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Physiological basis of interactive responses to temperature and salinity in coastal-marine invertebrate: implications for responses to warming

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Running head: Physiological basis of multiple stressor effects

ABSTRACT

Developing physiological-mechanistic models to predict species' responses to climate-driven environmental variables remains a key endeavour in ecology. Such approaches are challenging, because they require linking physiological processes with fitness and contraction or expansion in species' distributions. We explore those links for coastal marine species, occurring in regions of freshwater influence (ROFIs) and exposed to changes temperature and salinity. First, we evaluated the effect of temperature on haemolymph osmolality, and on the expression of genes relevant for osmoregulation in larvae of the shore crab *Carcinus maenas*. We then discuss and develop a hypothetical model linking osmoregulation, fitness, and species expansion/contraction towards or away from ROFIs. In *C. maenas*, high temperature led to a three-fold increase in the capacity to osmoregulate in the first and last larval stages (i.e. those more likely to experience low salinities). This result matched the known pattern of survival for larval stages where the negative effect of low salinity on survival is mitigated at high temperatures (abbreviated as TMLS). Because gene expression levels did not change at low salinity nor at high temperatures, we hypothesise that the increase in osmoregulatory capacity at high temperature should involve post translational processes. Further analysis of data suggested that TMLS occurs in *C. maenas* larvae due to the combination of increased osmoregulation (a physiological mechanism) and a reduced developmental period (a phenological mechanisms) when exposed to high temperatures. Based on information from the literature, we propose a model for *C. maenas* and other coastal species showing the contribution of osmoregulation and phenological mechanisms towards changes in range distribution under coastal warming. In species where the osmoregulatory capacity increases with temperature (e.g. *C. maenas* larvae), osmoregulation should contribute towards expansion if temperature increases; by contrast in those species where osmoregulation is weaker at high temperature, the contribution should be towards range contraction.

Keywords: *Carcinus maenas*, climate change, coastal zone, larva, mRNA expression, multiple stressors, osmoregulation, salinity, temperature.

INTRODUCTION

Climate change is driving multiple modifications in environmental conditions (e.g. temperature, humidity) of both terrestrial and aquatic organisms (Gattuso & Hanssen 2009, Helmuth et al. 2010, Gunderson et al. 2016, Boyd et al. 2018). Environmental change is multivariate and therefore organisms are exposed to the simultaneous action of several environmental drivers (Sokolova et al. 2012, Torres et al. 2019, 2020; previously referred as stressors: Folt et al. 1999, Crain et al. 2008, Piggott et al. 2015). Thus, environmental drivers can operate on biological systems (e.g. organisms) in an interactive mode and responses (e.g. survival) cannot be accurately predicted by evaluating the action of each driver in isolation (Crain et al. 2008, Kroeker et al. 2013, Piggott et al. 2015, Gunderson et al. 2016, Côté et al. 2016, Boyd et al. 2018). Interactive responses are classified in two general categories, synergistic or antagonistic. Relevant examples to climate change are for instance, synergistic effects of increased temperature combined with food limitation or elevated CO_2 (i.e. the combined effect of both stressors is larger than the sum of each separate effect: Giebelhausen & Lampert 2001, Schiffer et al. 2014, Przeslawski et al. 2015, Torres & Giménez 2020). By contrast, increased temperature and reduced salinity can operate antagonistically: i.e., warming appears to mitigate the negative effects of low salinity on the performance and fitness in some coastal marine organisms (Lange & Marshall 2017, Spitzner et al. 2019, Torres et al. 2020).

Currently there is a gap in our knowledge about the mechanisms that underlie organismal responses to multiple environmental drivers. The capacity to better understand and predict effects of climate change through multiple drivers requires knowledge of the mechanisms

79 driving such effects (Galic et al. 2018, De Laender 2018, Thompson et al. 2018, Orr et al.
80 2020). In particular, responses occurring at the individual, population or species levels are
81 based on physiological mechanisms driving tolerance, in addition to biotic interactions (Pörtner
82 2010, Somero 2010, 2011, Sokolova et al. 2012, Baert et al. 2016, Ames et al. 2020). Hence,
83 understanding physiological responses to multiple environmental drivers is the starting point
84 towards better models of biotic responses to climate change.

85 Coastal organisms, occurring in estuaries or regions of freshwater influence (ROFIs:
86 Simpson 1997), are frequently exposed to low salinity, which usually reduces organismal
87 performance (Kinne 1971, Anger 2003). ROFIs are coastal areas influenced by freshwater
88 runoff (associated with estuaries) and are widespread along the world coastal zones (e.g. North
89 European Seas, East coast of the Americas and SE Asia, West coast of Africa). ROFIs are
90 nursery areas for many economically important species. Predictions for changes in salinity at
91 ROFIs varies regionally (IPCC 2013) and seasonally (Robins et al. 2016) but all share the same
92 fate in one regard: temperature is expected to increase over the century. Thus, at the ROFIs,
93 warming will create a new environment within which organisms face additional stressors such
94 as reduced salinity. Hence, a key question is how such organisms will respond to reductions of
95 salinity in the context of increased temperatures. At present, there is no testable mechanistic
96 model predicting species' responses under such scenarios. More in general, reviews on the
97 combined effect of multiple drivers on performance and fitness bring contradictory results
98 about whether responses to temperature and salinity are predominantly antagonistic (Crain et
99 al. 2008) or synergistic (Przeslawski et al. 2015).

100 The aim of this paper is twofold: first, we explore the mechanistic basis of an antagonistic
101 effect of temperature and salinity on a model species. Second, we use our results and
102 information from the literature to propose a testable hypothetical model linking
103 osmoregulation, interactive effects of temperature and salinity on organismal performance, and

104 future changes in species distributions across coastal estuarine gradients in response to
105 warming. We first focus on the antagonistic effect called thermal mitigation of low salinity
106 stress (TMLS: Spitzner et al. 2019) which is common in coastal estuarine organisms (Kinne
107 1971, Anger 1991, Janas & Spicer 2008, González-Ortegón & Giménez 2014). TMLS is
108 defined as the mitigation of the negative effects of exposure to low salinity on performance or
109 fitness (e.g. survival) by increased temperatures. The critical point is that in species exhibiting
110 TMLS, moderate warming may mitigate the negative effect of low salinity on survival. We
111 studied the mechanisms driving TMLS in the larval stages of the shore crab *Carcinus maenas*.
112 The shore crab *C. maenas* is native to northern Europe and NW-Africa but is a successful
113 invader worldwide (Roman & Palumbi 2004, deRivera et al. 2007, Compton et al. 2010,
114 Leignel et al. 2014, Young & Elliot 2020). *Carcinus maenas* is also representative of a large
115 number of marine organisms that develop through a pelagic larval phase (Anger 2006, Spitzner
116 et al. 2018).

117 We focus on larval stages of *C. maenas* because we know that TMLS occurs in our study
118 population (Spitzner et al. 2019), and the target stages are osmoregulators (Cieluch et al. 2004);
119 yet, the effect of temperature on osmoregulation has never been studied in *C. maenas*. We are
120 interested in larvae because they are more sensitive to environmental variation compared to the
121 juvenile-adult stages (Pandori & Sorte 2019), and variation in survival controls recruitment
122 (Palumbi 2003, Giménez 2004, Cowen & Spounagle 2009). For *C. maenas*, the first and last
123 larval stages (zoea I and megalopa), as well as juveniles and adults, hyper-osmoregulate at low
124 salinity (Siebers et al. 1972, Cieluch et al. 2004); all these stages occur in coastal or estuarine
125 waters. In contrast, advanced zoeal stages (zoea II-IV) occur in open waters and do not hyper-
126 regulate at low salinities. Spitzner et al. (2019) found that increased temperatures (21-24°C)
127 experienced during the early larval stages can mitigate the effect of low salinity (20‰) on
128 larval survival and developmental time, as compared to larvae reared at temperatures

129 experienced by the local population (<20°C; North Sea, German Bight, Wiltshire et al. 2010).
130 Within a population, the strength of TMLS can vary due to parental influences, but evidence
131 for TMLS is found in populations of the Irish Sea (Nagaraj 1993, Torres et al. 2020) and in the
132 Pacific coast of N. America where it is an invader (Hines et al. 2004).

133 Several mechanisms may drive TMLS in *C. maenas* larvae (and other organisms). For
134 instance, more individuals may reach a given larval stage (or survive to maturity in other
135 species) because high temperatures reduce the time of exposure to a stressor; we define this as
136 "phenological mechanism" as it is based on a change in the timing of events in the life cycle
137 (Post 2020). In addition, there should be adaptive physiological mechanisms explaining the
138 response. Based on previous work on other species (Flügel 1963; Charmantier-Daures et al.
139 1988, Campbell & Jones 1989, Janas and Spicer 2008), we hypothesise that increased
140 temperatures cause an enhancement in the capacity of individuals for extracellular
141 osmoregulation. Extracellular osmoregulation (here referred to as "osmoregulation") is defined
142 as the active regulation of the concentration of osmotically active substances in the
143 haemolymph or blood (Péqueux 1995, Charmantier 1998, Lucu & Towle 2003, Henry et al.
144 2012, McNamara & Faria 2012, Lignot & Charmantier 2015, Rahi et al. 2018). Osmoregulation
145 is an adaptive mechanism keeping organisms at optimal functioning, maintaining for instance
146 the acid-base balance (Whiteley 2011, Whiteley & Taylor 2015), sustaining growth (Torres et
147 al. 2011), and perhaps contributing to tolerance to ocean acidification (Whiteley et al. 2018).
148 Osmoregulation is achieved through the active uptake of ions from the surrounding "diluted"
149 water by transport cells (ionocytes), located in the epithelium of specialised organs (gills in
150 adults; branchiostegites in larval stages of crustaceans: Cieluch et al. 2004, 2007). Ion uptake
151 occurs through the concerted action of several proteins including the enzyme Na^+-K^+-ATPase
152 (Lucu & Towle 2003, Thuet et al. 1988, Mackie et al. 2005, Cieluch et al. 2007, Torres et al.
153 2007, Ituarte et al. 2016), and ion co-transporters (e.g. Na^+-K^+-$_2Cl^-$ symporter). The action of

154 the above-mentioned proteins may involve several processes including the upregulation of gene
155 expression of those enzymes and co-transporters (Luquet et al. 2005, Serrano & Henry 2008,
156 Xu & Liu 2011, Ituarte et al. 2016, Faleiros et al. 2017).

157 We report on three experiments designed to determine the mechanisms driving the
158 mitigation effect produced by elevated temperature in larvae of *C. maenas* exposed to
159 moderately low salinities (TMLS). In the first and second experiments, we studied the effect
160 of temperature and salinity on the osmoregulatory capacity of zoeae I and megalopae,
161 respectively. In the third experiment, we evaluated the combined effects of salinity and
162 temperature on the levels of mRNA expression of the genes coding for the Na^+-K^+-ATPase and
163 the Na^+-K^+-$_2Cl^-$ symporter in zoeae I. Then, we discuss and integrate our results into a wider
164 framework and formulate a testable model showing the contribution of osmoregulation to range
165 expansion and contraction of coastal-estuarine species towards or away from ROFIs.

166 ## METHODS

167 ### Experimental design and procedures

168 Experiments were carried out with larvae hatched from berried females and megalopae
169 collected from the field at the island of Helgoland (North Sea, German Bight) during the
170 reproductive period in spring-summer (Experiments 1 and 2: osmoregulatory capacity;
171 Experiment 3: gene expression patterns). During embryogenesis, berried females were kept in
172 separated 2L aquaria in oxygenated and filtered (0.2µm) natural seawater (32.5‰) in a
173 temperature-controlled room at 18°C with a 12:12 h light:dark cycle. All experiments were run
174 following standard methods of larval rearing (Torres et al. 2011, Spitzner et al. 2019, Torres &
175 Giménez 2020) using filtered natural seawater, constantly aerated. Larvae were reared in
176 temperature-controlled rooms (at 15, 18, 21, 24°C) and at two acclimation salinities (see Table
177 S1 for experimental design). Salinity (expressed as salt content in "‰") was manipulated by

178 diluting natural seawater (32.5‰) with appropriate amounts of tap water and adjusting values
179 using a salinometer (see Table S2 for details on acclimation and test salinities and the
180 corresponding values of osmotic pressure expressed in mOsm kg^{-1}). Water was changed daily:
181 experimental glass bowls were rinsed and cleaned, larvae were fed freshly hatched *Artemia* sp.
182 nauplii, and dead individuals were removed from the cultures.

183 **Experiment 1: Osmoregulation in zoeae I**

184 We evaluated how temperature modified the osmoregulatory capacity (OC) in larvae reared
185 under different acclimation salinities and subsequently exposed to different tests salinities. OC
186 is defined as the difference between the osmolalities of haemolymph and of the external
187 medium at a given salinity under different conditions (Lignot et al. 2000). We therefore used a
188 factorial design (see Table S1 for details) considering (1) Acclimation salinities, i.e. those
189 experienced from hatching until initiation of the 24h exposure to the test salinities, (2) Test
190 salinities, i.e. those experienced during 24h previous to the sampling of haemolymph, (3)
191 Temperature experienced from hatching until time of sampling of haemolymph. The
192 acclimation salinities and temperatures were chosen based on a previous study showing TMLS
193 (Spitzner et al. 2019). The test salinities were those where larvae are known to significantly
194 hyper-regulate while survival rates are high (>60%: Cieluch et al. 2004); larvae reared in
195 seawater are near the osmotic equilibrium and thus OC ≈ 0 mOsm kg^{-1}.

196 To avoid effects associated with developmental processes related to the moult cycle,
197 osmoregulatory capacity was quantified after exposure to the test salinities in zoeae I at
198 intermoult (i.e. more than 50% of the moult cycle: period between hatching of zoea I and moult
199 to zoea II occurred at the acclimation salinities). Freshly hatched larvae were first assigned to
200 replicate groups of different acclimation salinities and temperatures (see Table S1), group-
201 reared in 500 ml glass bowls until the time of exposure to the test salinities at a density: 0.1

202 individual * ml^{-1} (see Experiment 3 for rearing details). Because acclimation salinity and
203 temperature affect developmental time (Table S3), we took special care to ensure that
204 organisms were sampled at intermoult at the appropriate time (see Table S3). Zoeae I from
205 each combination were assigned randomly to the two test salinities by placing individuals in
206 petri dishes for 24h. Previous experiments had shown that an exposure time of less than 24h is
207 sufficient for haemolymph osmolality to become stable in *C. maenas* larvae (Cieluch et al.
208 2004) and in other tested species (Charmantier 1998). The osmotic pressure of the test salinities
209 was expressed as osmolality (3.4‰ ≈ 100 mOsm kg^{-1}, 29.4 mOsm kg^{-1} ≈ 1‰, thus natural
210 seawater at 966±1 mOsm kg^{-1} = 32.5‰) which was determined with a micro-osmometer
211 (Model 3MO, Advanced Instruments, Needham Heights, MA, USA) using 20µl of each salinity
212 (Table S2).

213 Larvae used to quantify haemolymph osmolality were quickly rinsed in deionised water,
214 gently dried on a filter paper and submersed in mineral oil to avoid evaporation and desiccation;
215 the remaining water was then aspired using a micropipette. A second micropipette was inserted
216 into the heart in order to extract the haemolymph (sample volume ~ 30 nl). Haemolymph
217 osmolality was then determined with reference to the medium osmolality (i.e. test salinities)
218 using nanoosmometry (Kalber-Clifton nanoliter osmometer; Clifton Technical Physics,
219 Hartford, NY, USA), following Charmantier et al. (1998, 2002), and Cieluch et al. (2004). The
220 results were expressed as osmoregulatory capacity (OC), i.e. the difference between the
221 osmolality of the haemolymph and the medium (i.e. test salinities).

222 **Experiment 2: Osmoregulation in megalopae**

223 Osmoregulation in megalopae was quantified in larvae collected in the intertidal of
224 Helgoland in summer. We opted for field collections because the large number of larvae needed
225 for the experiments (80 megalopae = 4 temperatures x 2 acclimation salinities x 1 test salinity

x 10 megalopae) was difficult to obtain from cultures. Megalopae were taken from floating and benthic collectors deployed and collected daily, using standard techniques (Giménez et al. 2020). Collected megalopae were immediately transferred to the laboratory, assigned randomly to the different acclimation temperature and salinity treatments as in Experiment 1 (Table S1, Experiment 2), and reared following the same protocol as the zoeae I (see Experiment 1 for details) for 3 days, before exposure to the test salinities. At the appropriate time, megalopae (see Table S4 for n) were exposed to the test salinity for 24h and then used for quantification of osmoregulatory capacity following the same techniques as those used for zoeae I (see Experiment 1). Due to low availability of megalopae, we only used one test salinity (20‰).

Experiment 3: Expression of genes related to osmotic stress in zoeae I.

To assess the variation in the gene expression patterns caused by salinity changes in a warming environment, we selected two target genes encoding for the ion-transport enzyme Na^+-K^+-ATPase and the Na^+-K^+-$2Cl^-$ symporter (Towle & Weihrauch 2001), and used the elongation factor 1 and ubiquitin conjugating enzyme E2 L3 as reference genes (Oliphant et al. 2018). The gene expression was evaluated in zoeae I from four separate females collected from the intertidal of Helgoland. Each replicate for the determination of the expression of mRNA consisted of 125 pooled zoeae I after exposure to the acclimation salinities until intermoult as in Experiment 1 (see Table S3 for details). In order to obtain the appropriate number of larvae for ca. 10 replicates (n=10, as in the osmoregulation capacity measurements), the rearing of more than 10,000 larvae was required (n=10 implies rearing more than 10 x 125 = 1,250 individuals per treatment; 1,250 x 8 = 10,000 larvae). Since we were not able to rear such a large number of larvae simultaneously, we used larvae harvested from four different females and took 3 replicates per female per treatment (all females together: n=12 per treatment, 12,000 larvae were sampled). We are aware of the variation among females in the responses to

250 temperature, salinity and other stressors (Spitzner et al. 2019, Torres et al. 2020); therefore, we
251 maintained the factor "Female" as a random factor in our statistical analyses and present the
252 averaged values of all females as well as the data discriminated by female (see Results).

253 Upon hatching, larvae of each female were assigned at random to culture bowls for mass
254 rearing. The rearing conditions were chosen in the range of temperatures and salinities where
255 TMLS has been found (Spitzner et al. 2019), as for Experiment 1 (acclimation salinities: 25.0
256 & 32.5‰; temperatures: 15, 18, 21 & 24°C; see Tables S1 & S3 for details). Zoeae I were then
257 sampled from the mass cultures at intermoult (i.e. >50% of the moult cycle occurred under the
258 acclimation conditions) to avoid effects of the moult cycle (as for Experiment 1, see Table S3).

259 Three replicate samples (125 larvae each: see Tables S1 & S3 for details) were taken to
260 determine the mRNA level of genes related to ion-transport: Na^+-K^+-ATPase (*CamaNaK*) and
261 Na^+-K^+-$_2Cl^-$ symporter (*CamaCOT*) using elongation factor 1 (*CamaEL*) and ubiquitin
262 conjugating enzyme E2 L3 (*CamaUB*) as reference genes (following Oliphant et al. 2018). In
263 a PCR-clean environment, larvae were quickly rinsed in distilled water, gently blotted dry with
264 filter paper, placed in 1ml of RNAlater® to stabilize the RNA and immediately frozen at -80ºC
265 for later analysis. In addition, three replicates (200 larvae each) of freshly hatched zoea from
266 each female were collected to use as control samples.

267 Samples were quickly rinsed in DEPC-water to eliminate the RNAlater® and placed in PCR-
268 clean microcentrifuge tubes (kept at 4°C) containing TRIzol® (Invitrogen, Carslbad, CA). Total
269 RNA was extracted from each replicate of pooled larvae by homogenizing the tissue directly
270 in 500µl TRIzol® in a Qiagen TissueLyser LT (50 oscillations min^{-1}). Extractions were carried
271 out according to the manufacturer's instructions, except that the washing steps in 75% ethanol
272 were repeated twice. Extracted-RNA was resuspended in 20µl *RNAse*-free water before
273 removal of contaminating gDNA with Turbo DNA-free™ *DNAse* (Ambion, Austin, Texas)
274 following manufacturer's instructions. Total RNA concentration (714±SE 18 ng/ml) was

determined spectrophotometrically with a NanoDrop ND2000™ (Thermo Scientific, UK). Three µl of RNA from each extraction were reverse-transcribed using High Capacity™ cDNA synthesis kit reagents (Applied Biosciences, Carslbad, CA.) and random primers for 10 min at 25°C followed by 120 minutes at 37°C; the reaction was terminated by heating to 85°C for 5 minutes. The obtained cDNA was stored at -20°C until the quantitative-PCR was performed.

Standard curves or the quantitative-PCRs were developed for all genes (target: *CamaNaK* & *CamaCOT*, internal reference: *CamaUB* & *CamaEL*; sequences for all primers and probes in Table S5). Templates for standard curves were generated by reverse-transcription of RNA extracted from transport tissues (gills) from adult *Carcinus maenas* and appropriate dilution of the resulting cDNA. Two sets of multiplex Taqman PCRs were done in 96-well plates using the Bioline SensiFAST™ Probe Lo-ROX kit following manufacturer's instructions. The qPCR mix (8µl) consisted of 5µl SensiFAST Probe Lo-ROX mix, 0.4µl of each primer (400nM), 0.1µl probe (100nm), 1.2µl DEPC-water and 2µl cDNA (either standards for the calibration curves or unknowns). Selection of different reporter-fluors allowed reactions to run in multiplex with the following combinations (NaK-F & NaK-R and COT-F & COT-R; UB-F & UB-R and EL-F & EL-R; see Table S5 for details). Quantitative-PCRs (in duplicate plates as technical replicates) were run on an Applied Biosystems StepOne Plus™ machine using the following cycling parameters: 95°C for 2 minutes followed by 40 cycles of 5s at 95°C and 30 secs at 60°C. PCR amplification factor and efficiency were determined using the qPCR Efficiency Calculator Thermofischer Scientific (See Table S6 for details) with slopes calculated (GraphPad software) from the calibration curves performed with cDNA from gill tissues; standards were accepted only when amplification efficiency was >90%. Relative expression of target genes (*CamaNaK* and *CamaCOT*) was determined against the values from freshly hatched larvae as control using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001, Pfaffl 2006) modified to consider different amplification efficiencies and multiple reference genes

300 (Vandesompele et. al 2002, Hellemans et al. 2007). All data were normalized to the geometric
301 mean of the reference genes elongation factor 1-alpha (*CamaEL*) and ubiquitin conjugating
302 enzyme E2 L3 (*CamaUB*).

303 **Statistical analyses**

304 The OC data (Experiments 1 and 2) were analysed through 3-way factorial ANOVA
305 (Underwood 1997) with temperature, acclimation salinity, and test salinity as factors.
306 Preliminary tests showed that residuals approached normal distribution and variances were
307 homogeneous (Cochran-test). For the mRNA expression data (Experiment 3), we used mixed
308 modelling (Zuur et al. 2009) in order to control for the random variation associated with female
309 of origin. In that case, analysis was based on generalised linear squares modelling using the
310 *lme* function of the package *nlme* (Pinheiro et al. 2018), implemented in R (R Core Team 2013).
311 Thus, for the mRNA expression data, female of origin was considered a random factor in
312 addition to the acclimation temperature and salinity used as fixed factors in a factorial design.
313 Hypotheses for the mRNA expression data were evaluated through backward model selection;
314 selection of the best random model was carried out through restricted maximum likelihood
315 fitting while selection of the fixed terms was carried out through maximum likelihood. The
316 best model was chosen using the adjusted Akaike information criterion (AICc). For each
317 variable, the model showing the smallest AICc score was selected unless the lowest scored
318 model was more complex that the next scored model and the corresponding difference between
319 those models was ΔAICc <3; in that case we tested the models using likelihood ratio tests. We
320 also report p-values associated with those models (see Table S7, Supplementary material for
321 details).

RESULTS

Osmoregulation in zoeae I

All larvae, irrespective of the acclimation salinity or temperature, survived the exposure to the test salinities (=100% survival). The osmoregulatory capacity of zoea I larvae responded to the interactive effect of temperature and acclimation salinity (Fig. 1, S1), and it was not significantly affected by the test (medium) salinity (15 or 20 ‰). There was a strong effect of temperature: the osmoregulatory capacity at the highest temperatures (21 and 24°C) was about two to three times higher than that shown by larvae kept at 15°C. Acclimation to low salinities enhanced the positive effect of high temperature on the osmoregulatory capacity. In larvae acclimated to seawater, the osmoregulatory capacity was significantly higher for those individuals reared in 18°C as compared to 15°C, but it reached a plateau in the range 18-24°C at ca. 40-45 mOsm kg^{-1}. By contrast, in larvae acclimated to 25‰, the osmoregulatory capacity increased with temperature up to a maximum of 57 mOsm kg^{-1} at 24°C.

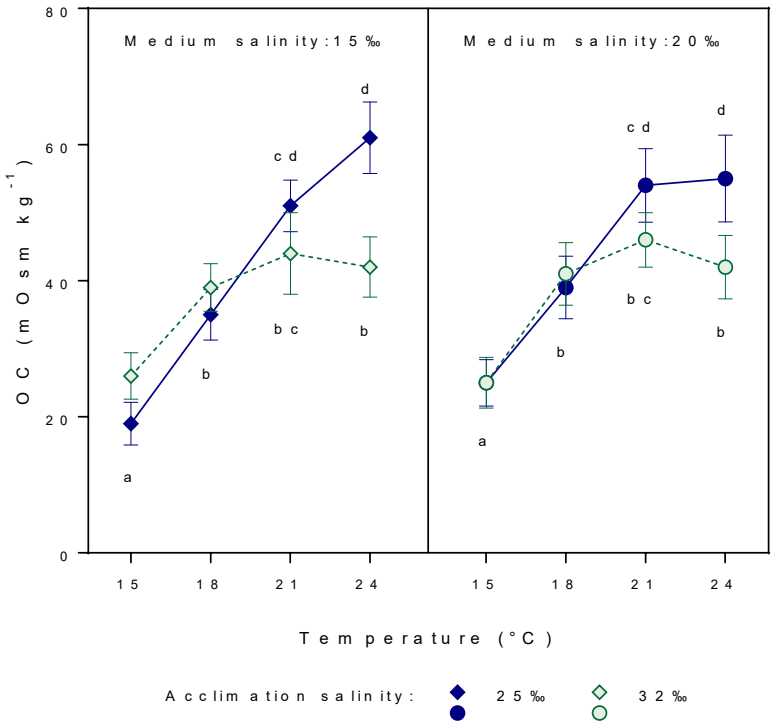

Figure 1. *Carcinus maenas*. Effect of temperature on the osmoregulatory capacity (OC) of zoeae I acclimated to 25.0‰ (blue symbols) and seawater (32.5‰, green symbols) during Experiment 1. OC was determined after exposure to the medium (test) salinities: 15.0 (diamonds in left panel) and 20.0‰ (circles in right panel). Values are shown as mean ± standard error (n=10). Different letters show significant differences among treatments.

Osmoregulation in megalopae

The osmoregulatory capacity (OC) of the megalopae increased with temperature, but was not significantly affected by the acclimation salinity (Fig. 2). OC increased almost linearly with temperature, from ~ 60 mOsm kg^{-1} at 15°C to 85 mOsm kg^{-1} at 24°C; i.e. temperature resulted in an increase of 40% in OC. By contrast, differences associated to acclimation salinity were not consistent across temperatures and resulted in a maximum of 12.5% increase from seawater to 25.0‰, occurring at 24°C.

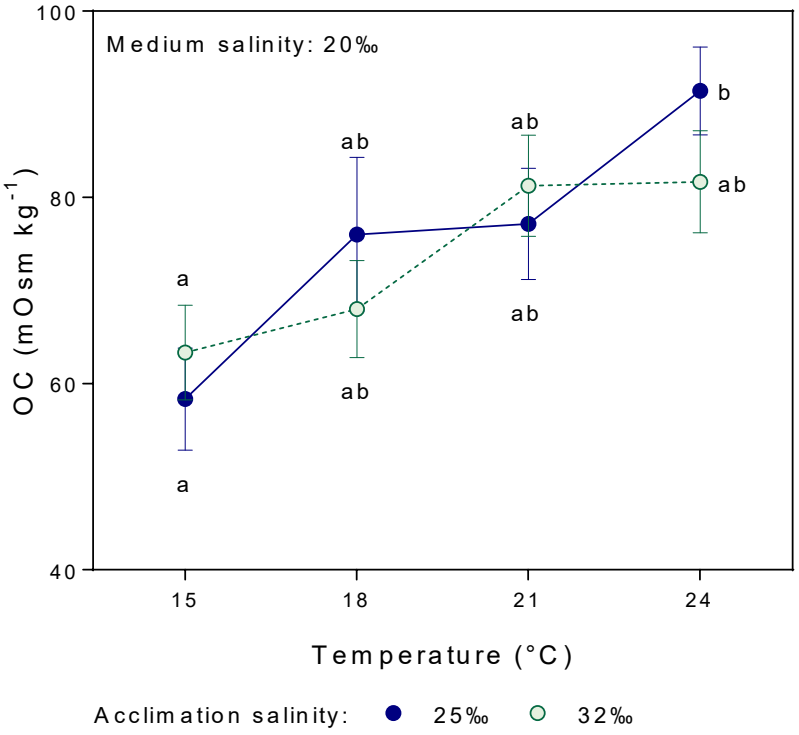

Figure 2. *Carcinus maenas*. Effect of temperature on osmoregulatory capacity of megalopae acclimated to 25.0‰ (blue circles) and seawater (32.5‰, green circles) during Experiment 2. OC was determined after exposure to the medium (test) salinity: 20.0‰. Values are shown as mean ± standard error (n=5-8, see Table S4 for details). Different letters show significant differences among treatments.

Gene expression patterns in zoeae I

For the enzyme Na^+-K^+-ATPase (NaK), low acclimation salinity reduced the average gene expression by about 13% (Fig. 3). Best models retained acclimation salinity (but not temperature) as the main driver of gene expression. There was also important variation in the response associated with the female of origin (Fig. S2, left panel; Table S7). Plots of average gene expression by female did not show a consistent effect of temperatures although the prevailing pattern was a decreased gene expression at low salinity (Fig. 3, left panel).

On average, gene expression of the Na^+-K^+-$_2Cl^-$ symporter (COT) peaked at 18°C and showed a decrease towards lower or higher temperatures with a minimum at 24°C (Fig. 3, right panel) where it achieved the largest decrease (ca. 26%). The best model retained temperature as the main driver of gene expression and also showed important variation in the response by female: plots by female of origin showed that gene expression peaked at 18°C (fem 1, 3, 4) or 21°C (fem 2) but decreased expression was found consistently at 15 and 24°C (Fig. S2, right panel).

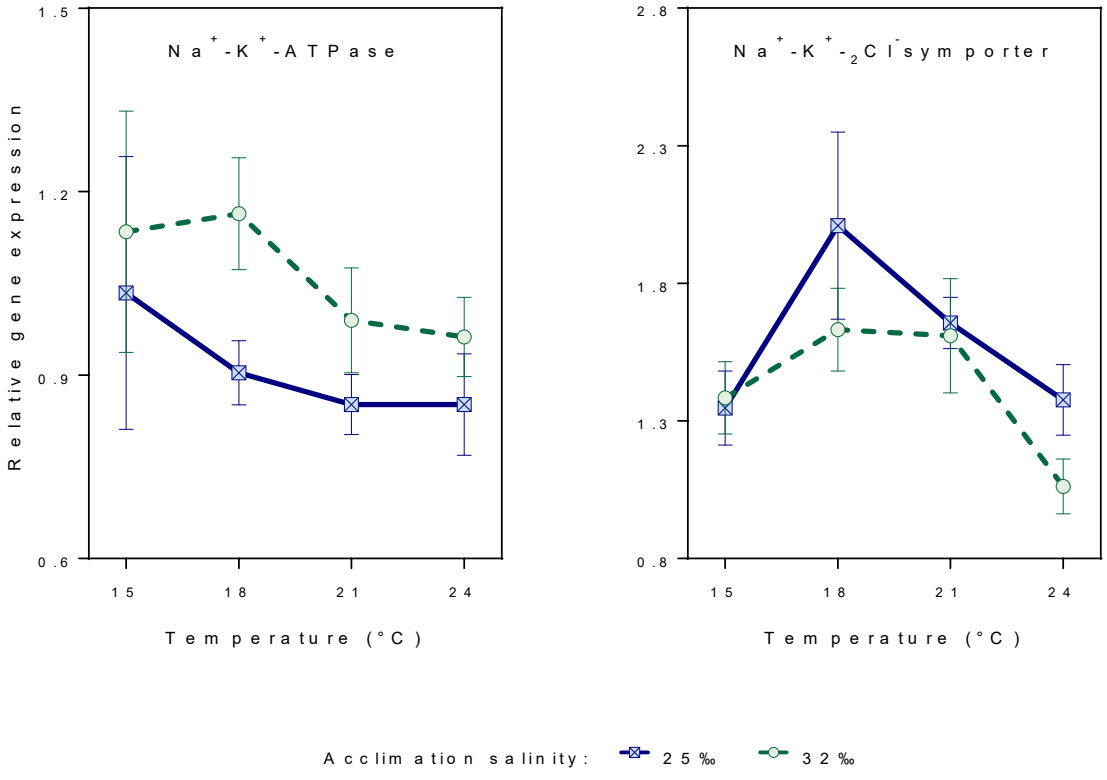

Figure 3. *Carcinus maenas*. Effects of acclimation salinity and temperature (Experiment 3) on relative expression of mRNA of Na^+-K^+-ATP (left panel) and Na^+-K^+-$_2Cl^-$ symporter (right panel) in zoeae I. Data are shown as average values ± SE (n=4 for all four females); acclimation to 25.0‰ is shown in blue and to natural seawater (32.5‰) in green. Data discriminated by female are shown in the Supplementary material (Fig. S2).

DISCUSSION

We found that increased temperatures enhanced the capacity to osmoregulate in the zoea I and megalopa (Experiments 1 & 2). We also found (in zoeae I) subtle changes in expression of Na^+-K^+-ATP and Na^+-K^+-$_2Cl^-$ symporter mRNA (NaK and COT, respectively) related to the acclimation salinity and temperature (Experiment 3). Because the results of the mRNA expression study were inconclusive, we still do not know which mechanisms operate at the cellular or molecular levels. However, the haemolymph osmolality appears to provide an integrative measure of the capacity to tolerate the combined effects of temperature and salinity. Information on osmoregulatory responses is important for the development of a predictive model for responses to warming and reduced salinity. However, such models require the establishment of links between physiology, performance, and species distribution (Ames et al. 2020). We divide the following discussion in four sections covering such links, for both the case of *C. maenas* and coastal estuarine species in general. We present a model showing the contribution of osmoregulation to fitness and species' distribution in response to climate-driven changes in temperature and salinity.

Thermally driven osmoregulation

The positive relationship between osmoregulatory capacity and increased temperature in both zoea I and megalopa of *C. maenas* matched previous observations in adult stages of coastal crustaceans (e.g. Williams 1960, Charmantier 1975, Hagerman & Uglow 1983, Campbell & Jones 1989, Janas & Spicer 2008). The determinations of the OC (Experiments 1&2) showed an important and rapid response to salinity after exposure of larvae of *C. maenas* to the test salinities (15 and 20‰) for 24h. In addition, measurements of OC in zoeae I showed that previous acclimation to low salinity (25‰) contributed to further increase the osmoregulatory capacity. Hence, it appears that osmoregulation results from responses to salinity at different

426 time scales. We can ascribe the increase in osmoregulation to physiological plasticity because
427 survival was >80% during the acclimation period (data not shown, see also Spitzner et al. 2019)
428 and 100% during exposure to the test salinities (i.e. there was little opportunity for phenotypic
429 selection). The two-threefold increase (ca. 20 mOsm*kg^{-1} at 15 °C to ca. 50-60 mOsm*kg^{-1} at
430 24 °C) in the osmoregulatory capacity found in *C. maenas* driven by temperature demonstrates
431 a considerable amount of physiological plasticity. The increase is also important in the light of
432 the existing information on interspecific variation in osmoregulatory capacity exhibited by
433 crustacean larvae. Available data for decapod crustacean larvae show that the osmoregulatory
434 capacity at comparable salinities (17‰) ranges between ~20 mOsm kg^{-1}, for coastal marine
435 species (including *C. maenas*) to 140-200 mOsm kg^{-1} found in larval (zoea I and megalopa)
436 stages of the Jamaican crab *Armases miersii*, developing in supratidal pools characterised by
437 strong variations in salinity (Charmantier et al. 1998). The values of 60-90 mOsm kg^{-1} found
438 in this study at 24°C (for zoeae I and megalopae, respectively), shifts *C. maenas* towards the
439 mid-range, characteristic of larvae of estuarine crabs which are released in (or return to)
440 brackish waters (Charmantier et al. 2002, Cieluch et al. 2007). For example, the
441 osmoregulatory capacity recorded for the first and last larval stages (zoea I and megalopa) of
442 the estuarine crab *Neohelice granulata* is 130-150 mOsm kg^{-1} at 17‰ (Charmantier et al.
443 2002). Thus, within the three patterns of ontogeny of osmoregulation described in crustaceans
444 (Charmantier 1998), variations in haemolymph osmolality values seem possible due to
445 physiological plasticity; testing this hypothesis in a strongly hyperosmoregulating species such
446 as *Armases miersii* would be worthwhile.

447 We evaluated, for zoeae I, if increments in relative mRNA expression for NaK and COT
448 would contribute to the mechanism underpinning the thermally driven osmoregulatory
449 plasticity (Experiment 3). As this is the first report on mRNA expression of NaK and COT in
450 larvae of *C. maenas*, we hypothesised that the response would be similar to that found in adult

451 gills (*C. maenas*: Jillette et al. 2011, Whiteley et al. 2018) and in other species (Serrano &
452 Henry 2008, Havird et al. 2013). We did not find evidence of such up-regulation but instead
453 mRNA levels were modestly down-regulated at low salinity, for both NaK and COT. In
454 addition, COT was slightly down-regulated at the highest tested temperature. Down-regulation
455 occurred at the same salinity (25‰) where we found significant increase in osmoregulatory
456 capacity (in Experiment 1: acclimation to 25‰ significantly increased the osmoregulatory
457 capacity at high temperatures as compared to acclimation in seawater) and where Whiteley et
458 al. (2018) found up-regulation in adult *C. maenas*. We propose four explanations for these
459 changes, although we think that only the last two explanations are likely to be correct. First,
460 the increase in levels of mRNA expression that occur in osmoregulatory tissues may be masked
461 when quantified using whole body samples. However, Lind et al. (2013) reported an increase
462 in expression of variants of Na^+-K^+-ATPase in cyprids from the euryhaline barnacle
463 *Amphibalanus improvisus* using samples of intact cyprids. Second, the time scale of the
464 transcriptional response is much shorter than the time scale of exposure to low salinity in our
465 experiment (3-5 days). If the transcriptional response in larvae is short, it is not consistent with
466 that of adults, where up-regulation was still observed after >3 months of exposure to low
467 salinity (Whiteley et al. 2018); in addition, it is not consistent with studies showing that the
468 transcriptional response can occur on a longer time scale (Faleiros et al. 2017, 2018). Third,
469 the signal may be too weak because zoeae I of *C. maenas* are poor osmoregulators when
470 compared to adults; at the tested low acclimation salinity (25‰), the osmoregulatory capacity
471 of juvenile/adult crabs is >10 times higher than that of larvae (Cieluch et al. 2004: 749 mOsm
472 kg^{-1} ~ 25‰ salinity). In addition, the tolerance to low salinity of *C. maenas* larvae is lower than
473 that of *A. improvisus* (down to 5‰: Nasrolahi et al. 2012); *A. improvisus* may be a strong
474 osmoregulator. Fourth, post-translational adjustments (enzyme kinetic behaviour: Corotto &
475 Holliday 1996) can occur more rapidly than transcriptional responses (Faleiros et al. 2017,

476 2018) and may explain the differences between osmoregulatory capacity and transcriptional
477 responses. Additional adjustments may also occur through neurohormonal control (Lucu &
478 Towle 2003) and translocation of enzymes from intracellular vesicular stores to the membranes
479 (McNamara & Torres 1999).

480 **Temperature, osmoregulatory capacity, and tolerance to low salinity**

481 The positive effect of temperature on osmoregulatory capacity in zoeae I and megalopae of
482 *C. maenas* (Figs. 1, 2) was consistent with the thermal mitigation of low salinity effect found
483 previously (Spitzner et al. 2019): larvae reared at the temperature range 21-24°C were more
484 tolerant to low salinities than those reared at 15°C. There are two potential groups of
485 mechanisms driving such responses. First, there is the effect of increased temperature that
486 shortens the developmental time ("phenological mechanism"): if instantaneous mortality rates
487 were constant, larval survival to a given stage (e.g. moulting to zoea II) should increase with
488 temperature simply because such larvae took less time to moult. Second, there is the effect
489 generated by adaptive responses to low salinity such as osmoregulation; such responses should
490 reduce the instantaneous mortality rate (here called "physiological mechanism"). Those
491 mechanisms are not mutually exclusive and they are likely operating simultaneously.

492 In order to check for evidence of a "physiological mechanism" we used data reported by
493 Spitzner et al. (2019, their Fig. 3) to calculate the instantaneous mortality rates from hatching
494 to moulting to zoea II. These data were produced with larvae from the same local population
495 as this study; it thus can be compared to the osmoregulatory patterns observed in Fig. 1. Such
496 calculations (Table S8) show that when TMLS occurred, the instantaneous mortality rates were
497 two times lower in larvae reared at high temperature than those reared in low temperature.
498 Hence, the "phenological mechanism" alone cannot explain TMLS and instead a
499 "physiological mechanism" should also play a role. We argue in favour of osmoregulation as

500 a potential “physiological mechanism”, because the capacity to osmoregulate provides
501 organisms a steady state of body fluids, which contributes to the maintenance of performance
502 and fitness (Péqueux 1995, Charmantier 1998, Charmantier et al. 2009, Whiteley 2011).

503 An important question is whether there is evidence between changes in osmoregulation and
504 TMLS. Our experiments do not provide a direct link because we had to measure
505 osmoregulation (and hence kill the larvae) before the TMLS arises. Instead, we have indirect
506 evidence in that TMLS occurs in the same population where we found the positive effect of
507 temperature on osmoregulatory capacity. Additional evidence is provided by studies in
508 temperate amphipods and shrimps (Kinne 1952, Williams 1960, Hagermann & Uglow 1983,
509 Janas & Spicer 2008), showing decreased osmoregulatory capacity and survival under the
510 combination of low salinity and low temperature. As stated by Kinne (1971) a disruption of
511 active transport processes is expected below at low critical temperature.

512 More in general, evidence of a link between osmoregulation and tolerance to low salinity is
513 widespread across adult crustaceans (Mantel & Farmer 1983, Péqueux 1995). Even small
514 increases in osmoregulatory capacity, as 20 $mOsm*kg^{-1}$, result in enhanced salinity tolerance,
515 particularly in weak osmoregulators. For example, in the copepod *Eurytemora affinis*, slight
516 increases in osmoregulatory capacity result in dramatic evolutionary shifts in adaptability to
517 low salinity media (Lee et al. 2012). Evidence for crustacean larvae is given for instance by
518 studies on stage-dependent patterns of osmoregulation, showing that the increased
519 osmoregulatory capacity is characteristic of species or stages that are able to survive over a
520 wide range of salinities (Charmantier 1998, Charmantier et al. 1998, Anger & Charmantier
521 2000, Anger et al. 2008). Another piece of evidence is the positive relationship between
522 osmoregulatory capacity and accumulation of reserves at low salinities existing in crustacean
523 larvae (Torres et al. 2011). There is also evidence showing that the osmoregulatory capacity
524 matches patterns of survival at the intraspecific level concerning the effect of salinity

525 acclimation (Charmantier et al. 2002). Overall, we conclude that there is sufficient comparative
526 evidence to propose a link between the effect of temperature on osmoregulation and on
527 tolerance to low salinities.

528 **Implications for climate change**

529 If the effects of temperature on osmoregulatory capacity and phenology (timing of events
530 such as moult or metamorphosis) are relevant to fitness, they may also drive changes in species'
531 distributions. From the standpoint of osmoregulation, responses should be species-specific
532 because osmoregulation can increase (Kinne 1971, Janas & Spicer 2008, this study), decrease
533 (Burton 1986, Weber & Spaargaren 1970) or show little response to temperature (additional
534 examples in Burton 1986). From the standpoint of the phenological effects, survival should
535 increase with temperature but such effect may vary across species depending on the slope of
536 the development-temperature curve. Phenological effects of high temperature (e.g. increased
537 survival to metamorphosis) may counteract negative effects of temperature on osmoregulation
538 (decreased survival) and may enhance positive effect of temperature on osmoregulation.

539 Concerning osmoregulation, there is evidence suggesting a link between osmoregulatory
540 capacity and species distributions in crustaceans. For instance, Weber & Spaargaren (1970)
541 noted differences between the effects of temperature on osmoregulation of species distributing
542 towards cold *vs.* warm latitudes: the shrimp *Crangon septemspinosa* shows a direct positive
543 effect of temperature on osmoregulation (Haefner 1969) and distributes further south than *C.*
544 *crangon* with an inverse effect of temperature (Flügel 1963). Weber & Spaargaren (1970) also
545 discussed cases showing how thermally driven osmoregulation varied in "winter" and
546 "summer" acclimated organisms in order to match the prevailing temperature conditions.
547 Similar patterns have been found in more recent studies: for instance, Janas & Spicer (2008)
548 found that osmoregulation at high salinities was impaired at low temperatures in large
549 individuals of the shrimp *Palaemon elegans*, which perform winter migrations off the coast of

550 Great Britain, but similar temperatures did not impair osmoregulation in smaller individuals,
551 which remained in intertidal pools.

552 There is additional evidence (provided by various sources), suggesting a link between
553 invasion of estuaries and the capacity to osmoregulate (Freire et al. 2008, Lee et al. 2012).
554 Another source of evidence is the strong match between patterns of ontogenetic migration in
555 marine crustaceans and concurrent ontogenetic changes in the capacity to osmoregulate (Anger
556 & Charmantier 2000, 2011, Anger et al. 2008, Charmantier et al. 1998, 2002, Cieluch et al.
557 2004, 2007). Those studies show that larval stages occupying or crossing water masses
558 characterised by low (and varying) salinity (estuaries, rivers and tidal pools) are osmoregulators
559 while those occurring in the open sea (stable higher salinity) are osmoconformers. The same
560 studies found that the match between the habitat and the osmoregulatory capacity is consistent
561 along the full life cycle: while marine species are consistently osmoconformers or weak
562 osmoregulators at all life phases, the patterns of osmoregulation vary between larval and
563 juvenile-adult phases depending on habitat. In synthesis, thermally driven changes in
564 osmoregulation and the ontogeny of osmoregulation appear to have evolved in order to match
565 the conditions experienced at each particular life phase or developmental stage. As stated by
566 Weber & Spaargaren (1970), the responses of osmoregulation abilities to temperature appear
567 to be adaptive to the prevailing conditions of temperature and salinity.

568 Hence, given the above comparative evidence, we propose a hypothetical model showing
569 the contribution of osmoregulation to responses to salinity and temperature in terms of fitness
570 and species distributions of coastal marine species of temperate ROFIs (Fig. 4); our model also
571 recognises the contribution of the phenological mechanism. Our predictions are that: (1) In
572 species where elevated temperatures have a positive effect on osmoregulation (e.g. *C. maenas*),
573 osmoregulation should contribute towards TMLS and range expansion towards brackish water
574 habitats (e.g. ROFIs or estuaries) in a warming scenario. (2) The phenological mechanism

should also promote TMLS and range expansion towards brackish water habitats in a warming scenario. (3) In species where elevated temperature results in decreased osmoregulatory capacity, osmoregulation should promote a synergistic negative effect of increased temperature and reduced salinity on fitness (abbreviates a TELS for "thermal exacerbation of low salinity stress"). In those species, and in a warming scenario, osmoregulation should contribute towards range contraction (i.e. away from brackish water habitats, unless the phenological mechanism prevails). All those predictions are valid only in case of moderate warming, as extreme temperatures would result in a dominating effect of thermal stress in the responses.

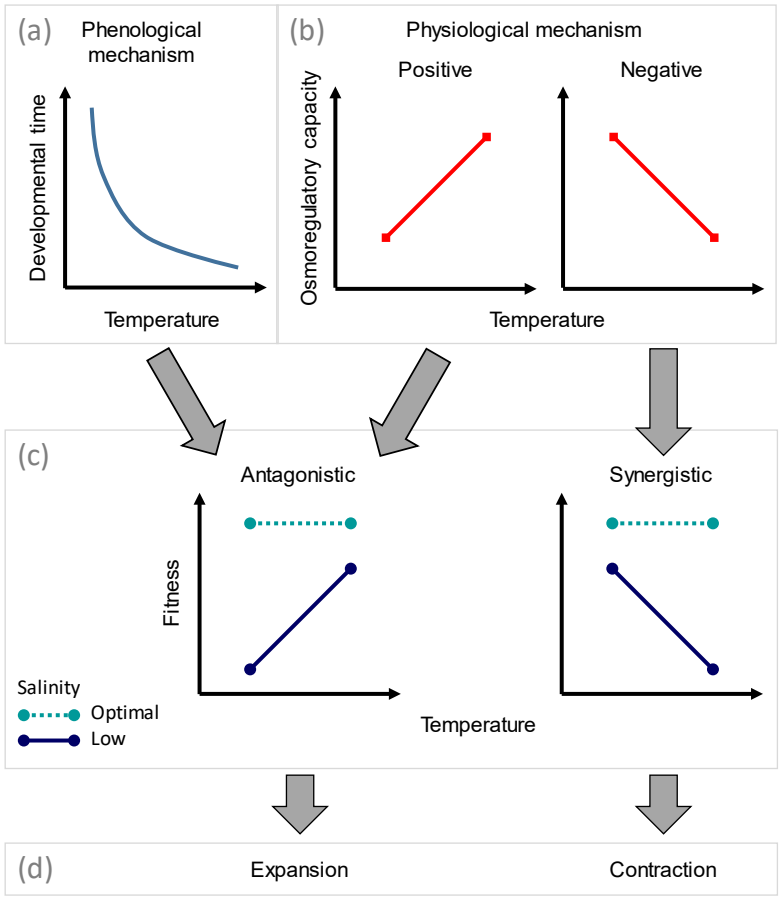

Figure 4. Model describing the contribution of phenological and physiological mechanisms to the responses to effects to temperature and salinity in terms of fitness and species' distributions. (a) Phenological mechanism: because increased temperature shortens the developmental time, individuals at high temperatures are exposed to low salinity for a shorter period; in consequence, survival-at-stage is higher and the overall response in (c) is antagonistic. (b) Physiological mechanism: when the osmoregulatory capacity (OC) increases with temperature, the fitness response to temperature and salinity in (c) should be antagonistic. When osmoregulatory capacity decreases with temperature, the fitness response in (c) should be synergistic and negative. (d) Contribution of fitness responses to changes in distribution changes in coastal-estuarine species: if temperature increases (and salinity remains constant), an antagonistic pattern should contribute towards expansion towards estuarine waters while the synergistic and negative responses should contribute towards range contraction. Both low

salinities and increased temperatures are assumed to be moderate (slightly sub-optimal), so as not to become dominant responses; otherwise, the trivial prediction is a dominating effect of either high temperature or low salinity, whichever is stronger.

Perspectives for model development

The proposed model needs to be tested and expanded, but it can be used as a framework to develop a more general model, considering factors other than osmoregulation. A possible expansion may consider the importance of intracellular regulation (Freire et al. 2008) and permeability to salts; the latter may change with temperature and impact performance, through effects on the amount of energy needed to invest in osmoregulation (Spaargaren 1975). An additional contributor is required to explain responses where the limiting factor is given at high temperatures: for instance, McLusky (1979) did not find evidence of temperature driving osmoregulation in mysids; yet those adapted to winter conditions had decreased survival in combination of low salinity and high temperatures. For those cases (not covered in our model nor in Fig. 4), low salinity and high temperature may impose limitations other than those associated to the uptake of osmotically active substances. The effect of osmoregulatory capacity on performance may be non-linear. For instance, in strong osmoregulators (e.g. amphipods) variations in the osmoregulatory capacity in response to temperature does not always correlate with survival in the laboratory (Dorgelo 1977), although such drops may impair performance in the field. Care should be taken into considering the "phenological mechanism" yet to be quantified.

In addition, by proposing the above-referred model, we do not intend to ignore the effects of additional factors on range contraction and expansion (Siren & Morelli, 2020); hence, we must emphasise that our model refers to contributions of osmoregulation and the proposed phenological mechanism. For instance, hypoxia and ocean acidification in coastal areas may become an important factor for performance of marine organisms and it may result from the

631 combined effect of warming and eutrophication (Vaquer-Sunyer & Duarte 2008, 2011, Glober
632 & Baumann 2016). Oxygen limitation for instance may result in changes in the "Pejus
633 temperature", i.e. critical thermal thresholds set by limitations in the capacity of circulatory
634 and respiratory systems to provide oxygen to tissues (Pörtner 2010). Limitations in oxygen
635 availability are likely to reduce the energy available for osmoregulation, resulting in inhibition
636 of enzymes responsible for ion transport (Lucu & Ziegler 2017). In consequence, under
637 combined effects of low salinity at high temperatures, oxygen limitation may range from
638 reductions in the strength of TMLS to the induction of TELS. The effect of ocean acidification
639 and salinity may vary among species; those able to osmoregulate appear to withstand moderate
640 levels of acidification (Whiteley et al. 2018). Biotic interactions such as competition between
641 native and exotic species may drive range contractions in native species (Epifanio 2013). In
642 the case of dispersive larvae, transport by currents play a central role in determining recruitment
643 of individuals to a given population (Connolly & Roughgarden 1999) and in mediating effects
644 of climate change on species distributions (Lo-Yat et al. 2011, Fuchs et al. 2020). Responses
645 to temperature and salinity are likely to define components of performance that are relevant for
646 e.g. competition and larval transport. For instance, competitive abilities can change according
647 to the temperature where such competition occurs (Tomanek & Helmuth 2002, Watz et al.
648 2019); comparative studies should provide insights into how the outcome of competition is
649 modulated by responses to combination of temperature and salinity. In addition, larval transport
650 in many crustaceans depends on circatidal or diel migration behaviour, which should be driven
651 by the capacity of larvae to accumulate and use energy for vertical swimming. We know that
652 crustacean larvae respond to temperature and salinity as stimuli (Epifanio & Cohen 2016);
653 however, swimming speed can be modified through physiological effects of both factors (Yu
654 et al. 2010, Sorochan & Metaxas 2017, Landeira et al. 2020). Overall, physiological
655 information provided by the model may be integrated for instance in metapopulation models

(Fordham et. al. 2013, Giménez et al. 2020) to consider processes occurring at the individual and population level.

The proposed model should also incorporate the importance of intraspecific variability in the responses to temperature and salinity, as driven by plasticity and selection (Charmantier et al. 2002, Lee et al. 2012). There is currently very little information about the magnitude of intraspecific variation in responses to temperature and salinity in coastal organisms. In *C. maenas*, two recent studies show that the magnitude of TMLS can vary among larvae originated from different females (Spitzner et al. 2019, Torres et al. 2020). In both *C. maenas* and other estuarine species, the tolerance to salinity is driven by the salinity conditions experienced by embryos (Laughlin & French 1989, Charmantier et al. 2002). Some of those responses appear to be adaptive: for instance in *N. granulata*, the "embryonic effect" produced by low salinity consists in an increased osmoregulatory capacity and increased survival of the first larval stage. However, in *C. maenas*, low salinity experienced during embryogenesis results in a pre-emption of TMLS (Torres et al. 2020). Hence, such type of intraspecific variation suggests that conditions existing in the benthic habitat can also affect the capacity of larvae to use estuarine waters. For *C. maenas*, moderately high temperature experienced during embryogenesis promotes TMLS (Torres et al. 2020); thus moderate increases in temperature both in the maternal and larval habitats may enable larvae the use of waters characterised by moderately low salinity.

Osmoregulatory responses will be relevant for crustaceans and most probably fish, i.e. those groups living in aquatic habitats that have evolved the capacity to osmoregulate (McCormick et al. 1997, Charmantier et al. 2009, Evans & Claiborne 2009). Research on the role of temperature in driving intracellular osmoregulation would be useful to formulate models for invertebrates that osmoconform at low salinities. Importantly, intraspecific variations in the capacity to osmoregulate in temperate species may also explain why we observe both

681 synergistic and antagonistic responses to temperature in marine species (Crain et al. 2008,
682 Przeslawski et al. 2015), i.e. because species differ in the nature of the response of
683 osmoregulation capacities to temperature (positive or negative response).

684 How osmoregulatory responses may drive survival in the field should depend on the
685 covariation of temperature and salinity in regions of freshwater influence (ROFIs). For
686 instance, in temperate ROFIs, spring-summer warming of the coastal zone causes shallow
687 waters characterised by reduced salinity to warm up in summer (Gunderson et al. 2016), but
688 cool-down in winter. In addition, climate change predictions for coastal areas vary regionally
689 concerning salinity (IPCC 2013), depending on projections of future freshwater runoff from
690 estuaries. Hence, how organisms respond to salinity and temperature will depend on the region,
691 species' phenology and ontogenetic patterns in osmoregulatory capacity. Usually, larval
692 development takes place in spring-summer while the timing of occurrence of juvenile and
693 adults depends on the species life-history and migration patterns. Across a sufficiently wide
694 range of temperatures, one would expect a unimodal survival curve driven by thermal tolerance
695 (Pörtner 2010), with location and spread varying depending on salinity. For instance, on the
696 latitudinal scale, the distribution of *C. maenas* is driven by temperature (Compton et al. 2010),
697 but the question here concerns the use of coastal waters of low salinity (e.g. estuarine plumes
698 or areas such as the Baltic Sea), which may enhance recruitment at the local scale. In invasive
699 species like *C. maenas* (Hines et al. 2004, deRivera et al. 2007), a moderate increase in
700 temperature should be particularly important for expansion in recently invaded areas, to
701 habitats characterised by low salinities.

702 **CONCLUSIONS**

703 In synthesis, we have found that increased temperature enhances the capacity to
704 osmoregulate in the osmoregulatory larval stages of the shore crab *C. maenas*; correlations

between osmoregulatory capacity and survival suggest that low temperature may constraint the capacity of osmoregulatory mechanisms to work properly and sustain haemolymph osmolality at appropriate levels. The increase in osmoregulatory capacity works along the phenological effect (increased temperature reduces the time of exposure to low salinity) in mitigating the effects of low salinity on survival.

In addition, we propose a model, for coastal-estuarine organisms, showing the contribution of osmoregulation and phenological mechanisms into fitness responses to temperature and salinity, as well as responses to warming. Osmoregulatory capacity is a promising integrative measure for understanding the diversity of responses to temperature and salinity and for the prediction of responses of coastal-estuarine organisms to warming. Tests and expansion of the model are needed to orient research towards the prediction of effects of climate-driven changes in temperature and salinity on species' distributions. Given the current status regarding climate change, the "plea for the study of temperature influence on osmotic regulation" done by Verwey (1957) is still valid after almost a century of research in osmoregulation, as it is the plea for the study of the role of temperature on performance at suboptimal salinities.

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Authors' contributions: GT, LG, GC and SH conceived the experimental design. GT performed the larval experiments, sampling and mRNA expression measurements. GC performed the osmoregulatory capacity determinations. GT and DW analysed the mRNA expression data; GT, LG and GC analysed the osmoregulation data. GT and LG led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Conflict of Interest: The authors declare that they have no conflicts of interests. The funding body did not influence the design of the study, the collection, analysis, and interpretation of data nor the writing of the manuscript.

Data availability statement: The datasets used and/or analysed during the current study are available from the Dryad Digital Repository (doi:10.5061/dryad.4tmpg4f97).

Compliance with Ethical Standards: The research presented in this paper complies with the guidelines from the directives 2010/63/EU of the European parliament and of the Council of 22nd September 2010 on the protection of animals used for scientific purposes.

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