimation of relative consumption rates (RCR) was done twice during the experimental period. The first assay was conducted during the first week and the final one in the third week of exposure. The individuals selected for consumption were starved for 24h prior to the consumption experiment.

Using the equation adopted by Gutow et al. (2014), amphipod consumption was calculated as \( C = W_i \ast (C_f / C_i) - W_f \); where, \( W_i \) and \( W_f \) are the initial and the final WW of the Ulva sp tissue, respectively, and \( C_i \) and \( C_f \) are the equivalent WW of the control pieces (biogenic controls). Relative consumption rates (RCR, g Ulva ww g\(^{-1}\) individual ww day\(^{-1}\)) were calculated as \( C / (W_M \ast t) \), where \( C \) is the Ulva consumed for each time interval (1d) and \( W_M \) is the final wet mass of individuals for each time interval.

2.7 Reproductive Traits

Following the pre-reproduction phase, individuals within each replicate were taken and sorted into couples to ensure mating and reproduction. Each replicate included one couple.

The reproductive behavior of G locusta involves a precopulatory guarding phase in which the male holds and carries the female. This ensures that insemination can occur as soon as the female mouls and is ready to release eggs into the marsupium (Maranhão and Marques, 2003). During this phase, couples were observed daily until females were separated from males and observed to be ovigerous. Embryonic development time followed the method by Maranhão and Marques (2003); the period from oviposition to release of juveniles from the brood pouch of females. Hence, ovigerous females were observed daily until the marsupium were noticed to be completely empty. As soon as females were observed with an empty marsupium, experimental units were filtered through 500µm and 250µm sieves to separate juveniles from females.

Fecundity of G. locusta was accounted as the number of juveniles released per female in each replicate. Following the separation from females, juveniles were straight away sacrificed and stored away in 70% ethanol for counting. Juveniles were counted using stereomicroscope (leica EZ4).

2.8 Quantification of Levonorgestrel (HPLC-DAD)

Quantification of the synthetic progestin hormone LNG by High Performance Liquid Chromatography (HPLC), followed the method employed by (Ribeiro et al., 2007) and (Beck et al., 2005) (figure 3; general scheme).
Water samples (2L: L₁ & 1L: L₂) were collected from experimental units at two time intervals. First sample collection was conducted 30mins after the first injection (T₀) and the second after 1h30 (T₉₀). Samples were then taken and filtered through a 47mm glass fiber filter. All samples were then acidified with sulfuric acid (H₂SO₄) to pH = 2 before the Solid Phase Extraction (SPE).
For the SPE, each sample was vacuum forced through a 200mg Oasis HLB cartridge that had initially been treated with 25ml dichloromethane: methanol (CH$_2$CL$_2$: CH$_3$OH, 50: 50, v/v), followed by 12ml of CH$_3$OH and 25ml of ultrapure Milli.Q water (UP). After all the samples had passed, cartridges were then washed with 25ml UP water followed by 1ml of CH$_3$OH and left under the vacuum forced filtration for 30mins to dry-out residual water. SPE extracts were then mounted on 1 g Sep-Pak silica cartridges (that had been initially washed with 10ml of CH$_2$CL$_2$: CH$_3$OH, 50: 50 v/v) after which 20ml of CH$_2$CL$_2$: CH$_3$OH (50: 50 v/v) was eluted strategically through the cartridges and collected in round bottomed glass test tubes. This final step collected eluates by gravity feed into their respective glass test tubes.

Samples were then left to evaporate in an oven at 40°C overnight before being transferred into vials and respective glass tubes sequentially washed with acetonitrile (250µL). Samples were further evaporated to dryness in a thermostatic bath under a nitrogen stream and further dissolved in 200µL of CH$_3$OH: CH$_3$CN (50:50, v/v). Finally, 20µL was injected into the HPLC-DAD system for analysis.

2.9 Data Analysis

Survivorship of individuals was compared between paired treatments by log-rank tests following the same procedure as Ko et al. (2014).

Data were first transformed to improve normality and homogeneity of variance. Data that still did not conform to the assumption of homoscedasticity were ranked-transformed before performing three-way ANOVA, following the same approach as (Ko et al., 2014). A 3-Way ANOVA on ranks (temperature x pH x LNG) was used to test for differences in the condition index of the organisms, consumption rates (3rd week), fecundity and embryonic development among the different treatments.

For the growth analysis, firstly, growth rates were estimated for the entire exposure period (between day 1 and day 21). Then, a 3-Way ANOVA (temperature x pH x LNG) was applied to test for differences in growth rates among the distinct treatments. A similar approach was applied to test differences in consumption rates (1st week) among treatments. Analyses were performed with STATISTICA 7 software (StatSoft, Tulsa, OK, USA).
CHAPTER THREE

3 RESULTS

3.1 Levonorgestrel Quantification

The actual concentrations of LNG were measured 30mn (T₀) and 90mn (T₉₀) immediately after the first injection. Table 2 shows that after 30 mn the measured concentrations were close to the nominal values. However, after 90 mn the concentrations decreased considerably, reaching approximately half of the initial value. The concentrations in the control and solvent control replicates were below the limit of detection (1.2 ng L⁻¹).

Table 2 – Levonorgestrel concentrations (ng L⁻¹) in waters during 21 days exposure. The water was sampled 30 minutes after the first injection (T₀) and 90 minutes later (T₉₀).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Nominal</th>
<th>T₀</th>
<th>T₉₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>18°C pH 8.1</td>
<td>C</td>
<td>-</td>
<td>&lt; 1.2</td>
</tr>
<tr>
<td></td>
<td>SC</td>
<td>-</td>
<td>&lt; 1.2</td>
</tr>
<tr>
<td></td>
<td>L1</td>
<td>10</td>
<td>11.4 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>L2</td>
<td>1000</td>
<td>928.5 ± 44.5</td>
</tr>
<tr>
<td>18°C pH 7.6</td>
<td>C</td>
<td>-</td>
<td>&lt; 1.2</td>
</tr>
<tr>
<td></td>
<td>SC</td>
<td>-</td>
<td>&lt; 1.2</td>
</tr>
<tr>
<td></td>
<td>L1</td>
<td>10</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>L2</td>
<td>1000</td>
<td>915 ± 84.8</td>
</tr>
<tr>
<td>22°C pH 8.1</td>
<td>C</td>
<td>-</td>
<td>&lt; 1.2</td>
</tr>
<tr>
<td></td>
<td>SC</td>
<td>-</td>
<td>&lt; 1.2</td>
</tr>
</tbody>
</table>
### Results

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>10</td>
<td>11.8 ± 0.6</td>
<td>&lt; 1.2</td>
</tr>
<tr>
<td>L2</td>
<td>1000</td>
<td>929.5 ± 43.1</td>
<td>393.5 ± 71.4</td>
</tr>
<tr>
<td>C</td>
<td>-</td>
<td>&lt; 1.2</td>
<td>&lt; 1.2</td>
</tr>
<tr>
<td>SC</td>
<td>-</td>
<td>&lt; 1.2</td>
<td>&lt; 1.2</td>
</tr>
<tr>
<td>L1</td>
<td>10</td>
<td>11.9 ± 0.6</td>
<td>4.6 ± 0.1</td>
</tr>
<tr>
<td>L2</td>
<td>1000</td>
<td>931 ± 101.1</td>
<td>354 ± 28.3</td>
</tr>
</tbody>
</table>

**22°C pH 7.6**

#### 3.2 Amphipod Survival

Warming condition (22°C, pH 8.1) was the one that caused the highest negative impact on the *G. locusta* survival, reaching the lowest values (30% - 50%) at day 21. On the other hand, ambient temperature under normocapnia (18°C pH 8.1) witnessed the highest survival rate (70% - 90%), while the acidification scenario (18°C pH 7.6) presented slightly lower values (60% - 90%) (Figure 4).

Finally, the warming and hypercapnic condition (22°C, pH 7.6) presented similar values to the acidification, except for the solvent control that was significantly different (log-rank test: 2.53, \( p = 0.011 \)). In addition, for each of the other conditions (temp. x pH) no significant differences were observed between control and LNG concentrations (log-rank test, \( p > 0.05 \)).

Moreover, comparing all control treatments, significant differences were observed between warming and ambient condition (log-rank, test statistic = 2.30, \( p = 0.020 \)) and also between warming and warming/hypercapnic treatment (log-rank, test statistic = 2.101, \( p = 0.035 \)). Also, significant differences were observed between L1 under warming and ambient condition (log-rank, test statistic: -2.45, \( p = 0.014 \)) and the acidification scenario (log-rank, test statistic: -2.89, \( p = 0.004 \)).
**Figure 4**: Survival (%) of *G. locusta* individuals exposed to different combinations of temperature, pH and LNG concentrations (n = 12). Abbreviations, C- control, SC- solvent control, L1- 10ngL⁻¹ LNG and L2- 1000ngL⁻¹ LNG.

### 3.3 Fulton Condition Index

The fulton condition index, at day 21, suggests that the fitness condition of *G. locusta* was more influenced by temperature than pH or LNG (Figure 5). Fitness condition of individuals exposed to warmer scenarios was significantly reduced (3-Way ANOVA on ranks: $F_{1, 115}= 6.658$, $p= 0.011$) compared to those exposed to ambient temperature. This pattern was more visible in the solvent control. Both LNG concentrations (L1 & L2) under ambient and warming temperatures showed a slight trend of reduced fitness under normocapnia compared to hypercapnic conditions. However, despite the aforementioned trend, LNG and pH had no significant effect on the fitness condition of *G locusta* (3-Way ANOVA on ranks: $F_{3, 115}= 0.76$, $p> 0.05$ and $F_{1, 115}= 0.43$, $p> 0.05$ respectively).
Figure 5: Fulton condition index (K) for G. locusta exposed to different combinations of temperature, pH and LNG concentrations at day 21. Values represent mean ± SD. Abbreviations, C- control, SC- solvent control, L1- 10ngL⁻¹ LNG and L2- 1000ngL⁻¹ LNG.

3.4 Amphipod Growth Rate

In general, growth rate seemed to be higher at ambient temperature under normocapnia than the other treatments (Figure 6). This trend was quite clear in the control treatments (18°C/normocapnia – 0.18mm day⁻¹, 18°C/hypercapnia – 0.16mm day⁻¹, 22°C/normocapnia – 0.14mm day⁻¹, 22°C/hypercapnia – 0.15mm day⁻¹). Moreover, growth rates were significantly lower under hypercapnic conditions (3-Way ANOVA, F₁,₁₂₅ = 7.60, p = 0.007) for both temperatures and under LNG exposure (3-way AONVA, F₃,₁₂₅ = 3.94, p = 0.009). The latter was more visible for the solvent control compared to the control.
3.5 Relative Consumption Rate (RCR)

The RCR by *G. locusta* during the 1st week (figure 7A) of exposure was substantially higher (more than 2-fold) compared to the 3rd week (figure 7B). In the first week, there was a clear tendency for a decline in the RCR with the increase of temperature for the control treatment, despite no significant differences were observed (3-way ANOVA, $F_{1,75} = 1.96, p = 0.165$) (Figure 6A). On the other hand, at 18°C the solvent control presented significant lower values than the control (3-way ANOVA, $F_{4,75} = 7.67, p = 0.0001$) while at 22°C no significant differences were observed between them. A significant interaction between temperature and LNG concentration was detected (3-way ANOVA, $F_{4,75} = 5.43, p = 0.002$). In addition, at 18°C, RCR tended to be lower in treatments exposed to LNG concentrations than in control while at 22°C an opposite pattern was observed.

During the 3rd week exposure, the RCR pattern was different from the 1st week (Figure 7B). For the control treatments, significant lower values were observed under hypercapnia (3-Way ANOVA on ranks, $F_{1,58} = 8.98, p = 0.040$). Also, a significant effect of the LNG was detected (3-way ANOVA on ranks, $F_{3,58} = 4.08, p = 0.012$) especially for the solvent control. Due to the lowest robustness of the analysis no significant differences were detected between multiple comparisons.
3.6 *G. locusta* Fecundity and Embryonic Development

*G. locusta* fecundity seemed to be affected mainly by two factors, temperature and the LNG concentration. Generally, number of newborns released by all females exposed to higher temperatures was significantly lower than under ambient temperature (3-way ANOVA on ranks, $F_{1,36} = 7.49, p = 0.009$) (Figure 8). Also, hypercapnia conditions seemed to negatively affect the fecundity at both temperatures, except for the highest LNG concentration (L2). LNG exerted a significant effect (3-way ANOVA on ranks, $F_{3,36} = 3.93, p = 0.016$), which was somewhat dose dependent under both temperatures. Moreover, these two factors (temp*LNG) seemed to impose a synergistic effect on the number of

Figure 7: RCR of *G. locusta* individuals at the (A) 1st week of exposure and (B) 3rd week of exposure. Values represent mean ± SD. Different letters indicate significant differences among treatments (3-Way ANOVA, Post-Hoc analysis $p < 0.05$).
newborns produced. However, no significant interaction was observed between them (3-Way ANOVA on ranks, $F_{3,36} = 0.22$, $p > 0.05$).

![Figure 8: Total number of newborns released by female *G. locusta* during the post-reproduction phase exposed to the different treatments. Values represent mean ± SD.](image)

*G. locusta* embryonic development was significantly lower (approximately half of the time) under warming conditions compared to the ambient temperature (3-way ANOVA on ranks, $F_{1,44} = 25.49$, $p < 0.001$) (Figure 9). This trend was more visible on control treatments but also evident in those exposed to LNG concentrations.

On the other hand, hypercapnic conditions seemed to have delayed embryonic development especially under ambient temperature. Additionally, a significant interaction between temperature and pH was detected (3-way ANOVA on ranks, $F_{1,44} = 6.44$, $p = 0.015$).
Figure 9: Embryonic development time of *G. locusta* exposed to the different treatments. Values represent mean ± SD.
CHAPTER FOUR

4 DISCUSSION

4.1 Survival, Fitness Condition and Growth

On the facet of global warming (+4°C) and ocean acidification (Δ 0.5pH units) (IPCC, 2014) coupled with human induced EDCs’, marine invertebrates’ are harshly challenged to adapt in a changing ocean. In the present study warming condition (22°C, pH 8.1) revealed as the condition that caused the highest mortality (50-70%) on the amphipod G. locusta compared to the other conditions (Figure 4). On this note, elevated temperature appears to be the factor controlling survival compared to pH and/or the synthetic steroid, LNG. Similar results have been reported by other studies on the effect of warming on the survival of crustaceans (Tlusty et al., 2008, Mayor et al., 2012, Pansch et al., 2014), echinoderms (Byrne et al., 2009, Nguyen et al., 2012), marine gastropods (Zippay and Hofmann, 2010) and other marine invertebrates (Byrne, 2011).

In addition, hypercapnic condition is known to suppress metabolic process in an organism causing delayed development and even mortality (Pörtner et al., 2004, Michaelidis et al., 2005, Carter et al., 2013). Results herein elucidate that under ambient temperature, hypercapnic condition (Δ pH 0.5 units) reduced overall survival relative to the ambient scenario (18°C pH 8.1). This could be a consequence of an inefficient acid-base regulation in extracellular and intracellular fluids creating a stressful internal homeostasis (Michaelidis et al., 2005, Fabry et al., 2008). Slow metabolism could have compromised the transport of essential ions necessary for physiological development and survival (Pörtner et al., 2004). However, under the combined effect of warming and acidification the mortality rate was much more reduced (17- 40%) compared to warming (22°C pH 8.1)
with exception of the solvent control. In addition, comparing the acidification scenario (18°C pH 7.6) with the warming and acidification condition, both presented similar results, with exception of solvent control that presented a totally different response. However, for the control treatment (without LNG) the mortality rates were lower under the combined effect of warming and acidification than under acidification alone. Similar results have been observed in previous studies (Miles et al., 2007, Sheppard Brennand et al., 2010, Byrne and Przeslawski, 2013, Cardoso et al., Submitted). Thus, our results suggest that 22°C may already exceed the thermal limits of the species; yet, under the combination of lower pH, the deleterious effect of warming seemed to be minimized.

Regarding the fitness condition of the amphipods, once again the temperature revealed to be the dominant factor. Individuals exposed to warmer temperature in general revealed a reduced fitness condition compared to those exposed to ambient temperature (Figure 5). This further consolidates the strong influence of temperature causing physiological stress in G. locusta. Consequently, poor fitness could have resulted from a reduced metabolic scope and reduced oxygen consumption that ultimately reduced growth rate and increased mortality (Dissanayake and Ishimatsu, 2011). With this in mind, Delorme and Sewell (2016) reported that the echinoderm Evechinus choroticus conditioned under elevated temperature obtained a poor fitness condition and ultimately disturbed gonad development (Delorme and Sewell, 2016). In addition, Rosa et al. (2014) who used the same condition index on the juvenile bamboo shark Chiloscyllium punctatum reported that, fitness condition was significantly reduced when exposed under warming and hypercapnia conditions. More important, interaction between warming and hypercapnia condition significantly reduced survival (44%) compared to warming and normocapnia condition (71%) (Rosa et al., 2014). The authors confirmed that warming had a greater impact on the fitness condition of juvenile C. punctatum than acidification. It is imperative to recognize that the effect of warming on fitness condition in the present study is concomitant with growth rate data (Figure 6).

It is important to highlight that despite that Fulton condition index is more often used with fish species (Froese, 2006, Todd et al., 2008, Rosa et al., 2014), it has also been applied to crustaceans (Moreira et al., 2015). However, the results obtained were not very satisfactory since they did not reflect the effects observed, for example on the survival. So, probably this index has some limitations or it is not the most appropriate for this particular species. It could happen that the organisms could die before the condition factor is affected. Or the frequent over-interpretation of a simple numeric reading of a length-weight relationship is not the most adequate way of estimating the fitness condition of an organism, particularly an amphipod.
G. locusta growth rate seemed to be affected in part by temperature, since according to our results, individuals exposed to higher temperature presented slightly lower values than under ambient temperature (Figure 6). This is in accordance with the previous results of survival and condition fitness of the organisms. However, pH and LNG seem to have had a higher negative effect on growth rates than temperature. According to the present results, individuals exposed to hypercapnia under both temperatures suffered a negative impact on the growth rates. Also, a significant negative effect of LNG was observed.

Concerning the effects of acidification, they are in agreement with other previous studies (Kurihara et al., 2008, Kroeker et al., 2010, Kroeker et al., 2013, Kroeker et al., 2014). According to Kurihara et al. (2008) pH 7.8 had no effect on the growth rate of the marine shrimp Palaemon pacificus. Additionally, at pH 7.6, growth rate and molting frequency was significantly reduced. In crustaceans molting is vital process modulated cooperatively by the sinus gland and the Y-organ (Subramoniam, 2000). Suppressed growth rate under hypercapnia condition noticed herein could be a consequence of depressed metabolic activity coupled with changes in extracellular and intracellular acid-base regulation (Michaelidis et al., 2005, Dissanayake and Ishimatsu, 2011). Also, imbalance of acid-base state can potentially disrupt the production of the active molting hormone 20-OH-ecdysone, which ultimately reduced growth rate (Michaelidis et al., 2005). However, slow growth process induced by hypercapnia could be further exacerbated by interaction with LNG.

Regarding the LNG and despite a significant negative effect was detected, a clear pattern was not visible in figure 5. Still, we could observe that under hypercapnic conditions and higher LNG concentrations (L2) lower growth rates were observed. A few synthetic xenostrogens and androgens have been reported as potential agonists and/or antagonists for, 20-OH-ecdysone (Rodríguez et al., 2007). Particularly, the androgen testosterone was reported by (Mu and LeBlanc, 2002) to have an anti-ecdysteroidal activity in the crustacean Daphnia magna. The authors did conclude that testosterone did not have any ecdysteroidal activity in the ecdysone-responsive cell line yet when administered together with the active 20-OH-ecdysone, the androgen was able to block the activity of 20-OH-ecdysone (Mu and LeBlanc, 2002). This interference resulted in delayed molting in D. magna, increased abnormalities, and reduced number of offspring (Mu and LeBlanc, 2002). Considering that LNG is a synthetic testosterone derivative (Kumar et al., 2015), it is likely that even at low concentration (Table 2) this synthetic androgen could have potentially interfered and disrupted the endocrine axis governing molting activity for G locusta.
4.2 Relative Consumption Rate (RCR)

RCR during the first week of exposure (Figure 7A) was more than 2-fold compared to the third week (Figure 7B) of RCR analysis. This result can be entirely related with the growth rate of the species. *G locusta* is characterized by a short life cycle with substantial growth rates for the first weeks of their life stage and subsequent gradual reduction until the end of the life span (Costa and Costa, 1999, Neuparth *et al.*, 2002). Thus, the obvious difference in RCR noticed here between 1st and 3rd week of exposure can be related to the fact that during the first week since individuals were younger and presented a higher growth rate, they also presented a higher metabolic rate which means that organisms needed to consume more to compensate the enormous amount of energy required for somatic development and ultimately growth. In addition, we could observe that under higher temperatures the consumption rates were lower, contrarily to our expectations.

According to the metabolic theory an increase in temperature will generally lead to an increase in metabolism, which stimulates processes like consumption, growth, reproduction and embryonic development (Brown *et al.*, 2004, O'Connor *et al.*, 2009). However, our result on RCR yields the contrary. It is likely that the 22ºC seems to be a lethal temperature (LT) for the species, inhibiting better fitness condition, growth and also consumption rates (Somero, 2002, Somero, 2010). Delorme and Sewell (2016) showed similar results on the marine echinoderm *Evechinus choroticus* whereby feeding rate was significantly reduced under elevated temperature. Their results on feeding rates correlated strongly with lower growth rates of individuals exposed under warming temperature (Delorme and Sewell, 2016). Our findings were also corroborated by other studies. For example, Nowicki *et al.* (2012) reported that extreme temperature beyond LT limit has also been responsible for a decline in consumption rate and foraging behavior in the juvenile teleost *Amphiprion melanopus*. Also, Werner *et al.* (2016) reported that grazing by *Idotea spp* and *Gammarus spp* on *Fucus vesiculosus* under warming condition was highly dependent on season and species identity. The former being crucial as experiments conducted in late summer triggered an enormous decline in grazing due to considerable collapse in the abundance of the two grazers (Werner *et al.*, 2016). The authors concluded that collapse in grazer abundance is a consequence of intolerant ability to survive under elevated temperature, which had likely exceeded the grazer LT limit (Werner *et al.*, 2016).

On the other hand, RCR during the third week of exposure was especially affected by hypercapnic condition (Figure 7B). Exposure to OA can result in metabolic depression in those species that are unable to regulate intracellular pH, which can lead to decreased feeding rates (Rosa and Seibel, 2008, Nowicki *et al.*, 2012, Kroeker *et al.*, 2014). Also, acidification condition may change the constituent elements in algal tissues (e.g., the ratio
between nitrogen and carbon) (Poore et al., 2013). Change in the nutritional constituent elements of algal feed may decrease its value for herbivores species. Thus, hypercapnic condition may have a more profound effect than predicted, bearing in mind its impact on both primary producers and grazers (Poore et al., 2013, Werner et al., 2016).

4.3 Fecundity and Embryonic Development

G. locusta fecundity was clearly affected by temperature and LNG concentration. According to our findings, the number of newborns released under warming conditions was significantly lower compared to ambient temperature (Figure 8). In addition, this negative effect was incremented under higher concentrations of LNG. Our findings can be a consequence of impaired fertilization (Byrne et al., 2009, Parker et al., 2010), abnormal larval development (Parker et al., 2009, Rahman et al., 2009, Nguyen et al., 2012) or even fitness condition of progenitors which is relevant for embryo survival (James et al., 2009). In our study we could verify that the fitness condition of individuals were suppressed under elevated temperature (Figure 5), which could result in smaller sized individuals and ultimately inability to produce newborns. Additionally, lower fitness condition could potentially threaten gonad development of progenitors (James et al., 2009). Delorme and Sewell (2016) reported that the echinoderm Evechinus choroticus conditioned under extreme heat stress observed lower feeding rate ultimately having a poor fitness condition. More important, gamete production of the progenitors of, E. choroticus was significantly inhibited and histological analysis showed empty gonads and/or lack of gametic material within gonads (Delorme and Sewell, 2016). Therefore, in the present study under extreme heat stress it is likely that organisms were exposed to a temperature beyond their LT limit, lowering fitness condition (Figure 5) and potentially affecting gonadal development and maturation of progenitors.

Also, our results revealed a synergistic effect between temperature and LNG concentration, which means that under the combined effect of higher temperature and higher LNG concentration more negative impacts on the reproduction were observed. Synthetic progestins have been recently reported harmful on reproduction in aquatic organisms (Fent, 2015). This is mainly due to its ability to interact endogenously with specific receptors causing major physiological transformation (Ellestad et al., 2014). LNG has been reported to react with androgenic receptors (AR) in teleost jeopardizing reproduction (Zeilinger et al., 2009) (effective concentration; ≥0.8ng/L⁻¹), egg production (Runnalls et al., 2015) (EC: 1 -10ng/L⁻¹), embryonic development (Hua et al., 2015) (EC: 33NG/L⁻¹) and gene expression (Ellestad et al., 2014, Hua et al., 2015) (1 – 33ng/L⁻¹).

According to our results, for the maximum concentration of LNG (L2), even after 90 minutes of hormone administration, the detected values (≥300ng L⁻¹) proved to be
harmful on teleost physiology. Androgen and estrogen receptors have been detected in amphipods (Köhler et al., 2007, Lewis et al., 2012, Lewis et al., 2015). Taking this into consideration, we can speculate that the LNG could interact with AR triggering endogenous modifications and possibly changing secondary sexual characteristics in progenitors in the present study. The probable consequence is deterred gonadal development, inhibition of molting and masculinized females. The aforementioned physiological endpoints have been reported for other synthetic steroid like xenoestrogens (Hutchinson et al., 1999, Watts et al., 2001, Watts et al., 2002, Vandenbergh et al., 2003, Watts et al., 2003). Still, no significant interaction between temperature and LNG was found in our study. It is plausible to suggest that elevated temperature may have enhanced LNG effect on *G. locusta* progenitors reproduction (Noyes et al., 2009, Hooper et al., 2013, Nikinmaa, 2013).

Nonetheless, our results did show that elevated temperature resulted in a much faster embryonic developmental process (Figure 9). Similar results have also been revealed in other crustacean like, *Nephrops norvegicus* (Styf et al., 2013) and *Gammarus locusta* (Neuparth et al., 2002) supporting the metabolic theory under elevated temperature (Brown et al., 2004). In this regard temperature may have benefited embryos and larvae for a shorter time period of development in their most vulnerable life-stage Yet, reported by Styf et al. (2013) that the early life stage development of *N. norvegicus* can only be accelerated within a specific thermal limit. Beyond this range follows developmental failure, lower number of offspring’s and high level of mortality (Styf et al., 2013). Concurrently, it has been reported that increasing temperature within thermal range masks the effect of acidification (Sheppard Brennand et al., 2010, Nguyen et al., 2012).

In the present study, acidification had no significant effect on the number of newborns released, despite a slight tendency for a decline has been observed under hypercapnia for both temperatures. Hypercapnia impairs sperm motility presenting an unsuccessful fertilization scenario (Christen et al., 1983). It can reduce sperm internal pH, which inhibits the activity of dynein ATPase activity causing sperm to be immotile (Christen et al., 1983, Schlegel et al., 2015). It is also plausible to imply that reduce sperm motility of *G. locusta* under hypercapnic condition resulted in a low chance of fertilization and hence low number of individuals (Figure 8). Nonetheless, despite these arguments, our analysis confirmed that elevated temperature statistically had a more deleterious effect than acidification (Byrne et al., 2009, Sheppard Brennand et al., 2010, Nguyen et al., 2012). In addition, we could observe that acidification slightly prolonged the embryonic development under ambient temperature (Figure 9). The detrimental impact of low pH on embryonic development has also been reported for oysters (Parker et al., 2009, Parker et
Discussion

al., 2010), sea urchins (Kurihara and Shirayama, 2004, Foo et al., 2012) and copepods (Weydman et al., 2012, Pedersen et al., 2013). Still, hypercapnia condition impairs fertilization (Kurihara et al., 2007, Talmage and Gobler, 2011, Davis et al., 2013), larval calcification (Byrne et al., 2013a, Byrne et al., 2013b) and molting process (Kurihara et al., 2007, Kurihara et al., 2008). Therefore, prolonged embryonic development under ambient temperature and hypercapnia in the present study, is likely attributed to a depressed metabolic scope leading to slower development (Dissanayake and Ishimatsu, 2011) and/or the direct impact of acidification on the gametes of progenitors (Christen et al., 1983, Schlegel et al., 2015).

4.4 Conclusions

Environmental conditions play a fundamental role on the proper physiological development and functioning of marine invertebrates. Temperature is the single most crucial environmental factor that determines successful reproduction, embryogenesis, larval development, swimming performance, foraging, oxygen consumption and even phenological interactions (Somero, 2002, Edwards and Richardson, 2004, Somero, 2010, Byrne and Przeslawski, 2013). This fact was demonstrated in the present study where temperature appeared to be the dominant factor all throughout measured endpoints. Recent studies have proven that near future warming scenario predicted for 2100 (+4°C) (IPCC, 2014) may inflict serious implications on marine invertebrates as it is expected to be beyond organism’s LT limit (Stenseng et al., 2005, Parker et al., 2010, Somero, 2010, Zippay and Hofmann, 2010). Concerning the combined effects of warming and acidification, previous studies have already demonstrated the negative effect of this interaction (Kroeker et al., 2010, Foo et al., 2012, Byrne et al., 2013a, Kroeker et al., 2013, Styf et al., 2013) however our study presented different results. Namely, for the G. locusta survival, it was observed that the negative effect of warming was slightly minimized under the combined effect of acidification.

Finally, the interaction between these two climate variables may very well govern the efficacy of LNG as an EDC (Noyes et al., 2009, Nikinmaa, 2013). To our knowledge, this study is the first one to highlight the effects of EDCs under a climate change scenario.

In the recent research programs, more attention has been given to the possible interaction of multiple stressors, which can represent a more realistic scenario and give us a more holistic view about ecosystem functioning. So, further research on this topic is very important in order to better understand the main interactive effects of climate change and EDCs on the structure and functioning of coastal ecosystems. This will have a great relevance in terms of management and conservation of these ecosystems.
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