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Interactive effects of increased temperature, pCO_2 and the synthetic progestin levonorgestrel on the fitness and breeding of the amphipod *Gammarus locusta*☆

P.G. Cardoso [a, *, 1], E.M. Loganimoce [a, 1], T. Neuparth [d], M.J. Rocha [a, b], E. Rocha [a, b], F. Arenas [c]

[a] Group of Histomorphology, Physiopathology and Applied Toxicology, Interdisciplinary Centre of Marine and Environmental Research (CIIMAR/CIMAR), University of Porto, Porto, Portugal

[b] Laboratory of Histology and Embryology, Department of Microscopy, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, Porto, Portugal

[c] Group of Aquatic Ecology and Evolution, Interdisciplinary Centre of Marine and Environmental Research (CIIMAR/CIMAR), University of Porto, Porto, Portugal

[d] Group of Endocrine Disruptors and Emergent Contaminants, Interdisciplinary Centre of Marine and Environmental Research (CIIMAR/CIMAR), University of Porto, Porto, Portugal

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A B S T R A C T

Given the lack of knowledge regarding climate change-chemical exposure interactions, it is vital to evaluate how these two drivers jointly impact aquatic species. Thus, for the first time, we aimed at investigating the combined effects of increased temperature, pCO_2 and the synthetic progestin levonorgestrel on survival, growth, consumption rate and reproduction of the amphipod *Gammarus locusta*. For that, a full factorial design manipulating temperature [ambient temperature and warming (+4 °C)], pCO_2 [normocapnia and hypercapnia (Δ pH 0.5 units)] and the progestin levonorgestrel (LNG: L1 – 10 ngLL^{-1} and L2 – 1000 ngLL^{-1}, control – no progestin and solvent control – vehicle ethanol (0.01%)) was implemented for 21 days. *G. locusta* was strongly negatively affected by warming, experiencing higher mortality rates (50–80%) than in any other treatments. Instead, growth rates were significantly affected by interactions of LNG with temperature and pCO_2. It was observed, in the short-term (7d) that under ambient temperature (18 °C) and hypercapnic conditions (pH 7.6), the LNG presence promoted the amphipod's growth, while in the medium-term (21d) this response was not observed. Relative consumption rates (RCRs), during the first week were higher than in the third week. Furthermore, in the first week, RCRs were negatively affected by higher temperature while in the third week, RCRs were negatively affected by acidification. Furthermore, it was observed a negative effect of higher temperature and acidification on *G. locusta* fecundity, contrarily to LNG. Concluding, the impact of increased temperature and pCO_2 was clearly more adverse for the species than exposure to the synthetic progestin, however, some interactions between the progestin and the climate factors were observed. Thus, in a future scenario of global change, the presence of LNG (and other progestins alike) may modulate to a certain level the effects of climate drivers (and vice-versa) on the gammarids fitness and reproduction.

1. Introduction

Future ocean conditions will challenge marine organisms with a variety of ecosystem-level stressors which will be exacerbated by global change (Byrne and Przeslawski, 2013; Boyd et al., 2016). Temperature and pCO_2 are among the most relevant environmental stressors that control the distribution and performance of marine species (Portner and Farrell, 2008; Kroeker et al., 2010; Byrne, 2011). As atmospheric CO_2 concentrations increase, ocean pH will tend to decline, almost 0.3–0.4 units by the year 2100, while global sea surface temperature is expected to increase 1.1–6.4 °C for the

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* Corresponding author.
E-mail addresses: pteixeira@ciimar.up.pt, gcardoso@ci.uc.pt (P.G. Cardoso).

[1] These authors contributed equally to this work.

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same period (Meehl et al., 2007). Besides these factors, organisms are exposed to a multitude of abiotic and biotic stressors (e.g. salinity, ultraviolet radiation, nutrients, chemical pollution) which can interfere with temperature and/or pCO_2 making the biological responses even more complex.

In the last years, literature regarding the combined impacts of acidification and warming has increased considerably, which allows us to better understand the complexity of responses associated to different species (Harvey et al., 2013). Besides climate change, there is a growing concern about some chemical compounds, like pharmaceuticals whose consumption has increased in the last years and tend to rise even more in a near future (Kummerer, 2009; Fent, 2015; Kay et al., 2017). Among the most critical pharmaceuticals are steroid hormones that may act as endocrine disruptor chemicals (EDCs). According to the literature, steroids are among the most mighty endocrine disrupters reaching aquatic ecosystems through excretion by humans and livestock and from their use as contraceptives (Orlando and Ellestad, 2014; Overturf et al., 2014; Fent, 2015; Kumar et al., 2015). Progestins or gestagens are a class of synthetic steroids with progestogenic activity, which have been scarcely studied as EDCs. Particularly, levonorgestrel is a synthetic progestin used as a contraceptive as well as post-coital contraception modality ("the morning after pill") that has been detected in the range of ng L^{-1} in effluents of sewage treatment plants, surface and ground waters and also in sediments (de Alda et al., 2002; Vulliet et al., 2007, 2008). Previous studies have already demonstrated its negative effects on reproduction and development of distinct aquatic species (Runnalls et al., 2013; Overturf et al., 2014). However, there is a complete lack of knowledge about the effects of climate stressors on the performance/behaviour of those steroids (and vice-versa) and consequent effects on aquatic ecosystems.

Beyond the effects of chemical and nonchemical stressors in aquatic organisms, environmental changes have the ability to modify the toxicokinetics of chemicals that consequently can have implications at organism and population level responses (Hooper et al., 2013; Di Lorenzo et al., 2015; Noyes and Lema, 2015). Interactive effects between climate change and chemical pollution are extremely complex, since on one hand, environmental variables can change the sensitivity of the organisms to the chemicals and on the other, the pollutants can modify the organisms' ability to respond to climatic conditions (Hooper et al., 2013; Noyes and Lema, 2015). Considering these facts and attending to the few works done on climate change-chemical exposure interactions (e.g. Jacobson et al., 2008; Di Lorenzo et al., 2015; Cardoso et al., 2017) it is vital to evaluate and understand the response of aquatic organisms to all those global change drivers. Crustaceans are ubiquitous in the aquatic ecosystem and are considered reliable bioindicators of contamination and environmental changes (Neuparth et al., 2002, 2014; Costa et al., 2005). Specifically, *Gammarus locusta* is a species with a strong ecological relevance and high sensitivity to contaminants, which associated to the short life cycle and ease to keep and breed in the laboratory, makes it a good model to assess the effects of contaminants along its life cycle (Costa et al., 2005). Therefore, in this study, we aimed to test the conjugation effects of temperature, pCO_2 and levonorgestrel stressors to the amphipod *Gammarus locusta* in order to contribute to the understanding of the vulnerability of this species to environmental changes. Here, we applied an integrative approach, considering multiple key endpoints at individual level, such as survival, growth rate, consumption rate and reproduction.

2. Materials and methods

2.1. Pharmaceutical

The standard levonorgestrel (LNG; CAS 797-63-7; purity = 99%) was purchased from Sigma-Aldrich (Steinheim, Germany). Stock solutions were prepared with analytical ethanol (CAS 64-17-5; purity ≥ 99.9%) supplied by Merck and stored in dark at −20 °C.

2.2. Amphipod collection and acclimation

The amphipod *Gammarus locusta* is a marine epibenthic crustacean that feeds mainly on green macroalgae (e.g. *Ulva* spp). and presents a vast geographical distribution along the European coastal systems, including the Portuguese coast (Costa and Costa, 2000). The individuals used in the experiment 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 by size and sub-adult individuals (with approximately 3–4 weeks) were selected for the experiment. During acclimation period (7 days), animals were fed with *Ulva* spp. 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 (1 cm) and pebbles in order to mimic the natural environment. Photoperiod was set to 18 h light: 6 h dark to simulate summer conditions. The organisms were acclimated under the ambient 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.

2.3. Experimental design

The experimental set-up followed a full factorial design manipulating temperature [ambient temperature and warming (+4 °C)], pCO_2 [normocapnia (pCO_2 = 400 μatm) and hypercapnia (pCO_2 = 1600 μatm; Δ pH 0.5 units)] and the progestin levonorgestrel (LNG: L1 − 10 ngLL^{-1} and L2 − 1000 ngLL^{-1}, control − no progestin and solvent control − vehicle ethanol (0.01%)) in a total of 16 treatments (Table 1). During exposure, the amphipods were maintained at the same salinity as during acclimation (33–35). In addition, the organisms were gradually exposed to the increase of temperature (1 °C/day) until reaching the highest temperature (i.e. 22 °C).

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,

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}).

Treatments	Condition
T1	18 °C, pH 8.1, C
T2	18 °C, pH 8.1, SC
T3	18 °C, pH 8.1, L1
T4	18 °C, pH 8.1, L2
T5	18 °C, pH 7.6, C
T6	18 °C, pH 7.6, SC
T7	18 °C, pH 7.6, L1
T8	18 °C, pH 7.6, L2
T9	22 °C, pH 8.1, C
T10	22 °C, pH 8.1, SC
T11	22 °C, pH 8.1, L1
T12	22 °C, pH 8.1, L2
T13	22 °C, pH 7.6, C
T14	22 °C, pH 7.6, SC
T15	22 °C, pH 7.6, L1
T16	22 °C, pH 7.6, L2

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