Universität Bremen

Responses of early-life stages of coastal marine invertebrates to different environmental variables

Noé Espinosa-Novo

Dissertation 2023

Responses of early-life stages of coastal marine invertebrates to different environmental variables

Dissertation

In fulfilment of the requirements for the degree of

Doctor of natural sciences (Dr. rer. Nat.)

at the Faculty 02- Biology and Chemistry

of the University of Bremen, Germany

Submitted on September 29th 2023 and reviewed by

Prof. Dr. Maarten Boersma

Prof. Dr. Martin Zimmer

Colloquium held on 30th November 2023

Supervised by

Prof. Dr. Maarten Boersma

POLMAR
Helmholtz Graduate School
for Polar and Marine Research

GEFÖRDERT VOM

Bundesministerium
für Bildung
und Forschung

AWI
ALFRED-WEGENER-INSTITUT
HELMHOLTZ-ZENTRUM FÜR POLAR-
UND MEERESFORSCHUNG

Thesis reviewers

First reviewer: Prof. Dr. Maarten Boersma
Shelf Sea System Ecology
Alfred-Wegener-Institut, Helmholtz-Zentrum für Polar-und Meeresforschung,
Biologische Anstalt Helgoland

Second reviewer: Prof. Dr. Martin Zimmer
Mangrove Ecology,
University Bremen, and
Leibniz Centre for Tropical Marine Research
Bremen, Germany

Examination Commission

Commission chair: Prof. Dr. Marko Rohlfs
Population and Evolution Ecology
University Bremen

Examiner: Prof. Dr. Maarten Boersma
Shelf Sea System Ecology
Alfred-Wegener-Institut, Helmholtz-Zentrum für Polar-und Meeresforschung,
Biologische Anstalt Helgoland

Examiner: Prof. Dr. Martin Zimmer
Mangrove Ecology,
University Bremen, and
Leibniz Centre for Tropical Marine Research
Bremen, Germany

Examiner: Dr. Christine Ewers
Evolution Ecology and Biological Invasions
Kiel, University
Kiel, Germany

Student member: Msc. Sheng V. Wang
PhD candidate at Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research and University of Bremen
Bremen, Germany

Student member: Verena Wutz
Master Student (Ecoloy) at the University of Bremen
Bremen, Germany

Summary

Quantifying species responses to the effects of changing environmental conditions is critical for a better understanding of how climate change affects invasion, expansion, and contraction of marine coastal species. Climate change is leading to modifications in the marine coastal environment, to conditions not experienced before; climate change results in that marine organisms experience simultaneous changes in several environmental variables (=drivers: e.g. temperature, salinity, food). How simultaneous changes in multiple drivers are experienced depend on species-specific traits (e.g. physiological tolerance, developmental time); for instance, co-occurring native and non-native species may experience and respond to climate change in different ways. In addition, within species, responses to multiple drivers may vary across populations and environmental gradients. The general objective of this thesis was to quantify the effects of environmental drivers (temperature, salinity and food limitation) on performance of native and non-native species with focus on larval stages and using crabs as model systems. There were two main objectives, first to compare native and non-native species in the responses to multiple environmental drivers and to quantify larval responses to temperature across their distribution range. I focused on larvae because they play a critical role in population dynamics: larvae are important for the dispersion and connectivity of populations, and are more sensitive to changes in environmental conditions than adults. I used three ecologically relevant species of coastal areas of the North Sea and North Atlantic Ocean as models: *Hemigrapsus sanguineus*, *Carcinus maenas* and *Hemigrapsus takanoi*. *C. maenas* is native to Europe; *Hemigrapsus* spp. are both non-native species in the European coast, where they coexist with *C. maenas* as juveniles and adults in the benthos. I used factorial experiments rearing larvae from hatching to megalopae at different combinations of temperature and other environmental drivers (salinity, food limitation). Larval performance was quantified as survival, duration of development, and growth. The first series of result show that both non-native (*Hemigrapsus* spp) species had higher performance (high survival, shorter duration of development and high growth rates) than the native *C. maenas* at higher temperatures and at moderately low salinities (18 – 24 °C, 20 – 25 ‰). These results are comparable to another non-native species in Europe, the Chinese mitten crab *Eriocheir sinensis*. In *H. sanguineus*, larvae show moderate level of tolerance to limited access to food at high temperature, which contrasted to the low tolerance shown in native *C. maenas*. Experiments and modelling show that the nature of the multiple driver response depends strongly on the metric used to measure time, where my emphasis is on biological time (time to metamorphosis). The results from the populations comparisons showed species and gradient-specific responses. For *H. takanoi*, distributed over a salinity gradient (North Sea -Baltic Sea), larvae from the North Sea populations always showed higher survival and faster development compared with those from the Baltic Sea. The population near the limit of the distribution showed very low survival, suggesting that subsidies or complex ontogenetic migration patterns are needed for population persistence. Results did not show genetic differentiation among the studied populations in the

mitochondrial cytochrome c oxidase subunit one gene (COI) suggesting that there is high connectivity among populations. For *C. maenas* distributed across a latitudinal gradient (South: Vigo, Spain; North: Bergen and Trondheim, Norway) and reared under different temperatures (range 6 to 27 °C in steps of 3 °C), there was little variation in survival and growth among populations. However, larvae from the Norwegian populations had a slightly shorter duration of development at low temperatures than those from Vigo, this response has an adaptive value in that it could sustain survival in scenarios of reduced temperature, by shortening the larval phase, when mortality rates are high. Besides, results from this experiment (as well as for the mentioned above) showed high intrapopulation variability in larval performance which has a potential to affect range expansion of the above-mentioned species. Variation in the responses of larval stages to the effects of different environmental drivers highlights the importance of using physiological descriptors to quantify the performance of marine invertebrates to changing environments. Larval responses vary in rates of survival but also in the duration of time to achieve metamorphosis, as well as the rate at which the organisms grow, with concomitant effects on post-metamorphic success, which in seasonal habitats may strongly depend on temperature. The results from the thesis highlight the importance of quantifying the responses of marine invertebrates to changing environmental conditions, considering different species and species distributed across different gradients as well as variations among and within species.

Zusammenfassung

Die Quantifizierung der Reaktionen von Arten auf veränderte Umweltbedingungen ist entscheidend für ein besseres Verständnis der Auswirkungen des Klimawandels auf biologische Invasionen, Ausbreitung und den Rückgang von Arten in Küstengewässern. Der Klimawandel führt zu Veränderungen in der marinen Küstenumwelt, und schafft Bedingungen, denen Organismen vorher nicht ausgesetzt waren. Der Klimawandel hat zur Folge, dass Meeresorganismen gleichzeitige Veränderungen mehrerer Umweltfaktoren (=Treiber: z. B. Temperatur, Salzgehalt, Nahrungsverfügbarkeit) erfahren. Wie die gleichzeitigen Veränderungen mehrerer Einflussfaktoren erlebt werden, hängt von artspezifischen Merkmalen ab (z. B. physiologische Toleranz, Entwicklungsdauer); so können z. B. gleichzeitig am gleichen Ort vorkommende einheimische und nicht einheimische Arten den Klimawandel auf unterschiedliche Weise erleben und darauf reagieren. Darüber hinaus können innerhalb einer Art die Reaktionen auf verschiedene Faktoren je nach Population und Umweltgradienten unterschiedlich ausfallen. Das Ziel dieser Arbeit bestand darin, die Auswirkungen von Umweltfaktoren (Temperatur, Salzgehalt und Nahrungslimitierung) auf die Leistungsfähigkeit einheimischer und nicht einheimischer Arten zu quantifizieren, wobei der Schwerpunkt auf Larvenstadien lag und Zehnfußkrebse als Modellsysteme verwendet wurden. Es gab zwei Hauptziele: Erstens sollten die Reaktionen einheimischer und nicht einheimischer Arten auf verschiedene Umwelteinflüsse verglichen und die Reaktionen der Larven auf die Temperatur in ihrem Verbreitungsgebiet quantifiziert werden. Ich habe mich auf Larven konzentriert, weil sie eine entscheidende Rolle in der Populationsdynamik spielen: Larven sind wichtig für die Ausbreitung und Konnektivität von Populationen und reagieren empfindlicher auf Umweltveränderungen als adulte Tiere. Ich verwendete drei ökologisch-relevante Arten der Küstengebiete der Nordsee und des Nordatlantiks als Model: Hemigrapsus sangineus, Carcinus maenas und Hemigrapsus takanoi. C. maenas ist heimisch in Europa; Hemigrapsus spp. sind beide gebietsfremde Arten an den europäischen Küsten, wo sie mit C. maenas als juvenile und adulte Tiere im Benthos koexistieren. In faktoriellen Experimenten wurden Larven vom Schlupf bis zum Megalopa-Stadium in Kombinationen von Temperatur und anderen Umwelttreibern (Salzgehalt und Nahrungslimitation) aufgezogen. Larvale Leistungsfähigkeit wurde quantifiziert als Überlebensrate, Entwicklungsdauer, und Wachstumsrate. Die erste Serie an Ergebnissen zeigten, dass beide gebietsfremden Arten (Hemigrapsus spp.) höhere Leistungsfähigkeit (höhere Überlebensraten, kürzere Entwicklungsdauer, höhere Wachstumsraten) als die einheimische Art C. maenas bei höheren Temperaturen und geringen Salzgehalten (18 – 24 °C, 20 – 25 ‰) aufweisen. Diese Ergebnisse sind vergleichbar mit jenen einer dritten gebietsfremden Art, der Chinesischen Wollhandkrabbe (E. sinensis). H. sanguineus Larven zeigen moderate Toleranz gegenüber limitiertem Zugang zu Nahrung bei hohen Temperaturen im Gegensatz zur niedrigen Toleranz, die die einheimische Krabbe C. maenas aufweist. Experimente und Modellierungen zeigen das die Natur der Antwort auf multiple Treiber davon abhängig ist, welche Metrik für Zeit verwendet wird. In dieser Arbeit liegt der Fokus auf

biologischer Zeit (Zeit bis zur Metamorphose). Die Ergebnisse der Populationsvergleiche zeigten Arten- und Gradienten-spezifische Antworten. Für H. takanoi, die entlang eines Salzgehalt-Gradienten (Nordsee - Ostsee) vorkommt, zeigten Larven aus Nordsee-Populationen grundsätzlich höhere Überlebensraten und schnellere Entwicklungsdauern als Larven aus der Ostsee. The Population an der Grenze des Verbreitungsgebiets zeigte sehr geringe Überlebensraten, was darauf hinweist, dass ein Ausgleich aus anderen Populationen stattfindet oder komplexe ontogenetische Migrationsmuster existieren um den Erhalt der Population sicher zu stellen. Die Ergebnisse zeigten keine genetische Differenzierung zwischen den untersuchten Populationen im mitochondrialen Cytochrom C Untereinheit I-Gen (COI) was auf eine hohe Konnektivität zwischen den Populationen hinweist. Für C. maenas wurden drei Populationen entlang eines latitudinalen Gradienten untersucht (Süden: Vigo, Spanien; Norden: Bergen und Trondheim, Norwegen). Larven wurden unter verschiedenen Temperaturen (6 ° bis 27 °C, in 3-Grad Schritten) aufgezogen und zeigten wenig Unterschiede in Überlebens- und Wachstumsraten zwischen den Populationen. Jedoch zeigten Larven aus den Norwegischen Populationen geringfügig kürzere Entwicklungsdauern in niedrigen Temperaturen als jene aus Vigo. Dies hat einen adaptiven Wert, als dass es Überleben in Szenarien mit reduzierter Temperatur sicherstellen könnte, da es die Larvalphase, in der die Sterblichkeit hoch ist, verkürzt. Dieses Experiment, wie auch das vorher beschriebene, zeigten hohe Variabilität in der Leistungsfähigkeit der Larven innerhalb der Populationen, was Auswirkungen auf Veränderungen des Verbreitungsgebiets der Arten haben könnte. Variation in der Antwort der verschiedenen Larvenstadien auf die Effekte von Umwelttreibern betont die Wichtigkeit physiologische Deskriptoren zu benutzen, um die Leistungsfähigkeit mariner Wirbelloser in einer sich verändernden Umwelt zu erfassen. Die physiologische Antwort von Larven auf Umwelttreiber variiert sowohl in Überlebensraten und Entwicklungsdauer bis zum Erreichen der Metamorphose, als auch der Wachstumsrate des Organismus, welche wiederum nachfolgende Effekte auf den Erfolg nach der Metamorphose haben kann. Dieser wiederum ist in von Jahreszeiten geprägten Habitaten sehr temperaturabhängig. Die Ergebnisse dieser Doktorarbeit betonen die Wichtigkeit die physiologische Antwort mariner Wirbelloser auf eine sich verändernde Umwelt zu quantifizieren und dabei Arten, Verbreitungsgebiete entlang von Umweltgradienten sowie die Variation zwischen und innerhalb von Arten zu betrachten.

TABLE OF CONTENTS

Summary i

Zusammenfassung iii

CHAPTER 1. GENERAL INTRODUCTION AND OBJECTIVES 1

Global climate change 1

Invasions and range shifts in species distribution 3

Organisms with complex life cycles 4

Gaps in knowledge 6

Model species 7

Thesis objectives 11

CHAPTER 2- Publication 1 15

CHAPTER 3- Publication 2 35

CHAPTER 4- Publication 3 51

CHAPTER 5- Publication 4 81

CHAPTER 6- Publication 5 121

CHAPTER 7. GENERAL DISCUSSION 149

Responses of native and non-native species to multiple environmental drivers 150

Responses of populations to environmental drivers across gradients 153

REFERENCES 157

CHAPTER 1. GENERAL INTRODUCTION AND OBJECTIVES

Global climate change

Global climate change refers to natural or human- induced changes in the state of the variabilities or properties of the climate, these changes persist for an extended period of time, usually decades or longer (IPCC 2023). Human-induced climate change has led to unprecedented impacts on the biosphere resulting in rising global atmospheric greenhouse gases which trap heat in the climate system, so the outgoing energy is lower than the energy entering the system and the Earth´s Energy Imbalance (EEI) is created (Hansen et al. 2005; IPCC 2013; Cheng et al. 2019). Earth temperature has risen by an average of 0.08 °C per decade since 1880; with 2022 being one of the warmest years, 0.86 °C warmer than the average in the 20th century (IPCC 2022; NOAA 2023). Atmospheric CO_2 has also been reported to be highest in recent years compared with the pre-industrial years (IPCC 2022). As a consequence of the steady increase in global temperature, global ice sheets are shrinking and sea level is rising (Duarte 2014; Lipczynska-Kochany 2018; Murshed and Dao 2022; IPCC 2022). Among all the changes related to global warming, the rate and intensity of cold and heat extremes (Donat et al. 2013) are the ones that show the most important variations, cold extremes are becoming less common while heat extremes are increasing (Fischer and Knutti 2015). Human-induced climate change has caused an increase in the average, frequency and duration of extreme heat waves and will be even more severe in the future (Meehl and Tebaldi 2004; Smale et al. 2019).

The oceans play an important role in the climatic system; they cover 71 % of the surface of the Earth and absorb and store ca. 90 % of the EEI as increased ocean heat content (Levituls et al. 2000; Hansen et al. 2005; Cheng et al. 2019). Global mean sea surface temperature has risen by 0.88 °C since the early 20th century, and most projections estimate that not only the sea surface temperature but also the temperature of the seafloor will continue to rise (Brito-Morales et al. 2020; Kwiatkowski et al. 2020). The ocean is also one of the largest natural reservoirs of carbon and has been buffering the changes in atmospheric carbon dioxide (CO_2) by absorbing up to 30 % of the total CO_2 released by human activities since the industrial era started (Sabine et al. 2004; Sabine and Tanhua 2010; Hoegh-Guldberg and Bruno 2010). Higher amounts of CO_2 released in the atmosphere mean higher amounts of CO_2 dissolving into the ocean with concomitant reduction in pH levels, affecting marine life, especially those organisms that rely on calcification (Hönisch et al. 2012; Kroeker et al. 2013). Another important aspect of climate change to consider is the velocity at which changes occur; the velocity of climate change and shift of seasonal timing are used as a measure to describe the pace at which organisms should move either in time or in place in order to follow the environmental changes (Burrows et al. 2011a). In this sense, a decrease in species richness in the Equator relative to an increase towards midlatitudes has been reported as a result of the warming of the ocean (Lenoir et al. 2020; Chaudhary et al. 2021). Simultaneously; marine heatwaves, defined as periods of time (> 5 days) of seawater

temperature is higher than a predefined threshold (Meehl and Tebaldi 2004; Hobday et al. 2016), have increased in frequency (by 17 %) and duration (by 34 %) since 1925 and are projected to continue increasing with global warming (Oliver et al. 2018).

Coastal marine ecosystems are ecologically relevant areas distributed all over the world; they are characterised by being highly productive, supporting high biological diversity and providing a number of ecosystem services (Costanza et al. 1997; Martínez et al. 2007; Melet et al. 2020). Coastal ecosystems are vulnerable to global warming, and in addition they are extremely vulnerable to human impacts which can drive degradation or even their collapse (Crain et al. 2009). Coastal zones are regions characterised by the influence of freshwater runoff (regions of freshwater influence, ROFIs, Simpson 1997); salinity in these areas is expected to vary seasonally and in the long-term as a consequence of global climate change in concert with temperature (Thompson et al. 2017; Tabari 2020; Rodrigues and Patil 2021). Seasonal variations are important in coastal areas as these habitats experience peaks of temperatures and variations in other environmental variables (Bates 2002) and the magnitude and variation of the change may depend on the region (Gunderson et al. 2016). Coastal organisms are usually adapted to these variable environments, but they often live near the edge of their physiological tolerances (Browne and Wanigasekera 2000; Stillman and Somero 2000). Their survival may depend on the changes in the environmental conditons (Pörtner 2001; Somero 2010) and under a scenario of global warming, coastal organisms may have to cope with these fluctuations but under increased temperatures.

Adaptation to ocean and coastal warming includes an array of biological responses from cells to ecosystems, and it is determined by the interplay of more than one environmental variable. Temperature is a key variable that drives various physiological processes including metabolism, enzymatic activity and gene expression. By altering these processes, ocean warming is expected to modify survival, growth, reproduction, migration, distribution, and species interactions (Angilletta 2009; Dahlke et al. 2017; Messmer et al. 2017; Pinsky et al. 2019, 2020; Saros et al. 2019). Rising temperatures could also affect ecological attributes such as larval dispersal, connectivity among populations, local adaptation, and speciation (O´Connor et al. 2007). Reduced developmental times of marine organisms may result in phenological mismatches, if for instance the effects of temperature are stronger in consumer than in resources, where the organisms develop under non-favourable food conditions (Durant et al. 2007). Coastal and marine organisms usually experience variabilities in more than one variable at the same time. For example increases in temperature combined with a decrease in oxygen availability is expected to result in the contraction of species distribution along the depth gradient (Deutsch et al. 2015). Warming and acidification impact negatively the process of photosynthesis, calcification, reproduction, and survival of many marine organisms (Harvey et al. 2013). The exposure to acidification and low oxygen availability may have a negative effect of the thermal tolerance of some organisms (Pörtner 2010).

Invasions and range shifts in species distribution

One of the most important responses of marine organisms to ocean warming constitutes the shifting in latitudinal and depth ranges (Perry et al. 2005; Mueter and Litzow 2008; Pinsky et al. 2020). Marine ectotherms are thermal- range conformers, as their latitudinal range matches their thermal tolerance (Sunday et al. 2012a). This means that their latitudinal ranges are primarily determined by their thermal tolerance limits (Stevens 1989). One would expect that, as a consequence of the close relationship between thermal tolerance and latitudinal range, marine organisms will be more sensitive to temperature changes at their poleward and equatorward range limits. Marine organisms have shifted poleward and equatorward range distribution limits towards higher latitudes as a response to ocean warming (Parmesan 2006; Poloczanska et al. 2013a, 2016), with the same frequencies for both boundaries (Sunday et al. 2012a). Climate velocity of species shift is set by the rate and direction of the changes in the isotherms through space (Ackerly 2009; Loarie et al. 2009), and it is a combination of both temporal and spatial rates of changes in temperature (Burrows et al. 2011a). Climate velocity differs among ecosystems and varies at local scales (Pinsky et al. 2013).

Those regions of the ocean that have seen rapid warming in a relatively short period of time have also shown changes at the species composition and community level (Poloczanska et al. 2016). For the species inhabiting areas close to the Equator, changes in ocean temperature usually results in a poleward shift, because those species tend to live closer to their upper thermal tolerance (Pörtner 2001; Stillman 2003; Pörtner and Knust 2007), implying that further thermal acclimation is not possible because of physiological constraints. Away from the equator change also occurred at various trophic levels (Reid et al. 2001; Beaugrand et al. 2002; Beaugrand and Reid 2012) in regions characterised by rapid changes in temperature. For example, in the European Atlantic Ocean, species distributions shifted poleward, in a process called “subtropicalization” (Montero-Serra et al. 2015). Towards the north of the European Atlantic ocean, in the Eurasian Arctic, the properties of the ocean are also changing due to enhanced inflow of warm Atlantic water, warming of surface temperature, and the retraction of the sea ice cover in a process called “Atlantification” or borealization of the Arctic ocean (Wassmann 2011a; Tsubouchi et al. 2021; Bacon et al. 2022). This phenomena is triggering changes in the biological communities of the Arctic due to the dispersal of subarctic communities towards the north, changes that are being reported in more than one trophic level (Wassmann 2011a; Kortsch et al. 2012; Eriksen et al. 2017; Vihtakari et al. 2018; Freer et al. 2022).

Species expansion and shifts in distributions also occur in the context of human-mediated introduction of species. Marine species have been transported by humans since the start of overseas travelling. However, it was only relatively recently that introduction of invasive species has increased exponentially due to the increase in global marine trade, construction of corridors, and aquaculture (Carlton 2002a; Hulme et al. 2008; Katsanevakis et al. 2013). Due to the fact that ocean warming is

exacerbating the establishment of species in environments where they are not native (Hulme 2017; Pyšek et al. 2020), understanding the mechanisms driving non-native species range expansion is of pivotal importance. The consequences of the establishment of non-native species in new habitats have been recorded in communities at a global scale (Simberloff 2009; Hulme 2017) but also, and importantly, at local scales in coastal marine ecosystems (Diederich et al. 2005; Smaal et al. 2009; Reise et al. 2017). The successful establishment and the consequent range expansion of non-native species may depend on ecological and evolutionary processes (Gurevitch et al. 2011a), with the potential of a species to invade, establish, and expand being dependent on species traits and the invasibility of local communities. For example, one would expect that those species with higher competitive abilities, tolerance to environmental variation, and high reproduction rates may be more successful at establishing and expanding their distribution ranges (Holdredge and Bertness 2011; Kelley 2014; Donelan et al. 2022). Given the state of the environment and all that it is changing it is very important to know how marine organisms deal with this. Benthic marine invertebrates with complex life cycles are a relevant group in this sense as their capacity to disperse and colonize new habitats depends strongly on their pelagic larval phase.

Organisms with complex life cycles

Many marine species have a complex life cycle with an adult benthic phase and a pelagic dispersive larval phase. For these species, dispersal ability depends on the dispersive larvae which determine the number of propagules colonising the benthic habitat (Simberloff 2009). Larvae of benthic invertebrates play therefore an important role in population dynamics by driving the connectivity among local populations (Cowen and Sponaugle 2009a), and persistence of populations after environmental disturbances (Cowen and Sponaugle 2009a; Pineda et al. 2009; Giménez et al. 2020a). Larvae are often more vulnerable to variations in environmental drivers (Harvey et al. 2013; Przeslawski et al. 2015; Pandori and Sorte 2019a) than juveniles and adults, but their tolerances to variations also change during ontogeny. This is particularly relevant in the context of climate change because larval responses to temperature and other drivers could drive most of the environmental responses exhibited by benthic invertebrates. Larvae are also subject to high predation risks and overdrift, that may have negative consequences for larvae settling in unsuitable environments.

In this thesis I focused on crustacean larvae given the above- mentioned environmental role and their sensitivity to environmental drivers. Pelagic larvae can either actively feed (planktotrophy) or consume their reserves accumulated in eggs (lecitotrophy) or in previous stages (endotrophy) (Anger 2001a). Crustacean larvae grow by species- specific number of successive moults which is also dependent on environmental conditions (Olesen 2018; Zeng et al. 2020; Möller et al. 2020). In the case of decapod crustaceans, zoeae metamorphose into a semi-benthic megalopa with the concomitant

settlement in the benthos (Forward et al. 2001; Gebauer et al. 2020); it is in the benthos where the megalopa undergoes further metamorphosis into the juvenile, and juveniles experience further metamorphosis into adults where reproduction occurs (Anger 2001a; Anger et al. 2020). The different changes highlighted along ontogeny represent different challenges that the organisms face at the moment of adapting to the different environments, failing to adapt to any of the environments may cause the collapse of a population.

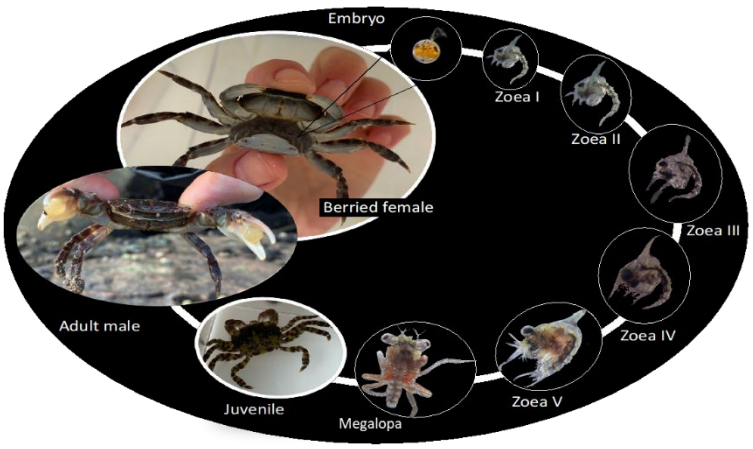

Figure 1. Life cycle of the crab species *Hemigrapsus sanguineus* as an example of complex life cycle. The pelagic phase is determined by the number of zoeal stages which is species-specific. This phase is followed by the semi-benthic phase, the megalopa which settles in the intertidal zone. The megalopa undergoes further metamorphosis in the benthos, into juveniles and adults.

Decapod crustacean larvae are excellent models to study variations in environmental conditions as they respond to many environmental drivers such as light, temperature, salinity, and food concentration among others (Forward et al. 2001; Anger 2006; Epifanio and Cohen 2016; Torres and Giménez 2020a; Cohen and Epifanio 2020; Giménez 2020). The study of larval development in decapod crustaceans has allowed for a better understanding of invertebrate responses to the combination of different environmental drivers, dispersal potential under different scenarios, population connectivity, and invasion biology; but it has also allowed a better understanding of ecophysiological process and adaptive mechanisms to environmental fluctuations under a warming scenario (Anger 2006; Torres et al. 2019; Giménez 2020; Anger et al. 2020).

In crustaceans like in many other invertebrates, processes occurring in one stage that affect survival and/or growth may have an impact on the performance of the subsequent life stages. For example, in some crustacean species the osmotic conditions experienced during embryogenesis determine the size of larvae at hatching (Giménez and Anger 2001a). Larvae of some species can also take alternative pathways during development, which vary in the number of stages and leading to

differences in the size and body mass at metamorphosis (Pestana and Ostrensky 1995; Giménez and Torres 2002). Which path to take depends on the initial larval biomass (Giménez and Torres 2002), food and osmotic conditions (Giménez and Torres 2002; Giménez and Anger 2003b). Developing through one or another pathway also has consequences for juvenile development; for instance, in the estuarine crab *Neohelice granulata*, juveniles originated from the short pathway were able to compensate the effects of the pre-settlement conditions (Giménez et al. 2004). Traits at metamorphosis into the benthic habitat have effects in the post-metamorphic life stages and in turn in recruitment success (Pechenik 2006a; Marshall et al. 2008; Torres et al. 2016b). Larvae metamorphosing with larger body mass have been shown to perform better in the juvenile phase (Pechenik 1999a; Giménez and Anger 2006), body mass is a plastic trait that is determined by duration of development and growth rates (Gotthard and Nylin 1995; Hentschel and Emlet 2000). In order to achieve larger body masses, organisms need longer developmental time spending more time in the planktonic environment with the consequential higher predation risk (Eckert 2003). In seasonal environments, extending the duration of development may have consequences in the environmental conditions the juveniles will experience later in the season (Jarrett 2003).

Gaps in knowledge

Despite all the information available regarding global change and its effect on the ocean, little is known about the responses of early life stages of organisms with complex life cycles that use the planktonic larval stages as the dispersive phase. Larvae may play a pivotal role in the process of invasion in new habitats, contributing to the propagule pressure (Simberloff 2009), and still we lack information on the responses of invasives relative to its native counterparts.

In this thesis I address the question of the role of effects of climate- driven environmental variables (environmental drivers) in larval responses, with special focus on the effect of higher temperatures in combination with other drivers. For this purpose, I used larvae of three coastal crab species (*Carcinus maenas*, *Hemigrapsus sanguineus* and *Hemigrapsus takanoi*) as model system (details on biology and distribution in section 5). These are coastal- estuarine species with wide distributions along the North Atlantic, North and Baltic Sea with different populations exposed to different conditions of temperature and salinity. While *C. maenas* is native to Europe, *H. sanguineus* and *H. takanoi* are non-native and coexist with *C. maenas* in the adult habitat.

There are two central points that need to be addressed:

1. The need to perform comparisons between native and non-native species in the responses to multiple environmental drivers (e.g. temperature, salinity, and food limitation). This is relevant to better understand the dynamics of species interactions and the process of invasion.

2. The need to quantify larval responses to temperature and other factors across the distribution range. This is based on recent evidence showing that species distributed across wide spatial scales may show different responses to the combined effects of environmental drivers due to local adaptation or phenotypic plasticity (de Villemereuil et al. 2016). The quantification of the different responses of the different populations to anthropogenic drivers may allow us to start to understand the effect of global change on evolutionary and physiological effects. Tolerance of larvae to changes in environmental drivers may be different between population depending on the distance to the distribution limits (Šargač et al. 2021b), tolerance of the larval stages may set limits to the distribution and / or range expansion of populations towards the poles, but also plasticity in the tolerance to cold conditions and ocean warming in the responses may allow range expansion (Carbonell et al. 2021b). In this regard, we do not have information on the thermal range in terms of survival and growth of the native species *C. maenas*, a species that due to ocean warming is expanding towards the north of the Atlantic Ocean.

This thesis is structured as follows: the next section of this chapter gives details on the model species and section 6 describes the objectives and gives a summary of the subsequent six chapters. Chapter 7 constitutes the general discussion.

Model species

This thesis uses decapod crustaceans as model organisms to study the responses to changing environmental drivers. Decapod crustaceans are one of the most diverse group of organisms, that are able to occupy different positions in the ecosystems during their different life stages (Anger et al. 2015; Olesen 2018; Möller et al. 2020). The species used in the thesis (presented below) are important benthic predators and omnivores, shaping benthic communities through trophic and horizontal interactions (Epifanio 2013; Geburzi et al. 2018; Young and Elliott 2020a).

a. Hemigrapsus sanguineus and Hemigrapsus takanoi

The Asian shore crab, *Hemigrapsus sanguineus* and the Japanese brush-clawed shore crab, *Hemigrapsus takanoi* are both native to east Asia, in particular to coastal waters of the Northwest Pacific (Takahashi et al. 1985; Fukui et al. 1989; Hwang et al. 1993; Asakura and Watanabe 2005; Yamasaki et al. 2011; Lee et al. 2013). Both species are common and abundant in intertidal zones . (Kikuchi et al. 1981; Fukui 1988; Saigusa and Kawagoye 1997; Asakura and Watanabe 2005; Marin 2013)

H. sanguineus invaded and established populations in the coasts of North America, North Europe, and the Adriatic and Black Seas (Schubart 2003; Micu et al. 2010), via ballast water (McDermott 1998b). In North America, it was first recorded in Delaware Bay in 1988 (Williams and

McDermot 1990), and it rapidly expanded northward to Schoodic Point in Maine and southward to Beaufort in North Carolina (McDermott 2000; Friedlander 2002; Delaney et al. 2008). Further expansion towards the north is determined by the summer temperature tolerance of the early stages (lower limit: 12 - 15 °C Epifanio 1998; Stephenson et al. 2009). In Europe, it was first introduced along the Dutch Delta system in 1999 via ballast water (Breton et al. 2002) and then spread northward in the North Sea and Scandinavia (Wolff 2005; Kerckhof et al. 2007; Dauvin and Dufossé 2011; Gittenberg et al. 2010; Gothland et al. 2013; Seeley et al. 2015; Karlsson et al. 2019) and southward to the coast of France (54° - 49 °N). Occasional observations have been reported for *H. sanguineus* in the Mediterranean and the Black Sea (Schubart 2003; Ben Souissi et al. 2004; Micu et al 2010; Ounifi-Ben Amor et al. 2015, 2016; GBIF.org 2023).

H. takanoi is also a successful invader in Europe, it was first recorded in 1993 in Bremerhaven, northern Germany (Gollasch 1998), in 1994 a reproducing population was found at La Rochelle in France (Noél et al. 1997). *H. takanoi* continued expanding and in 1996 it was reported from northern Spain to the Loire estuary (Noél et al. 1997; Noél and Gruet 2008) and in 1997 it was reported in the French coasts of Le Havre and along the English Channel (Gérard Breton et al. 2002; Dauvin et al. 2009; Dauvin 2009; Dauvin and Delhay 2010). *H. takanoi* was first reported in the North Sea in 2000 (Wolff 1998; Nijland and Beekman 2000) and expanded towards the Wadden Sea in later years (Obert et al. 2007; Gittenberger et al. 2010), and towards the Baltic Sea in 2014 and southern Great Britain in 2015 (Geburzi et al. 2015a; Wood et al. 2015; Ashelby et al. 2017). Ongoing expansion has been suggested as single individuals have been found on the coast of Sweden (Karlsson et al. 2019a).

As many other brachyuran crab species, *H. sanguineus* and *H. takanoi* have complex life-cycles comprised of a larval pelagic phase and benthic juvenile and adult phases. The duration of the reproductive season varies with latitude and depends on temperature in their native populations, lasting from 3 - 8 months, commonly from April to September (Takahashi et al. 1985; Fukui 1988; Miyajima et al. 2012; Miyajima and Wada 2017). In its invasive range in North America and Europe, the reproductive season of *H. sanguineus* was also reported to be in the warm months, May to September (McDermott 1999; Stephenson et al. 2009; Giménez et al. 2020a); for *H. takanoi*, in its invasive European population the same is true (van den Brink et al. 2012). The pelagic larval phase is comprised of 5 zoeal stages (Epifanio et al. 1998a; Epifanio 2013; Landeira et al. 2019). Duration of development depends on temperature among other environmental variables, mean duration of development from hatching to megalopa in *H. sanguineus* native range was reported to be 34 days at 22 °C (Kurata 1968) and 18 days at 25 °C (Hwang et al. 1993). In a study in a population in its invasive range in USA, duration of development from hatching to megalopa was 16 days at optimum temperature conditions (25 °C) and in the salinity range 20 - 30 ‰ and it extended at lower temperatures and salinity combinations. Larval survival was maximum at the combinations of high temperature and high salinity and at lower salinities it was determined by the temperature (Epifanio et al. 1998a). In the case of *H.*

takanoi survival to the megalopa stage in its native range is possible at salinities as low as 10 ‰ (Mingkid et al. 2006a), while in the invaded Baltic Sea survival to megalopa was only possible at salinities ≥ 20 ‰, and in the combination of 19 °C and 16 ‰ some larvae metamorphosed to megalopa and duration of development was only dependent on temperature (Nour et al. 2021a, 2022a). In both species, the pelagic larval phase is followed by a first metamorphosis to the megalopa, which settles in the benthos approximately between the end of July - end of August. For both species, the megalopa stage appears to be more sensitive to environmental drivers as in the case of *H. sanguineus* no survival to the juvenile stage was possible at temperatures lower than 20 °C and salinities lower than 25 ‰ (Epifanio et al. 1998a), and in the case of *H. takanoi* no survival to the first juvenile crab was found at 15 ‰ (Nour et al. 2021a).

Both *Hemigrapsus* spp show life-histories and ecological features that have probably helped in the process of invasion and establishment in novel environments (Geburzi and McCarthy 2018). They are diet generalist, predating and affecting populations of species in the invaded environment (Lohrer and Whitlatch 2002; Griffen and Byers 2009). Females have high fecundity rates, producing several broods per year in long spawning seasons (Fukui 1988; McDermott 1999; Gothland et al. 2014). Adults show broad salinity and temperature tolerances (Epifanio et al. 1998a; Lohrer et al. 2000; Nour et al. 2022a). They also have higher competitive abilities than its native competitors for food and space (Steinberg and Epifanio 2011; Geburzi et al. 2018)), lower number of parasites (Takahashi et al. 1997; McDermott 2011), and high dispersal potential (Hwang et al. 1993; Gothland et al. 2014).

b. Carcinus maenas

The European shore crab *Carcinus maenas* is native to the northeast Atlantic coast, from northern Africa to Norway, the British Isles and Iceland (Elner 1980; Carlton and Cohen 2003a; Roman and Palumbi 2004b; Darling et al. 2008). It is a global invader, and it has successfully established populations in all continents except Antarctica (Carlton and Cohen 2003a; Grosholz 2005; Young and Elliott 2020a); it is listed as one of the 100 worst invader species (Lowe et al. 2000). In its native and invaded ranges, it inhabits different hard and soft substrates, in the intertidal and subtidal zones, it is also found in brackish habitats like estuaries and other habitats as saltmarshes (Wenner 1985; Cohen et al. 1995a; Grosholz and Ruiz 1996; Jensen et al. 2002).

C. maenas has also a biphasic life-cycle, comprised of a planktonic larval phase and benthic juveniles and adults. The reproductive season usually lasts for some months (usually between May to September, in its native range in Europe) depending on the population (Baeta et al. 2005; Young and Elliott 2020a). The planktonic phase consists of four zoeal stages (Dawirs 1985b; Epifanio and Cohen 2016; Spitzner et al. 2019a; Šargač et al. 2021b, 2022a). Larvae can survive and successfully develop to megalopa under a wide range of temperature conditions, but development from hatching to megalopa is determined by environmental temperature and complete development can only occur at temperatures 9 °C (Dawirs 1985b; Dawirs et al. 1986). Larvae also tolerate wide ranges of salinity (10 - 44 ‰) for short periods and tolerance to low salinities increases with successive larval stages(Anger et al. 1998; Cieluch 2004); however, metamorphosis to megalopa is not possible if larvae are exposed chronically to salinities below 20 ‰ (Spitzner et al. 2019a). *C. maenas* larvae show an antagonistic response to the combined effects of high temperature and low salinities where the negative effects of low salinity are mitigated at high temperatures, termed "Thermal Mitigation of Low Salinity Stress" (TMLS) (Spitzner et al. 2019a; Šargač et al. 2022a), but the survival rates, developmental times and magnitude of the TMLS may vary within and among populations (Spitzner et al. 2019a; Torres et al. 2020a; Šargač et al. 2021b, 2022a). The pelagic larval phase is followed by the settling megalopa in a (first) metamorphosis, the megalopa settles in the benthic habitat and metamorphoses to the juvenile which undergoes subsequent moults until the adult stages(Moksnes 2002; Young and Elliott 2020a). In the case of *C. maenas*, the megalopa, juvenile and adult stages can tolerate greater salinity variations than larvae as they are able to osmoregulate (Cieluch 2004); however, larval stages are more tolerant to temperature changes than adults (Nagaraj 1993; deRivera et al. 2005).

Figure 2. Model species: *Hemigrapsus sanguineus* (adult male, left panel), *Hemigrapsus takanoi* (berried female, middle panel) and *Carcinus maenas* (adult male, right panel). Photos: *H. sanguineus* and *C. maenas*: Noé Espinosa-Novo; *H. takanoi*: Jan Phillipp Geißel.

Thesis objectives

The general objective of this thesis was to quantify the effects of environmental drivers in larval responses, with special focus on the effect of higher temperatures in combination with other environmental drivers. The focus was on larval survival and the integrated responses of development and growth rates (details given in the chapters) which, for benthic organisms, determine post-metamorphic survival (Giménez et al. 2004; Giménez 2010; Torres et al. 2016b). I conducted multiple drivers experiments, rearing larvae from hatching to metamorphosis to megalopa at different combinations of temperature and other environmental drivers (salinity and food limitation) following standard larval rearing procedures (Torres et al. 2021d).

In **Chapter 2** I focused on quantifying the combined effects of temperature and food limitation on performance and survival of larval stages of the invasive *H. sanguineus* in a local population in the German Bight (North Sea, Germany). I quantified the correlated responses of growth and development, driving size and reserves at metamorphosis and I compared the responses of the invasive *H. sanguineus* with results reported for a co-occurring population of native *C. maenas* under comparable laboratory conditions.

In **Chapter 3** the focus was on proposing a framework to understand and quantify responses to fluctuations in one or more climate-driven environmental variables, considering the importance of biological time as biological time may mediate the responses of organisms to fluctuations in the environment.

In **Chapter 4** covers the responses of different species to the combined effects of temperature and salinity. I quantified the responses of larval stages of *Hemigrapsus sanguineus* to different combinations of temperature and salinity and I reported the responses in terms of survival, development, and growth rates. I also compared the responses to those reported for the native European shore crab *Carcinus maenas* (Šargač et al. 2021b) and two other invasive species in Europe *Hemigrapsus takanoi* (Geißel et al. in prep) and *Eriocheir sinensis* (Anger 1991), obtained from experiments carried out under comparable conditions.

The objective of **Chapter 5** was to determine the combination of temperature and salinity regimes that allows for larval survival and development in different populations of H. takanoi, invasive in Europe, and quantify interactive effects of temperature and salinity at thermal regimes that are expected from future climate change. We also focused on determining the genetic structure of the populations, to quantify the genetic variability accounted for the degree of differentiation among the populations under study. Furthermore, this study should provide information on the possible limits of the range expansion of *H. takanoi* into the Baltic Sea.

In **Chapter 6**, I quantified the thermal tolerance of larval stages of the shore crab *C. maenas*, over a wide latitudinal range, by comparing 3 populations: one located in the north of Spain and two

Norwegian populations. The objectives of this study were to quantify the effect of temperature on larval survival, development, and growth for the three populations and to use data available from the German Bight (Helgoland, North Sea) and Cádiz (south of Spain) (Šargač et al. 2022a) to obtain a more comprehensive evaluation of the role of temperature on larvae of *C. maenas* along the European coast (35 °N - 65 °N latitude).

Publications and manuscripts

This thesis is based on the following papers:

Paper 1 (Chapter 2): **Espinosa-Novo, N.**, Giménez, L., Boersma, M. & Torres, G. On their way to the north: larval performance of *Hemigrapsus sanguineus* invasive on the European coast—a comparison with the native European population of Carcinus maenas. Biol Invasions 25, 3119–3136 (2023). https://doi.org/10.1007/s10530-023-03095-3

Paper 2 (Chapter 3): Gimenez Noya, L., **Espinosa-Novo, N.**, & Torres, G. (2022). A framework to understand the role of biological time in responses to fluctuating climate drivers. Scientific Reports, 12(1), [10429]. https://doi.org/10.1038/s41598-022-13603-5

Paper 3 (Chapter 4): **Espinosa-Novo, N.**, Giménez, L., Geißel, JP, Boersma, M. & Torres, G (2023). How to succeed in novel habitats? Invasive *Hemigrapsus sanguineus* as a case study of wide tolerance to temperature and salinity: comparisons with other invasive species and the native European species *Carcinus maenas*. In prep.

Paper 4 (Chapter 5): Geißel, JP., **Espinosa-Novo, N.**, Giménez, L., Ewers, C., Cornelius, A., Martínez-Alarcón, D., Harzsch, S. & Torres, G. Salinity and temperature during larval development determine the potential for range expansion into the Baltic Sea of the invasive crab *Hemigrapsus takanoi.* Submitted to Biological Invasions.

Paper 5 (Chapter 6): Geißel, JP. *, **Espinosa-Novo, N. ***, Giménez, L., Aberle N., van der Meer, G., Harzsch, S., Boersma, M., Torres, G. Variations in larval responses to temperature at the continental European scale in a global invader at home: the shore crab *Carcinus maenas*. In prep.

***shared first authorship**

My contribution to the papers:

Paper 1: I conceived the scientific idea and experimental design together with GT and LG. I performed the experiments with GT and I analysed the data with LG. I wrote the first draft of the manuscript and all the authors contributed to the writing of the manuscript and gave final approval for publication.

Paper 2: LG and GT developed the concept. GT and I performed the laboratory experiments. LG developed the mathematical equations and simulations. All the authors analysed the data. LG wrote the original draft. LG, GT and I edited the paper and improved it for publication.

Paper 3: I conceived the scientific idea and experimental design together with GT and LG. I performed the experiments with GT and JPG, and I analysed the data with LG and JPG. I wrote the first draft of the manuscript and all the authors contributed to the writing and improving of the manuscript.

Paper 4: JPG, NE-N, SH, LG, and GT conceived the experimental design. JPG, GT, CE, and AC collected the ovigerous females. JPG performed the experiments. JPG, NE-N, CE, DMA, and GT generated the data for the population genetics analyses. JPG, NE-N, CE, and LG analysed the data. JPG wrote the first draft. All authors contributed to the writing of the manuscript and gave final approval for publication.

Paper 5: JPG, NE-N, SH, LG, and GT conceived the experimental design. NE-N and GT collected the females in Spain. JPG, GT, LG, NA and GvdM collected the females in Norway. JPG, NE-N and GT performed the laboratory experiments. LG, JPG and I analysed the data. JPG and I wrote the first draft of the manuscript and all the authors contributed to the writing and improving of the manuscript.

CHAPTER 2

On their way to the north: larval performance of *Hemigrapsus sanguineus* invasive to the European coast- a comparison with the native European population of *Carcinus maenas*

Espinosa-Novo, N., Giménez, L., Boersma, M., Torres, G.

Biol Invasions
https://doi.org/10.1007/s10530-023-03095-3

ORIGINAL PAPER

On their way to the north: larval performance of *Hemigrapsus sanguineus* invasive to the European coast—a comparison with the native European population of *Carcinus maenas*

Noé Espinosa-Novo · Luis Giménez · Maarten Boersma · Gabriela Torres

Received: 3 March 2023 / Accepted: 21 May 2023

Abstract The Asian shore crab *Hemigrapsus sanguineus* has become invasive in North Europe and it co-occurs and competes with the native European shore crab *Carcinus maenas*. Both species develop through a feeding and dispersive larval phase characterised by several zoeal and a settling megalopa stage. Larvae of marine crabs are vulnerable to food limitation and warming has the potential to exacerbate the negative effects of food limitation on survival and growth. We quantified the combined effects of temperature and food limitation on larval performance (survival and growth) of *H. sanguineus* and we compared our results with those reported on performance of *C. maenas* larvae, under the same experimental design and methodology. Larvae from four females of *H. sanguineus* collected on Helgoland (North Sea) were experimentally reared from hatching to megalopa, at four temperatures (range 15–24 °C) and two food conditions (permanent vs. daily limited access to food). Larval survival of *H. sanguineus* was low at 15 °C and increased with temperature, in contrast to the high survival reported for *C. maenas* larvae in the range 15–24 °C. Food limitation reduced survival and body mass of *H. sanguineus* larvae at all temperatures, but without evidence of the exacerbating effect caused by high temperatures and reported for *C. maenas*. By contrast, high temperature (24 °C) mitigated the negative effect of food limitation on body mass on *H. sanguineus* larvae. Advantages of *H. sanguineus* over *C. maenas* appear especially under the increased temperatures expected from climate change.

Keywords Food limitation · Warming · Invasive versus native crabs · Survival · Growth rates

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10530-023-03095-3.

N. Espinosa-Novo (✉) · L. Giménez · M. Boersma · G. Torres
Alfred-Wegener-Institut Helmholtz-Zentrum für Polar- und Meeresforschung, Biologische Anstalt Helgoland, Ostkaje 1118, 27498 Helgoland, Germany
e-mail: Noe.Espinosa@awi.de

L. Giménez
School of Ocean Sciences, Bangor University, Menai Bridge LL59 5AB, UK

M. Boersma
University of Bremen, Bremen, Germany

Introduction

Increasing trade and travel in the last decades has led to biological invasions, i.e. the dispersion and introduction of many species to geographical areas located outside their native range (Hulmen and Weser 2011; Bailey et al. 2020). Dispersion of organisms due to anthropogenic activities occurs through shipping (ballast water, fouling), construction of corridors, aquaculture, fisheries, and food trade (Carlton 2002; Hulme et al. 2008; Molnar et al. 2008; Katsanevakis

Published online: 08 June 2023

et al. 2013). The introduction of a new species can lead to the decline in native species richness and abundances, loss of genetic variation in the native community, and an increase in the homogeneity of the invaded communities (Rahel 2000; Pyšek et al. 2012; Geburzi et al. 2018). Whenever the "introduced" species causes an impact on the host ecosystem, it is considered "invasive". Biological invasions are one of the most important threats to biodiversity after changes in land and sea use, animal exploitation, climate change, and pollution (Diaz et al. 2019; IPCC 2019). Invasive species affect the dynamics of native communities at different scales (Hulme 2017), by means of different interactions including competition, predation, and introduction of new diseases (Ruiz et al. 2000; Sakai et al. 2001; Jeschke et al. 2012). Biological invasions have been reported for most marine and estuarine habitats in the world (Katsanevakis et al. 2013; Chan and Briski 2017; Pyšek et al. 2020). Many hypotheses have been proposed to explain biological invasions and the success of the invaders (Simberloff and Von Holle 1999; Gurevitch et al. 2011; Kelley 2014), yet there is still little information on the processes involved in dispersion and establishment of alien species in novel habitats (Bailey et al. 2020; Rato et al. 2021).

Global climate change is causing an increase in the number and the impact of biological invasions (Hulme 2017; González-Ortegón et al. 2020; Pyšek et al. 2020), because of the steady increase in temperature (IPCC 2021), and the increased frequency of extreme events (e.g. heatwaves, Meehl and Tebaldi 2004; Smale et al. 2019). These changes combined with other human activities can act together to help the dispersal and establishment of new species. For example, the oyster *Crassostrea gigas* was first introduced in the North Sea for aquaculture purposes and was able to establish there due to above-average summer temperatures that helped the species to spread (Diederich et al. 2004; Smaal et al. 2009). Global warming has also led to the poleward expansion of many species (Sorte et al. 2010; Poloczanska et al. 2013; Giménez et al. 2020).

Global warming could affect both invasive species and their native competitors in different ways. How different species respond to changes in environmental variables (e.g. temperature) will ultimately determine individual performance and species interactions. Increases in temperature cause rises in metabolic demands, which should be met by food supply (Gillooly et al. 2001; Somero 2002). However, for organisms living in the marine pelagic realm, where the food distribution is patchy (Paffenhöfer et al. 1987; McManus et al. 2003) increased temperature combined with food limitation could impair growth and survival. The combined effect of food limitation and increased temperature may particularly impact life history stages characterised by high growth rates (Foster and Hirst 2012; Otto et al. 2020). Crustacean larvae are an example where high growth rates are sustained by high feeding rates, which in turn demand higher food availability (Anger 2001). In such case, one would expect that increased temperatures combined with limited access to food may cause reductions in growth rate and survival (Torres and Giménez 2020). However, the nature of the effect could vary between native and exotic competitors because of differences in thermal optimum ranges (Griffith et al. 2021). Hence, a critical question to understand current invasion and future population spread is how increased temperature combined with food limitation drive the performance of both native and exotic species.

The Asian shore crab *Hemigrapsus sanguineus* is native to the east coast of Asia (20–50° N) (Takahashi et al. 1985; Fukui et al. 1989; Hwang et al. 1993). It is one of the most abundant crab species on rocky beaches and occupies the upper and middle intertidal zones (Kikuchi et al. 1981; Fukui 1988). It has successfully invaded the coasts of North America, North Europe, and the Adriatic and Black Seas (Schubart 2003; Micu et al. 2010) via ballast water (Ai-yun and Yang 1991; Kraemer et al. 2007; Epifanio 2013). In northern Europe, *H. sanguineus* was first recorded in France and the Dutch Delta system in 1999 (Breton et al. 2002) and then spread to the North Sea and Scandinavia (Wolff 2005; Kerckhof et al. 2007; Dauvin and Dufossé 2011; Gittenberg et al. 2010; Gothland et al. 2013; Seeley et al. 2015; Karlsson et al. 2019). *H. sanguineus* was occasionally found in the Mediterranean and Black Sea (Schubart 2003; Ben Souissi et al. 2004; Micu et al 2010; Ounifi-Ben Amor et al. 2015, 2016; GBIF.org 2023). There are several factors likely to drive or limit the expansion of *H. sanguineus*, including the presence of congeneric competitors (e.g. US North Pacific, Steinberg and Epifanio 2011, Lord and Williams 2017) and low summer temperatures (Stephenson et al. 2009).

In the Atlantic coast of North America, the northern distribution limit of *H. sanguineus* is determined by the larval thermal tolerance to low temperatures (Epifanio et al. 1998; Stephenson et al. 2009). Hence, as a result of warming, the distribution of *H. sanguineus* is likely to expand further north (Epifanio 2013; Giménez et al. 2020). *H. sanguineus* co-occurs and competes with the shore crab *Carcinus maenas* in Europe and in North America. Both species overlap in diet and habitat use, with juvenile/adults of *H. sanguineus* outcompeting *C. maenas* in the use of space and resources. *H. sanguineus* also predates on *C. maenas* juveniles affecting their recruitment success (Lohrer and Whitlach 2002; Jensen et al. 2002; Geburzi et al. 2018). For example, in Southern New England *H. sanguineus* has significantly reduced the recruitment of *C. maenas* due to direct predation, leading to a decline in densities by 40–90% (Lohrer and Whitlatch 2002). In addition, when in sympatry individuals of *C. maenas* migrate towards the subtidal zone (Geburzi et al. 2018). The above-mentioned factors, help explain the displacement of *C. maenas* from environments where it was previously more abundant.

We compared the performance of *H. sanguineus* and *C. maenas* from the perspective of the larval phase for co-occurring populations of the German Bight (Helgoland, North Sea). We focus on larvae because larval survival and recruitment are critical contributors to the propagule pressure by *H. sanguineus* (Simberloff 2009) and for the persistence of populations of *C. maenas*. Propagule pressure, i.e. a group of individuals of a species arriving in a region to which they are not native, drives the establishment and spreading of invasive populations (Simberloff 2009). Marine larvae in particular, tend to be more sensitive to environmental fluctuations than juveniles or adults (Pandori and Sorte 2019) and larval survival is central to the recovery of populations after environmental disturbances (Cowen and Sponaugle 2009; Pineda et al. 2009; Giménez et al. 2020). In particular, differences in larval survival among co-occurring species may affect the balance of competition, either exacerbating or counteracting the outcome. In theory, counteracting effects may occur in cases of trade-offs between competition and dispersal abilities (Seifan et al. 2013). Because *H. sanguineus* was first reported in the German Bight very recently (2008- Scrosati et al. 2011, 2009- Jungblut et al. 2017), it is not clear yet whether *H. sanguineus* would be able to outcompete *C. maenas* at that local habitat. Unlike *C. maenas*, larvae of *H. sanguineus* cannot develop at temperatures below 13–15 °C (Epifanio 2013) which characterise the spring and early summers in the German Bight and coastal North Sea (Giménez et al. 2020). For *H. sanguineus*, larvae appear to be released when the temperature surpasses 15 °C which is early/ mid- June depending on the year (Giménez et al. 2020). In the case of *C. maenas*, larval release starts in May (Harms et al. 1994), but the full larval season of both species partially overlap. We know that under food limitation, zoea I of *H. sanguineus* is more tolerant to short thermal fluctuations than *C. maenas* (Giménez et al. 2021). However, over the entire larval phase and under low temperatures, food limitation may produce a stronger negative effect on survival of *H. sanguineus* than that observed in *C. maenas*.

Here we compared the responses of larvae of *Hemigrapsus sanguineus* and *Carcinus maenas* to food limitation under increased temperature, reared in comparable experimental conditions. We first quantified the combined effects of temperature and food limitation on survival and performance of larvae of *H. sanguineus.* For the first time, we documented growth and survival responses in any population of this species to food limitation over a wide range of temperatures. In particular, we quantified the correlated responses of growth and development, driving size, and reserves at metamorphosis, which for benthic invertebrates, are known to drive the performance of the post-metamorphic stages in the benthic habitat (Giménez et al. 2004; Pechenik 2006; Giménez 2010; Torres et al. 2016). Second, we compared our results on *H. sanguineus* with those obtained by Torres and Giménez (2020) for a co-occurring population of *C. maenas*.

Materials and methods

Animal husbandry, larval rearing and experimental design

Berried females of *H. sanguineus* (carapace width 15.8–17.2 mm) were collected on the island of Helgoland (North Sea, German Bight, 54° 10′ 40.9″ N 7° 53′ 32.4″ E) during their reproductive season (July–September) and transported to the laboratory.

Females were kept individually in 2-L aquaria with UV-treated filtered (0.2 µm) seawater (32.5‰) permanently aerated, in a temperature-controlled room at 18 °C (±0.5 °C) with a 12:12 h light: dark cycle. Females were fed every 3 days with shrimps (*Crangon crangon*) and water was changed daily to ensure high water quality at hatching.

The experimental setup comprised a factorial design, in which we exposed larvae obtained from each given female to different combinations of temperatures and access to food (following Torres and Giménez 2020). This experimental procedure was repeated four times, i.e. once for each of the hatches obtained from four different females, in order to assess potential variations in responses driven by maternal influence. Freshly hatched zoeae were distributed in 8 treatments (4 replicate vessels per treatment, see below for more details), combination of 2 levels of daily access to food (6 or 24 h/day, provided ad libitum) and 4 temperatures (15, 18, 21 and 24 °C). The different temperatures were chosen based on its natural variability in the German Bight: 15 and 18 °C represent temperatures recorded during the larval season of *H. sanguineus* (Giménez et al. 2020); this corresponds to summer temperatures around the local population (Wiltshire and Manly 2004). Temperatures > 20 °C are expected as the consequence of steady warming due to climate change (Schrum et al. 2016), and as the consequence of the expected increment in the frequency of warm summers (Christidis et al. 2015).

Experiments were carried out in temperature-controlled rooms and using natural UV-treated filtered (0.2 µm) seawater. When hatching occurred, 50 larvae were sorted into each of 500 mL rearing vessels (4 replicate vessels per treatment for each of the four females) in UV-treated filtered seawater at the temperature of hatching. Freshly hatched *Artemia* sp. nauplii (Great Salt Lake Artemia, Sanders, USA) were provided as food for the larvae in densities of ~ 5 nauplii/mL (Torres et al. 2021). In the treatment of limited access to food, *Artemia* sp. nauplii were available for 6 h each day (between 9 a.m. and 3 p.m., following Giménez and Anger 2005; Torres and Giménez 2020). By contrast, in the treatment of permanent access to food, *Artemia* sp. nauplii were available all day. Water in all treatments was changed daily following standard procedures for larval rearing (Torres et al. 2021). During the daily water change, live larvae were staged and recorded, dead ones were also recorded and removed from the experiments; in addition, we checked that remaining food was present in each rearing vessel.

Body mass and elemental composition (carbon and nitrogen) were measured in freshly hatched larvae (3 replicates, 50 zoea each at the start of each experiment) and in freshly moulted megalopae (sampled within 24 h after moulting). Carbon content is used as a proxy for lipid reserves, used by crustacean larvae to sustain periods of food limitation (Dawirs et al. 1986; Anger and Harms 1990); nitrogen content is used as a proxy for protein content (Dawirs 1986; Dawirs et al. 1986). The number of individual megalopae sampled in each of the 4-replicate rearing vessel was on average 5 (Table S1). Larvae were pipetted onto a filter, rinsed with distilled water, gently blotted dry with filter paper, and stored in pre-weighed tin cartridges at − 20 °C for later analysis. To determine the dry weight, samples were freeze-dried for 48 h and weighed using a microbalance (Sartorius SC2, precision 1 µg). Carbon and nitrogen content were then determined using an elemental analyser (vario MICRO cube CHNS analyser, Elementar Analysensysteme).

Data analysis

The response variables were survival, duration of development, body mass, elemental composition (carbon and nitrogen), and instantaneous growth at the megalopa. Survival to each zoeal stage was calculated as the percentage of survivors in relation to the number of organisms at the start of each experiment. Duration of development was calculated as the time elapsed from hatching to reach each developmental stage. Growth rates were estimated as $G = \log(W_f/W_0)/t$. In this formula W_0 is the average mass (dry weight, carbon or nitrogen) at hatching, W_f is the corresponding mass of each individual megalopa collected in each rearing replicate and t is the corresponding duration of development of each individual megalopa.

Mixed modelling was carried out in R (function lme and gls from package nlme, Pinheiro et al. 2018, R Core Team 2013) to assess the responses to the different combinations of food availability and temperatures on survival and duration of development. The models contained temperature and food

availability as fixed factors and female of origin as a random factor. We performed backwards model selection (Zuur et al. 2009) in two steps. In a first step, we tested the random terms using restricted maximum likelihood (REML), we compared the different models through the corrected Akaike information criteria (AICc) and ranked them. The model with the lowest AICc was selected for further analysis. When the difference between two models was $\Delta AICc < 3$ and the most complex model had the lower AICc we applied hypothesis testing (likelihood ratio tests). When the models differed significantly ($p < 0.05$), we chose the model with the lowest AICc and when the difference was not significant we chose the simpler model (with the lowest number of parameters). In a second step, the fixed terms were analysed through maximum likelihood (ML). For dry mass, elemental composition, and growth rates at the megalopa we did not get sufficient data in the food limited treatment at 15 °C for female 4 (F4). We therefore, analysed the data using two different starting models: (1) considering all females but without 15 °C and (2) considering all treatment combinations but without F4.

Data for survival was analysed in the logistic (Warton and Hui 2011) and logarithmic scale; as a first step, proportions (p) were re-scaled using the formula $p' = [p\ (50–1) + 0.5]/50$, to avoid inconsistencies associated to log (0) values. Logarithmically-transformed proportions were used to test the multiplicative model, whereby temperature and food limitation would act independently on the survival rates (Piggot et al. 2015). Survival responses consistent with a multiplicative model cannot be tested when the proportions are expressed in the logistic scale (Torres and Giménez 2020). Duration of development was analysed in the raw and the logarithmical scale to test if the effects were additive, multiplicative or interactive. Body mass and elemental composition were analysed in the raw scale. Tukey´s honestly significant difference tests (Tukey´s HSD) were performed to test differences among the different treatments.

Comparison performance *H. sanguineus* and *C. maenas*

We compared the performance of *H. sanguineus* and *C. maenas* at different temperatures and food conditions through two means. First, we compared the integrative response of body mass and developmental time. We calculated the ratios between the body mass under food limitation and those observed under permanent access to food for each temperature; the same calculation was made for duration of development. For comparison, these standardised values were plotted against those of *Carcinus maenas*. In this plot, the unit corresponds to the values of body mass (and duration of development) under permanent access to food at each temperature.

We also calculated the ratios of survival (S_R) and growth rates (G_R) between species, i.e. as $SR = S_H/S_C$ and $G_R = G_H/G_C$, where S_H and S_C are the survival to the megalopa of *H. sanguineus* and *C. maenas*, respectively and G_H and G_C the respective growth rates (from hatching to megalopa). We calculated average ratios and used simulations (details in: Supplementary material, "Materials and methods" section. Data analysis: details on model simulation) to incorporate the intraspecific variation in survival and growth associated to variations within and among families. First, for each species, we simulated 1000 values of the survival and growth rates. Survival was simulated from the statistical model fitted to the responses of both species to the different combinations of temperature and access to food; i.e. there were 1000 values for each combination of species, response variable, temperature, and food condition. Growth was simulated from an additional statistical model which also considers survival as covariate; this model incorporates correlations between average survival and growth associated to female-to-female variation in larval performance. The data and models used for *C. maenas* correspond to Torres and Giménez (2020). The model used for survival of *H. sanguineus* was that of Table S2; for growth we fitted an additional model based on female-to-female averages (Supplementary material, Materials and methods section. Data analysis: details on model simulation). Simulations were performed using the function *simulate* of the package nlme (R Core Team 2013) applied to the best fitted models for each variable. Second, for each combination of temperature and food condition a pair of values of survival and growth was randomly sampled (function *sample,* R Core Team 2013) for each female of each species. The ratio of survival (or growth) was calculated between two randomly chosen females (one per species). This procedure was repeated 4000 times in order to obtain distributions of ratios of survival (and growth) for each combination

of temperature and food condition. We then calculated the average of four ratios, comparing the performance of four hatches of *H. sanguineus* and four of *C. maenas*; this calculation takes into account that larvae obtained from four different females were used for each study. In addition, this procedure to calculate the average maintained the correlation between survival and growth. Using the simulated ratios of survival and growth, we calculated the centroid and the 90% confidence ellipses which were plotted for each combination of temperature and food (see Fig. 5).

Results

We first describe the survival, development and growth rates for *Hemigrapsus sanguineus* and we compare them to the data from *Carcinus maenas* already published (Torres and Giménez 2020, PANGAEA: https://doi.org/10.1594/PANGAEA.918056). The graphs showing the results corresponding to already published data of *C. maenas*, are highlighted with a grey background in Figs. 1, 2 and 3.

Survival, development, and growth

Survival to megalopa in *H. sanguineus* decreased towards lower temperatures and under limited access to food (Fig. 1, left panel); while at 15 °C, food limitation reduced survival by 74%, at 24 °C the survival reduction was 35%. Best models retained food availability and temperature operating in additive or interactive ways depending on the stage (Table S3), but with variations among larvae from different females (Table S4). Survival to megalopa under limited access to food ranged from 0 to 74% depending on temperature and female of origin, with 0% survival occurring at 15 °C and 24 °C for female 3 (Fig. S5). The effects of temperature and limited access to food on survival to zoeae II to IV were small, but increased for survival to ZV and especially to the megalopa (Fig. S6). The sensitivity to temperature varied among larvae from different females; survival at 15 °C was consistently low (13.2%) but survival at 24 °C ranged from 19 to 92% (larvae under permanent access to food) depending on the female (Fig. S5).

The combined effects of temperature and food limitation on the overall survival to megalopa (Fig. 1, left panel) were consistent with a multiplicative model (additive model retained in the logarithmic scale: Table S3). The multiplicative model can be illustrated considering the combination of 24 °C and permanent access to food as the optimal condition. In this case, the observed proportion of survivors under the combination of two stressors experienced simultaneously (15 °C and limited access to food: 0.05) is close to that expected

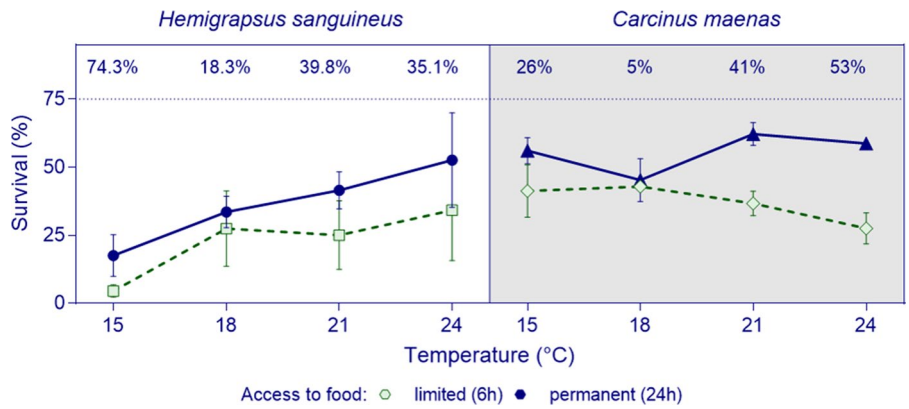

Fig. 1 *Hemigrapsus sanguineus* and *Carcinus maenas*. Average survival from hatching to megalopa as a response to temperature and food availability. Data shown as average values ± SE for the four females of origin. Permanent access to food: blue symbols and continuous line; limited access to food: green symbols with dashed line. Percentages on top (only when significantly different): percent difference in survival between permanent and limited access to food treatments for each temperature. Already published data for *Carcinus maenas* is presented in the right panel, grey background (Torres and Giménez 2020)

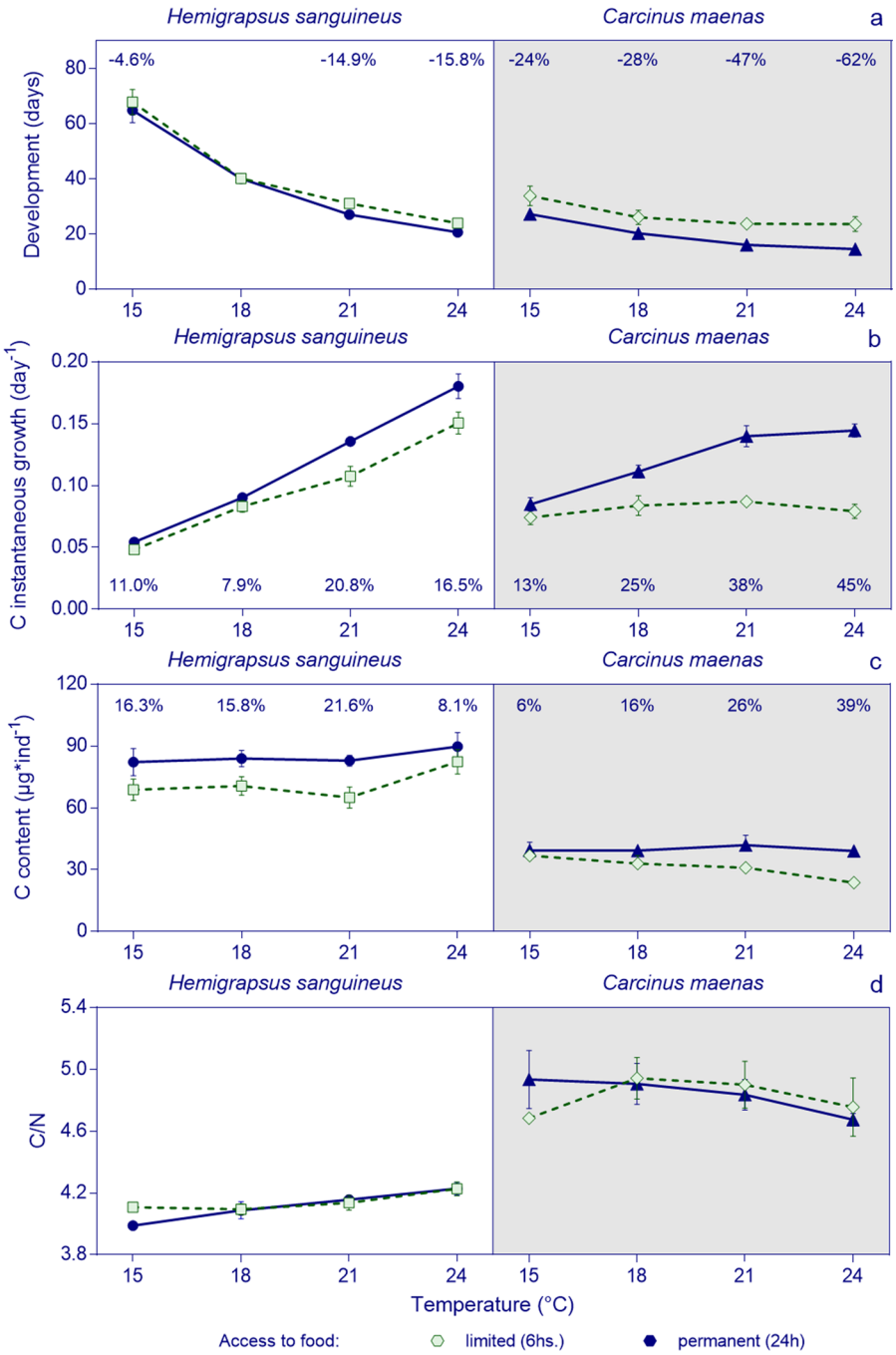

Fig. 2 *Hemigrapsus sanguineus* and *Carcinus maenas*. **a** Average duration of development. **b** Average growth rates. **c** Average carbon content. **d** Average C/N ratio. Data corresponds to the responses, from hatching to megalopa, to temperature and access to food. Data shown as average values ± SE. Symbols as in Fig. 1. Percentages on top or below (only when significantly different): percent difference in development time, C growth, C content, and C/N between permanent and limited access to food treatments for each temperature

by the product of the proportions observed when the stressors were experienced in isolation (limited access to food: 0.3; 15 °C: 0.2). On a female-by-female basis, the effects ranged from synergistic with a strong effect of temperature (F1 and F2) to antagonistic with a strong effect of limited access to food (F3 and F4).

The overall response of *H. sanguineus* was clearly different from that of *C. maenas* (Fig. 1). The increased survival of *H. sanguineus* with temperature irrespective of the food condition contrasts with the reduced survival of *C. maenas* under food limitation and high temperatures. The response of *H. sanguineus* to food limitation and temperature, consistent

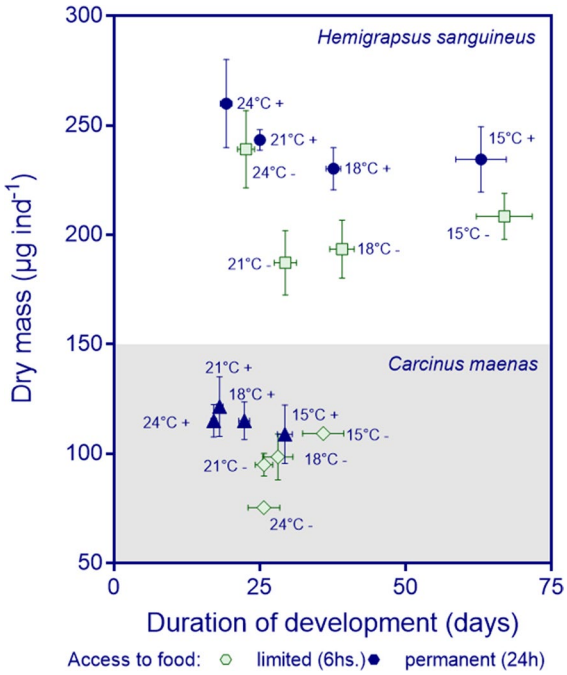

Fig. 3 *Hemigrapsus sanguineus* and *Carcinus maenas*. Integrated responses of body mass and duration of development under the different treatments of temperature and food availability. Data shown as average values ± SE for both variables. Symbols: permanent access to food is represented with blue symbols (indicated as '+') and limited access to food with green symbols (indicated as '−'), temperature is indicated in the graph next to the symbols

with a multiplicative model, also differs from the strong interactive effect shown by *C. maenas* where high temperatures exacerbated the effect of food limitation on survival (Fig. 1).

Duration of development to megalopa in *H. sanguineus* increased with decreasing temperatures in a non-linear pattern (Fig. 2a, left panel); the best model retained the interactive effect of temperature and limited access to food (Table S5). At low temperatures, the effects of food limitation were weak, producing a delay in the metamorphosis of fewer than 3 days (representing less than a 5% change between the two food conditions). However, at 21 and 24 °C the delay was longer than 3 days, representing 14–16% change (Fig. 2a, left panel). The effect of food limitation was weak at early stages and then it became stronger in the zoea IV (Fig. S7); best models retained temperature in development to stages ZII and ZIII, temperature and food availability operating in an additive way in development to ZIV, and interactively in development to ZV and megalopa (Table S5). In larvae from all females reared at 15 °C, there was an extra zoeal stage (zoea VI) regardless of food availability.

Carbon growth rates of *H. sanguineus* increased with increasing temperatures in both food conditions; food limitation caused a reduction in carbon growth rates, and the effect was stronger at higher temperatures (Fig. 2b, left panel); best models retained the interaction of food availability and temperature (Table S6). Similar effects were also found in terms of dry mass and nitrogen content (Fig. S8a, c). Exposure to food limitation resulted in a reduction in body mass, carbon and nitrogen content (Figs. 2c left panel, S8b, d), but the magnitude of the effect depended on temperature and varied among females. Best models retained food availability and temperature operating interactively (Table S6). Consistently for all females, the effect of food limitation on carbon content was strong in the range 15–21 °C as compared with 24 °C (e.g. 16% vs. 8% decrease in carbon content). Similar effects were found for dry mass and nitrogen content (Fig. S8b, d). Food limitation reduced carbon and nitrogen contents in similar proportions among temperatures resulting in comparable C/N ratios (Fig. 2d, left panel); the exception was 15 ºC where the reduction in nitrogen (19%) was higher than that of carbon (16%).

Duration of development and growth of *H. sanguineus* larvae were more affected by temperature which contrasts to those of *C. maenas*, that are more sensitive to food limitation (Fig. 2). In terms of duration of development, larvae of *H. sanguineus* were more sensitive to temperature than *C. maenas*, especially at low temperatures; at 15 °C *H. sanguineus* reaches the megalopa in ca 70 days while *C. maenas* needs ca 30 days (Fig. 2a). By contrast, limited access to food increased developmental time in a lesser extent in *H. sanguineus* (< 16% change between the two food conditions) than in *C. maenas* (> 20%). While growth rates of *H. sanguineus* increased with temperature irrespective of the food condition, those of *C. maenas* decreased with temperature but remained constantly low in the food limited treatment (Fig. 2b). *H. sanguineus* megalopa had higher carbon content and lower C/N ratios than *C. maenas* (Fig. 2d). The weakest effect of food limitation on carbon content found at the highest temperature in *H. sanguineus* contrasts to the pattern found in *C.*

maenas, where the effect was weakest at the lowest temperature (Fig. 2c).

Integrated growth responses to megalopa of *H. sanguineus* were characterised by a strong decrease in body mass under food limitation rather than a long delay in development. The delay in metamorphosis did not compensate the effects of food limitation on growth rates. At 24 °C and under permanent access to food larvae reached a maximum threshold of body mass (=260 µg/ind), and differences between food conditions were small (limited access to food=240 µg/ind). However, lower thresholds were reached at lower temperatures, especially under limited food availability (Fig. 3, upper side of graph). In addition, *H. sanguineus* larvae did not compensate for the effect of low temperature on body mass even under permanent access to food (Fig. 3, upper side of graph). Similar patterns characterised the integrated responses in terms of carbon and nitrogen content (Fig. S9).

The range of variation in the integrative response of *H. sanguineus* is much higher than that of *C. maenas* larvae (Fig. 3). However, when the duration of development and the body mass are standardised, the opposite pattern arises. Figure 4 compares the effect of food limitation on the integrated responses of body mass and developmental time, for each temperature, of both *Carcinus maenas* and *H. sanguineus*. Each variable (V: body mass or duration of development) was expressed for each temperature (T) as a ratio, R:

$$R_T = \frac{V_{T,L}}{V_{T,P}} \quad (1)$$

where the subindices L and P represent limited and permanent access to food, respectively. In this representation, the condition of permanent access to food is set to one irrespective of the temperature because the formula becomes:

$$R_T = \frac{V_{T,P}}{V_{T,P}} = 1 \quad (2)$$

Figure 4 shows that as compared with *C. maenas*, *H. sanguineus* extended the development in a small fraction in response to limited access to food. In *C. maenas*, the proportional reduction in body mass and delay in metamorphosis became larger with temperature (Fig. 4, see also Torres and Giménez 2020);

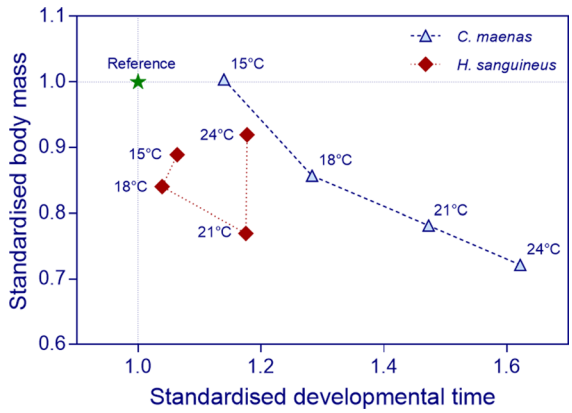

Fig. 4 *Hemigrapsus sanguineus* and *Carcinus maenas*. Summary of standardised responses to the different temperatures under food limited conditions (blue triangles and red diamonds, see Eq. 1). For the standardised values of body mass and developmental time, the values for permanent food conditions represent the unit (green star, see Eq. 2). At the lowest temperature tested, *C. maenas* extended the developmental time in response to food limitation; larvae compensated for the limited access to food, metamorphosing with high body mass. *H. sanguineus* did not compensate for the limited access to food and metamorphosed with lower body mass. Symbols: *C. maenas*: blue triangles, *H. sanguineus*: red diamonds. Data for *H. sanguineus* this study, data *C. maenas* from Torres and Giménez 2020

by contrast in *H. sanguineus* both reduction in body mass and delays in metamorphosis do not show any consistent trend.

The results of the simulated ratios of survival vs growth rates of both species showed that at most of the temperatures and food conditions (15 °C and 18 °C permanent and limited access to food and 21 °C limited access to food) larvae of *C. maenas* will be favoured; but at higher temperatures and especially under conditions of high access to food, larvae of *H. sanguineus* may be favoured (Fig. 5). The ellipses, indicating the importance of intraspecific variation in the performance of both species, show that *H. sanguineus* might perform better at higher temperatures, but it is not likely to outperform *C. maenas* at the lower temperatures that characterise the German Bight.

Discussion

There were three main findings out of our experiments. First, *Hemigrapsus sanguineus* is able to

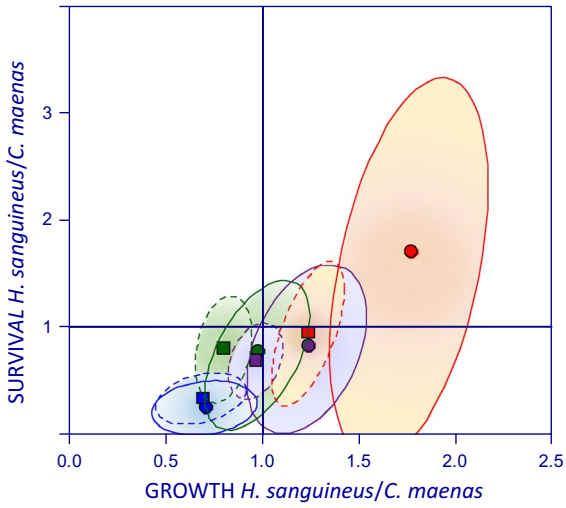

Fig. 5 *H. sanguineus* and *C. maenas* simulated survival and growth ratios under different food and temperature conditions. Squares and circles denote centroids of 95% confidence ellipses in shown colour. Data for permanent access to food (circles) shown with ellipses as continuous lines and for limited access to food (squares) as dashed lines. Blue: 15 °C, green: 18 °C, purple: 21 °C and red: 24 °C

metamorphose to megalopa under limited daily access to food over a wide thermal range (15–24 °C). Second, within the range of temperatures experienced in the local habitat (German Bight, North Sea), low temperature (15 °C) appears to be the primary limiting driver of larval performance, in terms of survival and growth. Third, when the performance is quantified in relation to that of *Carcinus maenas*, unfavourable scenarios for *H. sanguineus* are projected for temperature characterising cool summers. In turn, favourable scenarios are projected for summers characterised by long heatwaves and after coastal warming.

Temperature and food limitation

H. sanguineus larvae were able to metamorphose to megalopa under limited access to food over a wide range of temperatures, with some variations among families (i.e. larvae originated from different females). This is consistent with other studies on decapod crustacean larvae and strengthen the general hypothesis that such larvae can reach metamorphosis as long as they access food patches for a short period of time every day (Sulkin et al. 1998; Giménez and Anger 2005; González-Ortegón and Giménez 2014; D´Urban Jackson et al. 2014; Torres and Giménez 2020). Limited daily access to food is expected in habitats characterised by food patchiness (Paffenhöfer et al. 1987; Durham and Stocker 2012; Prairie et al. 2012; Robinson et al. 2021) and in scenarios where larvae perform diel or tidal migrations (Forward et al. 2001; Queiroga and Blanton 2005; dos Santos et al. 2008; Thygesen and Patterson 2019). In temperate latitudes such as the one of the German Bight, access to prey during night time should be limited to a few hours because *H. sanguineus* larvae develop in summer when the darker period is restricted to few hours. Hence, *H. sanguineus*, as other species including *C. maenas* should be able to survive the larval phase as long as they access prey for a limited amount of time every day.

Food limitation had important consequences in terms of body mass and reserves at metamorphosis to the megalopa as well as growth rates and developmental time. In species with complex life cycles, traits at metamorphosis are relevant for post-metamorphic survival and are the basis for trait-mediated effects of the pelagic environment on recruitment in the benthic habitat (Giménez 2004; Pechenik 2006; Marshall et al. 2008; Torres et al. 2016). Larvae of *H. sanguineus* under food-limited conditions metamorphosed to megalopae with reduced body mass at all temperatures, suggesting that impacts of food limitation are consistent across the thermal range expected for cool and warm summers. It is important to understand the mechanisms driving body size at metamorphosis as they should mediate effects of warming on species responses (Lowe et al. 2021; Song et al. 2021). While there is a good amount of information on mechanisms driving metamorphosis in species with complex life cycles (Werner 1988; Emlet 1988; Hentschel and Emlet 2000), only a handful of studies have investigated how such mechanisms respond to warming (D'Urban et al. 2014; Torres and Giménez 2020; Griffith et al. 2021). Body mass is a plastic trait, driven by variations in larval growth rates and developmental time, but body mass should vary within upper and lower thresholds set by fitness costs (Werner 1988; Gotthard and Nylin 1995; Hentschel and Emlet 2000; Gotthard et al. 2000). Beyond the upper threshold, costs are associated to the high growth rates needed to achieve a large body mass (e.g. predation). Longer developmental time contributes to larger body mass but also determines the period when

larvae are exposed to pelagic mortality risks (Eckert 2003) and the conditions experienced after settlement (Miron et al. 1999; Jarrett 2003). Because post-settlement conditions fluctuate in seasonal environments, the specific conditions experienced by juveniles are indirectly determined by the duration of the larval phase. For instance, in *H. sanguineus*, late settlement may result in reduced juvenile growth as individuals may miss most of the summer season where growth rates are enhanced by high temperatures in the intertidal zone. During summers, temperatures in the intertidal zone may boost growth because they are much higher than water temperatures given the exposure to sunlight during low tides (Stephenson 1942; Lewis 1964; Somero 2002). In addition, late settlement may expose individuals to cannibalism (Moksnes et al. 1997; Moksnes 2002, 2004) or predation by e.g. juvenile *C. maenas*. According to life history theory, costs associated to trait changes should drive the evolution of plastic responses to environmental variation; because of trade-offs associated to such costs, the less responsive traits should be those with higher associated costs (Gotthard and Nylin 1995; Gotthard 2000, 2004). In the case of *H. sanguineus*, larvae responded to food limitation mainly through reduction of growth rates; the extension of development time, found to partially compensate effects of food limitation in *C. maenas* (Torres and Giménez 2020) was rather short. By contrast, *H. sanguineus* larvae showed a strong plasticity to temperature by extending the development from < 20 days at 24 °C to > 60 days at 15 °C. Given that at the local population, *H. sanguineus* females appear to release larvae in early summer, only after temperatures reach 15 °C (Giménez et al. 2020), summers with water temperatures in the range of 15–18 °C would result in late settlement if larvae were to extend further the developmental time. Hence, in the range 15–18 °C and under food limitation, further extension of the larval phase may have higher fitness costs than metamorphosing with a smaller size (but profiting from a warm growing period).

Low temperatures

Our study is in line with others (Stepherson et al. 2009; Giménez et al. 2020), showing that low larval survival occurs at low temperatures (range 12–15 °C). Reduced body mass at metamorphosis found at 15 °C is expected because body mass at stage, peaks at temperatures where physiological performance is optimal (Anger 1998, 2001). Perhaps the body mass of megalopa of *H. sanguineus* would decrease at temperatures higher than 24 °C, as expected from the temperature-size rule (Atkinson 1994).

Under low temperature and food limitation, larvae hatching from all females showed a stronger reduction in nitrogen as compared to carbon. This is unlike previously observed responses to stressors in other species (Harms et al. 1994; Torres and Giménez 2020; Torres et al. 2021) where the main characteristic is a stronger reduction in carbon than in nitrogen content. In decapod crustaceans, carbon content is considered a proxy for lipid reserves (Anger and Harms 1990); hence, stress responses of that type are interpreted as a reduction in the accumulation of lipid reserves, but not in the proteins needed to sustain activity or the enzymatic machinery. Given that in decapods, nitrogen is a proxy for protein levels, we hypothesise that the reduction observed in *H. sanguineus* reflects a thermal limitation in the rate of protein synthesis. Protein synthesis accounts for a great proportion of the specific dynamic action (SDA, Brody 1964) in crustaceans, i.e. the energetic costs incurred by physiological processes related to feeding (including e.g. ingestion, digestion, assimilation and synthesis: Jobling 1993; Wieser 1994), which increase after a meal (Houlihan et al. 1990; Robertson et al. 2001). For example, in *Carcinus maenas*, protein synthesis accounts for 20–37% of the post-meal oxygen rise (Houlihan et al. 1990). Temperature affects rates of protein synthesis in fasted and inactive crustaceans (McMillan and Houlihan 1988; Whiteley et al. 1996; El Haj and Whiteley 1997). Hence, one would expect that rates of protein synthesis are compromised at lower than optimal temperatures, due to limitations in covering the associated costs (Whiteley et al. 1997, 2001). In this study, the lowest temperatures tested are suboptimal for *H. sanguineus,* but not for species that are native to North European Seas (e.g. D'Urban et al. 2014; Torres and Giménez 2020). Hence, the differences between *H. sanguineus* and e.g. *C. maenas*, in how C:N ratios respond to limited access to prey may reflect interspecific differences in the thermal tolerance range.

Performance of *H. sanguineus* relative to *C. maenas*

We found that larval performance (e.g. survival) of *H. sanguineus* at low temperatures is low in relation to that of *C. maenas* also at low temperatures (Fig. 1). This is relevant to understand the outcome of the balance between larval settlement, and competition between *C. maenas* and *H. sanguineus* in the benthos. *C. maenas* larvae can complete larval development at temperatures as low as 12 °C (Dawirs 1985; Nagaraj 1993). The larval season of both species partially overlap, *C. maenas* larvae are released from May onwards and megalopae settle in the intertidal zone from the end of June until the end of August (Giménez and Dirk, 2007). Interspecific competition occurs because juveniles of both species develop in the intertidal zone during summer (Geburzi et al. 2018) The balance of larval supply and competition of *C. maenas* and *H. sanguineus* must be evaluated considering both spring and summer conditions. For example, a large number of *C. maenas* juveniles would survive competition in years with strong settlement of *C. maenas* followed by weak settlement of *H. sanguineus* due to cool summers (temperature ~ 15 °C).

We hypothesise that summers characterised by long heatwaves and high temperatures due to warming would increase performance of *H. sanguineus* larvae and exacerbate current competitive advantages already exhibited by the juvenile and adult stages in the benthos (Lohrer and Whitlach 2002; Jensen et al. 2002; Geburzi et al. 2018). Marine heatwaves, i.e. periods of time (> 3–5 days) when temperatures are above a predefined threshold (Meehl and Tebaldi 2004; Hobday et al. 2016), can have drastic consequences in structure and functioning of marine ecosystems (Garrabou et al. 2009; Marbà and Duarte 2010; Wernberg et al. 2013; Mills et al. 2013) and those in the German Bight have become more frequent since the 1990's (Giménez et al. 2022). During summer heatwaves such as those occurring in 2018, water temperatures around the local population reached values above 18 °C (Giménez et al. 2020); temperatures in waters of the coastal Wadden Sea (where *C. maenas* and *H. sanguineus* co-occur) were much higher (BSH 2019).

Another important aspect considered in our analysis concerns the role of intraspecific variation in performance (IVP). IVP is common in invertebrate larvae (e.g. Appelbaum et al. 2014; Spitzner et al. 2019; Torres et al. 2020), and expected from genetic variation (Marshall et al. 2008; Durrant et al. 2013) or parental effects (Pond et al. 1996; Shama et al. 2014). Important IVP was found in this study as variation in survival, developmental time, and growth among larvae from different females (e.g. Fig. S5), which is also expressed in Fig. 5 as wide ellipses. The ellipses are spread across regions where the relative performance switches from being stronger in *H. sanguineus* to become stronger in *C. maenas*. The ellipses surround the area where 90% of the 1000 simulated events for each factor combination are located. Hence, Fig. 5 depicts the importance of the intraspecific variation in the performance of the species, as opposed to what means show; the ellipses show that, for example, there is a possibility for *H. sanguineus* larvae to be able to perform (i.e. survive and grow) better than *C. maenas* at low temperatures. Likewise for *Carcinus maenas*, there is room for their larvae to perform better than those of *H. sanguineus* at high temperatures under food limitation. Species coexistence is one of the important ecological consequences of intraspecific variation (Bolnick et al. 2011; Appelbaum et al. 2014).

In synthesis, larvae from the invasive crab *H. sanguineus* are able to complete their development under a wide range of temperatures and under daily limited access to food (for a period as short as 6 h), with variations among families; hence, *H. sanguineus* larvae should be able to survive starvation periods as long as they can access food patches for a brief time every day. *H. sanguineus* responds to food limitation through lengthening the development to a much smaller degree as compared to *C. maenas*; this occurs at expenses of metamorphosing to megalopa with lower body mass. It remains to be seen how such responses affect the balance of costs and benefits of metamorphosing with lower body mass but still profiting from the warm season increasing juvenile growth rates. *H. sanguineus* shows different responses to high temperatures and food limitation from the native *C. maenas* (Torres and Giménez 2020). Under cool summer conditions, we would expect limited survival of *H. sanguineus* larvae which could favour *C. maenas*. By contrast, under a warming scenario, *H. sanguineus* should benefit through high larval growth and survival rates. Under this scenario, increased rates of survival and growth in the pelagic habitat should

enhance propagule pressure of *H. sanguineus*, magnifying the effect produced by being the dominant competitor in the intertidal zone. Overall, our study emphasises the importance of integrative studies comparing the performance among native and invasive species across their life cycles, and extending our study towards the juvenile–adult phase. Such approach will help us to understand and predict effects of warming on species replacement.

Acknowledgements We are grateful to Julia Haafke (AWI) for performing the elemental analysis and Tobias Billens for help with larval rearing (University of Greifswald). We would also like to thank two anonymous reviewers whose comments and suggestions improved the quality of the work.

Author contributions GT, LG and NE-N conceived the experimental design. NE-N and GT performed the experiments. NE-N and LG analysed the data. NE-N wrote the first draft. All authors contributed to the writing of the manuscript and gave final approval for publication.

Funding Open Access funding enabled and organized by Projekt DEAL. The authors have not disclosed any funding. This study is part of NE-N doctoral dissertation and was supported by the Bundesministerium für Bildung und Forschung (Project MERGE; grant n° FKZ 01DN20002), Germany.

Data availability All data for this paper will be available from PANGAEA ®Data Publisher https://www.pangaea.de

Declarations

Conflict of interest The authors declare that they have no conflicts of interests.

Human or animal rights The research presented in this paper complies with national (Germany) and international laws (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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CHAPTER 3

A framework to understand the role of biological time in responses to fluctuating climate drivers

Giménez, L., **Espinosa-Novo, N.**, Torres, G.

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A framework to understand the role of biological time in responses to fluctuating climate drivers

Luis Giménez[1,2]✉, Noé Espinosa[1] & Gabriela Torres[1]

Understanding biological responses to environmental fluctuations (e.g. heatwaves) is a critical goal in ecology. Biological responses (e.g. survival) are usually measured with respect to different time reference frames, i.e. at specific chronological times (e.g. at specific dates) or biological times (e.g. at reproduction). Measuring responses on the biological frame is central to understand how environmental fluctuation modifies fitness and population persistence. We use a framework, based on partial differential equations (PDEs) to explore how responses to the time scale and magnitude of fluctuations in environmental variables (= drivers) depend on the choice of reference frame. The PDEs and simulations enabled us to identify different components, responsible for the phenological and eco-physiological effects of each driver on the response. The PDEs also highlight the conditions when the choice of reference frame affects the sensitivity of the response to a driver and the type of join effect of two drivers (additive or interactive) on the response. Experiments highlighted the importance of studying how environmental fluctuations affect biological time keeping mechanisms, to develop mechanistic models. Our main result, that the effect of the environmental fluctuations on the response depends on the scale used to measure time, applies to both field and laboratory conditions. In addition, our approach, applied to experimental conditions, can helps us quantify how biological time mediates the response of organisms to environmental fluctuations.

One of the biggest challenges faced by humanity is climate change[1–3]. A key characteristic of current climate change is the presence of extreme climatic events, i.e. strong fluctuating environmental conditions, manifested as storms, hurricanes and heatwaves. Hence, a challenge for ecologists consists in quantifying and predicting the responses of ecological systems (from populations to ecosystems) to such fluctuations[4–7]. Despite the emphasis in understanding ecology and evolution in fluctuating environments[8–11] most experiments concerning climate driven environmental variables focus on responses to constant conditions[11–13]. The currently growing body of work tackling responses to fluctuating environments highlights the complications in attempting to incorporate the role of variation in such environmental drivers[13–17] especially in dealing with their combined actions[18,19]. For instance, research on extreme events has identified five primary traits characterising heatwaves[20], which should be added to the effect produced by the average condition experienced during that event.

An additional layer of complexity is given by the biological time (e.g. time to metamorphosis, to reproduction, generation times), characterising biological systems[21]. Biological time is governed by the interaction between the environmental and physiological processes and plays a critical role in driving fitness, population dynamics and community structure[21–25]. Biological time is critical because of three reasons. First, responses (e.g. survival rates) to environmental conditions are driven by a number of developmental processes operating at different biological time scales[26–31], ranging from short (e.g. physiological acclimation) to medium (developmental plasticity) and long term (transgenerational plasticity, changes in gene frequencies). Second, if we characterise a fluctuation by its time scale, then the response will depend on the biological time characterising that species. From the perspective of organisms (e.g. with time scales ranging from those of from bacteria to trees), whether a fluctuation is long or short, is not determined by the chronological (= clock) time, but instead by its characteristic biological time[31]. Third, responses may be measured after a predetermined chronological time scale (e.g. at a given time in the year) or biological time scale (e.g. at maturity). The expression of biological responses in chronological

[1]Biologische Anstalt Helgoland, Alfred-Wegener-Institut, Helmholtz-Zentrum Für Polar- Und Meeresforschung, 27498 Helgoland, Germany. [2]School of Ocean Sciences, Bangor University, Menai Bridge LL59 5AB, UK. ✉email: Luis.Gimenez@awi.de

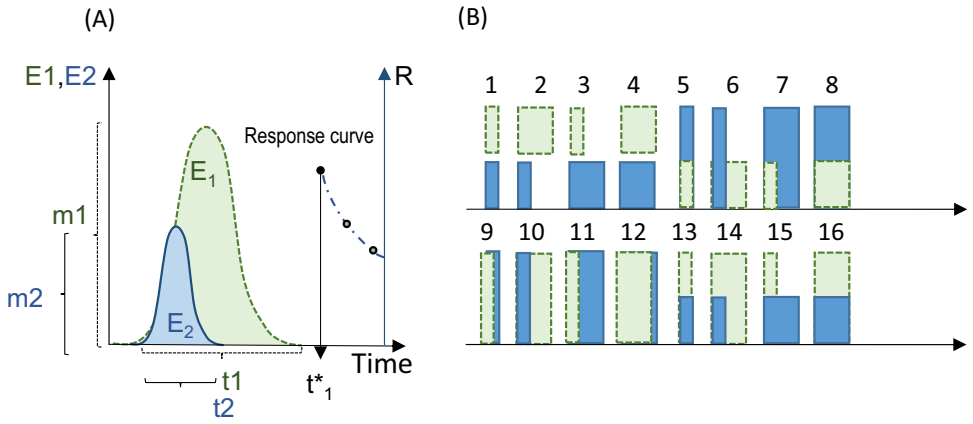

Figure 1. Experimental set up and equations. (**A**) A biological system (e.g. an organism) is exposed to two fluctuating environmental drivers (E_1, E_2). The fluctuations are characterised by predictors, i.e. the amplitudes ($m1$, $m2$) and time scales (t_1, t_2). Measurement of the response, R, are made at different times (t^*_1) after the fluctuations occurred (black circle); additional measurements may be carried out at other fixed times after t^*_1 (grey circles). (**B**) In a factorial experiment the process would be repeated so that observations are made for a minimum of two levels per predictor giving 16 factor combinations. In (**B**): vertical dimension = magnitude; horizonal dimension = clock time scale.

time is obviously important to understand long term changes in seasonal habitats. However, measurements on biological time scales are critical for understanding population dynamics and evolution because such number drives individual fitness and the population growth rate. This is especially important in species that experience habitat shifts (e.g. most bottom living marine invertebrates, insects, migratory fish, birds and mammals). In such species, the biological time is "reset" at critical stages (e.g. metamorphosis, onset of migration) because, after the habitat shift, organisms experience the environmental conditions of a new habitat and the conditions in the old habitat might become irrelevant. Overall, understanding and quantifying the actual response to climate driven fluctuations requires that we also understand how responses are modified with a change in the time reference frame (from chronological to biological).

We propose a framework to understand and quantify responses to fluctuations in one or more climate-driven environmental variables, considering biological time. We consider environmental fluctuations characterised by a magnitude (e.g. the amplitude) and a timescale (e.g. the period of the fluctuation or the time of exposure to a given magnitude: Fig. 1). The response is quantified at least once after the environmental drivers are experienced, with respect to chronological time or at a given life history event (e.g. at maturity). We use an experimental case and simulations to understand how biological responses to fluctuating environmental drivers are modified by the clock and biological time scale used to study the response. We structure this article as follows: First, we introduce definitions and a system of equations describing the biological responses. Second, the equations are explored using four specific cases. Third, in the methods section, we describe the experiments carried out to illustrate case 3.

Results

Mathematical theory. We consider a biological response (e.g. body size, survival, biodiversity) to two environmental drivers (i.e. any abiotic or biotic factor) but the same idea may be applied to a larger number of drivers. The response depends of a set of predictors consisting in the magnitudes (m_1 and m_2) and time scales of fluctuation of two drivers ($i = 1, 2$); in addition, the response is quantified at least once after the fluctuations have been experienced (Fig. 1a).

Time is defined using two different frames; chronological (= clock) time (measured by clocks) and biological time. For the "clock" time scales of the fluctuations (t_1, t_2) there are associated biological times (τ_1, τ_2). Likewise, for the clock time at which the response is quantified (t^*) there is an associated biological time (τ^*).

Biological time is the proportion of (clock) time needed to reach a life history event (e.g. moulting, maturity). Hence, for t_1, t_2 and t^* we obtain $\tau_i = t_i/D$ and $\tau^* = t^*/D$, (D = clock time needed to reach such life history event). We express the τ_i and τ^* in terms of a function $L = 1/D$. For instance, for t^* we obtain:

$$\tau^* = t^* \cdot L \tag{1}$$

where $L = L(\boldsymbol{\omega}) = D^{-1}(\boldsymbol{\omega})$ characterises the timing of a life history event (with units as the inverse of clock time units). L depends on the set of predictors $\boldsymbol{\omega}$ associated to the fluctuations; an important set of predictors will be defined by thermal fluctuations (the amplitude and time scales), which in ectotherm species have a strong influence on developmental time[32,33]. We find by differentiation that L provides the transform function between clock and biological time frames; for instance, if L does not depend on any t_i we have $L = d\tau/dt_i$.

The response is expressed as a function of the predictors defined above, as $R(m_1, m_2, t_1, t_2, t^*) = r[m_1, m_2, \tau_1(t_1), \tau_2(t_2), \tau^*]$. The contribution of each predictor to the response is better understood by the partial derivatives

with respect to each predictor; this defines a system of partial differential equations (PDE; Supplementary note 1) which expressed in matrix form give the following matrix equation.

$$\begin{bmatrix} \frac{dR}{dm_1} \\ \frac{dR}{dm_2} \\ \frac{dR}{dt_1} \\ \frac{dR}{dt_2} \\ \frac{dR}{dt^*} \end{bmatrix} = \begin{bmatrix} 1 & \frac{dm_2}{dm_1} & \frac{d\tau_1}{dm_1} & \frac{d\tau_2}{dm_1} & \frac{d\tau^*}{dm_1} \\ \frac{dm_1}{dm_2} & 1 & \frac{d\tau_1}{dm_2} & \frac{d\tau_2}{dm_2} & \frac{d\tau^*}{dm_2} \\ \frac{dm_1}{dt_1} & \frac{dm_2}{dt_1} & \frac{d\tau_1}{dt_1} & \frac{d\tau_2}{dt_1} & 0 \\ \frac{dm_1}{dt_2} & \frac{dm_2}{dt_2} & \frac{d\tau_1}{dt_2} & \frac{d\tau_2}{dt_2} & 0 \\ 0 & 0 & 0 & 0 & \frac{d\tau^*}{dt^*} \end{bmatrix} \cdot \begin{bmatrix} \frac{dr}{dm_1} \\ \frac{dr}{dm_2} \\ \frac{dr}{d\tau_1} \\ \frac{dr}{d\tau_2} \\ \frac{dr}{d\tau^*} \end{bmatrix} \quad (2)$$

In the PDE (Eq. 2), the left-hand side is a vector column of the derivatives of the response in clock time (**R**), with respect to each predictor; the right-hand side is the standard (= inner) product of a matrix (**M**) by a vector of the derivatives of the response in biological time (**r**), i.e. **R** = **Mr**. The matrix contains the derivatives of the predictors with respect to each other, with time both expressed in clock or biological scales; one can think of **M** as an object containing coefficients that transform **r** into **R** in the same way as a constant (= 1000) would transform kilometres into meters of distance. The large number of terms in **M** highlights the considerable diversity and the challenges in quantifying responses to multivariate environmental fluctuations. We show below how to use Eq. (2) to quantify the effect of fluctuating environmental drivers on biological responses, as mediated by biological time.

First, we note that **M** contains three groups of terms: (1) Terms accounting for situations where the magnitude of a driver affects the magnitude of the second driver (e.g. temperature drives oxygen concentration in aquatic habitats): these are dm_i/dm_j for any $i, j = 1, 2$. (2) Terms accounting for cases where the magnitudes and time scales of stressors are related: dm_i/dt_j and dm_i/dt_i. (3) Terms where biological time depends on the magnitude or time scale of the environmental fluctuation $d\tau_i/dt_j$ and $d\tau_i/dm_j$. The terms of groups (1) and (2) are zero when they are mutually independent, such as in a factorial experiment with orthogonal manipulation. We will set those to zero in the rest of this analysis.

Second, we note that for group (3) there are three scenarios: (3a) biological time does not depend on any environmental driver. This is the trivial case where biological time is proportional to clock time, not considered here; **M** is simplified to a diagonal matrix, i.e. with constants in the diagonal, and zero's otherwise leading to a single constant term per equation (3b). Biological time depends on the magnitudes of any or both drivers. In such case, τ_1 τ_2, *and* τ^* will be driven by the same equation: if $\tau_i = t_i \cdot L\ (m_1, m_2)$ we obtain $d\tau_i/dt_j = d\tau_i/dt_i = L\ (m_1, m_2)$. (3c) Biological time depends on the time scale of the fluctuations: in such case, differentiating Eq. (1) with respect to time, we obtain $d\tau_i/dt_i = L + t_i\, dL/dt_i$.

Here, we explore four special cases where the equations are simplified to highlight the importance of biological time in modifying the responses as compared to clock time. We start with the simplest case where there is a single environmental variable and then we consider cases with two variables. We focus on cases representing the most frequent experiments carried out on multiple driver research, i.e. factorial manipulations where terms of the groups 1 and 2 are zero.

Case 1: responses to the magnitude of a single variable. We start with the simplest case i.e. where the response is driven by the magnitude of a single driver, e.g. temperature (= m). Examples of this case are laboratory experiments quantifying the effect of temperature on body mass or survival of a given species, or mesocosm experiments quantifying effects of temperature on species richness where thermal treatments are kept constant over time. Here, the response is quantified at different times, both in the clock and biological frames. In such case we have $R(m, t^*) = r[m, \tau^*(m, t^*)]$ and the PDEs simplify to.

$$\frac{dR}{dm} = \frac{\partial r}{\partial m} + \frac{\partial r}{\partial \tau^*} \cdot \frac{d\tau^*}{dm} \quad (3)$$

From Eq. (3), and because $dR/dm \neq dr/dm$, we see that the response to the magnitude of the driver depends on a component quantifying the effect biological time: as long as $d\tau^*/dm \neq 0$ the time reference frame affects the observed effect of m on the response. The simulation illustrated in Fig. 2 shows a case where there are differences between the observed responses at clock vs biological times. In the simulated experiment, there is a strong effect of the magnitude of the driver on the response at clock time, but such effect is much less pronounced at biological time. By contrast, there is no effect when the response is measured in the biological time frame.

Equation (3) (details in Supplementary code 1) captures an obvious but important feature of experiments manipulating temperature over the development of ectotherms, for instance, from birth to metamorphosis; namely that there is no consistent definition of a simultaneous event across the different time frames. Experiments are usually stopped at different clock times because organisms must be sampled at the same biological time. All points located in the horizonal line in Fig. 3 represent simultaneous events, as defined in clock time occurring at different temperatures (e.g. whether an animal is dead or alive); however, simultaneous events occurring in biological time are represented by the points on the curve. Hence, Fig. 2 gives a geometric representation of such fact. Temperature as a driver of developmental rates[32] is a central candidate to produce responses that differ at clock vs biological time.

We explore further this case with an example where the response is expressed as a function of time and an instantaneous rate $\mu(m)$ quantifying for instance mortality, growth or biomass loss. For this example, we obtain $R(m, t^*) = r[\mu(m), \tau^*(m, t^*)]$. By differentiating in both sides, we get:

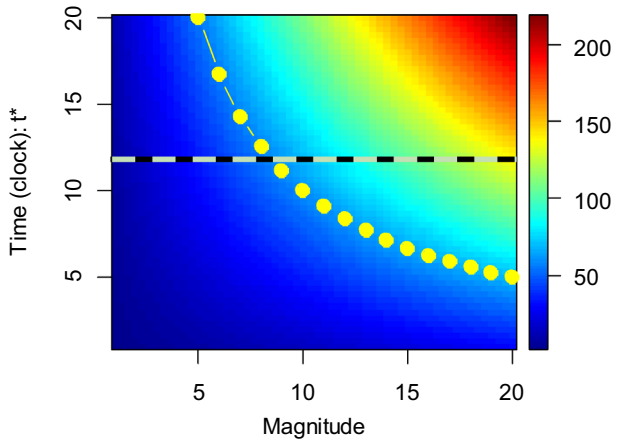

Figure 2. Case 1: Response to the magnitude of a single variable (m). Horizontal line: measurement taken at clock time $t^* = t^*_c$; note that, along the line, the response increase with m (it crosses the colour gradient). Curve with yellow circles: measurements taken at a constant biological time ($\tau^* c = 100$); along the curve, the response does not vary with m. The equations used were: $R = m(0.5t^*)$, $\tau^* = t$. m giving $r = 0.5$. τ^* not depending on m.

$$\frac{dR}{dm} = \frac{\partial r}{\partial \mu} \cdot \frac{d\mu}{dm} + \frac{\partial r}{\partial \tau^*} \cdot \frac{d\tau^*}{dm} \tag{4}$$

Equation (4) shows that m affects the response through two components: the instantaneous rate ($d\mu/dm$) and the biological time ($d\tau^*/dm$). We call the first component "eco-physiological" and the second component "phenological" (m drives the timing of a biological event, e.g. time to maturation). Those components are not evident if the response is expressed in clock time; otherwise we would obtain $dR/dm = \partial R/\partial \mu \cdot d\mu/dm$.

In order to better understand Eq. (4), consider an example where the response is biomass loss experienced by an organism during the process of migration (e.g. towards a feeding or reproductive ground); when the access to food during migration is very limited the result should be a decrease in body mass reserves through time. Let biomass (B) be modelled as an exponential decaying function of time and an instantaneous rate of biomass loss μ; let μ depend on temperature (= m) such that, $\mu = \mu(m)$. In such case we obtain:

$$B(m,t) = e^{-\mu(m)\cdot t^*} = e^{-\mu(m)\cdot \tau^*\left(m,t^*\right)} \tag{5}$$

By differentiation in both sides of Eq. (5) we get:

$$\frac{dB}{dm} = -e^{-\mu(m)\cdot \tau^*\left(m,t^*\right)} \left\{ \tau^* \cdot \frac{d\mu}{dm} + \mu \cdot \frac{d\tau^*}{dm} \right\} \tag{6}$$

Equation (6) shows the eco-physiological ($d\mu/dm$) and phenological components ($d\tau^*/dm$) within the brackets. If μ responds linearly to temperature, then $d\mu/dm$ would be represented by a constant quantifying the thermal sensitivity of biomass loss; the value of such constant would depend on physiological processes associated to use of reserves to sustain movement and the basal metabolic rate. Likewise, if τ^* responds linearly to temperature, the $d\tau^*/dm$ would be driven by a constant controlling the sensitivity of developmental time to temperature.

Because biomass is a trait that is central to fitness, Eq. (6) gives the indirect contribution of phenological and physiological responses to fitness. Assuming that fitness should be maximised, adaptive responses should involve the mitigation of negative effect of m on both components of Eq. (5), represented by the partial derivative of the right-hand term. For instance, organisms with the ability to minimise the eco-physiological effect (through e.g. a compensatory physiological mechanisms) or the phenological effect (e.g. shortening the exposure time) would complete the migration minimal loss of reserves.

By generalization, Eqs. (4–6) help us to provide biological meaning to the terms of the matrix **M**: any term of the form $d\tau^*/dm_j$, $d\tau_i/dm_j$ or $d\tau_i/dt_j$ represents the effect of an environmental driver on the timing of a phenological event; hence, they are phenological components. Terms that contain the effect of an environmental variable on an instantaneous rate are eco-physiological components. By substitution we find that the terms of the matrix in Eq. (2) can be classified in two categories according to whether the component is eco-physiological (E) or phenological (P):

$$\begin{bmatrix} E & 0 & P & P & P \\ 0 & E & P & P & P \\ 0 & 0 & P & P & 0 \\ 0 & 0 & P & P & 0 \\ 0 & 0 & 0 & 0 & P \end{bmatrix} \tag{7}$$

Case 2: multiple driver responses. Here we expand the previous case by looking at a response to the magnitude of two different drivers; i.e. keeping the levels of each driver constant over the duration of the experiment. Examples of this case are experiments quantifying the effect of temperature and nutrient load on body mass (e.g. in a rearing containers) or species richness (e.g. in mesocosms). This case is represented by the terms of first two rows of the matrix and the vectors of Eq. (2), with the terms of the remaining rows set to zero. Here, there are different scenarios, but we focus on the one highlighting the importance of biological time.

Consider a case where biological time depends on the magnitude of the first driver while the response is explicitly driven by the magnitude of the second driver (Fig. 4). For instance, the response may be the survival rate of a host organism exposed to different temperature and parasitic load. The response in clock time is described as $R(m_P, t^*)$. The driver controlling the biological time is temperature (m_T) while the parasitic load (m_P) controls survival. In such case, $\mathrm{d}\tau^*/\partial m_P = 0$, $dR/dm_P \neq 0$ and $dR/dm_T = 0$. Although by definition the response in clock time does not depend on m_T, it will do so in biological time. This is because, applying the matrix multiplication in Eq. (2), we obtain:

$$\frac{\partial R}{\partial m_T} = \frac{\partial r}{\partial m_T} + \frac{\partial r}{\partial \tau *} \cdot \frac{d\tau *}{dm_T} \tag{8a}$$

$$0 = \frac{\partial r}{\partial m_T} + \frac{\partial r}{\partial \tau *} \cdot \frac{d\tau *}{dm_T} \tag{8b}$$

$$\frac{\partial r}{\partial m_T} = -\frac{\partial r}{\partial \tau *} \cdot \frac{d\tau *}{dm_T} \tag{8c}$$

The second right-hand term in Eq. (8a) quantifies the effect of temperature on the response mediated by biological time. In order to better understand the responses, consider a simple linear response: $R = R_0 - m_P \cdot t^*$ and notice that, for a fixed clock time (t^*_c) the effect of the magnitude of parasitism is constant ($dR/dm_P = -t^*_c$); hence, the response can be understood, geometrically, as a flat surface with slope not depending on temperature. Now, note that under the specific conditions of our example, $r = R_0 - m_P \cdot \tau^*/L(m_T)$. Hence, for a fixed biological time (τ^*_c) we obtain $\partial r/\partial m_P = -\tau^*_c/L(m_T)$; i.e. the importance of the parasitic effect depends now on temperature. In addition, this example is valid for the case of additive effects of any two environmental drivers: assuming $R = R_0 - (a_1 \cdot m_P + a_2 \cdot m_T) \cdot t^*$ (a_1, a_2 are constants), we obtain $dR/dm_P = -a_1 t^*$; however, $\partial r/\partial m_P = -a_1 \tau^*_c/L(m_T)$. In words, additive effects observed in clock time become interactive in biological time. This is illustrated in the simulation (Supplementary code 2) depicted in Fig. 4: the response in clock time depends on a single driver (parasite load); however, the response in biological time is interactive, i.e. the effect of parasite load depends on temperature.

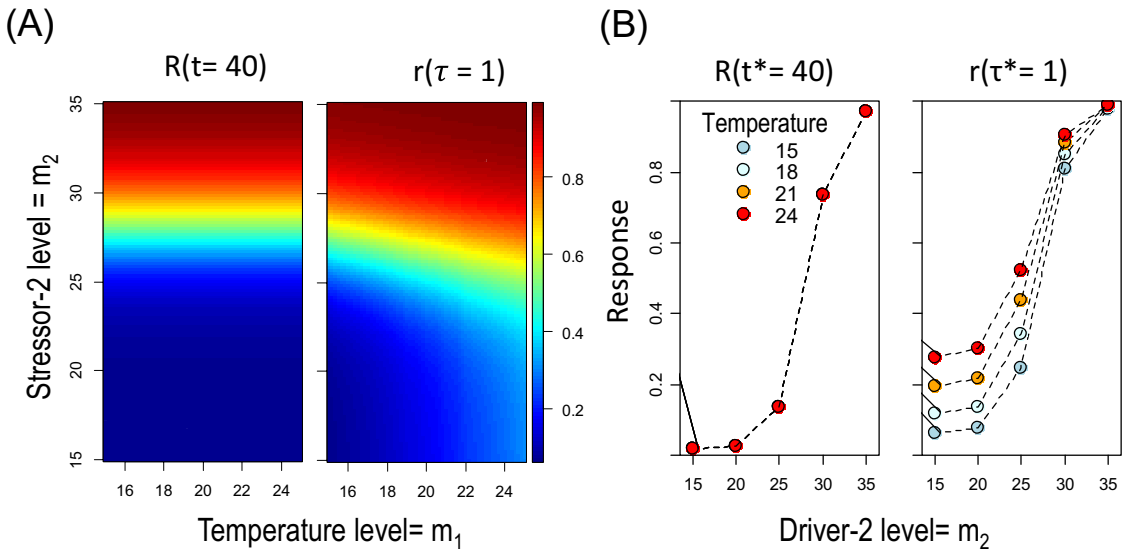

Figure 3. Case 2: Multiple driver responses. (**A**) Modelled responses (colour scale) at a specific clock ($t^* = 40$) and biological times ($\tau^* = 1$), showing an interactive effect only in the biological time frame. (**B**) Interaction plots of the responses for specific levels of temperature and a second driver showing that the effect high temperature mitigates the negative effect of the second driver on the response. The response was modelled with as a sigmoidal function $R = exp(-t^*\varphi)$ with $\varphi = 0.1[1 + exp(m_2/2)]^{-1}$ to produce a strong gradient in the range of $m_2 = 25–30$ units. The biological time was modelled based on the effect of temperature on the development of marine organisms[33] as so that $t^* = \tau^* exp[-22.47 + 0.64/(k(m_1 + 273)]$, i.e., using the Arrhenius equation with k: Boltzmann constant ($\approx 8.617\ 10^{-5}$ eV K^{-1}).

Case 3: role of clock and biological time scale of fluctuation. Previous examples did not consider, the time scale of the fluctuations as drivers of the response. Here we explore how a biological variable (= survival rate) responds to different levels of magnitude of a driver (= temperature) and to simultaneously changing the time scale of a fluctuation (from clock to biological time) of a second driver (= food limitation). As model, we use larval stages of a crab because there is sufficient information on the effect of temperature and food levels on survival and the timing of moulting[33,34].

We performed the so-called point-of-reserve-saturation experiment (PRS[35]), i.e. exposing groups of recently hatched larvae of the crab *Hemigrapsus sanguineus* to different initial feeding periods (= our time scale of fluctuation), after which they were starved until they either died or moulted to the second larval stage (Supplementary Fig. 1). *H. sanguineus* is originated from East Asia but has invaded the Atlantic shores of North America and North Europe[36,37]. This experiment was carried out at 4 temperature levels (15–21 °C), within the range of thermal tolerance of larvae of this species, i.e. where the magnitude of temperature does not affect survival[38,39]. In addition, because there is a single level of food limitation (= starvation), the magnitude of food limitation (m_F) is not considered as a variable in the example.

The response variable was the proportion of first stage larvae surviving the moulting event to the second stage, set to biological time $\tau^* = 1$. In response to different starvation periods (preceded by feeding), the survival shows a sigmoidal pattern[35], characterised by a parameter, PRS_{50}. This is the point of development where larval reserves are "saturated"; i.e. enough reserves have been accumulated during the previous feeding period to ensure survival and moulting to the next stage.

Under the conditions of the experiment, the survival proportion (= R) is driven only by the time scale of a fluctuation (here $t_1 = t$, $\tau_1 = \tau$ for simplicity), characterised by the starvation period; hence, $R = R(t) = r[\tau(t)]$ given that there is a single time of observation fixed to $\tau^* = 1$. Because biological time does not depend t, we get $L = d\tau/dt$ and:

$$\frac{dR}{dt} = \frac{\partial r}{\partial \tau} \cdot \mathcal{L}(m_2) \tag{9}$$

Equation (9) is represented in the PDE by the terms of row 3 and column 4 of **M** multiplied by the term of row 3 of the column vector **r**; $d\tau/dt = L(m)$, m represents the magnitude of temperature.

The relationship between biological time and temperature was best explained by a power function $D(T) = aT^b$ (Fig. 4A, Supplementary Table 1, Supplementary Fig. 2), in consistence with previous studies[36,40]. The interaction between starvation time and temperature was weak (Supplementary Fig. 3); best models retained starvation time only at 21 °C where the percentage of explained variance was still low ($R^2 < 0.2$). The full range of starvation times resulted in a variation of developmental time of < 2 days, while the full range of temperature used resulted in variations of 8 days (range 5–14 days); hence, we approximated the model as L depending on temperature as $L = 1/(aT^b)$.

Survival showed an S-shape pattern consistent with results found for other species[35]. When the starvation time was expressed in clock time ($PRS_{50} = t_{50}$ in days) there was a dilation/contraction effect of the response

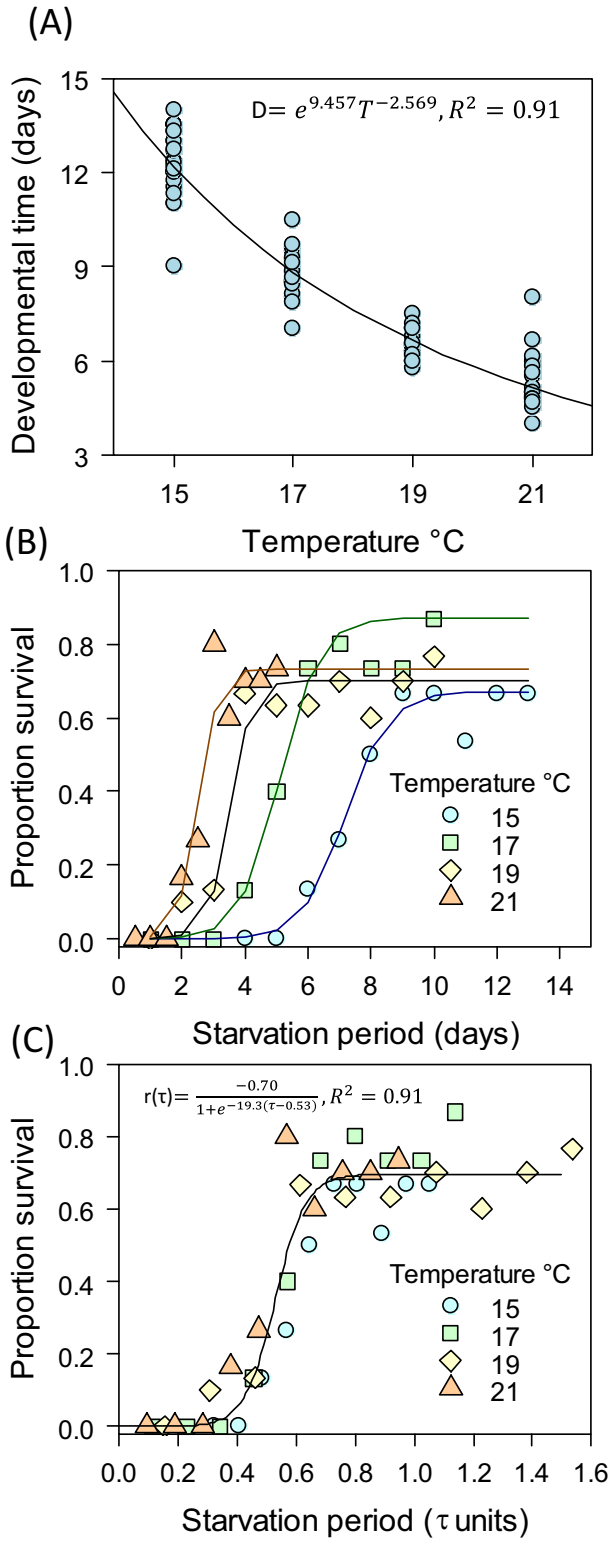

Figure 4. Case-3: Responses of food limitation. (**A**) Effect of temperature on the time needed by larvae to moult for developmental time, $D(T)$. (**B**) Proportion of survivors in response to temperature and the starvation period measured in clock time. Data were fitted with a Boltzmann sigmoidal function with parameters given in Supplementary Table 2. (**C**) Proportion of survivors vs time scale of starvation period in biological time. The equation obtained for the response in clock time (t), was $R\,(t,T) = -0.70/(1+f)$, with $f = 19.3[t \cdot e^{-9.457} \cdot T^{2.569}) - 0.53$, $L = 1/D(T)$.

curve, quantified by the PRS_{50} and driven by the effect of temperature on biological time (Fig. 4B, Supplementary Table 2). When time was expressed in biological time units ($PRS_{50} = \tau_{50}$), a single response curve explained 91% of total variation (Fig. 4C, Supplementary Table 3), irrespective of temperature. The estimate of parameters by temperature showed PRS_{50} in the range of 0.47–0.58% of moulting time with a slight decrease towards higher temperatures (Supplementary Table 4); the range of percent values found here is also consistent with findings in other species (40–60%)[35]. There were therefore important differences in the effect of temperature on estimates of PRS_{50} depending on the choice between time scale (Supplementary Table 5). In synthesis, in the biological time scale we found a simple function showing that the PRS_{50} was less responsive to a change in temperature than in clock time; we will address this point in the discussion in the context of physiological time keeping mechanisms.

Case 4: biological time depends on the time scale of the fluctuation. Here, we generalise the above cases by considering situations where both the magnitude and time scale of an environmental fluctuation drive biological time. For simplicity, we consider a single driver. In such case, $L = L(m,t)$ and the PDEs reduce to:

$$\frac{\partial R}{\partial t} = \frac{\partial r}{\partial \tau} \cdot \mathcal{L} + \frac{\partial r}{\partial \tau} \cdot t \cdot \frac{d\mathcal{L}}{dt} \tag{10a}$$

$$\frac{\partial R}{\partial m} = \frac{\partial r}{\partial m} + \frac{\partial r}{\partial \tau} \cdot t \cdot \frac{d\mathcal{L}}{dm} + \frac{\partial r}{\partial \tau *} \cdot t^{*} \cdot \frac{d\mathcal{L}}{dm} \tag{10b}$$

We now consider a response interpreted as a decay in performance of an organism, where longer time scales of the fluctuation increase the biological time, as expected for cases where organisms are exposed to suboptimal conditions (e.g. food limitation experienced over a given time scale). There are obviously many possible scenarios but for better understanding, we consider Cases 4A-D, where dL/dt is negative, reducing performance and where the functions linking m and t with L act additively or multiplicatively. Additive responses are will be illustrated with $L = [k_1/m + k_2/t]$, while multiplicative responses will be illustrated $L = k_3/mt$, with k_1, k_2 and k_3 as constants.

The first two cases focus on Eq. (10a), which may be considered as an extension of Case 3. Case 4A: additive response: in such case dL/dt depends only on t and we get that dR/dt and $dr/d\tau$ differ by a factor k_1/m:

$$\frac{\partial R}{\partial t} = \frac{\partial r}{\partial \tau} \cdot \frac{k_1}{m}$$

In addition, if L only depends on t, the result is that the response in clock time does not depend on the time scale of fluctuation ($dR/dt = 0$) because the terms of Eq. (10a) associated to L cancel out. However, the response in biological time does not need to be zero ($dr/d\tau$ may not be zero). For instance, assume that $r(m, \tau) = exp(-\omega \cdot m \cdot \tau)$, and $L = k_2/t$, with ω = constant. We obtain $R = exp(-\omega \cdot m \cdot k_2)$ and $dR/dt = 0$, while $dr/d\tau = -\omega \cdot m \cdot exp(-\omega \cdot m \cdot \tau)$.

Case 4B: Multiplicative response of m and t in L. In such case, $dR/dt = 0$ because the terms associated to L cancel out, while $dr/d\tau$ may not be zero.

The next two cases focus on Eq. (10b), which is an extension of cases 1 and 2. The effect of the time scale of the fluctuation depends again on how it relates, through L, to the effect of the magnitude of the fluctuation. We interpret the response as a decay in performance (or fitness), contributed by the "ecophysiological" and "phenological" components:

$$\frac{dR}{dm} = \frac{\partial r}{\partial \mu} \cdot \frac{d\mu}{dm} + \left\{ \frac{\partial r}{\partial \tau} \cdot t \cdot \frac{\partial \mathcal{L}}{\partial m} + \frac{\partial r}{\partial \tau^{*}} \cdot t^{*} \cdot \frac{\partial \mathcal{L}}{\partial m} \right\} \tag{11}$$

In Eq. (11), the phenological component (within the brackets) is driven by two terms. The expression $t \cdot dL/dm$ shows that the time scale of the fluctuation act as increasing the exposure to the suboptimal conditions and contributes to a further reduction in performance. Equation (11) takes different forms depending on whether the functions linking t and m with L are additive or multiplicative.

Case 4C: when the response is additive, the phenological contribution is proportional to the time scale of the fluctuation which then contributes to a further reduction of the performance:

$$\frac{dR}{dm} = \frac{\partial r}{\partial \mu} \cdot \frac{d\mu}{dm} - \frac{k_1}{m^2} \left\{ \frac{\partial r}{\partial \tau} t + \frac{\partial r}{\partial \tau^{*}} t^{*} \right\}$$

Case 4D. When the response is multiplicative, the phenological component responds non-linearly to the time scale of the fluctuation.

$$\frac{dR}{dm} = \frac{\partial r}{\partial \mu} \cdot \frac{d\mu}{dm} - \frac{k_3}{m^2} \left\{ \frac{\partial r}{\partial \tau} + \frac{\partial r}{\partial \tau^{*}} \cdot \frac{t^{*}}{t} \right\}$$

In both 4C and 4D the effect of the magnitude, m, on the response depends on whether the fluctuation is quantified in terms of clock or biological time. For example, when $L = k_1/m$ or $L = k_3/mt$, dR/dm does not depend on t while dr/dm depends on τ. Overall, Cases 4A-D further extend the relevance of cases 1 and 2 in understanding the effects of fluctuating environmental drivers on the responses.

Discussion and conclusions

We have introduced a mathematical framework to better understand and quantify the responses of biological systems to fluctuations in climate driven stressors. Central to that framework is the need to consider biological time as playing a role in driving ecological and evolutionary processes[20]. We did not consider the average magnitude of the fluctuation because responses to fluctuations can differ from responses to the average in two different ways. First, under extreme fluctuations critical tolerance levels may be surpassed leading to the collapse or irreversible shift of the biological system under study. The most obvious case is when the temperature surpasses critical tolerance levels leading to death[42] but experiencing less extreme levels may lead to negative carry-over effects or acclimatory responses[15], not present when average conditions are experienced. Second, when the relationships between environmental drivers (e.g. temperature) and biological responses (e.g. physiological performance) is non-linear, average conditions do not predict well the expected response to a fluctuating environment[13,17].

We started with the simple Case 1 of a single variable and no fluctuation. This case may be considered as trivial but helped as a step to understand more complex cases. For instance, through Case 1 we noted that when the matrix **M** of the PDE is not the identity matrix, simultaneous events in biological time are not so in clock time; this known observation in experimental research was then identified geometrically as the difference between a curve and straight lines when plotting the response in the space defined the magnitude of an environmental variable and the time scale of observation (Fig. 2). With further analysis of Case 1, we noted that the **M** contained two types of terms interpreted as eco-physiological and phenological effects. The specific example of the migrating organism provided further biological interpretation to the components of the differential equation. We focused on the negative effects of temperature (biomass loss) and then noted that adaptations should minimise either one or both the phenological and the physiological components if biomass loss were to be minimised. This is for instance the case of the evolution of early life histories of marine invertebrates to habitats characterised by limited food availability[43]. Where food is available, marine invertebrate tend to develop through feeding larval stages; however, where food is too limiting, most species develop through non-feeding larvae with abbreviated larval phase. In such case, the allocation of maternal reserves into eggs contributes to minimise both the eco-physiological a phenological components with respect to survival, because both the mortality rates and developmental time are independent of food availability.

In Case 2, we introduced the magnitude of a second variable, to explore more complex scenarios studied through factorial experiments carried out under constant conditions; i.e. not yet considering the time scale of a fluctuation. Here, the presence of an interactive response depended on the scale used to measure time. This finding is central to climate change biology, given the interest on interactive effects of multiple environmental variables on biological systems[12,18,19,44]. A critical question is whether effects are additive or whether they are antagonistic or synergistic. Additive effects refer to situation where the response can be modelled from the isolated effect of each single variable; many biological responses are however synergistic or antagonisitic[44]. Synergistic responses occur when the combined effects are larger than the expected contribution of each separate variable. Synergistic responses are critical when they are negative, for instance the combined effect of habitat loss and an environmental stressor, as they can drive ecosystem collapse. By contrast, antagonistic responses imply a mitigation effect. For management, it is essential to get the response right because resources for actions are limited and disrupting synergies may be considered a priority[51]. In such context our findings suggest that management depends on using the correct time frame to measure the response. Interactive effects also change when responses at low levels of organization (e.g. consumer resource functional responses) are used to predict those at higher levels (e.g. population dynamics[45]), because of the non-linear nature of the function mapping the response across levels. In our case there is a non-linearity in that the components of **M** are partial derivatives which depend on the predictors (i.e. the original functions are non-linear).

We used Case 3 to explore responses including the time scale of fluctuation and to better understand how experimental results are interpreted in the light of the PDE. We studied responses in larval stages because of the relevance of marine larvae in driving climate-change effects on marine organisms: most marine organisms (e.g. mussels, crabs, fish) develop through a pelagic larval stage, and larval dynamics affect species range[38,39], population dynamics[46], connectivity[47] and community structure[48]. Warming provides a new context where larvae need to cope with fluctuations in e.g. food abundance (or other variables) in a scenario of increased metabolic demands due to higher temperatures[49].

Case 3 highlighted the importance of understanding how temperature drives time keeping mechanisms in biological systems, understood as those responsible for setting the pace and regulating the timing of life history events[50]. For Case 3, the phenological component of the PDEs captured the effect of temperature on PRS_{50} which instead reflects hormonal control of the so called "D_0-threshold". This threshold is surpassed when moulting hormones are triggered and the premoult period starts[51]; after D_0, development proceeds at a rate that is independent of food levels and larvae will moult. Case 3 therefore highlights the importance of understanding how temperature drives the hormonal regulation of moulting, for the formulation of mechanistic models predicting survival. By extension, knowledge of role of hormones and other signalling mechanisms[50,52,53] should help the formulation of models in other species. In cases where organisms undergo acclimation, an important question is how the time scale of acclimation relates to time keeping mechanisms, including hormonal processes and metabolic rates[31]. Acclimation speed correlates negatively with body size, most likely driven by the positive effect of metabolic rates on acclimation speed[54]. Perhaps acclimation time, as a fraction of developmental time, varies little with temperature or alternatively, acclimation time and the developmental processes setting phenological events have different sensitivities to temperature.

In Case 4, we introduced effects of the time scale of a fluctuation on the function L and found equations that may be considered extensions of the previous cases. A critical question is to determine situations when responses may follow Cases 1–3, 4 or be further simplified into the trivial case. An important point is therefore to determine

how and when developmental time depends on the time scale of the fluctuation being experienced. For instance, we find that dependencies on degree days[41] fit within Cases 1–3 (Supplementary note 2, Supplementary Fig. 4). However, whether the developmental time is driven by time scale of the fluctuation depends on the timing of the fluctuation in relation to size thresholds reached as organisms grow[55–57]. Our work suggest that we need to understand how such thresholds relate to time-keeping mechanisms.

We used the PDEs to understand the importance of the choice of time scale, within an experimental setting. In addition, Case 3 suggest the set of conditions where simple models predict responses to environmental fluctuations. In the example, the response may be approximated by a function quantifying the relationship between temperature and biological time and a second function controlling the timing of the starvation period. Notice that under the range of temperatures considered, the transformation from clock to biological time led to a simple model with high predicting capacity (> 90% of explained variation) although it ignores the significant (but small) effects of food limitation on developmental time (Supplementary Fig. 3). In similar cases, data of the response from a narrow temperature range would give an approximation of $\partial r/\partial \tau$ when scaled in biological time. In that case, additional data on the effect of temperature on biological time may be used predict the response. An important point is the set of conditions where equation-8 may be used with safety. For our experimental system, we hypothesise that equation-2 had a good fit because the developmental time varied little with the time of starvation and the range of temperatures was within the so called "pejus range"[58] i.e. where survival was high irrespective of the magnitude of temperature ($dR/d\mathrm{m}_T \approx 0$). However, assumptions are not valid if the response fall within Case 4 or at temperatures beyond the pejus threshold where a change in temperature have strong effects on the response.

Under the conditions of equation-8, one may combine mechanistic sub-models as modules (e.g. for each of the partial derivatives). In Case 3, the first module is given by L which may be modelled from metabolic theories (MTE[32,33]). For instance, in the MTE, the effect of temperature on biological time, is represented by the Arrhenius equation, which instead will determine L. For the second module (represented by $\partial r/\partial \tau$), we can associate the response to hormonal control of development which drive the timing the switch of the sigmoid survival function. More in general, sigmoid responses are characteristic of populations or ecosystems exhibiting regime or phase shifts[59,60]. At the population level, phase shifts reflect an unstable equilibrium (saddle points) point driven by thresholds associated to density-dependent changes in mating and reproduction. Hence, the mechanisms associated to such phase shifts would be captured in the response function expressed on biological time scale (e.g. the generation time for population level responses).

There are two important points concerning relevance of our approach to characterizing biological responses to environmental fluctuations in the field. First, our main finding, i.e. that responses to environmental fluctuations depends on the scale used to measure time, is valid for both field and experimental conditions. The use of the experimental set up only facilitates teasing apart the independent contributions of each of the predictors (i.e. the magnitudes and time scales of fluctuations). However, whether one can directly apply the equations straight away to field conditions, depends on meeting the assumptions used to formulate the equations; in this sense our approach is not different from any other experimental approach and the usual recommendations apply[18]. There are three assumptions: (1) the right few predictors are identified (e.g. magnitudes and times scales of temperature and any other factor). (2) No covariation among predictors; (3) fluctuations characterised by well-defined values of predictors. A first challenge in field applications is the complexity shown by natural environmental fluctuations. For instance, real fluctuations (e.g. heatwaves) consist of a sequence of oscillations; in addition, fluctuations may be characterised by descriptors other than the period and amplitude (e.g. the rate of daily temperature increase in tropical habitats)[20]. A second challenge is that environmental variables covary in the field[12]. This include situations, not considered here, where different environmental variables fluctuate sequentially[12,31], and an additional time scale must be included in the PDEs (i.e. the one separating the fluctuations). Third, in the field, fluctuations may be characterised by means and variances of the predictor values and attempts to model the average biological response need to consider issues associated to non-linear responses[13,17]. However, the most likely scenario is that field observations inform the design of future experiments. For example, field studies can identify the main environmental variables, the most important traits characterising the fluctuations, whether fluctuations of different variables occur sequentially or simultaneously[12,18].

Another important question is what time frame should we choose. The selection of the appropriate reference frame will depend on the question asked by the researcher. In experiments aimed at determining the effects of environmental fluctuations on body size or survival at e.g. maturation, biological time will be the obvious choice. Biological time will be a choice in situations where organisms experience habitat shifts through the life cycle, for example in species where the larval habitat is aquatic and the adult habitat is terrestrial. In such case, once the aquatic larvae metamorphose to a terrestrial juvenile stage, the importance of the larval habitat conditions for the survival of the juvenile is likely to be low; hence, what matters are the conditions experienced as larvae up to the time of metamorphosis. There are however cases where the decision is less clear. For example, where larvae and adults coexist or where key environmental variables (e.g. weather conditions) can affect both the larval and adult habitat. Another example is the one given by, mesocosm experiments, used to study the effect of warming on populations and communities[18]; under warming, it is likely that populations fluctuate over more generations that in the absence of warming. If the number of generations is important, it will be helpful to analyse the responses considering both clock and biological time (i.e. after a fixed number of generations). The same experiment may be designed to understand the importance of a fluctuation occurring at a characteristic clock time scale, associated to e.g. seasonal fluctuations or 5-day long heatwaves; in such case, the scale of the fluctuation should be kept in clock time. Overall, we will profit from analysing the response in both time frames, as they will provide different pieces of information.

Methods

Experiments were carried out in automated fully programmable incubators (RUMED-EcoLine[R]). At each combination of temperature and starvation period, three replicate groups (10 larvae each) were kept in well oxygenated and filtered natural seawater (mesh = 1 µm, salinity = 32.5 PSU) in glass vials of 100 ml. Water and food were changed every day (except at 21 °C where food was checked every ~ 12 h, at 7:00 and 18:00 h); at such times larvae were checked for moulting or mortality (dead larvae were removed from cultures). Larvae were fed freshly hatched *Artemia* sp nauplii, provided *at libitum* (density 5 nauplii per ml) during the feeding periods.

The effect of temperature (T) or feeding period on developmental time (D) were evaluated through model selection[61,62] using general least squares for model fitting and Akaike information criterium (AIC) for model comparison. Analyses were carried out in R using the package nlme[61]. Four models were compared, i.e. linear ($D = -a \cdot T$+b), exponential ($D = a \cdot e^{-b \cdot T}$), power ($D = a \cdot T^{-b}$) and Arrhenius ($D = a \cdot e^{\frac{b}{(T+273)}}$), where a and b are constants. Models were fitted after appropriate transformations, log(D) for exponential, log(D)-log(T) for power and log(D) vs 1/(T + 273) for Arrhenius model.

Effects of initial feeding periods on survival were evaluated using the sigmoidal dose response function:

$$f(x) = f_m + \frac{f_M - f_m}{1 + e^{-(x - PRS_{50})/k}}$$

where f_m and f_M are the asymptotic minima and maxima respectively, k is the slope parameter and PRS_{50} is the timing of the inflection point where $f(PRS_{50}) = f(x_M)/2$. Model fitting was carried out by non-linear regression (in GraphPad Prism software), with feeding period expressed in both chronological and biological time scales. When the biological time scale was used, a single model was fitted to data from all temperatures. In that case, there were sufficient degrees of freedom to enable appropriate estimation of the four model parameters. In addition, separate models were fitted by temperature using at biological and chronological time. For those models, the estimation of some parameters was unreliable (e.g. extremely large confidence intervals); hence, we focused on estimating PRS_{50}, which is the parameter determining the time of the inflexion point in the curve. Therefore, we set $f_m = 0$ and $f_M = M$, with M being the average survival of the last three points. In addition, for 19 and 21 °C we set k to a constant value obtained after our initial attempt to estimate k.

Data availability

Data will be available in the portal PANGEA.

Received: 24 August 2021; Accepted: 9 May 2022
Published online: 21 June 2022

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Acknowledgements

Noe Espinosa is supported by the Bundesministerium für Bildung und Forschung (BMBF), Germany.

Author contributions

L.G. and G.T. developed the concept. N.E. and G.T. run the laboratory experiment. L.G. developed the equations and the simulations. L.G., N.E. and G.T. analysed the data. L.G. wrote the original draft. L.G., N.E. and G.T. edited the paper.

Funding

Open Access funding enabled and organized by Projekt DEAL.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-13603-5.

Correspondence and requests for materials should be addressed to L.G.

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CHAPTER 4

How to succeed in novel habitats? Invasive H. sanguineus as a case study of wide tolerance to temperature and salinity: comparisons with other invasive species and the native European species C. maenas

Espinosa-Novo, N., Giménez, L., Geißel, J. P., Boersma, M., Torres, G.

How to succeed in novel habitats? Invasive *H. sanguineus* as a case study of wide tolerance to temperature and salinity: comparisons with other invasive species and the native European species *C. maenas*

Noé Espinosa-Novo[1]*, Luis Giménez[2], Jan Phillipp Geißel[1,3], Maarten Boersma[1,4], Gabriela Torres[1]

[1]Biologische Anstalt Helgoland, Alfred-Wegener-Institut, Helmholtz-Zentrum für Polar- und Meeresforschung, 27498 Helgoland, Germany.

[2]School of Ocean Sciences, College of Environmental Sciences and Engineering, Bangor University, LL59 5AB Menai Bridge, UK.

[3]Department of Cytology and Evolutionary Biology, Zoological, Institute and Museum, University of Greifswald, Soldmannstrasse 23,17498 Greifswald, Germany

[4]University of Bremen, Bremen, Germany

*Corresponding author:

Noé Espinosa-Novo

Ostkaje 1118

27498 Helgoland

Email: Noe.Espinosa@awi.de

Noé Espinosa-Novo : 0000-0001-8469-3374

Luis Giménez : 0000-0002-1472-2915

Jan Phillipp Geißel : 0000-0003-2876-4777

Maarten Boersma : 0000-0003-1010-026X

Gabriela Torres : 0000-0003-0970-5990

Abstract

Quantifying species responses to the combined effects of multiple environmental drivers is critical for better understanding the effects of climate change on performance of native and invasive species. Here, we quantified the combined effects of temperature and salinity on survival and growth of the larval stages of the crab *Hemigrapsus sanguineus*, invasive to the North Sea coast. In addition, we compared the survival responses of *H. sanguineus* to those reported for two other invasive species in Europe, *Hemigrapsus takanoi* and *Eriocheir sinensis* and to the native European shore crab *Carcinus maenas*, reared under comparable experimental conditions. Larvae of *H. sanguineus* were reared at a combination of four temperatures (15 – 24 °C) and three salinities (20, 25, 32 ‰), from hatching to megalopa. Survival and growth rates showed complex responses to salinity and temperature. Larvae were able to metamorphose to megalopa with high survival and growth rates at high temperatures (18 – 24 °C) irrespective of salinity. However, at 15 °C successful development varied among salinities, and occurred through alternative developmental pathways (short or long) characterised by either five or six zoeal stages. The proportion of larvae developing through the long pathway was determined by the salinity (highest proportions at 32 ‰); those larvae metamorphosed to megalopa with increased body mass and carbon and nitrogen content. Larvae of *H. sanguineus* along with those from the other two invasive species to the North Sea coast, showed higher sensitivity to low temperatures and lower sensitivity to low salinity than larvae of *C. maenas*. At 15 °C and 20 ‰, larvae of *H. sanguineus* showed higher survival rates than those of *H. takanoi*, *E. sinensis* and *C. maenas*. The ability of *H. sanguineus* to successfully develop at moderately low salinities, along with its high performance at high temperature may enhance the likelihood of establishing populations in regions of freshwater influence.

Keywords:

Hemigrapsus sanguineus, *Hemigrapsus takanoi*, *Eriocheir sinensis*, *Carcinus maenas*, multiple environmental drivers, larval performance, developmental plasticity

INTRODUCTION

The vulnerability of an ecosystem to be invaded by non-native species depends on several factors, such as the number of potential invaders as well as the suitability of environmental conditions (Wasson et al. 2001; Carlton 2002b; Pyšek et al. 2020; Green and Grosholz 2021; Reise et al. 2023). Coastal marine ecosystems are particularly vulnerable to the introduction of non-native species as they are usually exposed to a high number of transport vectors (e.g. shipping, aquaculture, construction of corridors, food trade; Wasson et al. 2001; Nehring 2006; Madricardo et al. 2019). Global climate change is causing changes in environmental variables in marine ecosystems at a high pace (Burrows et al. 2011b; Amorim & Wiltshire et al. 2023) with temperature as the environmental driver that has seen the most dramatic increase in the last century (IPCC 2019). Changes in the different environmental drivers are projected to be different among the open ocean and coastal marine environments, with coastal environments facing greater changes (FitzGerald et al. 2008; Day et al. 2008; Day and Rybczyk 2019). Besides, as a result of increasing population and trade among countries, coastal marine ecosystems are being altered by human-made structures as docks and marinas, piers, seawalls among other constructions (Briggs 2012; Mineur et al. 2012); which create new habitats available for colonization by non-native species (Carlton 1996, 2000; Byers 2002). Thus, coastal habitats are considered invasion hotspots (Molnar et al. 2008; Dawson et al. 2017).

Biological invasions are considered a major aspect of human-induced global climate change, the effects that non-natives can cause in the receptor environment have not only consequences on the biodiversity of the environments, but they also have economic costs and they can even affect human health (Ruiz et al. 2000; Simberloff et al. 2013; Pyšek et al. 2020). Invasion success and establishment of species in new habitats are linked to life-history traits and physiological tolerances, with non-natives species having wider tolerances to environmental fluctuations, they are usually diet generalists, they have complex life cycles and they have higher fecundity rates than their native counterparts (Sakai et al. 2001; Hänfling et al. 2011). Global climate change also constitutes a major modification of marine coastal habitat conditions with an impact on biological invasions. Rising temperatures may offer non-native species the opportunity to invade and survive in habitats where this was not possible under previous conditions (Walther 2010; Hulme 2017). Because of the wider tolerances to environmental fluctuations shown by non-native species, it is expected that under a warming scenario non-native species will perform better than native species (Sorte et al. 2010b; Anacleto et al. 2014). Considering the susceptibility of coastal marine ecosystems to the propagule pressure exerted by shipping and traffic, and considering that the establishment of non-native species in new habitats depends on the capacity to survive and reproduce, one would expect that global warming may enhance colonization, establishment, survival, and growth of non-native species (Sorte et al. 2010b).

Warming of coastal marine ecosystems occurs in a background of fluctuations in other environmental variables, such as salinity (Bojariu and Reverdin 2002; Goberville et al. 2010; Charria et al. 2020). Salinity is one of the main drivers of drives reproduction, dispersal, and recruitment of organisms in marine, coastal and estuarine habitats (Anger 2003; Cieluch et al. 2004; Anger et al. 2006; Torres et al. 2006). The interactive effect of warming and salinity on organisms may be considered within two main scenarios. First, salinity may change (along with temperature) because of long-term changes in the frequency and magnitude of precipitations, river flooding and runoff (Thompson et al. 2017; Tabari 2020; Rodrigues and Patil 2021). Those changes can have direct impacts on local conditions and communities (Lee et al. 2022) but are likely to vary regionally. Second, salinity may not change in the long-term. However, in this scenario, the quantification of effects of salinity on responses of organisms and populations is important because low salinity must be tolerated in the new context of increased temperature. Importantly, the combined effects of temperature and salinity cannot be predicted from the separate effects of each factor because they do not usually operate on organisms in an independent manner. Instead, they produce interactive effects that are species-specific, with increased temperature either enhancing or weakening to low salinity (Spitzner et al. 2019a; Torres et al. 2021b). Considering the vulnerability of coastal ecosystems to human-induced environmental changes and the fact that they are regarded as invasion hotspots, studying the responses of native and non-native species becomes relevant in the context of increased temperature.

An example of an invasive species that has established populations in marine coastal ecosystems is given by the Asian shore crab *Hemigrapsus sanguineus* (Hwang et al. 1993; Stephenson et al. 2009; Epifanio 2013). *H. sanguineus* has invaded the Atlantic coast of Europe and North America. In Europe, *H. sanguineus* was first observed in the Dutch Delta system in 1999 (Breton et al. 2009) and then spread further southwest and northeast reaching the coast of Scandinavia (Wolff 1998; Dauvin et al. 2009; Epifanio 2013; Seeley et al. 2015; Karlsson et al. 2019b). In North America, it was first recorded in the 1980s, and since then it has spread and it can be found from North Carolina to Maine (35°- 45°N, Epifanio et al. 1998; Delaney et al. 2008; Stephenson et al. 2009). In both invaded ranges, *H. sanguineus* has caused the displacement of *C. maenas* which is native to Europe (Lohrer et al. 2000; Geburzi et al. 2018). *H. sanguineus* has a complex life cycle comprised of a pelagic larval phase with 5 zoeal stages (and a sixth under condition of low temperature, Espinosa-Novo et al. 2023), a settling megalopa and benthic juvenile and adult stages (Hwang et al. 1993; Epifanio 2013). Larvae are able to complete their development to megalopa at temperature conditions of at least 15 °C, the northernmost distribution of the species is determined by the larval tolerance to low summer temperatures (Stephenson et al. 2009; Gimenez et al. 2020).

A critical question concerns the larval responses to the combination of temperature and salinity. Larvae play an important role in population dynamics (Pineda et al. 2010) as they connect local populations (Cowen and Sponaugle 2009a). Larvae are considered a bottleneck in the ontogeny (Anger

2006); they are more sensitive to changes in environmental variables than juveniles or adults (Pandori and Sorte 2019a), and recruitment depends on the completion of the larval phase. We know that *H. sanguineus* is able to complete the larval development at salinities of at least 15 ‰ (as long as the temperature is ≥ 15 °C). In a population from the Atlantic coast of USA, optimum survival rates were found at the combination of high salinity and temperature while at low salinities survival was determined by temperature (Epifanio et al. 1998b). However, there are a series of important gaps in our knowledge of responses to temperature and salinity that are relevant to understand how larvae are likely to sustain local populations in regions of freshwater influence (Simpson 1997) which are extensive along the North European coast, e.g. in the Wadden Sea and the Skagerrak area (S Scandinavia) adjacent to the entrance to the Baltic Sea (Ducrotoy and Elliott 2008). In these regions, organisms must deal with the variations in salinity, but in the context of higher temperatures with the concomitant effects that multiple drivers acting in concert may have on survival of species. First, we do not know how larval growth is driven by temperature and salinity: this is important for fitness consequences beyond those expressed as survival patterns. For instance, variations in larval growth, reflected in the body size or level of reserves at the settling stage (e.g. megalopa) can have important carry-over effects at the juvenile stages (Pechenik 2006a; Gimenez 2010; Torres et al. 2016b; Giménez et al. 2020d). Second, longer developmental time during the larval phase will determine the time period when larvae will be exposed to the risk of predation (Eckert 2003). Third, the effects of temperature and salinity on duration of development drive the timing of larval settlement into intertidal zones, which in seasonal environments is critical for post-metamorphic growth. In seasonal habitats, post-metamorphic growth and survival is determined by the time horizons (Gotthard 2001), driven by fluctuations in temperature and in the activity of predators. Late settlement can result in those early juvenile stages growth primarily in autumn- winter, when reduced temperature would result in longer time to maturity or smaller size at maturity. The most effective growth strategy will vary according to the fitness costs and benefits of body size, developmental time, and growth rates. Currently, there is only data available limited to a single population from the US (Epifanio et al. 1998b) but responses of larvae to environmental drivers can vary considerably across populations (Šargač et al. 2021b, 2022a) and within populations. For example, for our study population of *H. sanguineus* i.e. Helgoland (North Sea, German Bight), we know that larval responses to temperature and food limitation vary considerably among larvae from different females in the range of temperatures of 18 - 24 °C (Espinosa-Novo et al. 2023a). We also know that *H. sanguineus* larvae are able to hyper- osmoregulate over the entire larval phase at high temperatures and low salinities (Torres et al. 2021a). This capacity to survive and develop over a wide range of temperatures and salinities may by be advantageous for *H. sanguineus* at the moment of arriving in a new environment.

Here, we quantified the responses of *H. sanguineus* larvae to different combinations of temperature and salinity conditions in a full factorial experiment. We first report the responses in terms

of survival, development, and growth rates. Second, we compared the responses of *H. sanguineus* larvae to those reported for the native European shore crab *Carcinus maenas* (Šargač et al. 2021b) and two other invasive species in Europe *Hemigrapsus takanoi* (Geißel et al. in prep) and *Eriocheir sinensis* (Anger 1991), obtained from experiments carried out under comparable conditions. We focus on the responses of larvae from native and invasive species that coexist in the benthic habitats as juveniles and adults, as the performance of species is also related to the species that coexist and interact with them. In a previous study in the North Sea, *H. sanguineus* larvae showed different patterns in the responses to food limitation and increasing temperatures than the native species *C. maenas* (Espinosa-Novo et al. 2023a), which in turn will determine species interaction in the benthos as growth and development have a direct impact on the performance of the benthic stages (Gimenez et al. 2004; Torres et al. 2016b).

MATERIALS AND METHODS

Animal husbandry and larval rearing

Females with early-stage embryos were collected from the intertidal shore of the island of Helgoland (North Sea, German Bight, 54°10'40.9" N 7°53'32.4" E) during their reproductive season (July- September) and transported to the laboratory (Biologische Anstalt Helgoland, Alfred-Wegener-Institut Helmholtz-Zentrum für Polar- und Meeresforschung). Upon arrival to the laboratory, berried females were kept in separated 2-L aquaria with UV-treated filtered seawater (2 µm, 32 ‰), in a temperature-controlled room at 18 °C with a 12:12 h light: dark cycle. They were fed twice a week with shrimp (*Crangon crangon*) and the water from each aquarium was changed daily to ensure high-quality hatching conditions.

Experiments were repeated 5 times, using larvae from a different female for each repetition to account for variation among larvae coming from different females. Freshly hatched larvae from each of the 5 different females were randomly assigned to 12 treatments, combination of 3 salinities (20, 25 and 32 ‰ corresponding to seawater) and 4 temperatures (15, 18, 21 and 24 ºC), in a factorial design. Larvae were reared in groups of 10 individuals in 60-mL rearing glasses in 3 replicates per combination of temperature and salinity. The different combinations of salinity and temperature were chosen based on natural occurring ranges in the German Bight. Salinities in the German Bight usually vary between 20 - 33 ‰, depending on distance to the Elbe and Weser rivers (Bils et al. 2012). Salinities of 20 and 25 ‰ represent conditions of osmotic stress for the larvae (Torres et al. 2021d). Temperatures of 15 and 18 ºC are commonly experienced by larvae in the German Bight in summer and spring (Wiltshire et al. 2010), temperatures between 21 and 24 ºC represent possible warming scenarios as the temperature is expected to increase in the next years (IPCC, 2021; Amorim & Wiltshire et al. 2023).

Experiments were carried out in temperature-controlled rooms and using natural filtered (2 µm) and UV-treated seawater (32 ‰). In order to obtain salinities for the different treatments, natural seawater was diluted with the appropriate amount of tap water and controlled using a conductivity meter (WTW Cond 3110 SET1). Experimental water was stored in tanks in the corresponding experimental temperatures and salinity was monitored daily to ensure that it remained constant during the experiments. Water in the treatments was changed daily following standard procedures for larval rearing (Torres et al. 2021d). During the daily water change, the number of larvae and the stage were recorded, dead larvae were also recorded and discarded. Larvae were fed daily with *ad libitum* freshly hatched *Artemia* sp. in a concentration of ~ 5 nauplii/mL (Torres et al. 2021d).

Body mass and elemental composition (carbon and nitrogen) were quantified in freshly hatched larvae randomly chosen at the start of the experiment (50 zoea I in 3 replicates) and in freshly moulted megalopa (sampled within 24 h after moulting). All sampled individuals were pipetted onto a filter, gently rinsed with distilled water, blotted dry with filter paper and stored in pre-weighed tin cups at -20 °C for later analysis. To determine the dry mass, samples were freeze-dried for 48 h and weighed using a microbalance (Sartorius SC2, precision 1 µg). Carbon (C) and nitrogen (N) contents were then determined using an elemental analyser (vario MICRO cube CHNS analyser, Elementar Analysensysteme).

Data analysis

Cumulative survival to each zoeal stage was calculated as the percentage of survivors in relation to the number of organisms at the start of each experiment. Cumulative duration of development to each zoeal stage was determined as the time needed to reach the next developmental stage, considering the duration of development for the previous stages. Growth rates were estimated as $G = \log (W_f / W_0)/t$, where W_0 is the average mass (dry mass, carbon or nitrogen) at hatching, W_f is the corresponding mass of each individual megalopa collected in each rearing replicate and t is the corresponding duration of development of each individual megalopa.

Mixed modelling was used to quantify the combined effects of temperature and salinity on the response variables: survival, developmental time, dry mass, elemental composition (carbon and nitrogen) and instantaneous growth rates from hatching to megalopa. We applied backward model selection (Zuur et al. 2009) based on the second order Akaike information criterion (AICc). Model fitting was carried out with generalised least squares, using the package "nlme" (function lme and gls, Pinheiro et al. 2018, R Core Team 2013). The models contained temperature and salinity as fixed factors and female of origin as a random factor. The analyses were carried out in two steps: in a first step model selection of the random terms was performed by comparing models after restricted maximum likelihood (REML) and the model with the lowest AICc was selected for additional analysis. In those cases when

ΔAICc < 3 and the most complex model had the lowest AICc, we used hypothesis testing (likelihood radio tests, LRT) to test for model suitability: when the difference was significative ($p < 0.05$) the model with the lowest AICc was chosen; when the difference was not significative, we chose the simplest model (i.e. the model with the lowest number of parameters). In a second step, the fixed terms were tested through maximum likelihood (ML).

For the data analysis of survival, we first re-scaled the proportions (p) using the equation $p' = [p (n-1) + 0.5]/n$, where *n* is the number of larvae at the start of the experiment (n = 10 individuals) to avoid inconsistencies of log (0) values. Survival proportions were then transformed to the logistic (Warton and Hui 2011a) and logarithmic scales. The logarithmic transformation is used to test for the multiplicative effect as a null model, as the multiplicative model is used to test if salinity and temperature operate independently in the survival rates (Piggott et al. 2015a; Torres and Giménez 2020a). Duration of development was analysed in the raw and logarithmic scales to test for the additive, multiplicative (additive in the log-scale), or interactive (both scales) effects. For dry mass, elemental composition, and growth rates at the megalopa we did not get sufficient samples in larvae reared at 15 ºC and 20 ‰ for the female 5 (F5). In order to analyse the data, we used two different starting models: (1) excluding the treatment 15 ºC, but considering all the females and (2) excluding F5, but considering all the treatments. In order to determine differences among treatments, we performed Tukey´s HSD (honestly significant difference) post hoc test.

Comparisons between native and invasive crabs occurring in the North Sea coast

We compared the responses of larvae of *Hemigrapsus sanguineus*, the two other invasive crabs known in the North Sea (*H. takanoi* and *Eriocheir sinensis*) and the native species *Carcinus maenas*. *H. takanoi* has a recent history of invasion of the North Sea (since 1993) and occurs in intertidal areas (Geburzi et al. 2018). *E. sinensis* has an longer invasion history (more than 80 years, Dittel and Epifanio 2009): larvae develop in open waters but adults occur in rivers and perform ontogenetic migrations to the coastal areas where larvae are released (Anger 1991). We used survival data, obtained from experiments carried out under comparable conditions in the same laboratory (Biologische Anstalt Helgoland, Alfred-Wegener-Institut Helmholtz-Zentrum für Polar- und Meeresforschung) using similar diet (freshly hatched *Artemia* sp. Nauplii provided *at libitum*), salinities, and temperatures, and following the same rearing protocol (Torres et al. 2021d). Data of *C. maenas* were obtained from (Šargač et al. 2021), data of *Hemigrapsus takanoi* were obtained from Geißel et al. (in prep), both based on berried females collected in the local population of Helgoland as it was the case of *H. sanguineus*. Data of *E. sinensis* were obtained from Anger (1991): in this case, the adult population originates in the Elbe River, females release larvae at the Elbe mouth and larval development takes place in the German Bight, in areas adjacent to Helgoland (Anger 1991); thus, when the experiments were performed,

berried females were collected at the Elbe mouth and transported to Helgoland until hatching. For *H. takanoi*. and *C. maenas* temperature and salinity rearing conditions coincide fully with those of *H. sanguineus*; for *E. sinensis* there were only data available corresponding to larval rearing at 15 and 18 °C.

In order to compare the interactive responses to temperature and salinity, we used the space state approach (SSEA: Giménez et al. 2021). In SSEA, responses are viewed as showing the state of a system (here a group of organisms) as points in a space defined by the magnitude of a response to a combination of environmental variables (here salinity and temperature). For two variables, the space is defined by three axes quantifying the additive effect of each variable (axes 1 and 2) and the interactive effects (axis 3). SSEA works much as a principal component analysis in that one can visualise patterns produced by a large number of data points (Fig. 1). In SSEA, one can visualise interactive responses in a large number of treatment combinations and experimental replications. SSEA is also helpful in our case where larvae show different environmental optima. For instance, larvae of *C. maenas* are better at tolerating lower temperatures (complete larval development is achieved at 12 °C, Dawirs et al. 1986) than *H. takanoi* (high mortalities at 15 °C: Geißel et al. in prep) and *H. sanguineus* (this study). Hence, we cannot compare interactive responses among species based on the standard classification system (e.g. as either antagonistic or synergistic) because species do not share an optimal combination of temperature and salinity. Instead, we define a "reference" condition, against which we centre the responses observed at other temperature-salinity combinations. We first define responses, $R_{(x,y)}$, as logarithmic transformed survival proportions with x = temperature and y = salinity. Second, we take $R_{(0,0)}$ as the response at the reference temperature-salinity combination, here given by 15 °C and seawater (32 ‰); $R_{(T,0)}$ is the response at a temperature $T \neq 15$ °C (e.g. T = 18 °C) and the reference salinity, $R_{(0,S)}$ is the response at the reference temperature and a given salinity $S \neq 32$ ‰ (e.g. S = 20 ‰), and $R_{(T,S)}$ is the response at the chosen temperature-salinity combination away from the reference (e.g. T = 18 °C and S = 20 ‰). Third, the responses are centred by computing the differences: $D_{1(T)} = R_{(T,0)} - R_{(0,0)}$; $D_{2(S)} = R_{(0,S)} - R_{(0,0)}$ and $D_{3(TS)} = R_{(T,S)} - R_{(0,0)}$; the values $D_{1(T)}$ and $D_{2(S)}$ give the magnitude of the effects of temperature or salinity operating alone (f_1 and f_2 respectively), with respect to the reference condition; their sum gives the expected additive effect of both variables, $D_{1(T)} + D_{2(S)} = A_{(T,S)}$. Fourth, the magnitude of the interactive effect is computed as $G_{(T,S)} = D_{3(TS)} - A_{(T,S)}$. This procedure was repeated for any combination of temperature and salinity and for larvae of each female separately (i.e. the reference treatment is contingent to the female of origin). Figure 1 gives the 2-D projections of the 3-D-SSEA representation (f_1, f_2, g) with selected types of interactive responses (interaction plots given in the outer grey boxes), represented as stars with roman numbers set on different quadrants. In addition, lack of response ($f_1 = f_2 = g = 0$) would result in a star situated on the origin of the coordinate frame while additive effects ($g = 0$) will result in stars plotted on the axes f_1 and f_2 (not shown in Fig. 1).

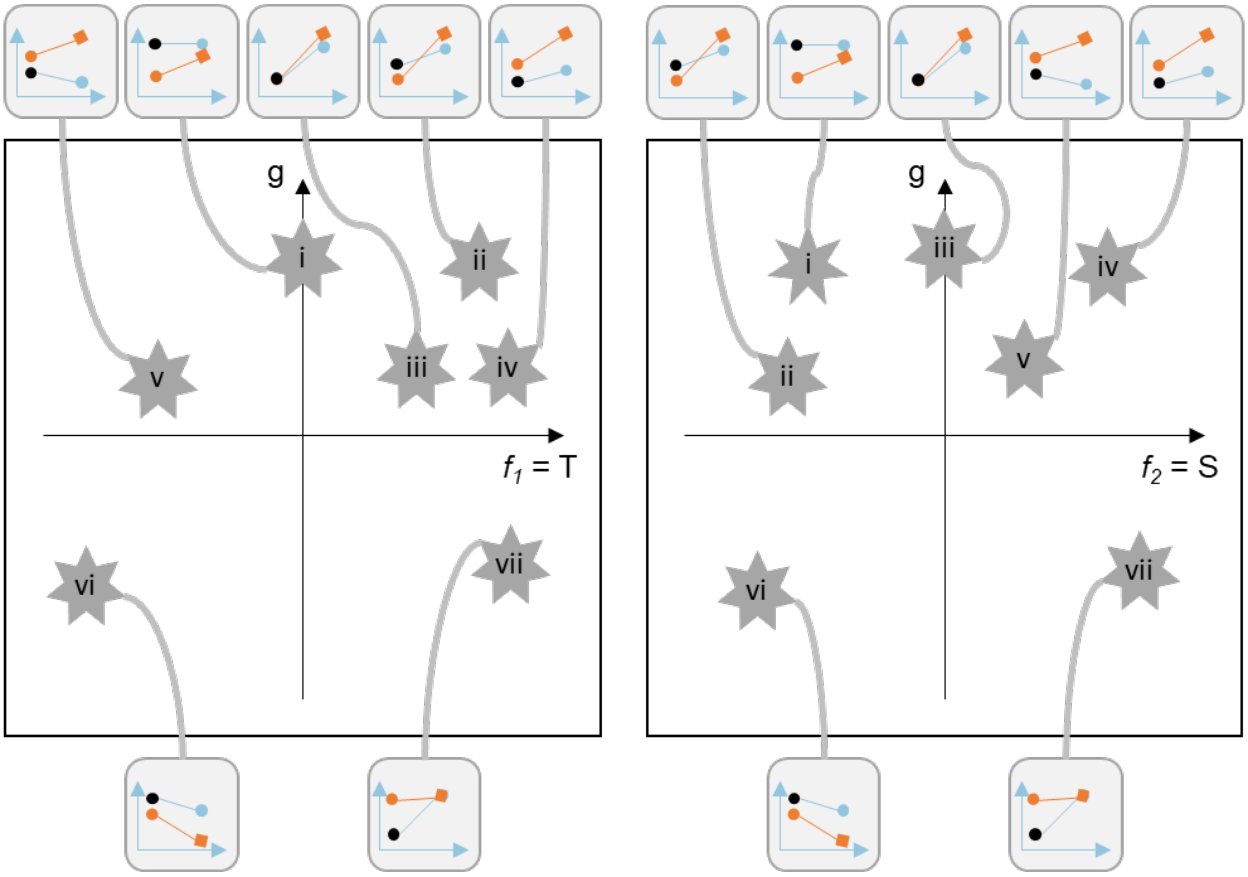

Figure 1. SSEA representation: 2-D projections (modified from Giménez et al. 2021) showing the position as roman numbers inside stars, which correspond to selected interactive effects of temperature and salinity on survival (interactions plots as grey outboxes). The axes are as follows: f_1: effect of temperature; f_2: effect of salinity; g: interactive effect. In the interaction plots (grey outboxes), the response at the reference treatment is represented as black circles (SW and 15 °C), blue circles are the response to temperature (SW and 18, 21 or 24 °C), orange circles are the response to salinity (20 or 25 ‰ and 15°C), and orange squares are the responses to salinity and temperature (20 or 25 ‰ and 18, 21 or 24 °C). The representation captures a great variability in the responses expected from the different species. For example, triple negative effects ($f_1 < 0, f_2 < 0$, g < 0) of temperature, salinity, and interaction are represented by the star vi (located in the bottom left quadrant of both graphs). On the contrary, triple positive ($f_1 > 0, f_2 > 0$, g > 0) are represented by the star iv (located in the upper right quadrant of both graphs). In cases where one of the variables has no effect, $f_1 = 0$ or $f_2 = 0$, the representation will include one of the stars on the g- axis as shown by the star i for the temperature graph ($f_1 = 0$), and the star iii for the salinity graph ($f_2 = 0$). When the effect is additive (g = 0), the points are in the plane defined by f_1 and f_2 (not shown).

RESULTS

Survival, duration of development, and growth rates

Larval survival to megalopa varied little among females and was mostly driven by temperature; highest survival rates were found at the highest temperature treatments irrespective of the salinity (Fig. 2). At the highest temperatures (21 and 24 ºC), survival was consistently higher than 65 % and without evidence of salinity effects. For larvae reared at 18 ºC and salinity 25 ‰, survival was still high (68 %)

and it dropped in the other two salinities (only between salinity 20 and 25 ‰ differences were significant, $p < 0.01$). When larvae were reared at 15 °C, the negative effect of salinity was stronger (Fig. 2): survival was higher at 25 ‰ (ca. 50 %) than at 20 ‰ (< 15 %). Best models retained temperature and salinity operating in an additive or an interactive way depending on the stage (except in zoea III where temperature was retained), in the intermediate stages (II- IV) the effect of salinity was weak (survival > 75 %); in stage zoea V the survival dropped down to 60 % if larvae were reared at 20 ‰ and 32 ‰ at 15 °C (Fig. S1). Survival to megalopa was explained by the interactive model (Table S1); the effects of low salinity occurring at 15 ºC appear mitigated (or reversed) at 21 and 24 ºC.

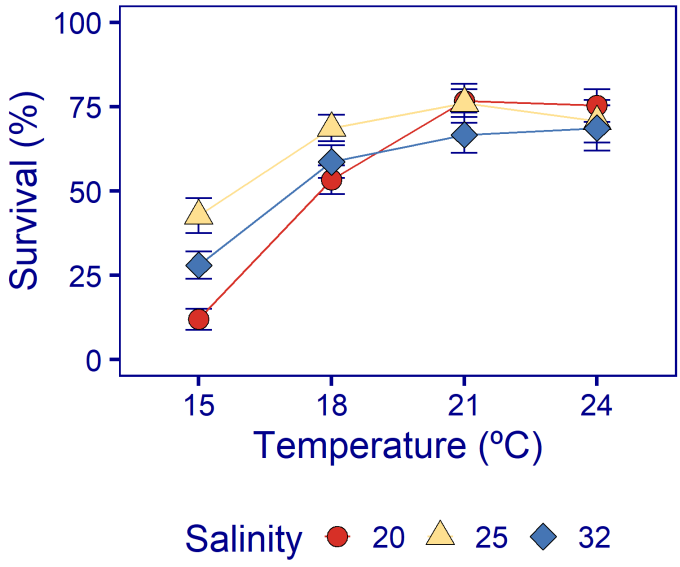

Figure 2. *Hemigrapsus sanguineus*. Average larval survival from hatching to megalopa for the combined effects of temperature and salinity. Data shown as average survival ± SE for the females of origin (n = 5). Symbols represent the different salinity treatments; red circle: 20 ‰, blue triangle: 25 ‰ and green diamond: 32 ‰.

Duration of development to each larval stage increased non-linearly with exposure to low temperatures, irrespective of salinity and with little variations among females (Fig. 3a). The best models retained temperature in the duration of development to zoea II and V, temperature and salinity operating in an additive way to zoea III and IV, and interactively in the duration of development to megalopa (Fig. S2). In larvae reared at 15 °C, duration of development ranged between 46 - 72 days (20 ‰: 60.5 ± 2.8, 25 ‰: 54.3 ± 0.97, 32 ‰: 61.4 ± 0.74 days). In some individuals, development occurred through an alternative pathway with an extra zoeal stage (zoea VI). For larvae reared in 32 ‰, the duration of the zoeal phase was longer than in the other two salinity treatments ($p < 0.0001$). In larvae reared at 18 °C and 32 ‰, there was also an extension of the zoeal phase compared to the other two salinity treatments ($p < 0.0001$).

Instantaneous growth rates, in terms of dry mass, increased with increasing temperatures irrespective of salinity (Fig. 3b); best models retained additive effects of temperature and salinity, but

the effect of salinity was small (Table S2). Similar effects were found for growth rates in terms of carbon and nitrogen content (Fig. S3 a, c). Larvae reared at 20 ‰ showed reduced body mass (Fig. 3c), and carbon and nitrogen content Fig. S3 b, d) in the lower temperature treatments (15, 18 and 21 °C) compared to 32 ‰ (reduction of 23, 13 and 8 %, respectively). At 15 °C and 32 ‰, larvae reached a maximum of dry mass as well as carbon and nitrogen content (Figs. 3c, S3 b, d). C:N ratios showed variations among the different treatments; at 15 °C and 20 ‰, the reduction in carbon compared to nitrogen was larger than in 20 and 25 ‰ (Fig. 3d). In 24 °C and 32 ‰, we observed the opposite trend: i.e. an increase in the carbon content when compared to 20 and 25 ‰ (Fig. 3d).

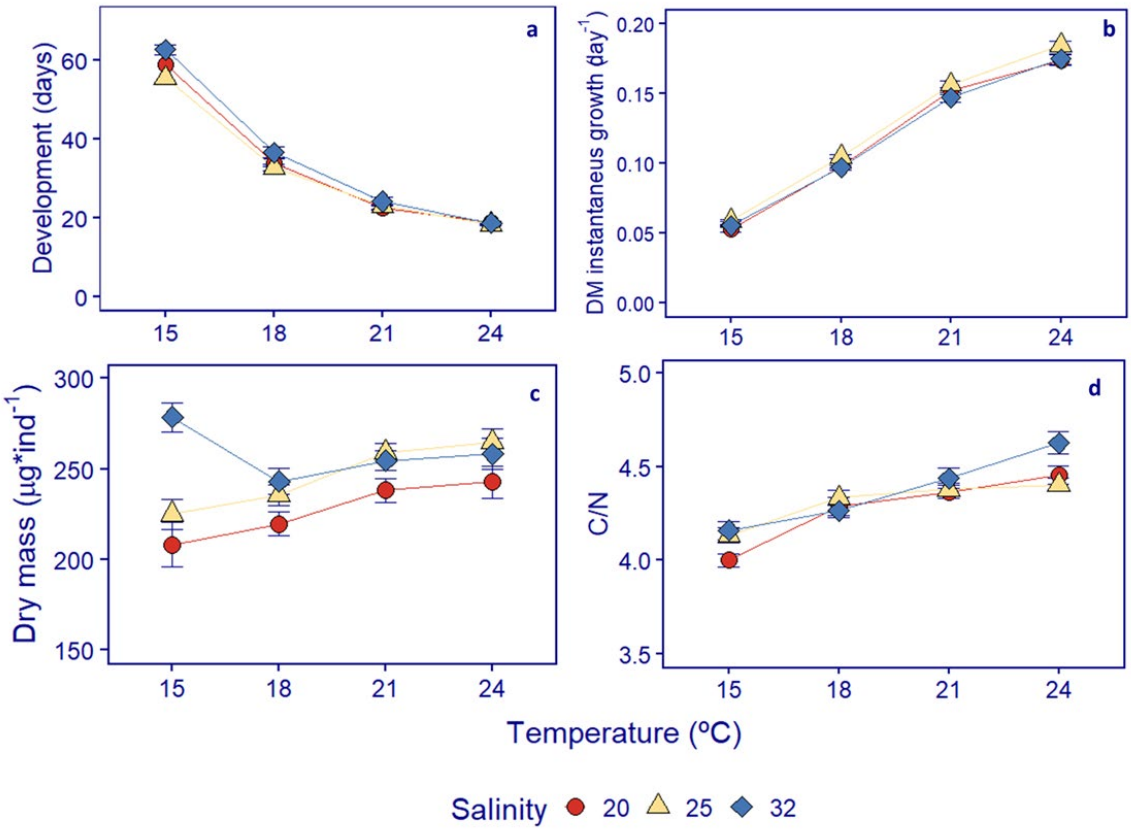

Figure 3. *Hemigrapsus sanguineus*. Average responses to the combination of temperature and salinity in terms of (a) duration of development, (b) instantaneous growth rate, (c) dry mass, and (d) C/N ratio. Data shown as averages ± SE for the females of origin (n = 5); symbols as in Fig. 1.

Integrated growth responses showed a general pattern where body mass correlated negatively with developmental time, especially due to the effect of temperature (Figs. 3, S4). The exception was that larvae reared at 15 °C and 32 ‰ reached the megalopa stage with the highest body mass. The lower body mass observed at a given salinity was not associated with an extension of the developmental time, except for larvae reared at 24 °C; for instance, at 15 and 18 °C, larvae reared at lower salinities reached metamorphosis to megalopa with smaller body mass and at an earlier time than those reared in 32 ‰.

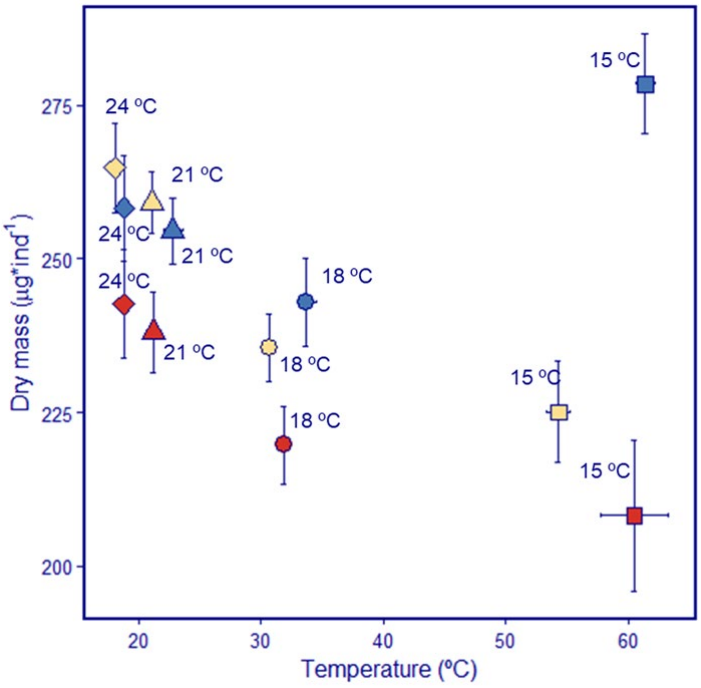

Figure 4. *Hemigrapsus sanguineus*. Integrated responses in terms of dry mass and duration of development to megalopa under the different combinations of rearing temperatures and salinities. Data shown as averages ± SE for the females of origin (n = 5). Symbols: 15 °C is represented with squares, 18 °C with circles, 21 °C with triangles, and 24 °C with diamonds; 32 ‰ is represented in blue, 25 ‰ in yellow, and 20 ‰ in red.

Comparisons between native and invasive crabs occurring in the North Sea coast

In this section, we compare larval responses to salinity and temperature of *H. sanguineus* with other invasive crabs (*H. takanoi* and *Eriocheir sinensis*) and the native *Carcinus maenas*. Survival responses to the combined effects of temperatures and salinities varied among species. Larvae of all invasive crabs, and in particular *H. sanguineus* show better survival when reared at 20 ‰ than the native *C. maenas*, especially at high temperatures. By contrast, *C. maenas* larvae reared in 32 ‰ show a consistently better performance than the invasive species, especially at low temperature. In synthesis, all invasive species are highly tolerant to moderately low salinities while the native is more tolerant to low temperatures.

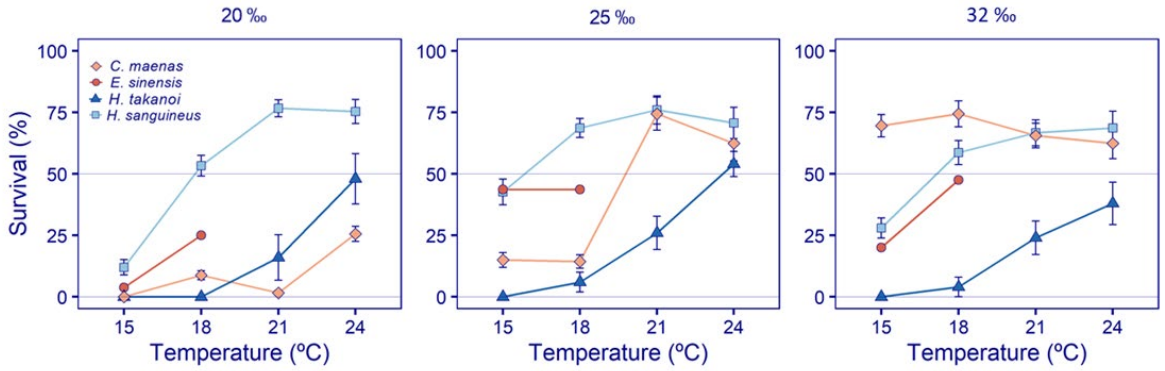

Figure 5. Survival from hatching to megalopa as a response to different combinations of temperature and salinity. Data shown as average survival ± SE (when available). Symbols in the graphs represent the different species: *H. sanguineus* is shown in light blue squares, *H. takanoi* in dark blue triangles, *C. maenas* in orange diamonds, and *E. sinensis* in red circles. Salinities are plotted in the different panels: 20 ‰ left panel, 25 ‰ middle panel and 32 ‰ in the right panel.

We used the state-space representation to explore differences in the nature of the interactive survival responses (in logarithmic scale) to temperature and salinity. In the representation, each point in figure 5 corresponds to the survival response of a group of larvae originated from each particular female to a specific temperature-salinity combination. The responses were computed using the survival in seawater at 15 °C as the reference (corresponding to the origin of coordinates, i.e. survival at 15 °C and 32 ‰ = 0); positive values reflect increases in survival and negative ones reflect decreases. In the representations, species are segregated from left to right irrespective of the temperature, salinity, or female of origin. The native *Carcinus maenas* segregates to the left of the panels showing little sensitivity to temperature (all points lie on the interactive axis of Fig. 5a) and that the negative effects of low salinity are mitigated at high temperatures (Fig. 5b: points in the upper left quadrant). By contrast, the exotic species, including *H. sanguineus* segregate to the right quadrants of Fig. 5a showing the positive effect of high temperatures, while they cluster around the centre of origin in Fig. 5b showing less sensitivity to salinity than *C. maenas*. The three invasive species show positive interactive responses (i.e. towards the direction of the effect of temperature: upper right quadrant) and negative interactive responses (i.e. opposite the direction of the effect of temperature: lower right quadrant), reflecting a change in the optimal salinity. *H. takanoi* differs from the other two invasive species as it shows very little sensitivity to salinity, but it responds to the interaction salinity-temperature (Fig. 5b).

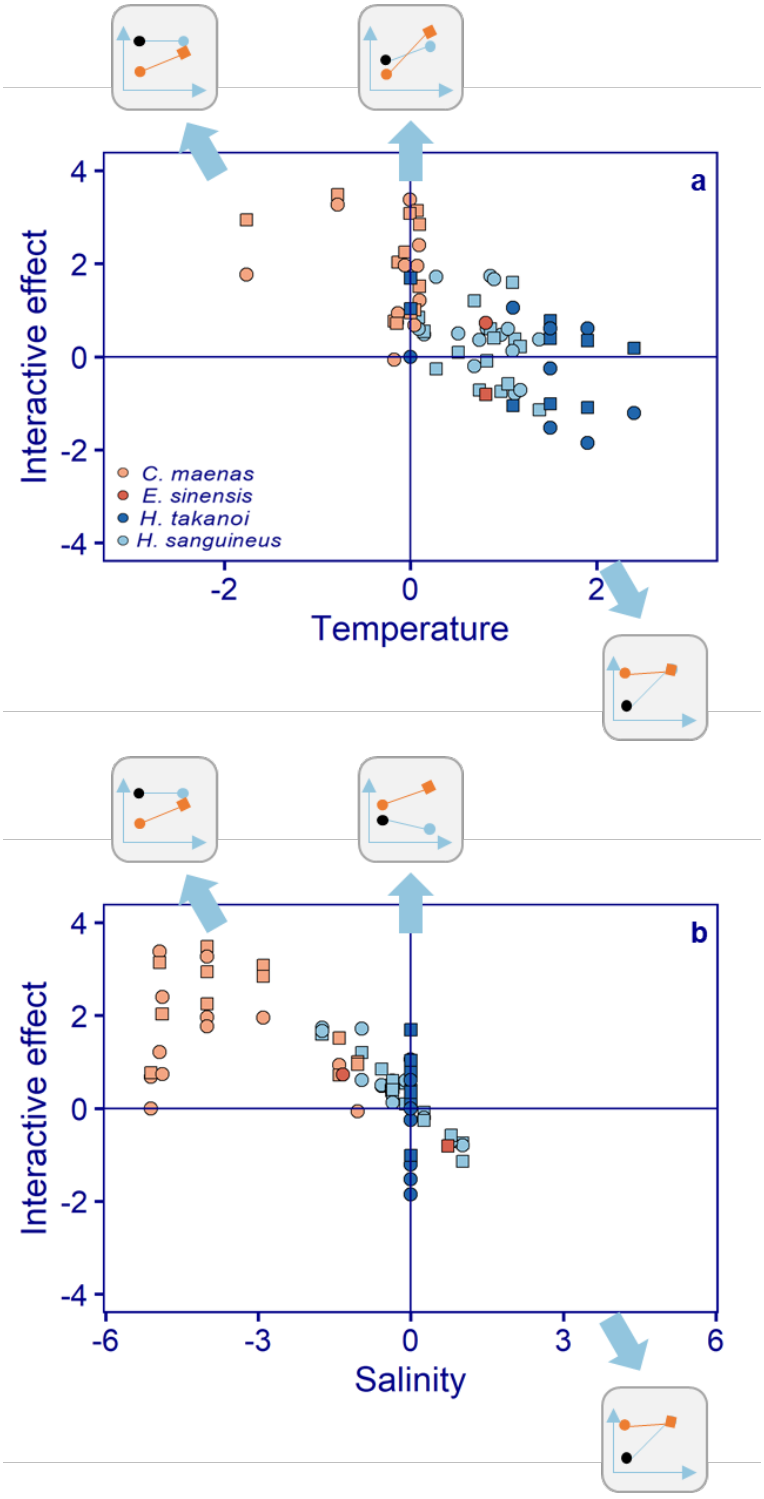

Figure 6. SSEA representation: representation of the survival response of larvae reared under different combinations of temperature and salinity. The representation is given in 2-D plots defined by the interactive effects (y-axis) and **(a)** temperature effect (x- axis) and **(b)** salinity effect (x-axis). The reference condition was the combination of 15 °C and seawater. In the plots, each symbol represents the response of a group of larvae, originated from each female, to a combination of temperature and salinity. Positive values represent conditions when survival increased, and negative values show conditions where survival decreased. Symbols in the graphs represent the different species: *H. sanguineus* is shown in light blue squares, *H. takanoi* in dark blue triangles, *C. maenas* in orange diamonds, and *E. sinensis* in red circles. Salinity 25 ‰ is represented by squares and salinity 20 ‰ by circles. Outbox graphs refer to examples in Fig. 1.

DISCUSSION

We quantified the combined effects of temperature and salinity on survival and the integrated responses of duration of development, body mass and elemental composition (C and N content), and growth rates during the ontogeny of the invasive crab *Hemigrapsus sanguineus* in a local population in the German Bight in the North Sea. Larvae of *H. sanguineus* showed complex responses to temperature and salinity as the consequence of developing through an alternative pathway characterised by different numbers of zoeal stages. Larvae were able to metamorphose to megalopa under the range of temperatures and salinities studied (15 - 24 °C and 20 - 33 ‰). The relative wide range of salinity tolerance is consistent with the known capacity of the larvae to osmoregulate during ontogeny (Torres et al. 2021a); larvae that show a wide range of salinity tolerance tend to be very little affected when exposed to low salinities (Charmantier 1998; Torres et al. 2011). However, high mortality occurred at the lowest temperature (15 °C) where we observed larvae developing through a long developmental pathway characterised by six zoeal stages, instead of five. When the survival of *H. sanguineus* was compared with other invasive and a native species, we found different patterns of responses to the combinations of temperatures and salinities tested. The three invasive species were more responsive to temperature, while the native species responded negatively to low salinities, but this negative effect was mitigated at high temperatures.

Responses of *H. sanguineus* to the combined effects of temperature and salinity

In general, we found that larval tolerance of *H. sanguineus* to low salinity is stronger at higher temperatures. Maximal survival occurred at 25 ‰, which coincides with high osmoregulatory capacity during ontogeny (Torres et al. 2021a); the ability of larvae to metamorphose to megalopa at 20 ‰ was modulated by temperature as found in a population in the southern Atlantic Bay, USA (Epifanio et al. 1998b). These results are in line with other studies showing lower larval survival at low temperatures in *H. sanguineus,* in its invasive range (Stephenson et al. 2009; Giménez et al. 2020a; Espinosa-Novo et al. 2023a). Development followed the expected pattern in its relation to temperature (Anger 2001a; Spitzner et al. 2019a; Torres and Giménez 2020a; Šargač et al. 2021b; Espinosa-Novo et al. 2023a), but it was characterised by a strong sensitivity to low temperature. The stress effect of low temperature was also manifested as development through an extra zoeal stage.

There was a short extension in the time to metamorphosis in response to low salinity, except at the lowest temperature (15 °C). Because growth rates were only slightly affected by low salinity, the result was that larvae metamorphosed to megalopa with reduced body mass at low salinities especially at 15 °C. The main reason for the difference between body mass in seawater *vs* lower salinities (found at 15 °C) is that in seawater a large proportion of larvae (ca. 63 %) that passed through the extra stage

(zoea VI) survived to megalopa. This longer pathway could also explain why, larvae reared in seawater at 15 °C metamorphosed to megalopa with a higher body mass than those reared at higher temperatures. The extension of the larval phase of *H. sanguineus* (ca. 7 days) is consistent with patterns found in other decapod species (e.g. *Neohelice granulata*), where the development through a longer pathway allowed larvae to feed and grow to larger megalopae (Giménez and Torres 2002). Development through alternative pathways is characteristic of shrimps (Anger 2001a; González-Ortegón and Giménez 2014a), and it is less common in brachyuran crabs except in the Grapsoidea (Anger 2001a) which includes *H. sanguineus*. The capacity of larvae to switch to an alternative developmental pathway when the conditions are not optimal, may be considered an adaptive response where organisms prioritise maintenance and growth over morphogenesis and it has been reported in a number of invertebrates under conditions of stress (Anger 2001a; Giménez and Torres 2002; Giménez and Anger 2003b; González-Ortegón and Giménez 2014a). Therefore, one could rank the level of stress responses based on the combination of the proportion of larvae reaching megalopae through the different pathways and the body mass of the megalopa (Fig. 7). At high temperatures (18 - 24 °C) and in all tested salinities (20 - 33 ‰) the stress level may be considered minimal; a great proportion of larvae survived (> 50 %) and all metamorphosed to megalopa following the short pathway, achieving a moderately high body mass (Fig. 7a). Moderate to high stress was progressively experienced at 15 °C and at salinities 25, 32, and 20 ‰: (1) at 25 ‰, survival was moderately low and about a third of the larvae metamorphosed via the long pathway; on average megalopa reached a low body mass (Fig. 7b). (2) At 32 ‰, a lower proportion of larvae survived, mostly by developing through the long pathway, resulting in the highest average body mass (Fig. 7c). (3) At 20 ‰ survival to zoea V was already very low and none of the larvae that passed through zoea VI metamorphosed to megalopa, and their body mass was low (Fig. 7d). It is important to mention that the extension of the planktonic larval phase has consequences associated to the timing of metamorphosis and body size of juvenile stages, because early juvenile stages are characterised by high mortality rates relative to advanced stages (Hunt and Scheibling 1997; Gosselin and Qian 1997). These consequences include a stronger tolerance to starvation (Giménez et al. 2004) and lower exposure to size- dependent predation (Moksnes 2004), which may be traded off against negative effects of late settlement (Klein Breteler 1976).

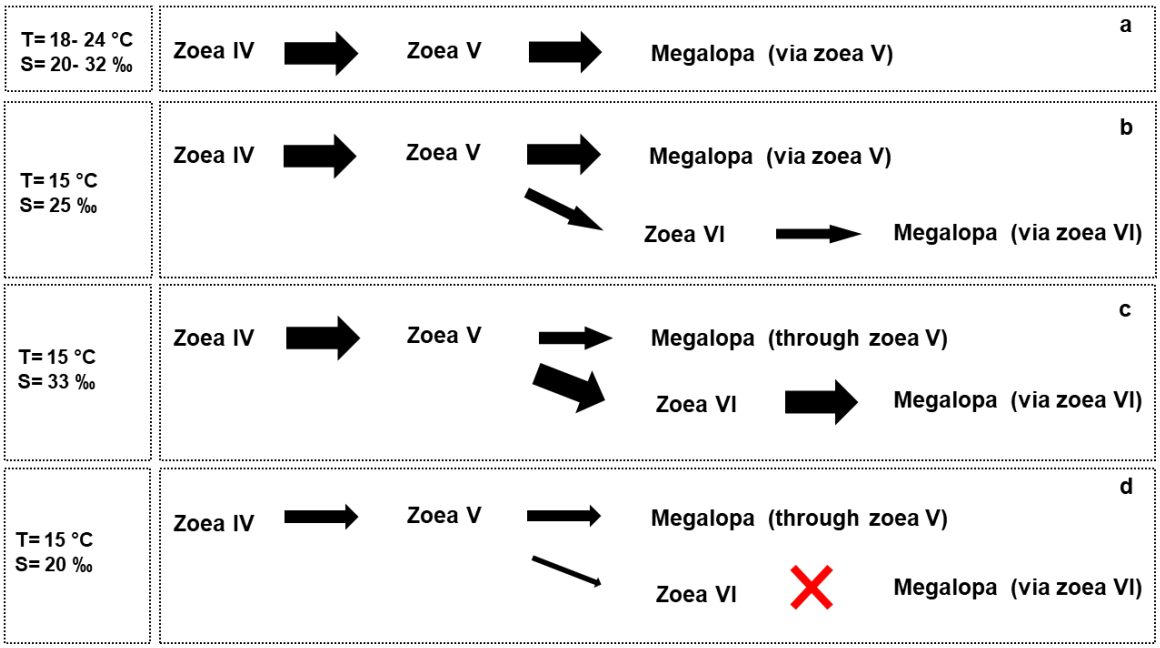

Figure 7. Model of the different responses to the interactive effect of temperature and salinity on larval development of *H. sanguineus*. (a) Pathway followed by larvae reared at all salinities (20 - 32 ‰) at the range 18 - 24 °C, representing minimal level of stress. (b) Pathway followed by larvae reared at 15 °C and 25 ‰, representing moderately low level of stress. (c) Pathway followed by larvae reared at 15 °C and 32 ‰, representing medium level of stress. (d) Pathway followed by larvae reared at 15 °C and 20 ‰, representing the highest level of stress, as a very low proportion of larvae reached stage V and none of the larvae that reached stage VI metamorphosed to megalopa (represented by the red cross). The width of the arrows represents the proportion of larvae that reached the different stages.

In larvae reared at high temperatures (18 - 24 °C), growth rates as well as body mass and reserves were only slightly affected by salinity. Understanding the mechanisms shaping the responses of organisms to salinity and temperature, is of particular interest under a global warming scenario. Here, it is important to consider the capacity of *H. sanguineus* larvae to osmoregulate, which occurs over the whole larval phase (Torres et al. 2021a). Osmoregulation is a mechanism by which organisms actively regulate the concentration of osmotically active ions (Charmantier 1998; Henry et al. 2012; McNamara and Faria 2012; Lignot and Charmantier 2015). We know that larval stages able to osmoregulate show a wider salinity tolerance than those that do not osmoregulate, and thus are less sensitive to exposure to low salinity in terms of growth rates (Torres et al. 2011). Importantly, the osmoregulatory capacity, which relies on the activity of the enzyme Na^+K^+-ATPase (Torres et al. 2007), can increase with temperature (Torres et al. 2021b); and we know that *H. sanguineus* is characterised by a strong osmoregulatory capacity at 24 °C during ontogeny (Torres et al. 2021a) where it achieves higher growth and survival rates. For the coastal North Sea, the only other crab species with comparable

osmoregulatory capacity during ontogeny is another invasive crab, *Eriocheir sinensis* (Cieluch et al. 2007).

Comparisons between native and invasive crabs occurring in the North Sea coast

We found that the survival responses of *H. sanguineus* to the combined effects of temperature and salinity resemble that of the other invasive crab species known for the North Sea. This is highlighted for instance, by the space-state representation where symbols corresponding to exotic species clustered separated from those of *C. maenas*. All three non-native species share an increased tolerance to low salinity in the range 20 - 25 ‰ which is not shown in the native *C. maenas* (survival ≤ 25 % at 20 ‰). This difference suggests that the drifting larvae of non-native species could occupy, at least temporarily, a coastal niche associated to estuaries and regions of freshwater influence where *C. maenas* larvae would struggle to survive. Niche opportunities (Shea and Chesson 2002) often arise when the traits of exotic species differ from the native species (Olden et al. 2006). Traits present only in invasive species provide an opportunity to occupy empty niches and establish populations in new environments (González-Ortegón et al. 2010; Azzurro et al. 2014; Tapkir et al. 2023). For example, *H. sanguineus* is already present in the Skagerrak area at the entrance of the Baltic Sea (Karlsson et al. 2019b) and *H. takanoi* has established populations in the Baltic Sea (Geburzi et al. 2022a). In addition, *E. sinensis* has established populations in the Baltic Sea where it is able to reproduce (Otto and Brandis 2011) and larvae hatch in lower parts of estuaries (Anger 1991).

Larvae of both *Hemigrapsus* spp. share an important sensitivity to low temperature (especially at 15 °C), where *C. maenas* shows a very high survival; in addition, larval survival of the *Hemigrapsus* spp. increased with temperatures (range 18 - 24 °C). This highlights the potential important role of global warming in facilitating the process of invasion through increased larval survival. Temperature is one of the most important environmental variables controlling physiological and ecological processes, as well as the evolution of ectotherms (Nguyen et al. 2011; Pinsky et al. 2019). Increases in water temperature can aid in the process of invasion via direct effects on survival and growth, giving the invasive species an advantage over the natives (Sorte et al. 2010b) but also by changing the time of recruitment of native and invasive species (Stachowicz et al. 2002).

We also found slight differences among larvae from the different exotic species, for example at low salinities larval survival of *H. sanguineus* increased strongly with temperature (reaching 50 % survival at 18 °C) while that of the congeneric *H. takanoi* increased in survival towards the highest temperature (reaching 50 % survival only at 24 °C). The pattern found in *H. takanoi* for Helgoland (Fig. 5) is also consistent with the pattern found for a local population in the island of Sylt, in the Wadden Sea (Geißel et al. in prep.). The responses of *E. sinensis* appear to be intermediate between that of *H. sanguineus* and *H. takanoi* at the tested temperatures (15 - 18 °C). Similar to *H. sanguineus*, *E. sinensis*

is able to osmoregulate over the entire larval phase (Cieluch et al. 2007), which may help explaining the higher survival found at moderately low salinities.

In synthesis, we found that *H. sanguineus* larvae showed complex survival and growth responses to temperature and salinity characterised by an extended larval phase through an additional zoeal stage at low temperatures. This pattern explains the non-linear response of body mass to temperature in seawater, and the increased body mass at 15 °C and seawater as compared to larvae reared at lower salinities. This is the first time *H. sanguineus* is reported to have successfully completed the development through an alternative pathway. *H. sanguineus* and *E. sinensis* are the only crab species in the North Sea exhibiting this type of developmental plasticity. In addition, the wide tolerance of *H. sanguineus* to moderately low salinities appear to be characteristic of larval stages of the invasive crabs of the North Sea coast. This tolerance, along with the increased performance at higher temperatures is likely to contribute to range expansion and of non-native species as a consequence of warming.

SUPPLEMENTARY MATERIAL

RESULTS

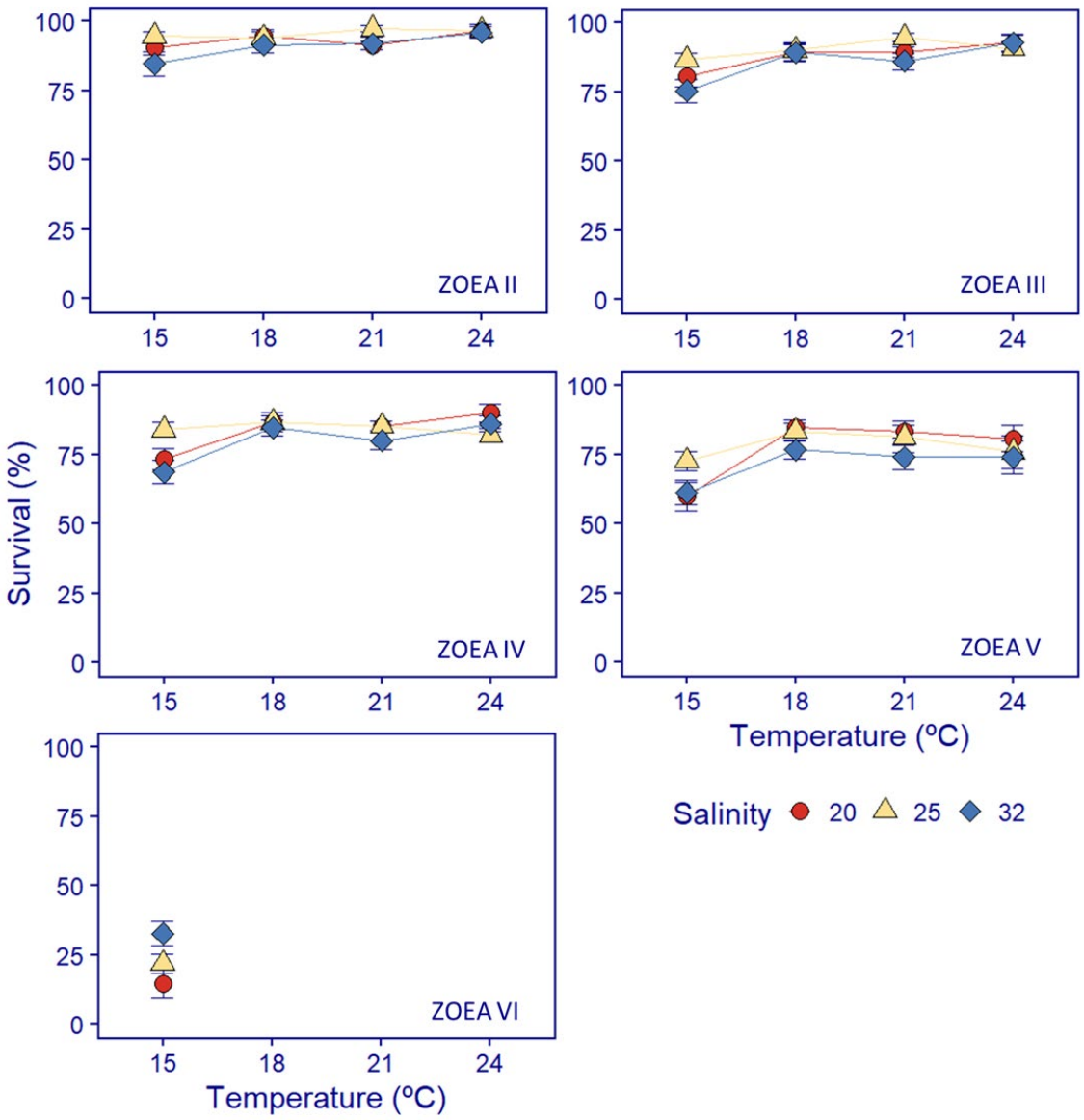

Figure S1. Larval average survival from hatching to zoea I, II, III, IV, V and VI for the combined effects of temperature and salinity. Data shown as average survival ± SE considering the five females of origin. Symbols in the graphs represent the different salinity treatments; red circle: salinity 20 ‰, yellow triangle: salinity 25 ‰ and blue diamond: seawater salinity (33 ‰).

Table S1. Model selection for larval survival of *Hemigrapsus sanguineus* from hatching to megalopa as a response to different temperature and salinity conditions. Data was analysed in the logistic and logarithmic scales. Akaike information criteria (AICc) was used to perform model selection. Symbols: Abbreviations: ♀: female of origin; S: salinity condition; T: temperature, ZII: zoea II, ZIII: zoea III, ZIV: zoea IV, ZV: zoea V, and M: megalopa, respectively). Highlighted in bold: the best overall model (containing both the best random and fixed term).

Model selection	Scale									
Random (REML)	Logistic					Logarithmic				
	ZII	ZIII	ZIV	V	M	ZII	ZIII	ZIV	V	M
♀:S:T	-270	-204	-131	-90	-48	**-228**	-143	-37	32	266
♀:T	-280	-211	-137	-93	-49	-238	-152	-44	32	248
♀:S	-279	**-221**	**-148**	**-107**	**-61**	-235	**-160**	**-53**	**15**	246
♀	**-280**	-214	-142	-98	-54	-237	-155	-48	27	**243**
Fixed (ML)										
S:T	-348	-282	-204	-161	**-112**	-302	-217	-104	-30	**213**
S+T	**-354**	**-286**	-205	**-166**	-100	**-308**	**-221**	-106	-33	246
T	-350	-283	**-204**	-162	-91	-303	-218	**-105**	**-31**	255
S	-348	-276	-196	-149	-64	-302	-212	-97	-17	361
Null	-344	-273	-196	-145	-54	-297	-208	-97	-14	364

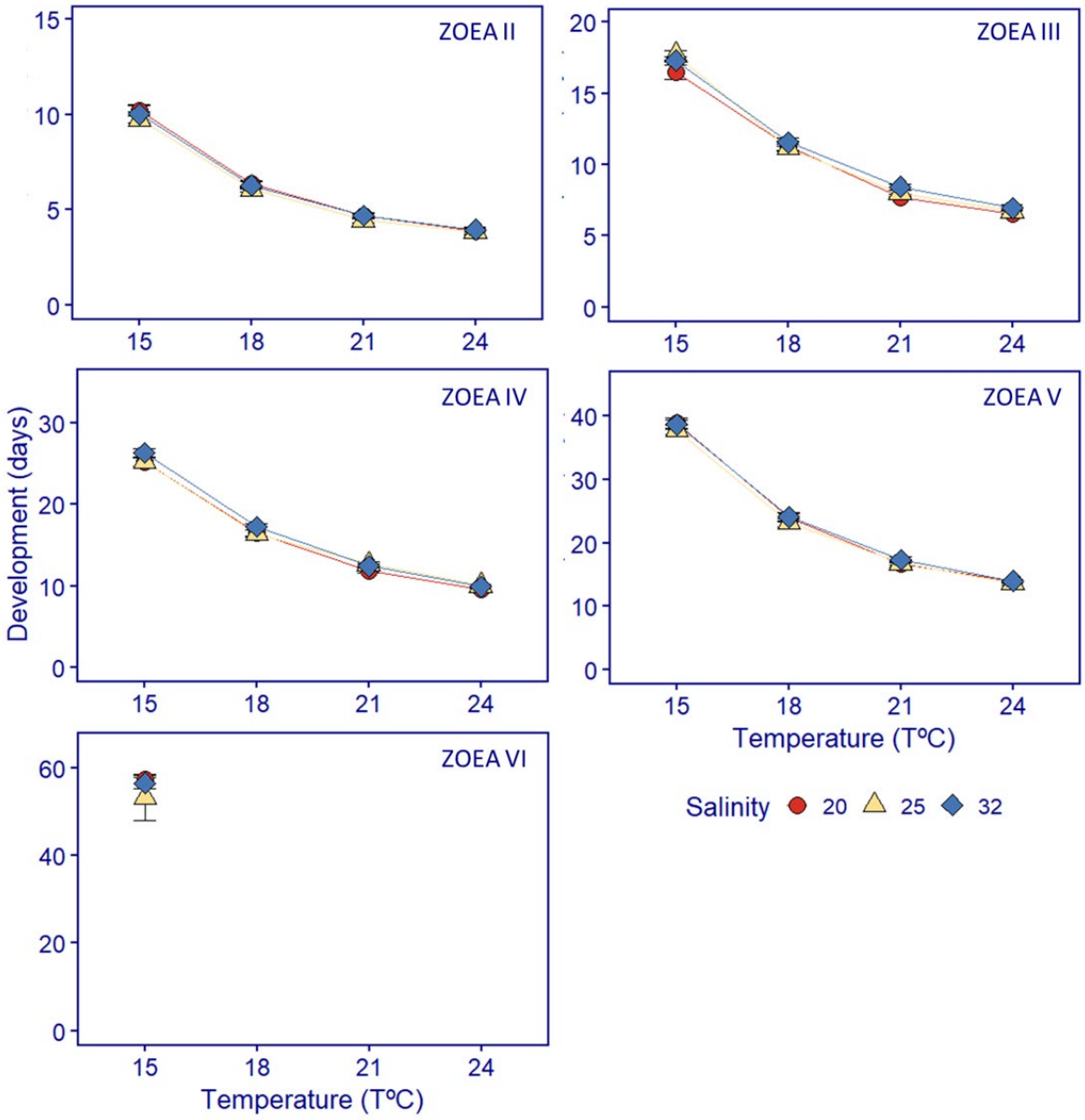

Figure S2. Larval average development from hatching to zoea I, II, III, IV, V and VI for the combined effects of temperature and salinity. Data shown as average duration of development ± SE considering the five females of origin. Symbols in the graphs represent the different salinity treatments; red circle: salinity 20 ‰, yellow triangle: salinity 25 ‰ and blue diamond: seawater salinity (33 ‰).

Table S2. Model selection for larval development of *Hemigrapsus sanguineus* from hatching to megalopa as a response to different temperature and salinity conditions. Data was analysed in the raw and logarithmic scales. Akaike information criteria (AICc) was used to perform model selection. Symbols: Abbreviations: ♀: female of origin; S: salinity condition; T: temperature, ZII: zoea II, ZIII: zoea III, ZIV: zoea IV, ZV: zoea V, and M: megalopa, respectively). Highlighted in bold: the best overall model (containing both the best random and fixed term).

Model selection	Scale									
Random (REML)	Raw					Logarithmic				
	ZII	ZIII	ZIV	V	M	ZII	ZIII	ZIV	V	M
♀:S:T	443	550	642	730	**528**	-261	-268	-301	-	-240
♀:T	456	564	650	743	621	-204	-224	-271	-330	-214
♀:S	**423**	**538**	**621**	**709**	533	**-276**	**-282**	**-318**	**-377**	**-254**
♀	451	564	646	738	619	-209	-226	-276	-335	-219
Fixed (ML)										
S:T	409	531	621	715	**538**	-340	-347	-385	-448	**-319**
S+T	397	**527**	**616**	705	552	**-352**	**-354**	**-391**	**-458**	-314
T	**398**	533	622	**707**	556	-348	-342	-383	-455	-289
S	456	594	685	790	582	-281	-296	-328	-385	-257
Null	458	600	692	792	587	-277	-284	-320	-382	-232

Table S3. Model selection for dry mass (DW), carbon (C) and nitrogen (N) content per individual and instantaneous growth in terms of dry mass (IgDW), carbon (IgC) and nitrogen (IgN) and C/N ratio in Hemigrapsus sanguineus larvae. Akaike information criteria (AICc) was used to perform model selection. Symbols: Abbreviations: ♀: female of origin; S: salinity condition; T: temperature, ZII: zoea II, ZIII: zoea III, ZIV: zoea IV, ZV: zoea V, and M: megalopa, respectively). Highlighted in bold: the best overall model (containing both the best random and fixed term).

Note: Model without including data for larvae reared at 15 °C

Random (REML)	AICc						
Term	DW	C	N	IgDW	IgC	IgN	C/N
♀:S:T	4506	3587	2349	-2252	-2264	-2258	189
♀:T	4495	3579	2342	-2166	-2180	-2178	190
♀:S	4496	3579	2343	-2157	**-2272**	-2265	188
♀	**4491**	**3575**	**2339**	**-2262**	-2180	-2177	**180**
Fixed (ML)							
Full factorial	4544	3608	2347	-2353	-2358	-2352	142
S+T	**4538**	**3602**	2343	**-2360**	**-2365**	**-2359**	145
T	4549	3604	2345	-2333	-2341	-2232	**143**
S	4552	3604	**2341**	-1781	-2333	-2327	150
Null	4562	3605	2342	-1778	-2310	-2301	147

Table S5. Model selection for dry mass (DW), carbon (C) and nitrogen (N) content per individual and instantaneous growth in terms of dry mass (IgDW), carbon (IgC) and nitrogen (IgN) and C/N ratio in Hemigrapsus sanguineus larvae. Akaike information criteria (AICc) was used to perform model selection. Symbols: Abbreviations: ♀: female of origin; S: salinity condition; T: temperature, ZII: zoea II, ZIII: zoea III, ZIV: zoea IV, ZV: zoea V, and M: megalopa, respectively). Highlighted in bold: the best overall model (containing both the best random and fixed term).

Note: Model without including data for female 5.

Random (REML)	AICc						
Term	DW	C	N	IgDW	IgC	IgN	C/N
♀:S:T	3481	2763	1831	-1826	-1848	-1848	80
♀:T	3463	2746	1814	-1745	-1761	-1768	77
♀:S	3465	2747	1815	-1830	**-1856**	**-1853**	77
♀	**3459**	**2742**	**1810**	**-1913**	-1764	-1770	**72**
Fixed (ML)							
Full factorial	**3534**	**2791**	**1825**	-2036	-1882	-2091	23
S+T	3541	2799	1830	**-2043**	**-1882**	**-2099**	24
T	3564	2807	1836	-2009	-1832	-2063	**23**
S	3562	2804	1833	-1288	-1046	-2048	66
Null	3578	2809	1839	-1279	-1043	-2013	63

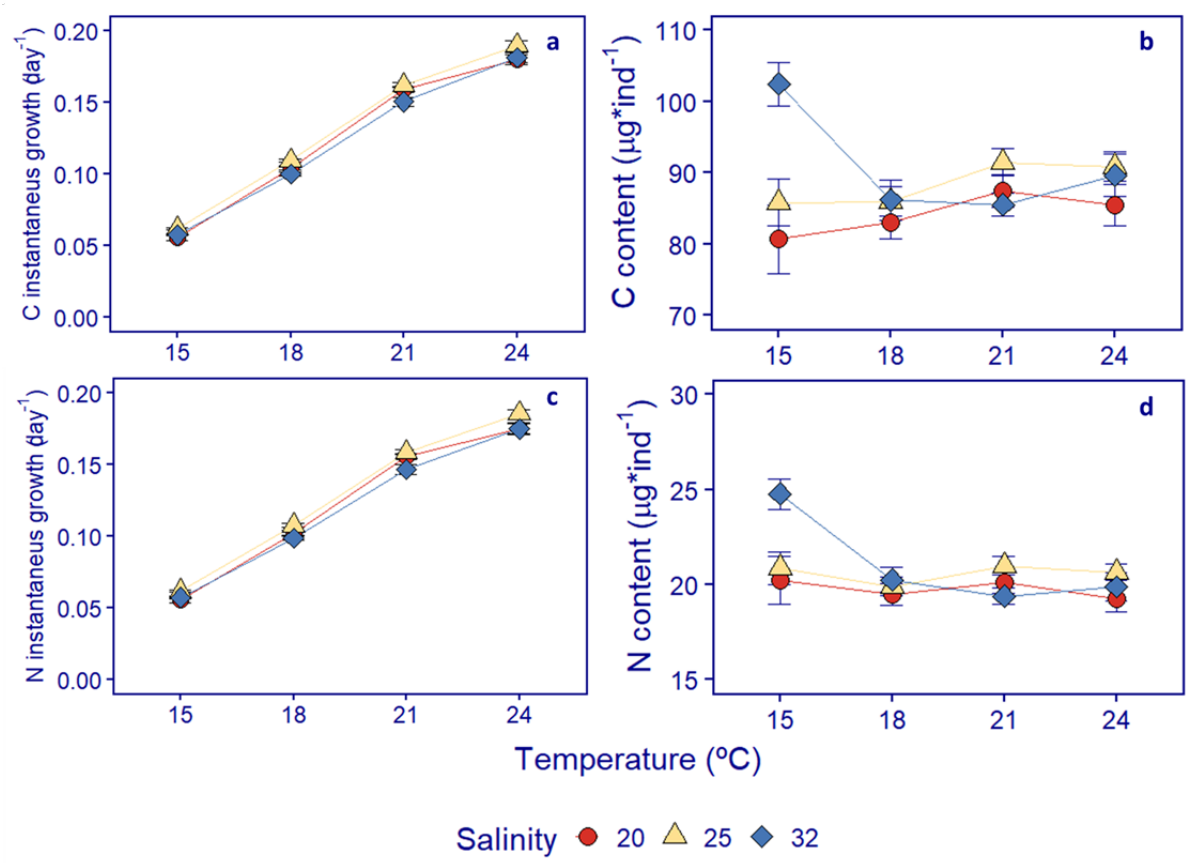

Figure S3. Average responses to the combination of temperature and salinity in terms of: (a) carbon growth rates, (b) carbon content, (c) nitrogen growth rates, (d) nitrogen content. Data shown as average values ± SE considering the five females of origin. Symbols in the graphs represent the different salinity treatments; red circle: salinity 20 ‰, blue triangle: salinity 25 ‰ and green diamond: seawater salinity (33 ‰).

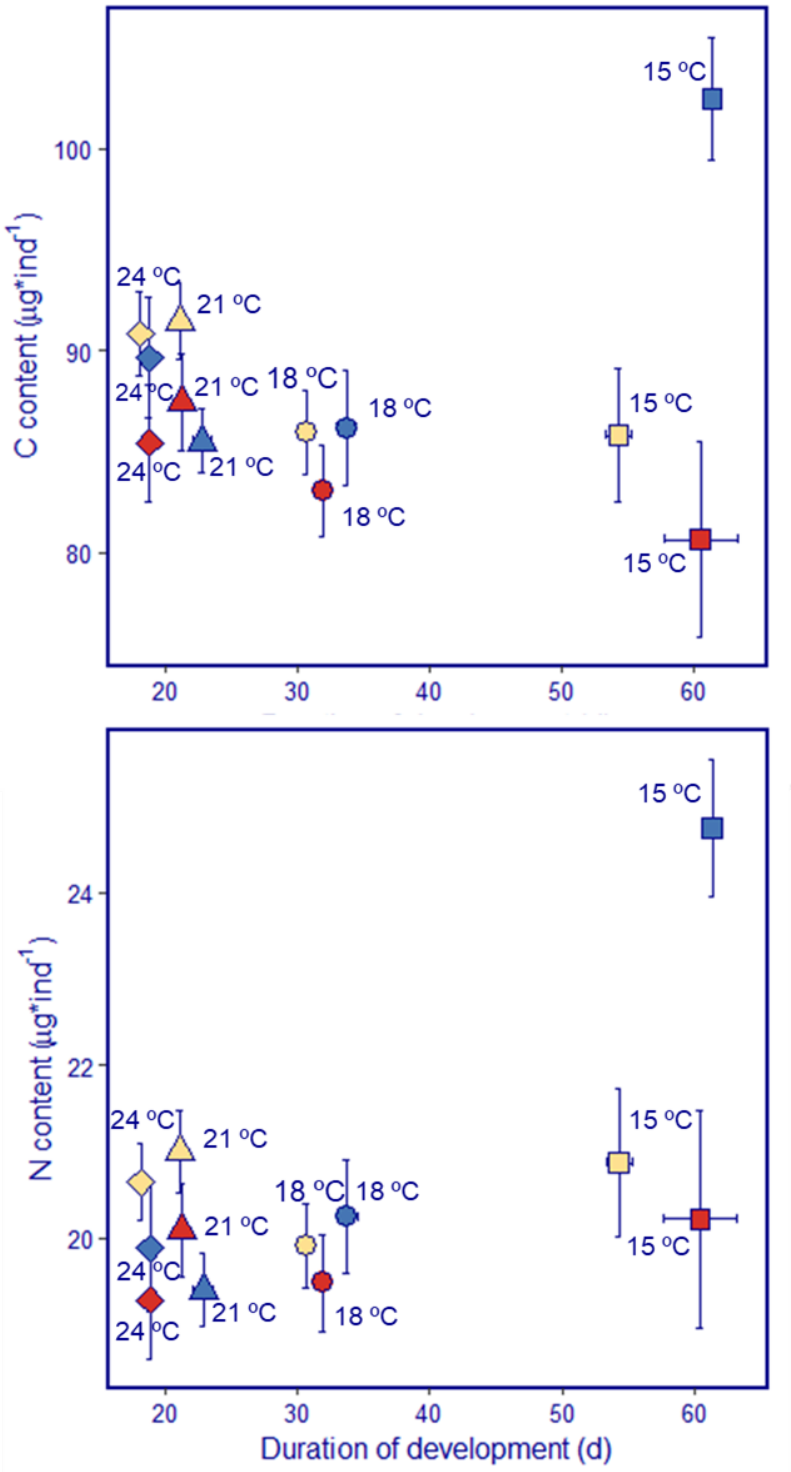

Figure S4. Integrated responses in terms of carbon content (upper panel) and nitrogen content (lower panel) and duration of development to megalopa under the different combinations of temperature and salinity. Data shown as average values ± SE considering the five females of origin. Symbols: 15 °C is represented with the squares, 18 °C with the circles, 21 °C with the triangles and 24 °C with the diamonds. Sea water salinity corresponds to colour blue, 25 ‰ to colour yellow and 20 ‰ is represented in red.

CHAPTER 5

Salinity and temperature during larval development determine the potential for range expansion into the Baltic Sea of the invasive crab *Hemigrapsus takanoi*

Geißel, J. P., **Espinosa-Novo, N.**, Giménez, L., Ewers, C., Cornelius, A., Martínez-Alarcón, D., Harzsch, S., Torres, G.

Salinity and temperature during larval development determine the potential for range expansion into the Baltic Sea of the invasive crab *Hemigrapsus takanoi*

Jan Phillipp Geißel[1,2], Noé Espinosa-Novo[2], Luis Giménez[2,3], Christine Ewers[4], Annika Cornelius[5], Diana Martínez-Alarcón[6], Steffen Harzsch[1], Gabriela Torres[2]

Authors' affiliations:

[1]Department of Cytology and Evolutionary Biology, Zoological Institute and Museum, University of Greifswald, Greifswald, Germany.

[2]Alfred-Wegener-Institut, Helmholtz-Zentrum für Polar- und Meeresforschung, Biologische Anstalt Helgoland, Helgoland, Germany.

[3]School of Ocean Sciences, College of Environmental Sciences and Engineering, Bangor University, Menai Bridge, UK.

[4]Kiel University, Zoological Museum, 24105 Kiel, Germany.

[5]Wadden Sea Station Sylt, Alfred-Wegener-Institut, Helmholtz-Zentrum für Polar- und Meeresforschung, List/Sylt, Germany

[6]UMR-MARBEC, Université de Montpellier, CNRS, Ifremer, IRD, Place Eugène Bataillon, 34095 Montpellier, France

*Corresponding author:

Jan Phillipp Geißel

janphillipp.geissel@uni-greifswald.de

Abstract

We studied the potential of a recent invader, the Asian brush-clawed crab (*Hemigrapsus takanoi*), to expand its distribution range further into the Baltic Sea. *H. takanoi* has been documented in the southwestern Baltic Sea since 2014. The ability to persist and further expand into the Baltic Proper will depend on their potential to sustain all stages of their complex life cycle, including pelagic larvae, under the Baltic Sea's conditions. Range limits may be established by the tolerance to low salinity, which in addition may be affected by seawater temperature. A key question is whether local populations at the distribution limit (within the Baltic Sea) show increased tolerance to low salinities and hence promote further expansion. We quantified the combined effects of salinity (10 - 33 ‰) and temperature (15 - 24 °C) on four populations of *H. takanoi* (two from the Baltic and two from the North Sea). We found substantial differences in larval performance between the populations from the Baltic and North Sea. Larvae from the North Sea populations always showed higher survival and faster development compared with those from the Baltic Sea. Only weak evidence of elevated tolerance towards low salinity was found in the larvae from the Baltic Sea populations. In addition, larvae from the population located near the range limit (Neustadt) showed very low survival under all tested salinity-temperature combinations and no evidence of increased tolerance to low salinity. There was no apparent genetic differentiation among the studied populations in the mitochondrial cytochrome c oxidase subunit one gene (COI) implying high connectivity among the populations. For populations to persist near the low salinity range limit, it appears that subsidies are needed from other populations or complex ontogenetic migrations to areas of higher salinity are required to enable moderate larval survival and recruitment.

Keywords:

Hemigrapsus takanoi - Intraspecific trait variation - Larval performance - Multiple stressors and drivers - Phenotypic physiological plasticity - Thermal tolerance

INTRODUCTION

Human-mediated climate change (Poloczanska et al. 2013b; García Molinos et al. 2016; Boersma et al. 2016; Burrows et al. 2019) and widespread biological invasions (Gurevitch et al. 2011b; Chan and Briski 2017) have major impacts on marine ecosystems. Invasive alien species (IAS) are an important component of global environmental change. IAS may cause modifications in community composition, and can be a major driver of local biodiversity loss (Bellard et al. 2016) as well as economic loss (Haubrock et al. 2021b, a; Henry et al. 2023). Climate change is considered an important driver of the success of biological invaders in aquatic ecosystems (Sorte et al. 2013; Gallardo et al. 2016); hence understanding the mechanisms of range expansion has become a priority (Gurevitch et al. 2011b). The successful conquest of new habitats should depend fundamentally on ecological and evolutionary processes (Gurevitch et al. 2011b) originating in/depending on the physical characteristics of the new habitat as well as the traits and performance exhibited by established species which are potential competitors, predators or parasites.

As witnessed by long term data series (Boersma et al. 2016; de Amorim & Wiltshire et al. 2023), global change in marine ecosystems affects environmental drivers such as water temperature, salinity, pH, and oxygen content (Poloczanska et al. 2013b; García Molinos et al. 2016; Boyd et al. 2018a). However, future fluctuations in environmental factors as a consequence of human influences will differ between open ocean, estuarine, and near-shore sites with coastal areas typically experiencing larger fluctuations (Duarte 2007; Hofmann et al. 2011). Semi-enclosed seas such as the Baltic and North Sea are particularly susceptible to the effects of global change and are expected to be increasingly affected by rising surface temperatures and freshening by increased river runoff (Gräwe et al. 2013; Hiddink et al. 2015; Robins et al. 2016). The Baltic Sea, for example, was recently dubbed a “time machine” for the future coastal ocean (Reusch et al. 2018). It is characterised by relatively low salinity, which varies spatially and temporally, depending on the proximity to the North Sea, river inflow, freshwater runoff, precipitation, and seawater inflow events through Skagerrak and Kattegat. Typically, in the Baltic Sea, the salinity decreases from west to east (Lehmann et al. 2022). Therefore, considering the expected changes of salinity and temperature increase, organisms living in coastal habitats and regions of freshwater influence, will face new combinations of environmental drivers. Furthermore, intertidal ecosystems, e. g. in the North Sea, have already shown particularly pronounced and rapid changes in response to anthropogenic influences so that focusing research efforts on the study of intertidal organisms in general can provide new insights into the physiological effects of global ocean change (Somero 2002; Helmuth et al. 2006a, b).

An example of a recent introduction and expansion in coastal-estuarine areas is given by the Japanese brush-clawed shore crab (*Hemigrapsus takanoi),* originated from SW Asia. *H. takanoi* has invaded the coast of Northern Europe and is currently distributed from the English Channel to the SW

Baltic Sea. This species is currently considered an ecological threat in European waters, exhibiting a potentially population-destabilising functional response towards blue mussels under Baltic Sea conditions (Theurich et al. 2022). *H. takanoi* was first found in Europe 1993 on a ship's hull in Bremerhaven, Germany (Gollasch 1999) and the first individuals were recorded in 1994, in the intertidal in La Rochelle, France (Noël et al. 1997). In the Wadden Sea, *H. takanoi* was first discovered in the Netherlands and later in Germany and Denmark (Geburzi et al. 2015b). In the Baltic Sea, it was recorded from 2014 onwards, first in the south-west (Kiel fjord) and then further east (Mecklenburg Bight) and north (Skagerrak and Kattegat).

As in other marine species, larval stages of *H. takanoi* are likely to play a central role in the process of invasion, as a contributor of the propagule pressure (Johnston et al. 2009). This is highlighted by the fact that larvae of *H. takanoi* have been found in ballast water of ships in the Arctic archipelago of Svalbard (Ware et al. 2016). Populations of sea bottom (=benthic) marine invertebrates, such as *H. takanoi*, are considered "open" in the sense that they are structured as a series of local populations of adult stages connected through larval dispersal (Caley et al. 1996; Armsworth 2002). Larvae play a central role in population connectivity and recovery from disturbance (Cowen et al. 2006a; Giménez et al. 2020b); however, larval stages are more sensitive to variations in environmental conditions than adults are. Hence, in scenarios of environmental change, larvae may represent a bottleneck for population persistence (Sorte et al. 2010a, 2018; Pandori and Sorte 2019b). Theory predicts that on distribution limits, narrow ranges of larval tolerance may result in that populations are not self-sustaining and instead are subsidised by human-mediated dispersal or natural dispersal on years when conditions are appropriate (Dauphinais et al. 2018; Giménez et al. 2020b). Hence, a central point for the establishment of *H. takanoi* concerns the capacity of the early stages to tolerate thermal and salinity conditions and hence develop self-sustaining populations across the North Sea-Baltic Sea gradient. We currently have information on the larval tolerance to temperature and salinity for two populations of *H. takanoi* (Japan: Mingkid et al. 2006; Kiel fjord, SW Baltic Sea: Nour et al. 2021, 2022). However, recent studies have highlighted important variability in environmental tolerance among invertebrate larvae from different females (Durrant et al. 2013a; Applebaum et al. 2014a; Spitzner et al. 2019b) and among populations distributed over environmental gradients (Sanford et al. 2006; Nasrolahi et al. 2012; Baldanzi et al. 2018; Šargač et al. 2021a). In particular, for *H. takanoi*, the current data on the lower limit of salinity tolerance (15 ‰) does not match the observed distribution of local adult populations: large numbers of adult crabs are found in areas of the Baltic Sea characterised by salinities below the known larval tolerance limit (Fig. 1a), e.g. Kiel fjord (salinity ~15 ‰) or Neustadt (salinity ~10 ‰) (Nour et al. 2020; Geburzi et al. 2020a). Prior research only examined salinity tolerance at a single temperature (24° C, Mingkid et al. 2006, 20° C, Nour et al. 2021) or at two temperatures (19 ° and 23 °C, Nour et al. 2022) but ignored the interactive effects of temperature and salinity. Multi-driver studies in the native crab *Carcinus maenas* showed interactive effects of salinity and temperature where

negative effects of low salinity on survival was mitigated at high temperatures (abbreviated as TMLS) (Spitzner et al. 2019b; Torres et al. 2021c; Šargač et al. 2021a). Furthermore, TMLS would be explained by the combination of an increased ability to osmoregulate of the osmoregulatory larval stages (Torres et al. 2021d), with the phenological effect (shorter development implies reduced time of exposure to low salinity) occurring at high temperatures. To account for interactive effects and future warming scenarios we used a mechanistic approach (Boyd et al. 2018) to assess the response to low salinity in multi-driver set-up. In addition, there seems to be a high level of genetic diversity within populations of *H. takanoi* from both the North and Baltic Seas (Geburzi et al. 2020a) that could be reflected in variability in responses to temperature and salinity among larvae from different females. A critical piece of information will be given by the quantification of larval performance in larvae from different females and populations located both at and away from the edge of the distribution range, as well as the quantification of genetic differentiation among those specific populations.

We carried out a multi-population study of *H. takanoi*, by focusing on populations of the North Sea and SW Baltic Sea. To assess potential local adaptations to low salinity we included a population located near the distribution limit in the Baltic Sea (Neustadt) and the first established Baltic Sea population (Kiel) (Fig. 1a). We compared those to two North Sea island populations (Helgoland and Sylt) that are exposed to seawater conditions year-round. We quantified the larval responses to temperature and salinity and determined the genetic structure of the populations under study. The experiments on larval responses aimed at: (1) Determining the combination of temperature and salinity regimes that allows for larval survival and development. (2) Quantifying interactive effects of the temperature and salinity, at thermal ranges that are also expected from future climate change. The quantification of genetic variability accounted for the degree of differentiation between the populations under study. Specifically, genetic homogeneity in concert with differences in larval performance would suggest either maternal effects or rapid local adaptation across the introduced range.

MATERIALS AND METHODS

Female collection and maintenance, larval rearing

Ovigerous females of *Hemigrapsus takanoi* were collected from four locations of the North and Baltic Sea coastal areas during one reproductive period (Fig. 1a). During low tide, ovigerous females from the North Sea were collected by hand in the intertidal, from under rocks and small boulders at the islands of Helgoland and Sylt (seawater conditions: 33 ‰). Ovigerous females from the Baltic Sea (Kiel and Neustadt) were collected scraping the fouling communities in and close to marinas using a scraping scoop (mesh size 0.5 mm). Females were found in water depths of 1 - 2 m in clumps of blue mussel (*Mytilus edulis*) on the floor on fine sediment or on artificial walls e.g. of floating pontoons and waterside promenades in Baltic Sea water (Kiel: 15 ‰ and Neustadt: 9.5 ‰). After

collection, females (from Sylt, Kiel, and Neustadt) were transported to the laboratory on Helgoland (Alfred-Wegener-Institut, Helmholtz-Zentrum für Polar- und Meeresforschung Biologische Anstalt Helgoland) in individual 1 L-containers filled with 500 ml water from the collection site. The individual containers were placed inside Coleman® coolers to ensure temperature stability during transport. Earlier studies showed that transport stress is negligible with this procedure (Šargač et al. 2021a, 2022b), thus no transport stress was simulated for animals collected on Helgoland.

In the laboratory, females were kept until hatching at 18 °C in individual 5 L-aquaria in natural UV-treated water at salinities corresponding to those at their respective sampling site: Helgoland & Sylt (33 ‰), Kiel (15 ‰) and Neustadt (10 ‰) and a 12:12 h light-dark cycle. Females were fed frozen shrimps (*Crangon crangon*) twice per week; water was changed daily to ensure high water quality at hatching.

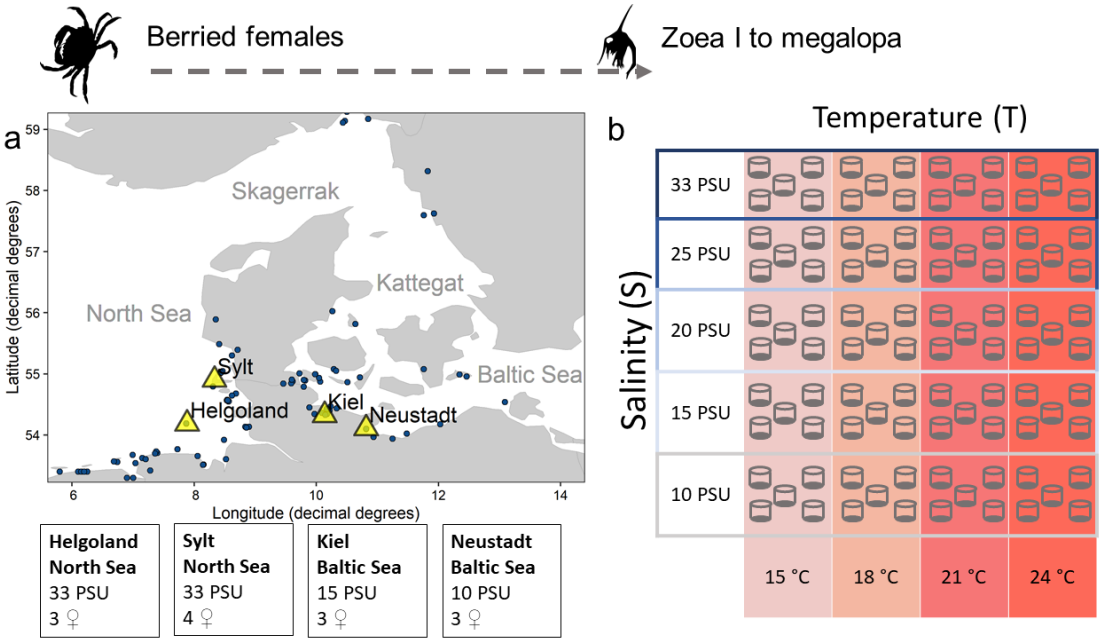

Figure 1. a) Map of the collection sites (yellow triangles) for the four tested populations on Helgoland and Sylt (North Sea), and Kiel and Neustadt (Baltic Sea); blue points represent presence data from GBIF.org (GBIF 2022). b) Experimental design to study the responses of larvae of *H. takanoi* from four different populations to different temperature conditions. Larvae of *H. takanoi* were reared from hatching to megalopa at four different temperatures: 15, 18, 21, and 24 ºC, represented in the picture from light red (15 ºC) to darker red (24 ºC) and 5 salinities: 10, 15, 20, 25, 33 ‰ represented by the borders from grey (10 ‰) to dark blue (33 ‰). Larvae were reared in 5 replicates of 10 individuals each.

Freshly hatched larvae from each female were assigned randomly to one of 20 treatments. The experimental design consisted of a factorial design based on the combination of 4 temperatures: 15, 18, 21, and 24 °C and 5 salinities: 10, 15, 20, 25, and 33 ‰ (Fig. 1b). The chosen salinities range from the

salinity found in Neustadt (10 ‰) to that found in Helgoland and Sylt (33 ‰). The chosen temperatures cover the range of temperatures typical for summer and those expected due to warming (Belkin 2009; Gräwe et al. 2013; Reusch et al. 2018; de Amorim & Wiltshire et al. 2023).

Survival and duration of development, dry mass, carbon and nitrogen content, and growth rates were assessed after exposure to the experimental conditions mentioned above. Each treatment consisted of five 60 ml glass-beakers: ten larvae were randomly allocated to each replicate beaker. Experiments were repeated with larvae originated from 13 different females (i.e., Sylt: 4, Helgoland: 3, Kiel: 3, and Neustadt: 3).

Larval rearing was performed following(Torres et al. 2021d) in temperature-controlled rooms (± 1 °C) with a 12:12 h light-dark cycle. Natural seawater was UV-treated, filtered (2 µm mesh size), and aerated seawater was used for the experiments. Experimental salinities were obtained by diluting seawater with appropriate amounts of tap water. Water and food (*Artemia sp.* nauplii *ad libitum*, Great Salt Lake Artemia) were changed daily. During the daily water change, dead larvae and moults were discarded and the larval stage of survivors was determined.

Elemental analysis

We quantified dry mass and elemental composition (i.e., carbon and nitrogen content) for freshly hatched zoea I and freshly moulted megalopa. For each experiment (i.e., for larvae from each female of origin), we sampled 3 replicates of 50 zoea I, as well as all the obtained megalopa. Furthermore, freshly hatched zoea I originated from additional females were also sampled (giving the following total number of females: Helgoland: 6; Sylt: 6; Kiel: 6; Neustadt: 4). During sampling, larvae were rinsed with distilled water, gently blotted dry with tissue (Kimtech® delicate task wipes), placed in a pre-weighted tin cartridge and stored at – 20 °C for further analysis. To quantify the dry mass, samples were freeze-dried for 48 h (Christ Alpha 1-4 freeze-drier) and weighed using a microbalance (Cubis ® MCA2.75S-2SOO-M Sartorius Lab Instruments GmbH & Co. KG). Carbon and nitrogen content were determined using an elemental analyzer (Vario MICRO cube CHNS analyser, Elementar Analysensysteme).

Population genetics analyses (DNA extraction, amplification, and sequencing

We amplified and sequenced a 618-base-pair (bp) fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene, using the universal primers (LCO1490 and HC02198, (Folmer et al. 1994)). Sequences were obtained from 68 crabs (Helgoland: 6, Sylt: 24, Kiel: 19, Neustadt: 19) that were preserved in 97% ethanol and stored at -20 °C prior to molecular analysis. Total DNA was extracted from pereiopod muscle tissue using the Qiagen DNeasy® Blood & Tissue kit following the

manufacturer's protocol for tissue samples except for the last step where DNA was eluded by adding 100 µl of elution buffer and centrifuged at 10.000 rpm. DNA purity and concentration were assessed using the Nanodrop Spectrophotometer (NanoDrop ONE ThermoFisher). DNA was used as a template for Polymerase chain reaction (PCR) amplification using PuReTaq® Ready-To-Go™ polymerase (Cytiva). All PCRs were carried out in a 25 µl reaction mix containing 2 µl DNA, 1 µl BSA and, 2.5 µl (5 µM) of each forward and reverse primer. The amplifications were carried out by a (Mastercylcler®) Eppendorf thermocycler with a program that consisted of 2 min at 94°C followed by 35 cycles of 0.5 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C, and a final extension of 10 min at 72 °C. The PCR products were Sanger sequenced in forward and reverse direction at the Institute of Clinical Molecular Biology in Kiel (IKMB).

Data analysis

The response variables were cumulative survival, duration of development, instantaneous growth rate, and body mass and elemental composition at metamorphosis. Cumulative survival was calculated as proportion of larvae surviving from hatching to a given life stage. To avoid situations of log(0) values, data were transformed using the formula $p' = [p(N-1)+0,5]/N$, where N is the number of larvae in the respective replicate (= 10). The proportion of survivors was then transformed into logarithmic and logistic scales. Duration of development was defined as time elapsed from hatching until the next stage (e.g. duration of development to the megalopa). Body mass (B) was determined as dry mass (DW), and carbon (C) and nitrogen content (N) in freshly hatched zoea I and freshly moulted megalopa. The instantaneous growth rate was calculated as $\log(B_M/B_{ZI})/D$, where B_{ZI} is the body mass parameter (DW, C, or N) of the freshly hatched zoea I, B_M is the body mass parameter of the megalopa, and D the duration of development to the megalopa.

The experimental design for larval rearing was factorial with 3 fixed and orthogonal factors: population (P), salinity (S), and temperature (T). Female of origin (F) was nested in the interaction of salinity and population, as a random factor. Statistical analysis was carried out in R (version 4.2.2) using a backwards model selection approach (Zuur et al. 2009) based on generalised least squares. We used the packages "nlme" (Pinheiro et al. 2019), and the functions "lme" and "gls". Model selection took place in two steps. First, models for random terms were fitted with restricted maximum likelihood (REML). Second, the model with the best random structure was refitted using maximum likelihood (ML), and selection was carried out of the fixed structure. In both cases, model selection was based on the corrected Akaike information criterion (AICc).

For survival, the best models retained all 2-way interactions (see Results). For the subsequent exploration of effects, we fitted quadratic models to the survival data instead of performing multiple

comparisons using a post-hoc test. In particular, we used polynomial models to estimate the temperature and salinity at which survival was maximized at each population.

For duration of development, dry mass, elemental composition, and growth we needed to perform multiple separate analysis strains as not all larvae metamorphosed to megalopa at all factor combinations (e.g. we had low survival in Baltic populations) and the design became disconnected. For duration of development, four separate statistical analysis were carried out as follows: (1) focus on three salinities (20, 25, and 33 ‰), two temperatures (21 °C and 24 °C) and three populations (Helgoland, Sylt, and Kiel). (2) Focus on all populations but at one salinity (25 ‰) and two temperatures (21°C and 24°C). (3) focus on three salinities (20, 25, and 33 ‰), three temperatures (18, 21, and 24 °C) but only for Helgoland and Sylt populations; (4) Including all temperatures, but only one salinity (33 ‰) and the populations from Helgoland, Sylt, and Kiel. Likewise, four separate analyses were conducted for body mass and growth: (1) With focus on two salinities (20 and 25 ‰), two temperatures (21 and 24 °C), and three populations (Helgoland, Sylt, and Kiel). (2) Focus on two salinities (25 and 33 ‰), three temperatures (18, 21, and 24 °C), and two populations (Sylt and Helgoland). (3) Including all populations but at only one salinity (25 ‰) and temperature (24 °C). (4) Including all populations but only one combination of salinity (20 ‰) and temperature (21 °C).

To assess genetic differentiation, the COI sequences were visually inspected for sequencing mistakes, assembled, aligned, and trimmed using the bioinformatics software Geneious Prime (Ver.2020.0.3 (Kearse et al. 2012)). All subsequent population genetic analysis were conducted in the R environment (R version 4.2.2; 2022). Haplotype network was produced using the R packages ggplot2 (Wickham 2016), scatterpie (Guangchuang 2022), and rworldmap (South 2011). Population differentiation was calculated using Jost´s D and PhiST with the packages “adegenet” (Jombart 2008) and statistical parsimony (Templeton et al. 1992) in the “pegas” (Paradis 2010) followed by bootstrapping with 1000 replicates.

RESULTS

For simplicity, we use the term “North Sea populations” for larvae obtained from Sylt and Helgoland and “Baltic Sea populations” for those obtained from Kiel and Neustadt. However, we do not claim that these animals represent the whole respective seas. Likewise, we refer to the animals from one sampling site as local populations without inferring that these “populations” are separated or distinctive in terms of connectivity or genetics.

Larval survival, duration of development, and growth rates

Larval survival varied considerably among populations and it was contingent on temperature-salinity combinations (Fig. 2); best statistical models retained interactions between population, temperature, and salinity (Table S1). Larvae from North Sea populations reared in the salinity range 20 - 33 ‰ showed an increasing trend in survival towards the highest temperatures (Fig. 2), with quadratic models indicating a maximum survival at the maximum temperature tested, 24 °C (Fig. 3). This pattern was already observed at the zoea II and then exacerbated in the late zoea stages (Fig. S1). By contrast, survival was consistently low at 10 and 15 ‰, irrespective of temperature, and at 15 °C irrespectively of salinity for all populations studied.

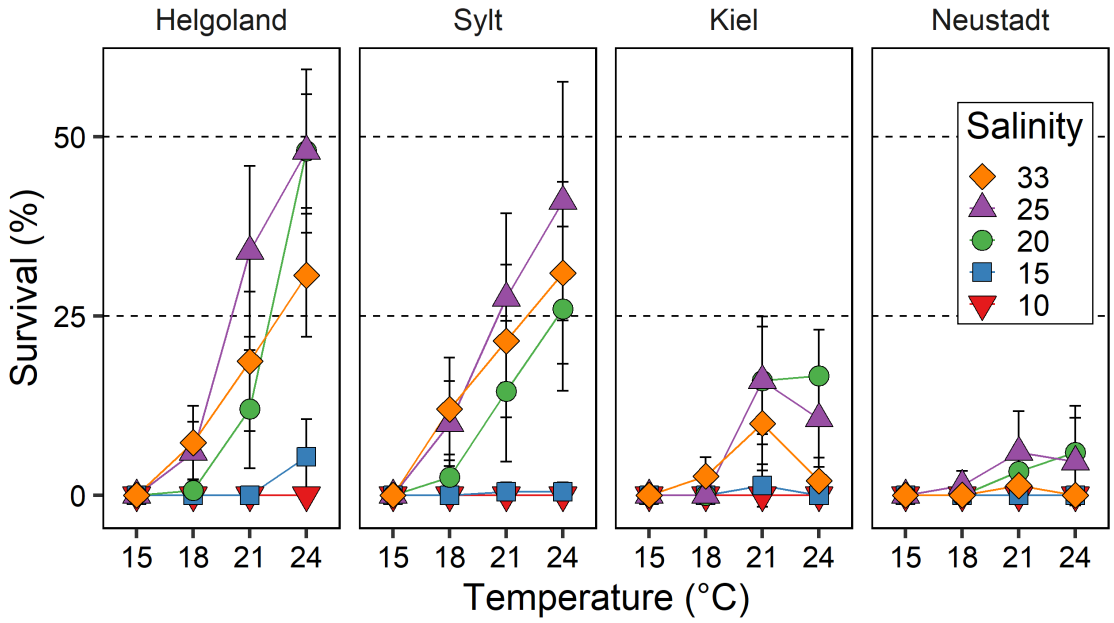

Figure 2. *Hemigrapsus takanoi*. Average survival from hatching to megalopa as a response to temperature and salinity discriminated by population. Comparison between populations from the North Sea: Helgoland and Sylt (left panels) and the Baltic Sea: Kiel and Neustadt (right panels) by temperature (15, 18, 21, 24 °C) and salinity (33 ‰, orange diamonds; 25 ‰, purple upwards triangles; 20 ‰, green circles; 15 ‰, blue squares, 10 ‰, red downwards triangles). For the North Sea populations, the habitat/embryonic salinity was 33 ‰, for Kiel 15 ‰ and for Neustadt 10 ‰. Data shown as means ± SE for larvae produced by each female from each population (n = 3 or 4).

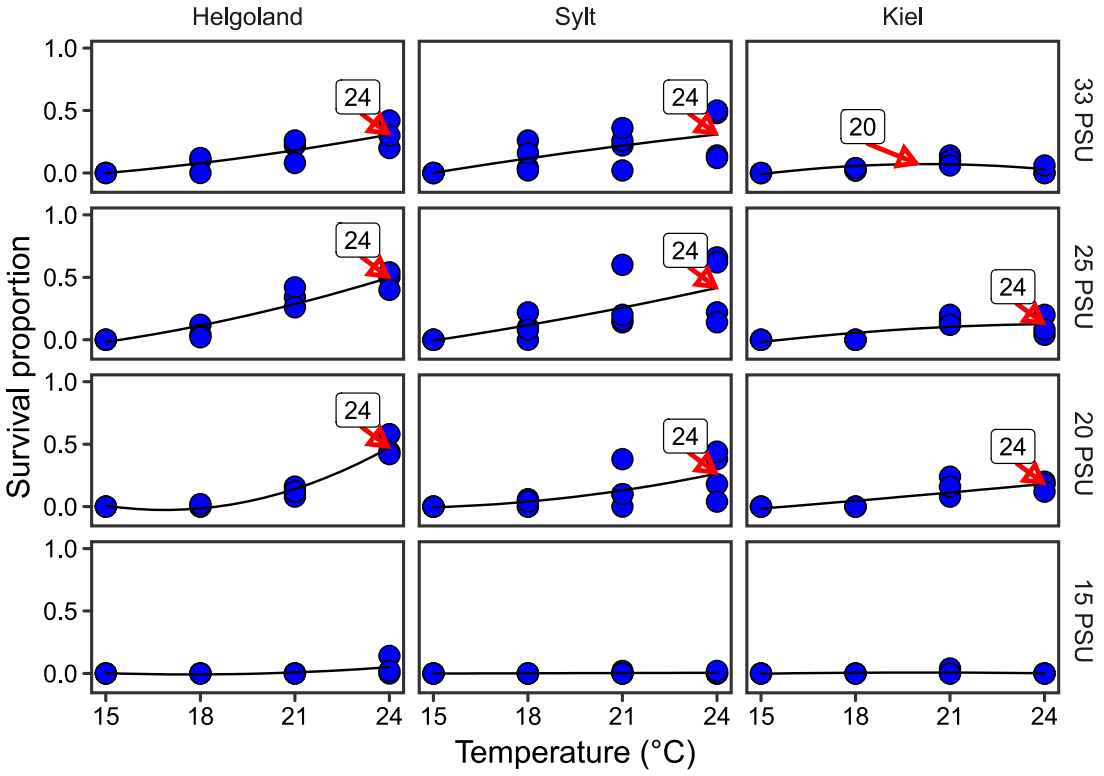

Figure 3. Quadratic polynomial models fitted to survival to the megalopa in response to temperature (°C) for different combinations of salinity and population. Models with significant trends for quadratic terms are marked with an arrow and label indicating the temperature of highest predicted survival by salinity and population. Blue points indicate mean observed survival by female. No predictions could be made for the Neustadt population due to the low number of survivors and the resulting high number of zeros in the data. For the North Sea populations, the habitat/embryonic salinity was 33 ‰, for Kiel 15 ‰ and for Neustadt 10 ‰.

When reared in the salinity range 20 – 33 ‰, larvae from the Baltic Sea populations had reduced survival (Fig. 2), with those from the Kiel population showing a response to temperature that was intermediate between the one found for larvae from Neustadt (very low survival, < 10%) and the one exhibited by the North Sea populations (> 25%). Importantly, in the Baltic populations, larval survival was consistently low at salinities 10 and 15 ‰, which are within the salinity range experienced by adults in the natural habitat (i.e., SW Baltic Sea). For the Kiel population, survival of larvae reared at 21 and 24 °C, had an estimated maximum at a slightly lower salinity than those of the North Sea populations (Fig. 4); the difference between those salinities was small (~1 ‰ difference between Kiel and Helgoland) and the survival curve was flat, showing that survival varied little within that range. In addition, the estimated salinity giving the maximum survival in Kiel (24 - 25 ‰) was much higher than those surrounding the local population (~15 ‰). Larvae from Neustadt showed lower survival than those from the Kiel population already from zoea II (Fig. S1).

In addition, for the North Sea populations, there was an important variation in survival among larvae from different females, with some females producing larvae showing high survival at almost all conditions tested. However, there were no survivors to megalopa at 15 °C, irrespective of female of origin, population or salinity. Low survival at 15 °C was found for larvae hatching from most females, already at the zoea II, especially at 10 ‰ (Fig. S1). Additionally, at 10 ‰, there were no survivors to zoea III, irrespective of female of origin, population, and temperature (Fig. S1). In some treatments (e.g. larvae from the North Sea populations reared at 21 °C, Fig. S2, Table S2), a small percentage of larvae (< 20 %), developed through an additional zoea VI before metamorphosing to megalopa.

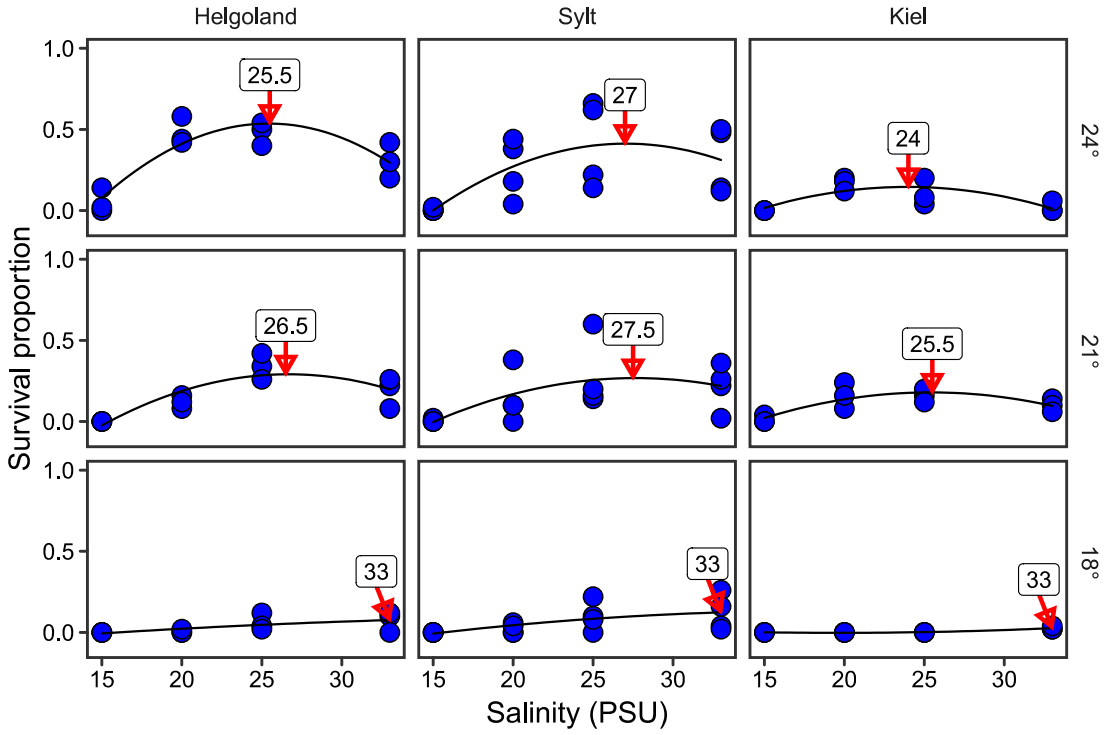

Figure 4. Quadratic polynomial models fitted to survival in response to salinity (‰) for different combinations of temperature and population. Models with significant trends for quadratic terms are marked with an arrow and label indicating the salinity of highest predicted survival by temperature and population. Blue points indicate mean observed survival by female. No predictions could be made for the Neustadt population due to the low number of survivors and therefor high number of zeros in the data. For the North Sea populations, the habitat/embryonic salinity was 33 ‰, for Kiel 15 ‰ and for Neustadt 10 ‰.

Average dry mass (DW), carbon (C) and nitrogen (N) content of freshly hatched zoea I varied considerably among larvae originating from different females within each population. In general, averages followed a trend (Fig. 5) with higher body mass in larvae from Helgoland and Sylt and lowest in those from Neustadt, although the best models did not retain population as an explanatory variable.

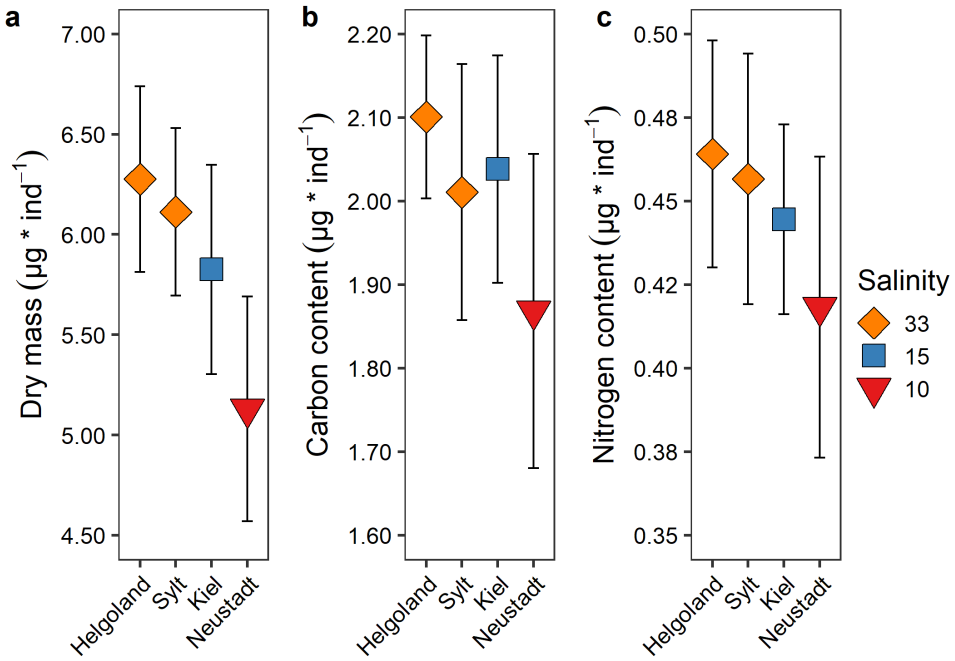

Figure 5. *Hemigrapsus takanoi*. Average dry mass (a), carbon (b) and nitrogen content (c) of freshly hatched zoea I, from four populations (North Sea: Helgoland and Sylt; Baltic Sea: Kiel and Neustadt). Symbols indicate salinity experienced during embryonic development (reflecting the salinity at the collection site): 33, orange diamonds; 15, blue squares and 10, red triangles. Data shown as means ± SE for larvae produced by each female of each population (n = 3 or 4). For the North Sea populations, the habitat/embryonic salinity was 33 ‰, for Kiel 15 ‰ and for Neustadt 10 ‰.

Dry mass (Fig. 6a), carbon and nitrogen content of megalopa (Fig. S4) varied among populations as well as among temperature-salinity combinations. Dry mass increased with temperature and salinity with a maximum at 25 or 33 ‰ depending on population (interactions population by salinity, and temperature by salinity retained the best models: Table S7). When larvae from the Neustadt population were reared at 24 °C, the dry mass of megalopa appeared to be slightly lower but population was not retained in the best model (Table S7). For carbon and nitrogen content, the additive term was retained in the model (Fig. S8).

Duration of development to megalopa (Fig. 6b) decreased with increasing temperatures (range 18 – 24 °C; no larvae survived at 15 °C), following a nonlinear pattern which varied among populations. The best model retained interactions of salinity by population and temperature as a main factor (Table S6). At 18 °C, larvae from the North Sea populations developed in a shorter time (30 - 40 days) than those from the Baltic Sea populations around (45 - 55 days). There was no evidence of a consistent effect of salinity on duration of development (tested range: 20 – 33 ‰; Table S6). For the Baltic Sea populations, duration of development was longer in seawater than for the North Sea populations (20 - 37 days depending on temperature for North Sea and 29 - 50 days for the Baltic Sea, respectively). For larvae of the North Sea populations, there were no clear effects of salinity on duration of development;

for larvae of the Kiel population, lower salinity (20 and 25 ‰) caused a reduction in duration of development. This reduction was slightly stronger at 24 °C than at 21 °C but we did not find strong evidence in favour of retaining the 3-way full factorial model (ΔAIC =3: Table S6). For the Helgoland population, the effect of temperature was smaller than for the Sylt population and development, especially in the lower temperatures, was comparably faster (Fig. 6b).

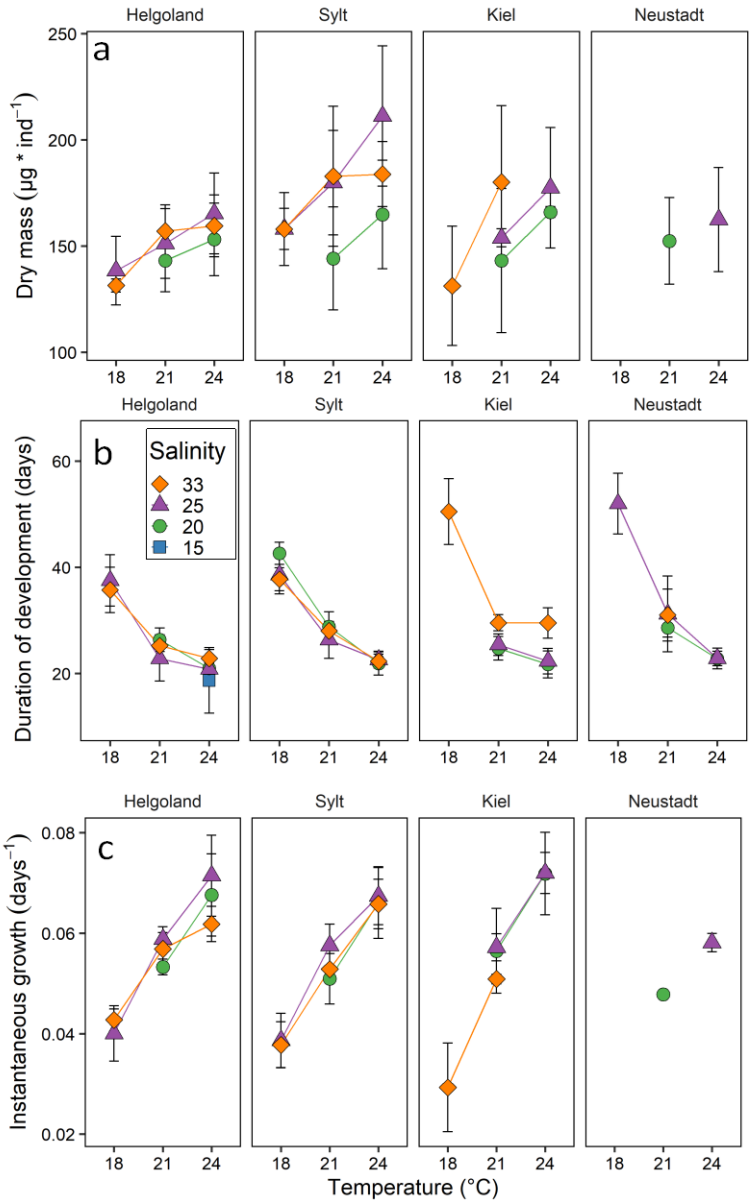

Figure 6. *Hemigrapsus takanoi*. Dry mass (a), duration of development (b), and instantaneous growth rate in terms of dry mass (c) from hatching to megalopa. Comparison between populations from the North Sea (Helgoland and Sylt) and the Baltic Sea (Kiel and Neustadt) by temperature (18, 21, and 24 °C) and salinity (33 ‰, orange diamonds; 25 ‰, purple upwards triangles; 20 ‰, green circles; 15 ‰, blue squares, 10 ‰, red downwards triangles). Data shown as means ± SE for larvae produced by each female of each population (n = 3 or 4). Means based on a single value were removed (see Fig. S3 in supplementary materials). For the North Sea populations, the habitat/embryonic salinity was 33 ‰, for Kiel 15 ‰ and for Neustadt 10 ‰.

Instantaneous growth rate to megalopa (in terms of dry mass) increased with temperature for larvae from all populations (Fig. 6c, Table S10). There was no evidence of a consistent effect of salinity or population of origin on instantaneous growth rates; the best model retained the interaction between salinity and population (Table S10) or the three 3-way interaction (Helgoland and Sylt populations). We did not find evidence of survival rates being predictors of growth rates, i.e., populations with higher survival rates did not show higher growth rates.

There was a negative relationship between duration of development and body mass (dry mass, carbon and nitrogen content) of the megalopa (Fig. 7, Table S11): larvae that reached the maximum dry mass (or reserves), did so in a shortest time (i.e., those reared at 24 °C). When reared at lower salinities, larvae did not seem to compensate by extending the duration of development, in order to maintain dry mass; instead, larvae reached metamorphosis with different dry mass but at similar times across salinities.

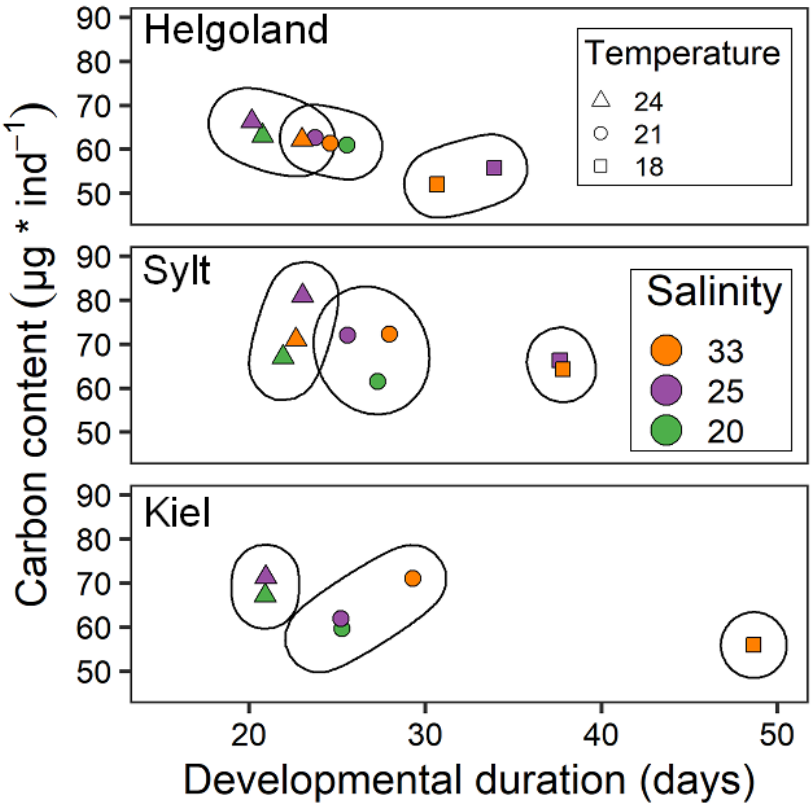

Figure 7 *Hemigrapsus takanoi*. Integrated responses of carbon content and duration of development of megalopae from three populations (Helgoland, Sylt, Kiel) reared at three temperatures and four salinities. Salinities (‰) are shown by colour (33: orange, 25: violet, 20: green), and temperature (°C) is shown as symbols (24: triangles, 21: circles, 18: squares) and surrounded by ellipses for easier identification. Data from Neustadt are not shown due to the low number of survivors. For the North Sea populations, the habitat/embryonic salinity was 33 ‰, for Kiel 15 ‰ and for Neustadt 10 ‰.

Molecular analysis

We found six mitochondrial haplotypes, of which four are shared haplotypes and two are private haplotypes. Two haplotypes (H1 and H2) were present in samples from all four populations, i.e., shared between all populations. The haplotype H3 was found in one animal from Neustadt and one from Sylt and H4 was found in two animals from Neustadt and one from Helgoland, i.e., both were shared between North and Baltic Sea populations. Only two haplotypes were private for one population each (H6: Neustadt; H5: Sylt). No significant differences between the populations were found. Neither the PhiST nor Jost's *D* did show significant differentiation among populations (Tables S12 to S16).

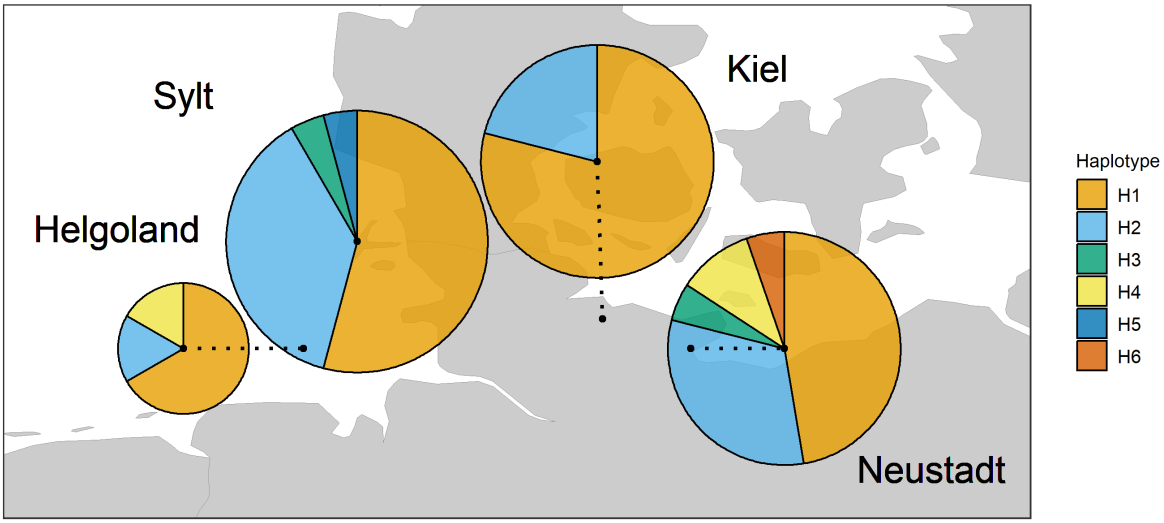

Figure 8. *Hemigrapsus takanoi*. Map of plotted frequencies of the found haplotypes in the four assessed populations: Helgoland and Sylt (North Sea), and Kiel and Neustadt (Baltic Sea). The size of the circles is relative to the sample size (Helgoland: n=6; Sylt: n= 24; Kiel: n=19; Neustadt: n=19); colours indicate the respective haplotype. The pies for Helgoland, Kiel, and Neustadt are offset from their coordinate to avoid overlap – offset is indicated by the dashed line.

Discussion

We found that survival and growth responses to temperature and salinity, in larvae of *Hemigrapsus takanoi* (non-native to the European coast) vary substantially among populations of the North and Baltic Seas. Survival was particularly low in larvae hatching from females collected in the south-western Baltic Sea populations (Kiel and Neustadt), where salinities are low. By contrast, larvae hatching from females from the North Sea populations generally exhibited higher survival; with larvae growing at higher rates and achieving higher dry mass at higher temperatures. These patterns emphasize the role of temperature and salinity in the dynamics of invasions and the importance of quantifying intraspecific variation in larval performance. The role of increased temperatures for the development of

self-sustaining populations of species with pelagic larval dispersal has been demonstrated (Giménez et al. 2020b; Griffith et al. 2021a; Espinosa-Novo et al. 2023b): higher temperature led to higher survival, shorter duration of development, and increased dry mass and in some cases also higher reserves. Larger body size in combination with shorter duration of development could have knock on effects on juvenile survival and growth. In addition, larval dry mass and size may be predictors of post metamorphic performance, with larger individuals performing better (Pechenik 1999b; Giménez 2006; Torres et al. 2016a). Furthermore, because faster developing larvae would settle earlier in the season, juveniles should experience the summer temperatures for a longer period and juvenile growth could be enhanced. Moreover, salinity could play an important role considering the differences in tolerance to low salinities between the invasive crab *H. takanoi* and the native crab *Carcinus maenas* (Šargač et al. 2021a) that coexist in the benthic habitat.

The level of intraspecific variation in larval survival, duration of development, and growth found for larvae of *H. takanoi*, emphasises the importance of quantifying intraspecific variation in responses to multiple drivers both among populations (Šargač et al. 2022b) and within populations (Spitzner et al. 2019b; Torres et al. 2020b; Espinosa-Novo et al. 2023b). We observed a slight shift in the optimal salinity between populations form the North Sea and the Baltic Sea and reduced duration of development at moderately low salinities in Kiel as compared to the North Sea populations. Given the fact that *H. takanoi* was only recorded in the Baltic Sea since 2014 (Geburzi et al. 2015b) it would be still early to see heritable physiological shifts as observed in other marine crustaceans establishing in low salinity habitats (Lee et al. 2011). Furthermore, our results also highlight the fact that, when evaluating performance of invasive species, extrapolations based on data obtained in native populations are risky and could be misleading, especially for local populations existing at the distribution limit, where suboptimal conditions experienced by adults might impact larval performance.

An interesting scenario was that larvae produced in Baltic Sea populations would show signs of local adaptation to low salinity either through a shift in the optimum or higher degree of euryhalinity than those of the North Sea. Local adaptation would contribute to the formation of self-sustaining populations, which increases connectivity and favours range expansion through source-sink dynamics (Giménez et al. 2020b). However, in addition to the depressed survival, the (slight) shift in the optimal salinity (Fig. 3 Kiel *vs.* North Sea populations) and the reduced duration of development (Fig. 6b) were contingent on temperatures (≥ 21 °C) that may not (yet) be experienced in the Baltic Sea for sufficiently long periods. The response to salinity was consistent with reports from a native Japanese population (Mingkid et al. 2006b) and from a previous study on the population in Kiel (Nour et al. 2021, 2022). In addition, our population genetic analysis showed no evidence of a clear separation between the

populations and several shared haplotypes. Although we had a restricted number of samples, our genetic results are consistent with those of Geburzi et al. (2020, 2022), based on a much larger sample size and on nine polymorphic microsatellites. In their study, animals from Neustadt appeared more distinct from the North Sea populations than the Kiel population. Our results based on mitochondrial sequence data do not support this result statistically, although we did find one private haplotype in Neustadt and two haplotypes that were absent from the other investigated site in the Baltic Sea. These may be the signature of multiple introductions at this site. Multiple introductions in Europe (Markert et al. 2014; Makino et al. 2018) could be an explanation for surprisingly high diversity and lack of founder effect (Roman and Darling 2007). While multiple introductions are often considered advantageous for the success of non-native populations (i.e. they increase genetic diversity and provide novel substrate for adaptive evolution), the above-mentioned differences in larval performance provide only a very weak evidence consistent with local adaptation. Besides, the larvae from Neustadt performed poorly under any condition. Maternal effects might explain the observed responses: females from Neustadt may be more stressed than females from the other sites. In addition, low salinity experienced during embryogenesis may result in poor larval performance as shown for the European shore crab *C. maenas* (Šargač et al. 2021a). From the perspective of larval success, the establishment of self-sustaining populations into the Baltic proper would not be expected unless the salinity rises above 15 ‰ for sufficiently long periods during the reproductive phase of *H. takanoi* (i.e., when temperatures are ≥ 18 °C). At sites characterised by summer salinities > 15 ‰, the window for successful larval release and survival should be wider especially under warming scenarios. This assessment is consistent with findings of brood development and egg maturation in the Wadden Sea (van den Brink et al. 2013) where *H. takanoi* might be benefitting from rising water temperatures. It is also important to mention that barcoding or microsatellites focus in few specific genes, therefore other approaches such as high-throughput sequencing could complement this data to examine genetic structure and adaptation in the invasive crab *H. takanoi* to examine populations spanning the native and invasive ranges

Given the low performance at low salinity, except perhaps at high temperatures, a critical point is whether populations of the Baltic Sea are maintained through subsidy from the North Sea or (in addition) through successful larval development in areas of the Baltic Sea, where salinities are higher, for example in deep waters (Corell et al. 2012). An initial assessment can be performed combining our experimental results with field data of temperature and salinity (Fig. 9). For instance, based on our experiments, moderate survival in the Kiel fjord is likely (salinity >15 ‰) with the caveat that temperatures should be ≥ 21 °C for at least the 20 days needed to complete the zoeal development (see Fig. 6b) under these conditions. By contrast, in the Bay of Neustadt, and the greater Bay of Lübeck, survival until megalopa appears unlikely under the salinities on site (< 15 ‰). Because tolerance to low salinities (< 33 ‰) is restricted to high temperatures, larval success would be possible only if the

phenological window of larval development matches the windows of high temperature (in summer) and salinities are >15 ‰. However, in the Bay of Neustadt, salinities higher than 15 ‰ only occur in winter and springtime, when storms force North Sea water masses into the Baltic Sea (Lehmann et al. 2022). Thus, currently there is a mismatch between the larval physiological phenotype and the environmental conditions at Neustadt. We therefore hypothesize that adults of *H. takanoi* at the distribution limit studied here (Mecklenburg Bight) are part of a (demographic) sink population (Pulliam 1988) or individuals perform ontogentic migrations. In such a case, subsidies may occur through two nonexclusive scenarios: (1) Adults from populations located at areas of high salinity disperse into areas of low salinity. (2) Larvae or adults are introduced into areas of low salinity by human mediated transport, e.g. in the fouling community of ships and boat hulls. Alternatively, such populations are sustained through a third mechanism: (3) Export strategy (Queiroga and Blanton 2004): i.e., early larval stages migrate to (or females release larvae in) areas, characterized by higher salinity, where larvae develop to the megalopa or juvenile stage, which then recolonize areas of low salinity. Such ontogenetic migration could occur between Neustadt and e.g. the Fehmarn Belt area (Fig. 9) where salinities are higher. Larval transport would depend on currents (e.g. surface currents from Neustadt to Fehmarn Belt: Mittelstaedt 2003); zoea I larvae from *H. takanoi* are known to migrate from near-shore hatching sites to more open waters (e.g. in the Kiel fjord: (Geburzi 2018)). The elucidation of the actual mechanisms requires field studies quantifying abundance of *H. takanoi* larvae along the SW Baltic.

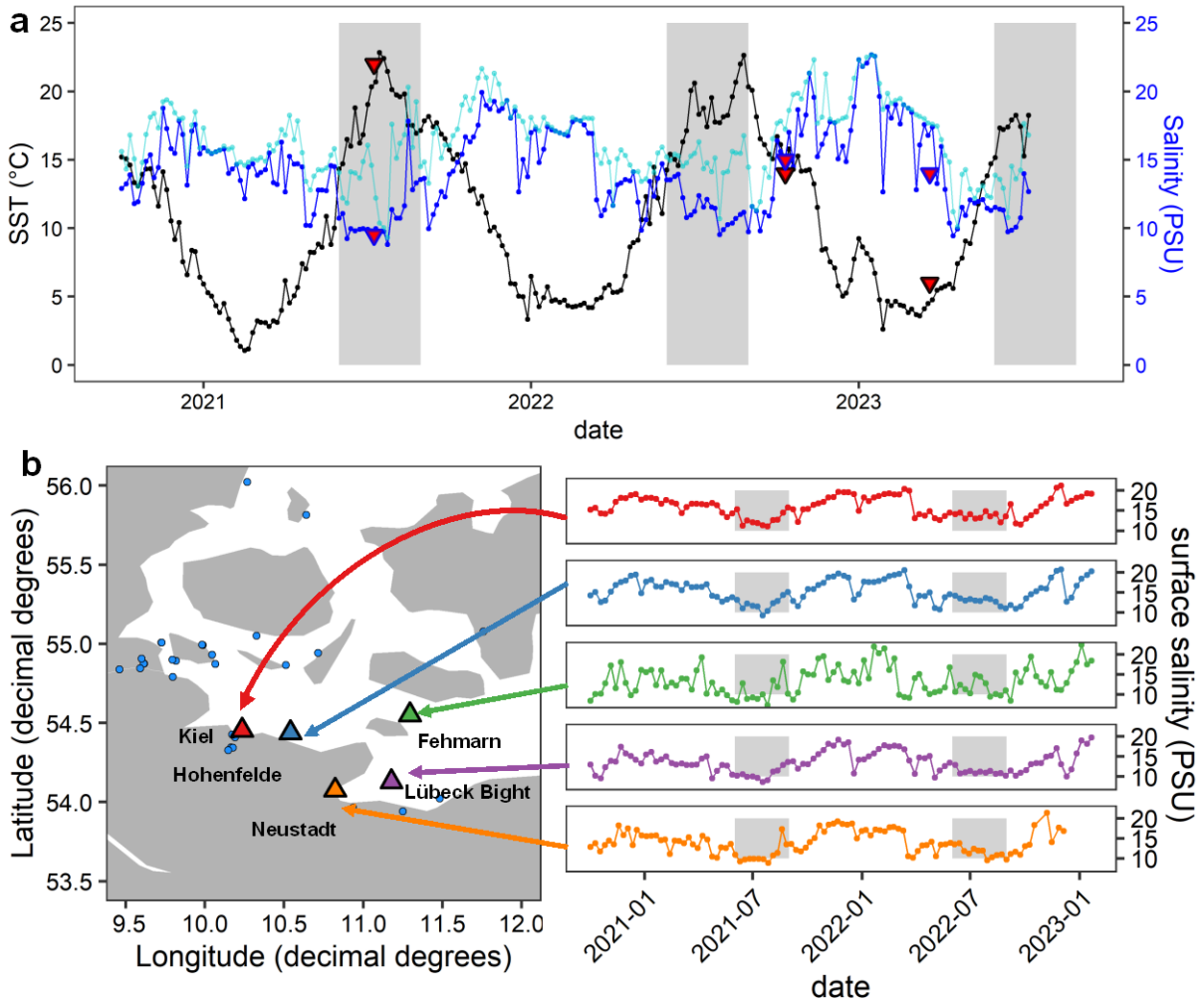

Figure 9. (a) Graph showing sea surface temperature (SST) (°C) in black dots, sea surface salinity (SSS) in dark blue dots, and sea bottom salinity (SBS) (‰) in Neustadt in light blue dots. Red triangles indicate in situ measurements during our field collections. (b) Map of selected locations in the south-western Baltic Sea (between Kiel and Neustadt) and the corresponding annual sea surface salinity (‰) fluctuations. Grey shadowed areas show summer periods (1st of June until 31st of August). Data obtained from Copernicus "Baltic Sea Physics Analysis and Forecast" (Generated using E.U. Copernicus Marine Service Information; https://doi.org/10.48670/moi-00010).

Another important question is how *H. takanoi* compares with other species distributed along the North Sea – Baltic Sea gradients. Larvae of *H. takanoi* showed a similar pattern to that reported *C. maenas* for the Baltic, i.e., depressed survival at low salinities at all temperatures which is contrary to the patterns found in the North Sea (Šargač et al. 2021). In *C. maenas*, there is evidence of post-zygotic maternal effects whereby exposure of embryos to low salinity affects negatively the adaptive responses

to low salinity, and thus survival (Torres et al. 2020; Šargač et al. 2021). However, exposure of embryos to low salinity can also increase larval performance at low salinity (e.g. *Neohelice granulata*, Giménez and Anger 2003), contributing to metapopulation connectivity (Giménez 2003). Furthermore, dry mass, and carbon and nitrogen content of freshly hatched larvae, was substantially lower for both Baltic populations than for the North Sea populations, and particularly low for larvae from Neustadt. In other species, larval survival, development, and growth are correlated to the dry mass, and elemental composition at hatching (Giménez and Anger 2001b, 2003a; González-Ortegón and Giménez 2014b; Torres et al. 2020b). Perhaps, exposure to low salinity causes a reduction in the investment of reserves into eggs, which in turn impacts larval performance. However, the limitation in tolerance found in *H. takanoi* larvae (and also in *C. maenas,* Šargač et al. 2021) is not completely unexpected: larval stages have usually narrower ranges of environmental tolerance than adults (Pandori and Sorte 2019b); in addition, range expansions (and invasions) into low salinity habitats are very challenging for species of marine origin (Ojaveer et al. 2010; Nasrolahi et al. 2012; Paiva et al. 2018).

In synthesis, our study shows a strong gradient, from the North to the SW Baltic Sea, in the capacity of *H. takanoi* larvae to develop, and a general inability to survive until metamorphosis in areas where salinity is 15 ‰ or lower. Surviving individuals at low salinities showed depressed growth and reduced body mass at metamorphosis, which is likely to compromise post-metamorphic survival. There was no apparent genetic differentiation among the studied populations, which could be underpinned by constant introduction of the species. Monitoring of populations, including that of larval stages in the plankton will be central to determine if populations are maintained by a larval export strategy or through alternative mechanisms.

Acknowledgements

We are grateful to Julia Haafke (AWI) for performing the elemental analysis, Sabrina Hüpperling and Pia Steingrübner (both Universität Greifswald) for their help collecting crabs and rearing larvae, and Jonas Geburzi (ZMT & Harvard University) for advice on sampling sites and access. We thank the Institute of Clinical Molecular Biology in Kiel for providing Sanger sequencing as supported in part by the DFG Clusters of Excellence “Precision Medicine in Chronic Inflammation" and "ROOTS". We thank T. Naujoks, Dr. D. Langfeldt and Dr. B. Löscher for technical support. This study was conducted using E.U. Copernicus Marine Service Information https://doi.org/10.48670/moi-00010.

This study is part of JPG and NE-N doctoral dissertations. JPG was supported by the Deutsche Forschungsgemeinschaft (Research Training Group 2010: RESPONSE) at the University of Greifswald, in a collaboration with the Alfred-Wegener-Institute. NE-N was supported by the Bundesministerium für Bildung und Forschung (Project MERGE; grant n° FKZ 01DN20002), Germany. DMA was funded by the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska Curie grant agreement (101023801) at the French National Centre for Scientific Research (CNRS).

Author contributions

JPG, NE-N, SH, LG, and GT conceived the experimental design. JPG, GT, CE, and AC collected the ovigerous females. JPG performed the experiments. JPG, NE-N, CE, DMA, and GT generated the data for the population genetics analyses. JPG, NE-N, CE, and LG analysed the data. JPG wrote the first draft. All authors contributed to the writing of the manuscript and gave final approval for publication.

Data availability

All data for this paper will be available from PANGAEA ®Data Publisher https://www.pangaea.de/

Declarations

Conflict of interest: The authors declare that they have no conflicts of interests.

Human or animal rights

The research presented in this paper complies with national (Germany) and international laws (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.

SUPPLEMENTARY MATERIAL

SURVIVAL & DURATION OF DEVELOPMENT

Table S1 - *Hemigrapsus takanoi*. Results of model selection (AICc values) for larval survival (logarithmic transformed data) in response to population (P), salinity (S), temperature (T), and female of origin (F). Maternal influences, represented by F, is a random factor nested within the interaction P * S. The remaining three factors are fixed and form a 3-way factorial design. Restricted maximum likelihood (REML) fitting was used for model selection on random terms. The best model, i.e. model with lowest AICc is in **bold**.

Model selection	**Logarithmic**					
	Zoea II	**Zoea III**	**Zoea IV**	**Zoea V**	**Zoea VI**	**Megalopa**
Random (REML)						
F * T * S	**1369**	1391	1392	**1436**	1266	**1242**
F * S	1401	**1388**	**1385**	1437	1264	1264
F * T	1441	1435	1427	1480	1264	1292
F	1453	1437	1424	1477	**1260**	1294
Fixed (ML)						
3-way (full model)	1315	1312	1304	1367	1148	1162
2-way factorial	**1297**	**1301**	**1287**	**1336**	**1143**	**1148**
without T : S	1312	1307	1289	1350	1165	1209
without T : P	1294	1330	1314	1340	1156	1164
without S : P	1307	1318	1320	1362	1163	1160
REML	1339	1361	1352	1397	1237	1218

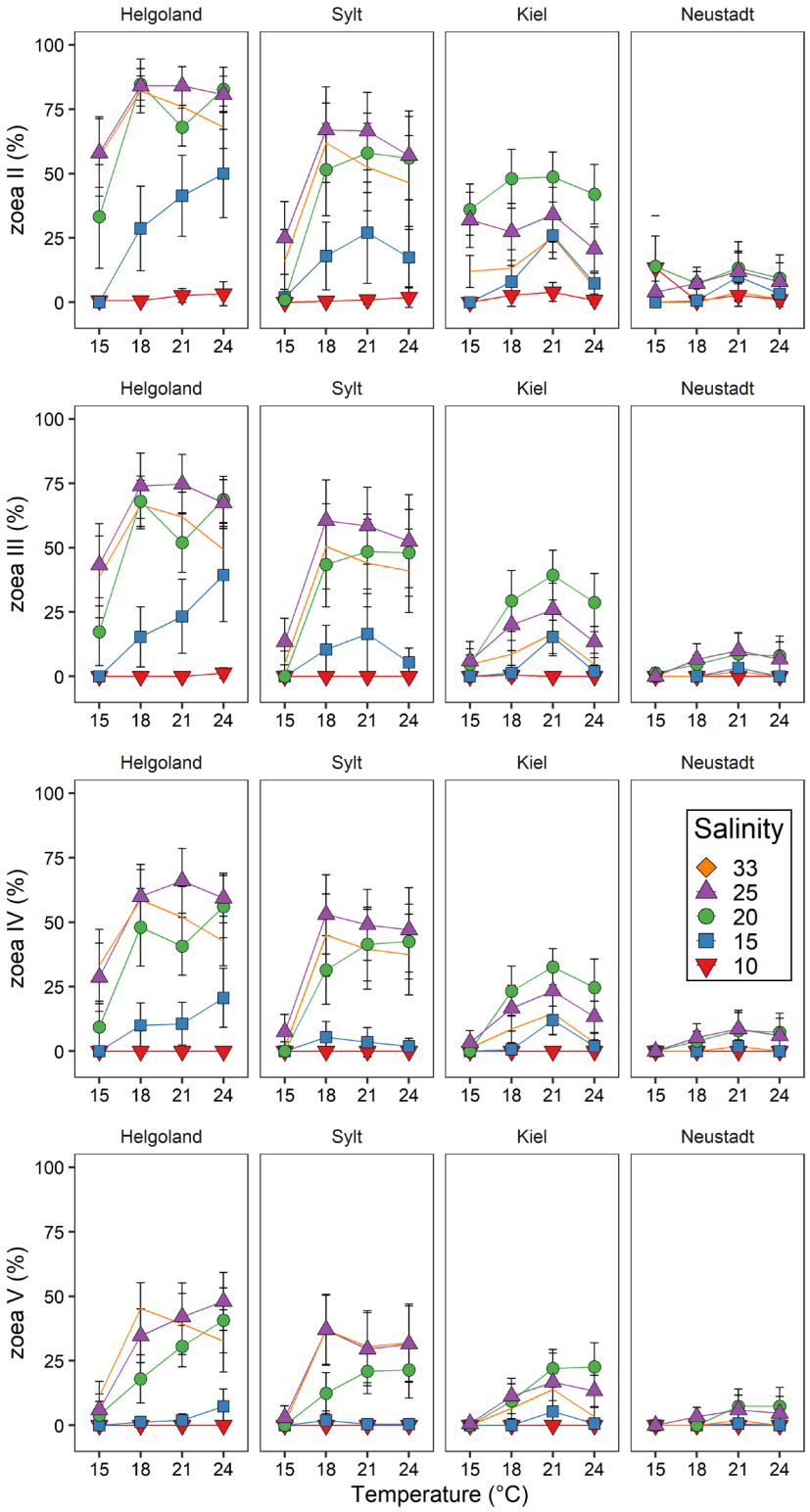

Figure S1 - *Hemigrapsus takanoi.* Cumulative survival to Zoea II - V discriminated by population. Comparison between populations from the North Sea: Helgoland and Sylt (left panels) and the Baltic Sea: Kiel and Neustadt (right panels) by temperature (15, 18, 21, 24 °C) and salinity (33 PSU, orange diamonds; 25 PSU, purple upwards triangles; 20 PSU, green circles; 15 PSU, blue squares, 10 PSU, red downwards triangles). Data shown as means ± SE for larvae produced by each female from each population (n = 3 or 4).

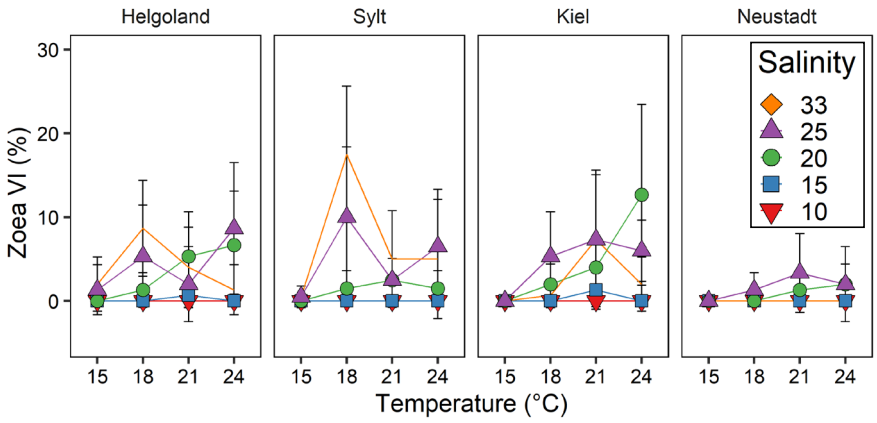

Figure S2 - *Hemigrapsus takanoi*. Cumulative survival to the extra zoeal stage Zoea VI discriminated by population. Comparison between populations from the North Sea: Helgoland and Sylt (left panels) and the Baltic Sea: Kiel and Neustadt (right panels) by temperature (15, 18, 21, 24 °C) and salinity (33 PSU, orange diamonds; 25 PSU, purple upwards triangles; 20 PSU, green circles; 15 PSU, blue squares, 10 PSU, red downwards triangles). Data shown as means ± SE for larvae produced by each female from each population (n = 3 or 4).

Table S2 - *Hemigrapsus takanoi*. Proportion of larvae going through Zoea VI.

Temperature	**Salinity**	**Survival (%)**			
(°C)	**(PSU)**	**Helgoland**	**Sylt**	**Kiel**	**Neustadt**
24	33	1.3	5	2	0
	25	8.7	6.5	6	2
	20	6.7	1.5	12.7	2
	15	0	0	0	0
21	33	4	5	7.3	0
	25	2	2.5	7.3	3.3
	20	5.3	2.5	4	1.3
	15	0.7	0	1.3	0
18	33	8.7	17.5	0.7	0
	25	5.3	10	5.3	1.3
	20	1.3	1.5	2	0
	15	0	0	0	0
15	33	2	0.5	0	0
	25	1.3	0.5	0	0
	20	0	0	0	0
	15	0	0	0	0

Table S3 - *Hemigrapsus takanoi*. Model selection for the cumulative survival to the respective larval stages.

Model selection	Logarithmic					
	Zoea II	**Zoea III**	**Zoea IV**	**Zoea V**	**Zoea VI**	**Megalopa**
Random (REML)						
F * T * S	**1369**	1391	1392	**1436**	1266	**1242**
F * S	1401	**1388**	**1385**	1437	1264	1264
F * T	1441	1435	1427	1480	1264	1292
F	1453	1437	1424	1477	**1260**	1294
Fixed (ML)						
3-way (full model)	1315	1312	1304	1367	1148	1162
2-way factorial	**1297**	**1301**	**1287**	**1336**	**1143**	**1148**
without T * S	1312	1307	1289	1350	1165	1209
without T * P	1294	1330	1314	1340	1156	1164
without S * P	1307	1318	1320	1362	1163	1160
REML	1339	1361	1352	1397	1237	1218

Table S4 - *Hemigrapsus takanoi*. Output of the quadratic model for survival to megalopa.

Population	Temperature (°C)	Parameter estimates	squared
Helgoland	18	3.743422	0.3652062
Helgoland	21	**29.218965**	0.5161292
Helgoland	24	**24.929302**	0.683872
Sylt	18	-6.362619	0.33058
Sylt	21	**32.53312**	0.3056924
Sylt	24	**29.766488**	0.3852895
Kiel	18	**16.689944**	0.7622541
Kiel	21	**24.912166**	0.5443811
Kiel	24	**22.190524**	0.4054225

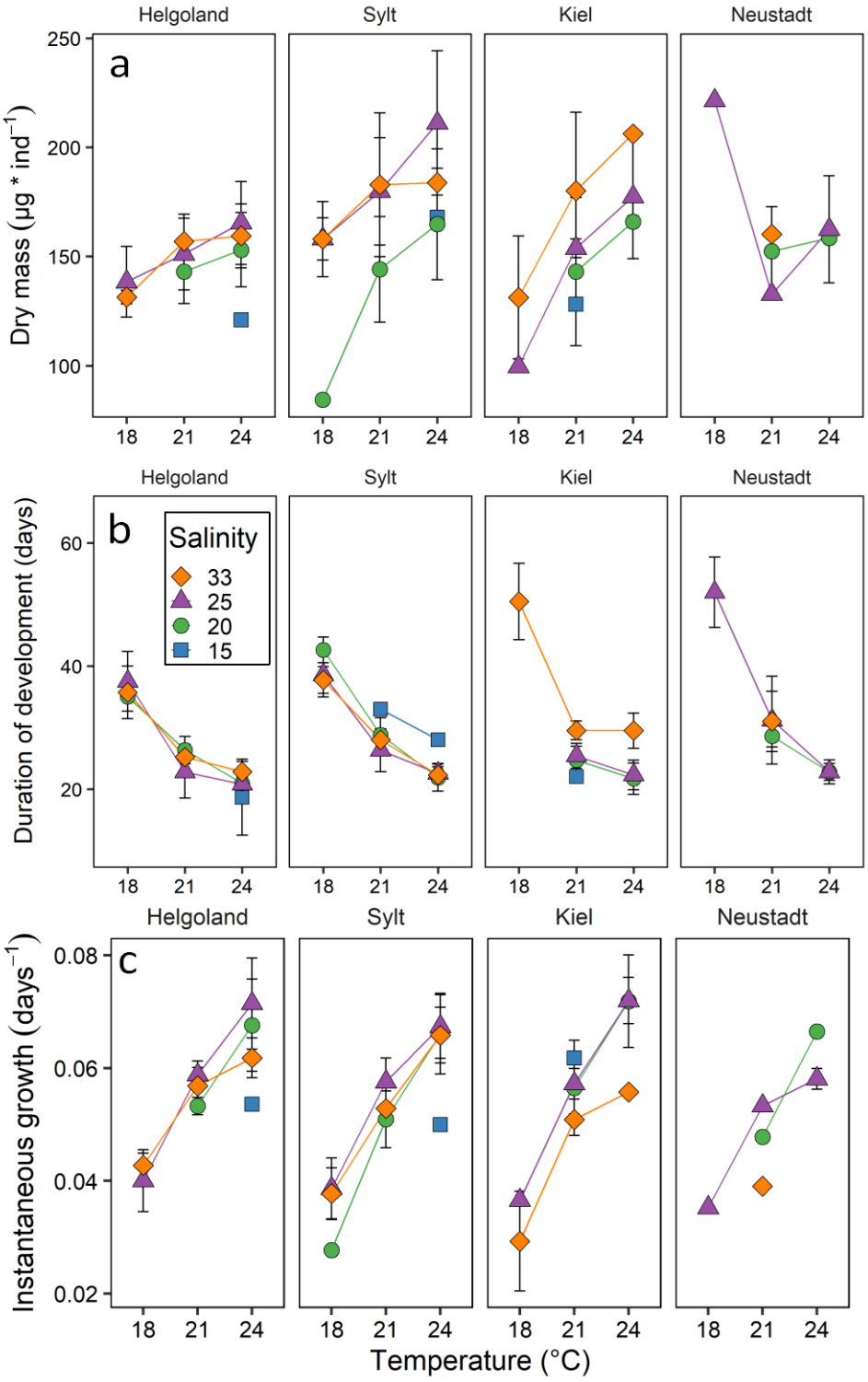

Figure S3 - *Hemigrapsus takanoi*. Dry mass (a), duration of development (b), and instantaneous growth rate in terms of dry mass (c) from hatching to megalopa. Comparison between populations from the North Sea: Helgoland and Sylt (left panels) and the Baltic Sea: Kiel and Neustadt (right panels) by temperature (15, 18, 21, 24 °C) and salinity (33 PSU, orange diamonds; 25 PSU, purple upwards triangles; 20 PSU, green circles; 15 PSU, blue squares, 10 PSU, red downwards triangles). Data shown as means ± SE for larvae produced by each female from each population (n = 3 or 4

Table S5 - *Hemigrapsus takanoi*. Output of the quadratic model for survival to zoea II by population and temperature.

Population	Temperature (°C)	Parameter estimates	squared
Helgoland	18	27.3421	0.7917365
Helgoland	21	27.15006	0.8527571
Helgoland	24	24.763	0.8579222
Sylt	18	29.38825	0.5020796
Sylt	21	25.73538	0.461222
Sylt	24	25.86896	0.4034664
Kiel	18	22.43256	0.3878893
Kiel	21	22.88808	0.6344496
Kiel	24	21.79229	0.5074884

Table S6 - *Hemigrapsus takanoi*. Model selection for duration of development after moult to megalopa.

	Analysis 1	**Analysis 2**	**Analysis 3**	**Analysis 4**
Model selection	Most complete T <21°C removed S < 20 PSU removed Neustadt excluded S*P*T	P*T T <21°C removed, at 25 PSU	P*T Neustadt & Kiel excluded all temperatures in seawater	T*P Neustadt excluded, at 33 PSU
Random (REML)				
F * T * S	3286		2287	
F * S	3280		2280	
F * T	3280	1499	2282	1046
F	**3279**	**1497**	**2278**	**1041**
Fixed (ML)				
3-way (full model)	3306		**2297**	
2-way factorial	3309	1512	2309	**1062**
without T : S	3309			
without T : P	3306	**1511**		1093
without S : P	3316			
without T		1512		
without P		1556		
without T:S & T:P	**3306**			
BEST	T + S + P + S:P	P	T*P*S	T + P + T:P + S:P +T:S

Table S7 - *Hemigrapsus takanoi*. Model selection for dry mass after moult to megalopa.

	Analysis 1	**Analysis 2**
Model selection	25 & 20 PSU for 3 populations Neustadt excluded for 21 & 24°C most complete	33 & 25 PSU for 18, 21 & 24°C Sylt & Helgoland
Random (REML)		
F * T * S	4502	4133
F * S	4500	4126
F * T	4500	4128
F	**4497**	**4124**
Null model		
Fixed (ML)		
3-way (full model)	4572	4198
2-way factorial	4568	4194
without T : S	4567	4196
without T : P	**4566**	**4192**
without S : P	4569	4193
without T:S and T:P	**4566**	
without T:P and S:P		**4191**
BEST	T + S + P + S:P	T + S + P + T:S

Table S8 - *Hemigrapsus takanoi*. Model selection for carbon content after moult to megalopa.

	Analysis 1	**Analysis 2**
Model selection	25 & 20 PSU for 3 populations Neustadt excluded for 21 & 24°C most complete	33 & 25 PSU for 18, 21 & 24°C Sylt & Helgoland
Random (REML)		
F * T * S	3613	3304
F * S	3611	3298
F * T	3612	3300
F	**3609**	**3296**
Null model		
Fixed (ML)		
3-way (full model)	3660	3346
2-way factorial	3656	3342
without T : S	3655	3342
without T : P	**3654**	**3340**
without S : P	**3654**	**3340**
without T:S and T:P	3652	3340
without T:P and S:P	3652	3338
without T:S and S:P	3653	3340
without S:P, T:P, T:S	**3652**	**3338**
BEST	T + S + P	T + S + P

Table S9 - *Hemigrapsus takanoi*. Model selection for nitrogen content after moult to megalopa.

Model selection	**Analysis 1** 25 & 20 PSU for 3 populations Neustadt excluded for 21 & 24°C most complete	**Analysis 2** 33, 25 PSU for 18, 21 & 24°C Sylt & Helgoland
	Random (REML)	
F * T * S	2329	2110
F * S	2326	2106
F * T	2326	2106
F	**2324**	**2103**
Null model		
	Fixed (ML)	
3-way (full model)	2338	2117
2-way factorial	2334	2113
without T : S	2334	2112
without T : P	**2332**	2114
without S : P	2333	**2111**
without T:S and T:P	2331	2112
without T:P and S:P	2330	2112
without T:S and S:P	2332	**2110**
without S:P, T:P, T:S	**2330**	2112
BEST	T + S + P	T + S + P+ T:P

Table S10 - *Hemigrapsus takanoi*. Model selection for instantaneous growth rate to megalopa.

	Analysis 1	**Analysis 2**
Model selection	25 & 20 PSU for 3 populations Neustadt excluded for 21 & 24°C most complete	33 & 25 PSU for 18, 21 & 24°C Sylt & Helgoland
Random (REML)		
F * T * S	-2712	-2580
F * S	-2714	-2584
F * T	**-2717**	-2582
F	-2716	**-2584**
Null model		
Fixed (ML)		
3-way (full model)	-2839	**-2710**
2-way factorial	-2841	-2702
without T : S	-2840	-2702
without T : P	**-2845**	-2706
without S : P	-2838	-2702
BEST	T+S+P+T:S+S:P	FULL

Table S11 - *Hemigrapsus takanoi*. Model selection (analysis 3 and 4) for dry mass, carbon and nitrogen content and instantaneous growth after moult to megalopa.

	Dry mass		**Carbon**		**Nitrogen**		**Growth (DW)**	
	Analysis 3	Analysis 4	Analysis 3	Analysis 4	Analysis 3	Analysis 4	Analysis 3	Analysis 4
Model selection	25 PSU 24°C all pop	20 PSU 21°C all pop	25 PSU 24°C all pop	20 PSU 21°C all pop	25 PSU 24°C all pop	20 PSU 21°C all pop	25 PSU 24°C all pop	20 PSU 21°C all pop
Random (REML)								
F	1654	533	1320	434	853	287	-958	-320
Fixed (ML)								
population	1683	562	1341	453	862	295	**-996**	-357
NULL	**1683**	**555**	**1339**	**447**	**860**	**289**	-995	**-361**

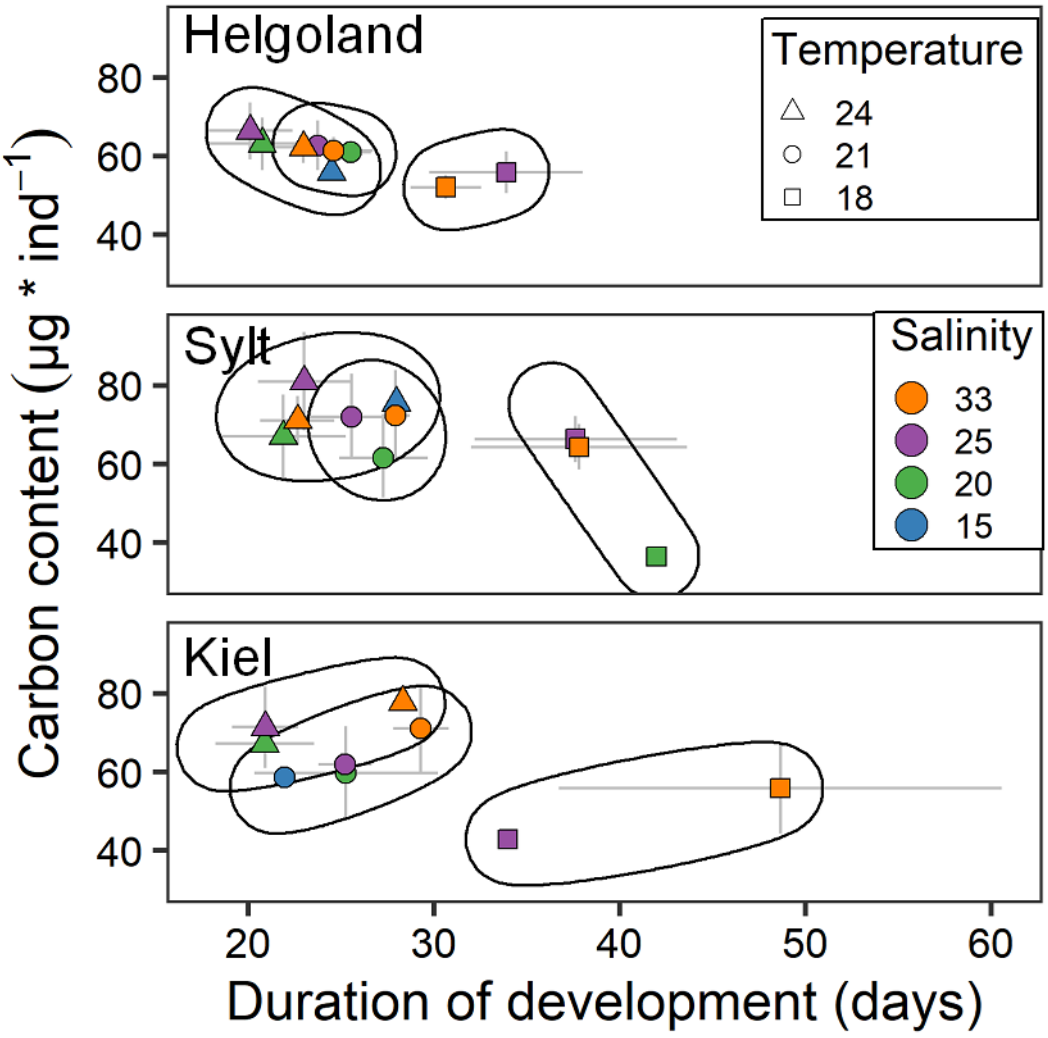

Figure S4 - *Hemigrapsus takanoi*. Integrated responses of carbon content and duration of development for megalopae from four populations (Helgoland, Sylt, Kiel, and Neustadt) and four temperatures and five salinities. Symbols: salinity (33 PSU: orange, 25 PSU: purple, 20 PSU: green, 15 PSU: blue) and temperature (18 °C: squares, 21 °C: circles and 24°C: triangles).

Table S12 - *Hemigrapsus takanoi*. Haplotype frequencies of the sampled populations showing frequencies of haplotype occurrence by population.

	Population			
Haplotype	Helgoland	Sylt	Kiel	Neustadt
	n = 6	n = 24	n = 19	n = 19
1	4	13	15	9
2	1	9	4	6
3	0	1	0	1
4	1	0	0	2
5	0	1	0	0
6	0	0	0	1

Table S13 - *Hemigrapsus takanoi*. JOST D values for statistical analysis of molecular differentiation.

JOST D				
	SYLT	NEUSTADT	KIEL	HELGOLAND
SYLT	0	0.00421926	0.000729812	-0.002336449
NEUSTADT	0.00421926	0	-0.001856752	-0.006802516
KIEL	0.000729812	-0.001856752	0	-0.006727633
HELGOLAND	-0.002336449	-0.006802516	-0.006727633	0

Table S14 - *Hemigrapsus takanoi*. JOST D p-values for statistical analysis of molecular differentiation.

JOST D p-values				
	SYLT	NEUSTADT	KIEL	HELGOLAND
SYLT	0	0.7	0.5	0.8
NEUSTADT	0.7	0	0.7	1
KIEL	0.5	0.7	0	1
HELGOLAND	0.8	1	1	0

Table S15 - *Hemigrapsus takanoi*. phi-values for statistical analysis of molecular differentiation.

phi				
	SYLT	NEUSTADT	KIEL	HELGOLAND
SYLT	0	0	0.01443486	0
NEUSTADT	0	0	0	0
KIEL	0.01443486	0	0	0
HELGOLAND	0	0	0	0

Table S16 - *Hemigrapsus takanoi*. phi p-values for statistical analysis of molecular differentiation.

phi p-values				
	SYLT	NEUSTADT	KIEL	HELGOLAND
SYLT	0	0.714285714	0.324675325	0.388611389
NEUSTADT	0.714285714	0	0.36963037	0.729270729
KIEL	0.324675325	0.36963037	0	0.666333666
HELGOLAND	0.388611389	0.729270729	0.666333666	0

Table S17 - *Hemigrapsus takanoi*. phi p-values for statistical analysis of molecular differentiation.

phi p-values				
	SYLT	NEUSTADT	KIEL	HELGOLAND
SYLT	0	0.714285714	0.324675325	0.388611389
NEUSTADT	0.714285714	0	0.36963037	0.729270729
KIEL	0.324675325	0.36963037	0	0.666333666
HELGOLAND	0.388611389	0.729270729	0.666333666	0

CHAPTER 6

Variations in larval responses to temperature at the continental European scale in a global invader at home: the shore crab *Carcinus maenas*

Geißel, JP. *, **Espinosa-Novo, N. ***, Giménez, L., Aberle N., van der Meer, G., Harzsch, S., Boersma, M., Torres, G. in prep.

***shared first authorship**

Variations in larval responses to temperature at the continental European scale in a global invader at home: the shore crab *Carcinus maenas*

Jan Phillipp Geißel[1,2*], Noé Espinosa-Novo[1*], Luis Giménez[1,3], Nicole Aberle[4,5], Gro van der Meer[6], Steffen Harzsch[2], Maarten Boersma[1, 7], Gabriela Torres[1]

Authors' affiliations:

[1]Alfred-Wegener-Institut, Helmholtz-Zentrum für Polar- und Meeresforschung, Biologische Anstalt Helgoland, Helgoland, Germany.

[2]Department of Cytology and Evolutionary Biology, Zoological Institute and Museum, University of Greifswald, Greifswald, Germany.

[3]School of Ocean Sciences, College of Environmental Sciences and Engineering, Bangor University, Menai Bridge, UK.

[4]Institute of Marine Ecosystem and Fishery Science, University of Hamburg, 22767 Hamburg, Germany.

[5]Biologisk stasjon, Norwegian University of Science and Technology, 7491 Trondheim, Norway.

[6]Austevoll Research Station, Institute of Marine Research, 5392 Storebø, Norway.

[7]University of Bremen, Bremen, Germany.

***joint first authors**

Corresponding author:

Noé Espinosa-Novo

Alfred-Wegener-Institut, Helmholtz-Zentrum für Polar- und Meeresforschung, Biologische Anstalt Helgoland, Helgoland, Germany

noe.espinosa@awi.de

Abstract

Range contraction and expansion, as a consequence of climate change may be enhanced or hampered by among-population variability in thermal tolerance. We quantified performance (i.e. survival, development, and growth) of larvae of the shore crab *Carcinus maenas*, at different temperatures (range 6 to 27 °C in steps of 3 °C), in populations located towards the limits of the European distribution range (South: Vigo, Spain; North: Bergen and Trondheim, Norway). Environmental conditions experienced by larvae can have strong effect on populations persistence because thermal tolerance is usually narrower in larvae than in adults. We hypothesized that larvae from Norway would show increased tolerance to low temperature (potentially enhancing expansion) while those from Vigo would show increased tolerance to high temperature (hampering contraction). Larval survival and growth at low and high temperatures varied little among populations, in spite of the wide latitudinal range covered in this study; little to no survival was observed at 6 and 27 °C for all populations. The observed patterns were also consistent with responses reported for populations located at the centre of the European distribution range (Helgoland, Germany) and at invaded locations (Pacific and Atlantic coasts of USA). Variations in larval survival and growth, observed over such a wide spatial scale were overridden by variations among larvae originated from different females within each population. However, larvae from Norway had a slightly shorter duration of development at low temperatures than those from Vigo, which may be adaptive in high latitudes as a way to shorten the larval phase. Larvae from Vigo and Norway showed lower survival at high temperature (24 °C) than those reported for southern Spain (Cadiz), which might also indicate increased thermal tolerance in populations located right at the southern European distribution limit. Hence, we found low potential for among population variability to affect range expansion and contraction but high potential for within population variation to affect those processes.

Keywords:

Carcinus maenas - Intraspecific trait variation - Larval performance – Latitudinal variation - Phenotypic physiological plasticity - Thermal tolerance

INTRODUCTION

Severe impacts on marine ecosystems have been caused by human-induced climate change (Burrows et al. 2011c; Poloczanska et al. 2013b; García Molinos et al. 2016; Boersma et al. 2016) and biological invasions (Gurevitch et al. 2011b; Chan and Briski 2017). Temperature is one of the most important physical changes directing species distribution, population persistence, and growth in an ecosystem and therefore drives community composition and diversity (Wiltshire and Manly 2004; Wiltshire et al. 2015; Litchman and Thomas 2023). Particularly in the European seas important changes are expected as the water temperature rises 2-3 times faster than in the global averages (Mackenzie and Schiedek 2007; Belkin 2009; Isaksen et al. 2022; Amorim and Wiltshire et al. 2023). In the south of the European Atlantic coast, observed warming leads to community shifts and species introductions termed "subtropicalization" (Montero-Serra et al. 2015). Towards the equatorial side of the distribution range, species may retract due direct negative effects of temperature or competition with warm-adapted species arriving from the south. Towards the Arctic, the rise in sea surface temperature, the decline of sea ice, and the inflow of Atlantic water masses lead to the retraction of Arctic species and the introduction of boreal Atlantic species. This process is known as "Atlantification of the Arctic" (Wassmann 2011b; Kortsch et al. 2015). Over the full distribution range, coastal and intertidal ecosystems, exhibit particularly pronounced and rapid changes in response to anthropogenic drivers (Somero 2002; Helmuth et al. 2006a, b).

Many coastal intertidal invertebrates develop through a complex life cycle, characterised by a pelagic larval phase (Levin and Bridges 1995; Pechenik 1999b). Larvae are dispersive as they can drift in the water column for periods of variable duration, with most planktonic feeding larvae requiring in the order of weeks to develop to the semi-benthic megalopa, depending on temperature (McConaugha 1992; Shanks 2009; Álvarez-Noriega et al. 2020). Given their dispersive nature, much of the natural process of changes in species distribution should occur through larval dispersal, either facilitated or limited by temperature and current patterns, among other factors (Cowen et al. 2006a; Cowen and Sponaugle 2009b). In addition, because of their small size, planktonic larvae can be transported in great quantities in the ballast water of ships, hence contributing to conquering of other habitats (Williams et al. 1988; Rilov and Crooks 2009; Verna et al. 2016; Ware et al. 2016). In higher latitudes (and regimes of lower temperatures) many invertebrate taxa show a trend of shorter or no pelagic larval duration, brooding, increased egg size and non-feeding larvae (Mileikovsky 1971; Morgan 2020). Larval dispersal contributes to the connectivity among populations; along latitudinal gradients, thus tolerance to high or low temperature may set a limit to the capacity to endure increased temperature or establish new populations in the poleward distribution limits. Temperature is critical for survival and growth, as for other ectothermic organisms (Somero 2005; Sokolova et al. 2012; Tepolt and Somero 2014; Sokolova 2021). Furthermore, because the larval tolerance range to environmental variables is narrower than that of adults (Pandori and Sorte 2019b), it is likely that a bottleneck in dispersal is set at the larval

phase (Cohen et al. 1995b; Carlton and Cohen 2003b; Cowen and Sponaugle 2009b; Giménez et al. 2020b). For instance, in the European shore crab, *Carcinus maenas*, complete larval development is limited to temperatures > 9 – 12 °C and is restricted to spring-summer (Dawirs 1985a; deRivera et al. 2007), while both embryos and adults tolerate much lower temperatures. Larvae from North American populations do not appear to develop successfully to megalopa above 22.5 °C (deRivera et al. 2007), but those from European populations complete the larval phase at temperatures as high as 24 °C (Spitzner et al. 2019b; Šargač et al. 2021a, 2022b). Hence, understanding the process of range expansion in coastal species will require a quantification of larval thermal tolerance, especially towards limits of the distribution range. This need is highlighted by the fact that larval tolerance to environmental stressors appears to differ between populations near *vs*. away from the distribution limits (Šargač et al. 2021a; Geißel et al. in prep.). As part of the poleward range expansion cold tolerance and plasticity in response to low temperatures can evolve which allows further expansions (Carbonell et al. 2021a).

The European shore crab *Carcinus maenas* is a key coastal species, likely to experience poleward expansion, initially through larval dispersal. *C. maenas* is native to the European Atlantic coast and is a well-known global invasive species present on the shores of all continents except Antarctica (Roman and Palumbi 2004a; Leignel et al. 2014; Young and Elliott 2020b). It is an opportunistic omnivorous decapod crustacean and an ecosystem engineer (Klassen and Locke 2007). When introduced into new habitats it has been shown to cause the restructuring of food webs (Cordone et al. 2023): i.e. serving as a new food item for apex predators (Yorio et al. 2020), as well as being a predator reducing the densities of many abundant taxa (Grosholz and Ruiz 1995).

Both in North and South America, poleward range expansions of *C. maenas* have been observed over the past 20 years (Hidalgo et al. 2005; deRivera et al. 2007; Yamada et al. 2017; Yorio et al. 2020). In North America, poleward range expansions would be part of an ongoing biological invasion (Cohen et al. 1995b; Behrens Yamada et al. 2022). Both the Arctic and the North-western Atlantic (North and Barents Seas) are warming at unprecedented rates and this is expected to continue (Polyakov et al. 2005; Mackenzie and Schiedek 2007; Wassmann 2011b; Ingvaldsen et al. 2021). In the European Arctic, the potential expansion following environmental change (particularly ocean warming) and potentially local adaptations would make *C. maenas* a “neonative” species (Essl et al. 2019). *C. maenas* has a complex life cycle with four planktonic zoeal stages and a semi-benthic megalopa stage at the end of the pelagic larval development (Williams 1967) followed by the benthic crab stages. The pelagic larvae are the main dispersive stages known to travel with currents up to 200 km (Domingues et al. 2012).

Species distribution models based on average summer/winter temperatures and observations of *C. maenas* in the invasive range failed to predict the presence of the northernmost-native populations (e.g. Norway, Compton et al. 2010). In addition, the northern range edge in the US east coast is populated by crabs with haplotypes from northern Europe (Roman 2006). This leads to the hypothesis that northern European populations might exhibit, not yet quantified local cold adaptations driving the

invasion potential in new habitats, including the potential expansion along the coast of Northern Norway and Russia. There is a growing body of work showing that intraspecific variation in response to suboptimal conditions root in phenotypic plasticity and evolutionary adaptation (Chevin et al. 2010; Simons 2011; Healy and Schulte 2012; Reusch 2014; Boyd et al. 2018a). This variation among conspecifics contributes to how *eurytherm* a species is and drives both their potential to persist in a rapidly changing native environment but also their success as an invasive species (Kuo and Sanford 2009; Kelly et al. 2012). A critical point is that we do not have information on the thermal range of survival and growth in larvae of *C. maenas* of European populations away from the North and Irish Seas. Most of the available information on the effect of temperature on survival and growth for European populations comes from a local population in the German Bight (Helgoland: 54 °N Dawirs 1985, Spitzner et al. 2019), the Baltic Sea (Šargač et al. 2021), the Irish Sea (Mohamedeen and Hartnoll 1989) and southern Spain (Šargač et al. 2022). However, information about the thermal tolerance limits of *C. maenas* larvae for European populations is available only for the German Bight and the Irish Sea, covering a narrow range of habitat temperatures and latitudes. Yet, information about thermal tolerance over a wide latitudinal range, is central to get a better quantification of the potential to cope with increased temperatures towards the south and for poleward expansion and invasions and worldwide invasion. A comparison of populations from Germany and one located near the southern European distribution limit (Cádiz: Šargač et al. 2022) has highlighted a diversity of larval responses to temperature, likely to contribute to the potential of invasion of coastal areas across the globe.

The objective of this study is to quantify thermal tolerance of larvae of the shore crab *Carcinus maenas*, over a wide latitudinal range, by providing information from two Norwegian and a Spanish population. This was achieved by performing a multi-population study, quantifying the effect of temperature on larval survival, development, and growth, in two populations of Norway (Bergen and Trondheim, Western Norway: 60 and 63 °N – nearly 10 ° north of the northernmost population with available data, Helgoland) as compared to a population of Northern Spain (Vigo) as a reference. We also complement our study with previously published data from the German Bight (Helgoland) and Southern Spain (Cádiz) to obtain a more comprehensive evaluation of the role of temperature on *C. maenas* larvae along the European coast (from 35 °N latitude to 65 °N latitude). We quantified the effects of temperature on survival and performance of larvae originating from three populations spanning > 20 ° of latitude. For the first time, we documented growth and survival responses in populations of this species from the northern range of the distribution range and covering a wide range of temperatures (6 °C to 27 °C in steps of 3 °C). In particular, we quantified the integrated responses of carbon-based growth rates and development, determining the body mass of megalopa, which for benthic invertebrates, are known to drive post-metamorphic survival in the benthic habitat (Giménez 2004a; Pechenik 2006b; Torres et al. 2016a). In addition, we compared our results to survival and performance of larvae from (seawater) populations studied in the past (Šargač et al. 2021, 2022, and unpublished

data). Quantification of the variation among populations in survival and physiological and developmental responses shall shed light on the degree of intraspecific variation across the native range. Differences between populations in the rates may indicate differences in the sensitivity to the drivers across different physiological processes and hint towards the action of local adaptations in combination with phenotypic plasticity, driven by effects of the parental habitat.

MATERIALS AND METHODS

Collection of organisms

Berried females of *Carcinus maenas* (carapace length: 29.0 - 62.5 mm) were collected during their reproductive season, from three locations along their distribution range in Europe (Fig. 1a): Vigo (Spain, coordinates: 42°07'08.4" N 8°49'19.0" W), Bergen (Norway, coordinates: 60° 23′ N, 5° 20′ E) and Trondheim (Norway, coordinates: 63° 26′ 24″ N, 10° 24′ 0″ E). The seasonal range of average monthly sea surface temperatures of the chosen sites are as follows: Vigo, 13 - 18 °C; Bergen, 5 - 16 °C; Trondheim, 5 - 15 °C. The temperatures at the month of collection were: Vigo, 14 - 15 °C; Bergen, 15 - 16 °C, Trondheim, 14 - 15 °C (source: https://www.seatemperature.org). Females from the Vigo population were hand-collected by divers in the subtidal zone of the sand bar Punta Ladeiras, in the mouth of the Miñor river; and transported to the Estación de Ciencias Mariñas de Toralla (ECIMAT). Females from Bergen were hand-collected on beaches and rocky shores across the Austevoll archipelago and transported to the aquarium facilities of the Institute of Marine Research/Havforskningsinstituttet, Forskningsstasjon Austevoll. In Trondheim, females were collected within the Trondheim fjord and on its outskirts near the Slettvik field station, Norwegian University of Science and Technology (NTNU) and animals were kept in the Trondhjem Biological Station (TBS NTNU). These two locations were known from preliminary observations to harbour shore crabs in dense populations which ensured availability of berried females during the reproductive season. Prior to the transport to the Marine Biological Station at Alfred-Wegener-Institute (Helgoland, Germany), berried females were kept in aquaria with aeration, or in flow-through systems using natural seawater (water temperature and salinity corresponding to their respective sampling site, range 32 - 35 ‰). For the transport, animals were individually placed in plastic containers, partially filled with water from their respective sampling site and a wet towel. The containers were then placed in a Coleman® cooler box to ensure constant temperatures during transportation. Upon arrival to the laboratory, females from each population were placed in aerated aquaria with natural filtered and UV-treated seawater from Helgoland (32.5 ± 1 ‰) at the temperatures recorded during collection at the sampling sites (Vigo: 15 ºC, Bergen and Trondheim: 12 ºC). Animals were fed frozen shrimps (*Crangon crangon*) twice per week; water was changed daily to ensure high water quality at hatching.

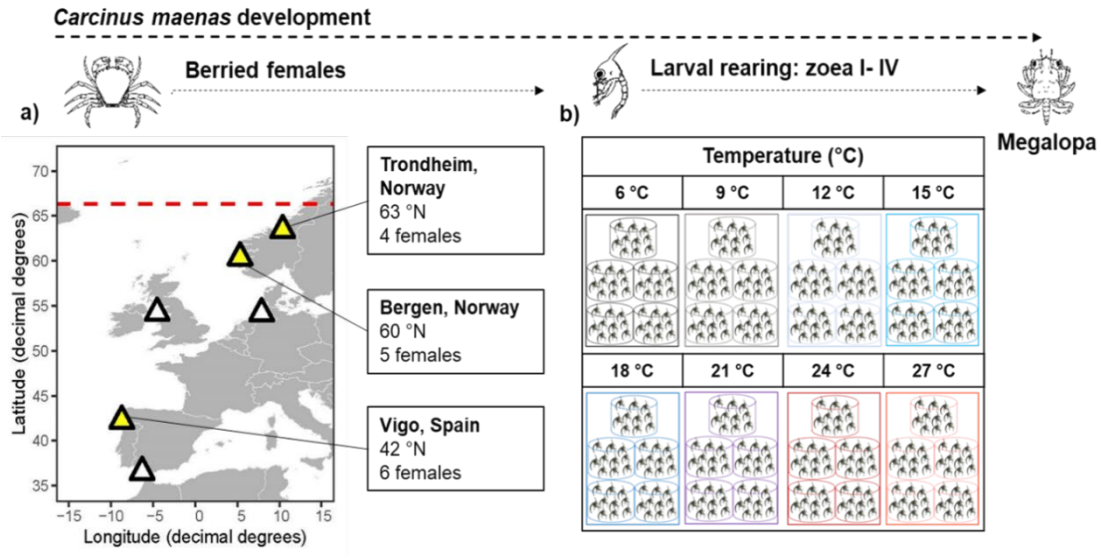

Figure 1: a) Map illustrating the sampling sites along the European Atlantic coast. Sampling sites are shown by yellow triangles, white triangles show sites were previous information is available (see discussion). The red dashed line indicates the Arctic circle at 66° 33' N. b) Experimental design to study the responses of larvae of *Carcinus maenas* from three different populations: Vigo (Spain), Bergen and Trondheim (Norway), to different temperature conditions. Larvae of *C. maenas* were reared from hatching to megalopa at eight different temperatures: 6, 9, 12, 15, 18, 21, 24 and 27 ºC, represented in the picture from light grey blue (6 ºC) to red (27 ºC). Larvae were reared in 5 replicates of 10 individuals each. Figure modified after Šargač et al. 2022.

Experimental set-up and larval rearing

Larval performance (i.e. survival, duration of development, and growth rates) was quantified in larvae reared from hatching to megalopa at eight temperatures, from 6 to 27 °C at 3 °C intervals (Fig. 1b). Freshly hatched larvae from each female were assigned to each treatment in five replicates of ten randomly assigned larvae in a 60 ml glass bowl each. The experiments were repeated with larvae from a total of 15 females (number of females: Vigo = 6; Bergen = 4; Trondheim = 5). We repeated the experiments with larvae from different females because variation in performance among larvae from different females can be large (Spitzner et al. 2019). Hence, repetition of experiments, with larvae originated from different females, is essential to reach a level of generality in the results. This experimental design allowed for the evaluation of the combined effects of population of origin and temperature during larval development. The experimental temperatures were chosen to match the span of conditions the larvae could experience in their respective natural environments.

Experiments were conducted in temperature-controlled rooms (± 1 °C) with a 12:12 h light: dark cycle. UV- treated and filtered (mesh size: 2 µm) natural seawater (33 ‰) was used in the experiments. Larval rearing was performed following standard rearing techniques (Torres et al. 2021e). Water in the experiments was changed daily; during the daily water change larvae were checked for

moults; exuviates and dead organisms were recorded and discarded from the experiments. Larvae were fed *ad libitum* with fresh *Artemia sp.* in a concentration of ~ 5 nauplii/mL (Great Salt Lake Artemia).

Body mass, elemental composition (C and N content), and growth rates of larvae, were quantified from samples taken at the start and at the end of the experiments. In crustaceans, larval carbon content is considered a proxy for lipid reserves and they are more sensitive to environmental variation than nitrogen content which is usually a proxy for proteins (Dawirs et al. 1986; Dawirs 1987; Anger and Harms 1990). Freshly hatched zoeae I (3 replicates of 50 larvae each) were randomly sampled at the start of the experiment and each freshly metamorphosed megalopa was sampled within 24 h after metamorphosis. Larvae were gently pipetted onto a filter and rinsed with distilled water, carefully blotted-dry with paper and stored for posterior analysis in pre-weighted tin cups at -20 ºC. Prior to the elemental composition analysis and in order to quantify dry mass, samples were freeze-dried (for 48 h, Christ Alpha 1-4 freeze drier) and weighed (microbalance, Sartorius MCA2.7S-2S00-M, precision 1 µg). Elemental composition (carbon and nitrogen content) was quantified using an elemental analyser (vario MICRO cube CHNS analyser, Elementar Analysensysteme).

Data analysis

We calculated cumulative survival to each zoeal stage as the percentage of survivors relative to the number of organisms at the beginning of each experiment; and cumulative duration of development to each larval stage as the time required to reach the next developmental stage, considering the duration of development of the previous stages. Instantaneous growth rates were calculated as $G = \log (W_f / W_0)/t$, where W_0 is the average mass (dry mass, carbon, or nitrogen) at hatching, W_f is the corresponding mass of each megalopa collected in each rearing replicate, and t is the time it took each corresponding larva to reach the megalopa stage.

The combined effects of temperature and population of origin were quantified using mixed modelling(Zuur et al. 2009). The response variables were survival, duration of development, elemental composition, and growth rates. The models consisted of temperature and population of origin as fixed factors, and female as a random factor. Model analysis was performed applying backward model selection (Zuur et al. 2009) based on the second-order Akaike information criterion (AICc). The package "nmle" (function lme for and gls, Pinheiro et al. 2019, R Core Team 2013) was used for model fitting with generalized least squares. Selection of models was performed following two steps; in a first step, the random terms were compared through Restricted Maximum Likelihood (REML) and in a second step the fixed terms were compared through Maximum likelihood, after refitting of the model with the best random structure. The model with the lowest AICc was always selected; in those cases where ΔAICc < was 3 and the most complex model had the lowest AICc, hypothesis tests (likelihood-ratio tests, LRT) was used to test for model selection: (i) when the models differed significantly ($p <$

0.05) we chose the model with the lowest AICc, and (ii) when the difference was not significant, we chose the simplest one. To detect differences between treatments, we performed the Tukey´ HSD (honestly significant difference) post hoc test. For survival analysis we use the whole temperature range (from 6 - 27 °C), but for the rest of the statistical analysis we use the results from 12- 24 °C as at 6 °C there was no survival to megalopa and at 9 and 27 °C only a few larvae reached the megalopa stage.

For survival proportions, data were rescaled to avoid situations of log(0) values; rescaled proportions were computed as p´= [p (n-1) + 0.5]/n, where n is the number of larvae at the beginning of the experiment (n = 10 individuals). Rescaled proportions were transformed to logistic (Warton and Hui 2011b) and logarithmic scales and data were analysed in both scales. We used the logarithmic transformation to test the multiplicative effect as a null model, as the multiplicative model to test if temperature and population of origin have independent effects on the rates of survival (Piggott et al. 2015b; Torres and Giménez 2020b). We analysed duration of development following two approaches: in the first one we treated temperature as a factor and in the second approach, temperature was analysed as a continuous variable.

RESULTS

Elemental composition of zoea I hatched larvae

Mean dry mass of zoea I larvae varied between 10.0 ± 2.0 µg*ind^{-1} in larvae from Vigo and 12.3 ± 2.9 µg*ind^{-1} in larvae from Trondheim (Fig. 2a), but we did not find statistical evidence of variation among populations nor for the case of C:N ratios (Fig 2d). We found statistical evidence of differences in carbon and nitrogen content between larvae from the Vigo (3.0 µg*ind^{-1}) and the Norwegian populations, which showed similar levels of carbon content (3.7 and 3.6 µg*ind^{-1}, respectively; Fig. 2b). Nitrogen content was significantly lower in Vigo (0.7 µgr*ind^{-1}) in comparison with Trondheim (0.9 µg*ind^{-1}, $p < 0.05$; Fig. 2c).

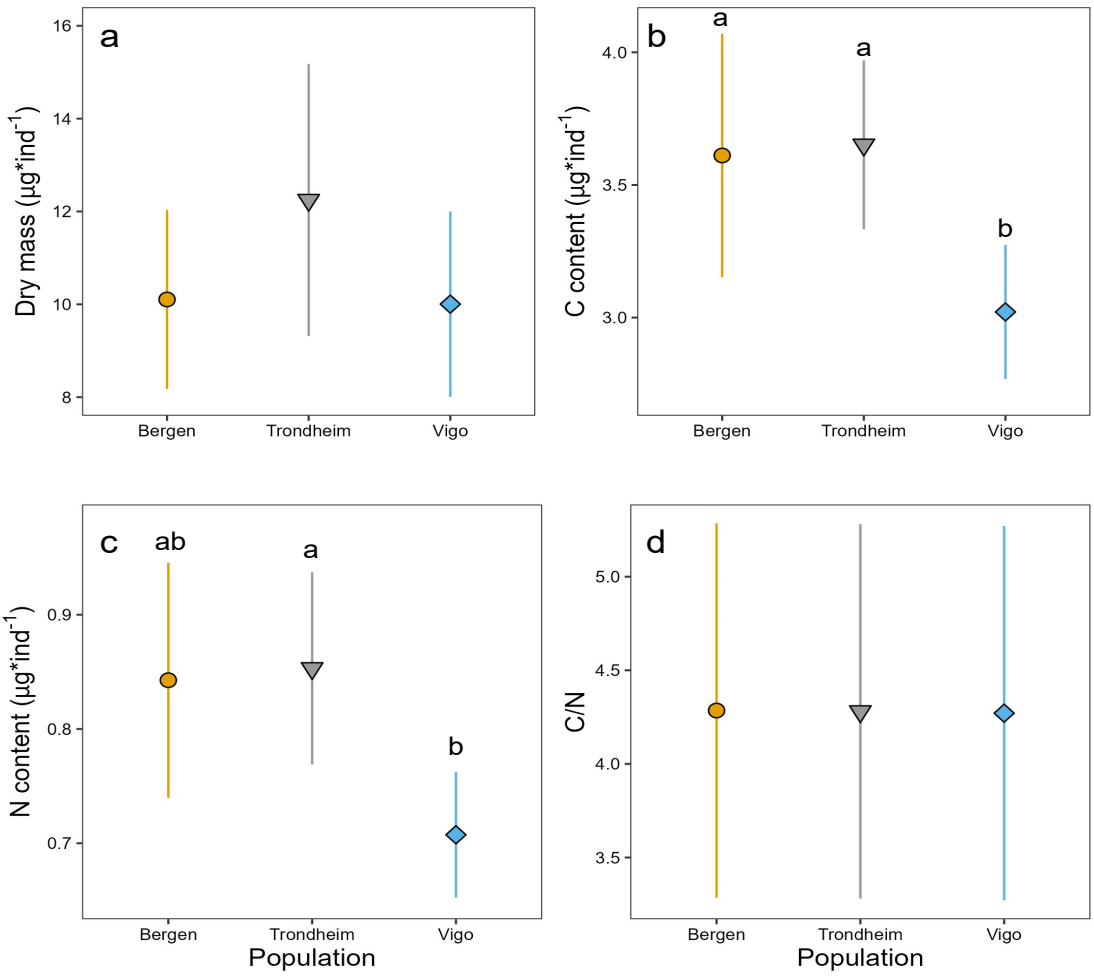

Figure 2: Dry mass (a), carbon (b) and nitrogen (c) content and C/N ratio (d) of freshly hatched zoeae I originated from females of three populations: Vigo, Bergen and Trondheim. Data presented as mean values ± SE of larvae produced by each female of origin. Symbols represent larvae from different populations; orange circles: Bergen, grey triangles: Trondheim and light blue diamonds: Vigo. Different letters represent significant differences between populations.

Survival, duration of development, and growth rates to megalopa

Larval survival to megalopa depended on temperature and varied among females (Fig. 3, Table S1: best model retained temperature in the fixed structure). For all three populations no metamorphosis to megalopa occurred below 9 °C (Fig. 3), and only one larva metamorphosed to megalopa at 9 °C (from Vigo). Also, in all populations survival was consistently low at 27 °C. In the range 12 – 24 °C: there was some variation among populations but it was overridden by the variability in survival among larvae from different females (Fig. 3: note error bars). From 12 °C, survival increased with temperature and reached optimal values in the range 15 - 21 °C. While survival for Vigo peaked

in the range 15 - 21 °C, for Bergen and Trondheim the peak was at 15 °C. Survival then decreased at higher temperatures and low survival rates were recorded at 24 and 27 °C for all three populations (Fig. 3).

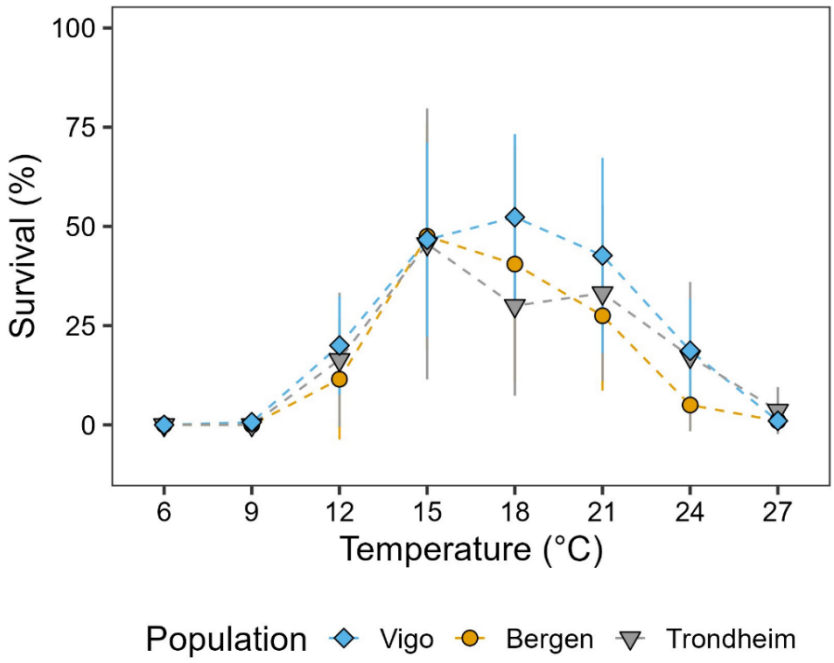

Figure 3: Average survival from hatching to megalopa of *Carcinus maenas* larvae reared under different temperatures, originated from females of three populations: Vigo, Bergen and Trondheim. Data presented as mean values ± SE of larvae produced by each female of origin. Symbols as in Fig. 2.

Duration of development to megalopa decreased with increasing temperatures in a non-linear pattern; best models retained the interaction of temperature and population, when temperature was treated as a factor (Table S2) and as a continuous variable (Table S3). The two Norwegian populations showed reduced duration of development as compared to Vigo, as evidenced by parameter estimates obtained when temperature was treated as a continuous predictor (Fig. S1). Duration of development reached a plateau in the range 21 - 27 °C of 16 - 18 days for all three populations (Fig. 4a).

Carbon growth rates increased with temperature for the three populations up to 21 ºC when it reached a plateau (Fig. 4b); the best model retained the temperature by population interaction (Table S3). Growth rates in terms of dry mass showed similar patterns to carbon growth rates; by contrast nitrogen growth rates for the Vigo and Bergen populations decreased at the highest temperatures and the model retained the effect of temperature (Table S3 and Fig. S2 b, d). Carbon content showed some variations among temperatures and populations: for Vigo it was generally constant in the range 12 - 21 ºC but it decreased at the highest temperatures; Bergen showed maximum values at 12 ºC and 15 ºC in

comparison to the other two populations ($p < 0.05$), and carbon content decreased at the highest temperatures; and it was generally constant for Trondheim in the range of temperatures studied (Fig. 4c). The best model retained temperature and population operating in an additive way (Table S3). Dry mass and nitrogen content showed similar patterns to the described above, but both were low for larvae from Vigo reared at 12 ºC when compared to those from Bergen and Trondheim ($p < 0.05$). Dry mass and nitrogen content tended to decrease at higher temperatures for Vigo and Bergen (Fig. S2 a, c). The best models retained temperature and population operating in an additive way (Table S3). C/N ratios were generally constant in the range 12 - 24 ºC for all three populations (Fig. 4d), at 27 ºC the ratio was higher for the Vigo population where there was a reduction in the nitrogen content of the megalopae, best model retained population (Table S3).

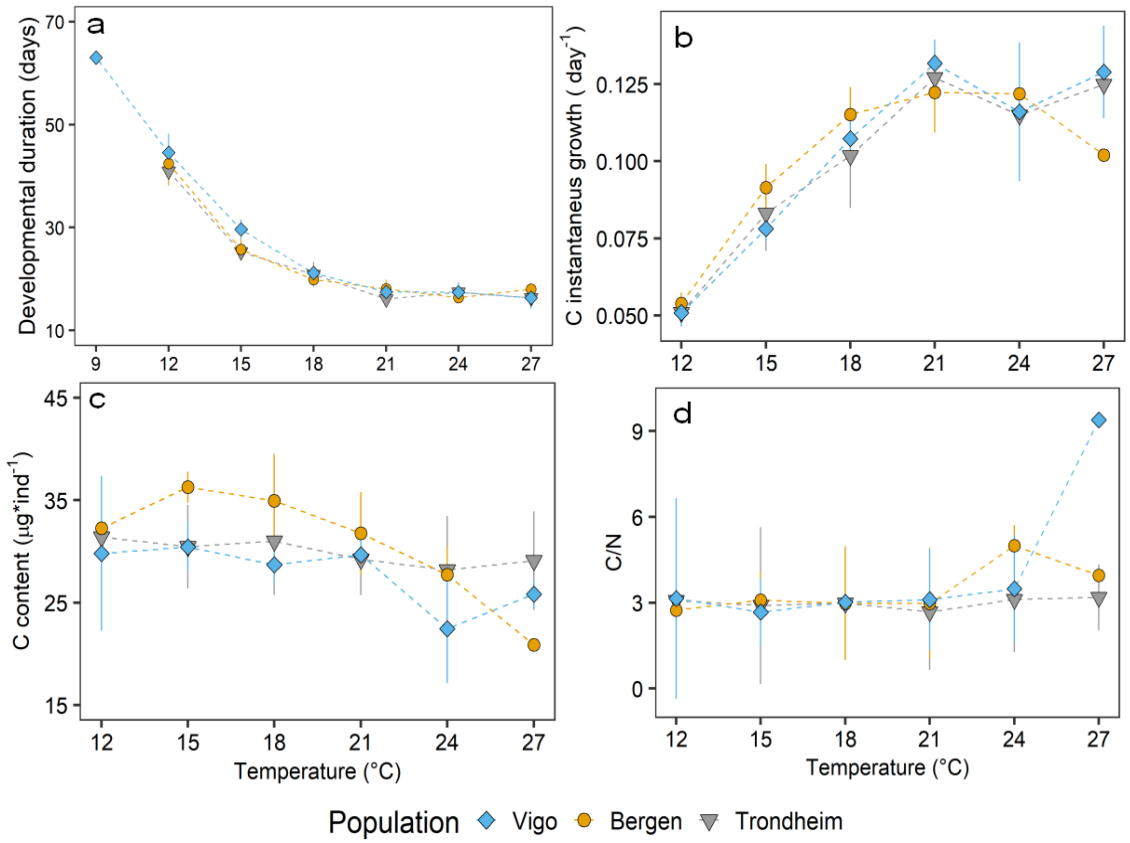

Figure 4: (a) Average duration of development to reach megalopa. (b) Average growth rates in terms of carbon at megalopa. (c) Average carbon content of megalopa. (d) Average C/N ratio of megalopa. Data represented in the graphs correspond to larvae of *Carcinus maenas* reared from hatching to megalopa under different temperatures, originated from females of three populations: Vigo, Bergen and Trondheim. Data presented as mean values ± SE of larvae produced by each female of origin. Symbols as in Fig. 2.

Integrated growth responses to megalopa were characterised by an extension of the larval phase at the lowest temperature tested. At 12 ºC and 15 ºC, larvae metamorphosed to megalopa after >

40 days and ca. 30 days, respectively; however, with high dry mass (30 - 36 µg*ind^{-1}). At 18 and 21 °C, when duration of development was shorter than at lower temperatures, larvae still metamorphosed with high dry mass; however, such pattern was not sustained at 24 and 27 ºC (Fig. 5). These reductions in dry mass reflect the fact that shorter durations of development were not compensated by the higher growth rates needed to attain higher dry mass. Integrated growth responses for carbon and nitrogen showed similar patterns as those shown for dry mass (Fig. S3).

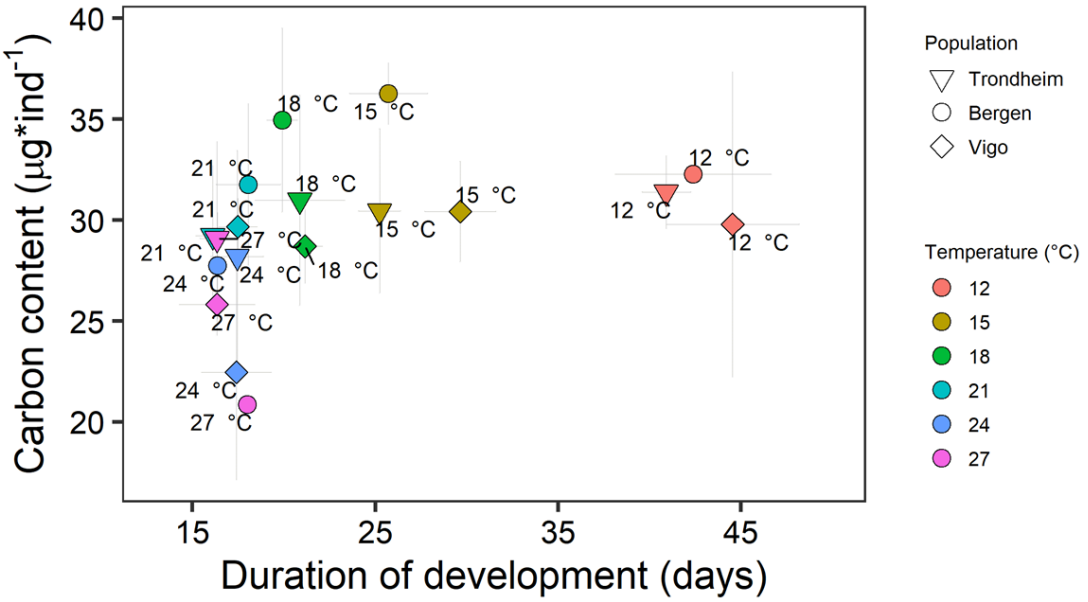

Figure 5: Integrated responses of carbon content and duration of development of larvae of *Carcinus maenas* reared under different temperatures, from hatching to megalopa, from larvae originated from females from three populations: Vigo, Bergen and Trondheim. Data presented as mean values ± SE of larvae produced by each female of origin. Symbols: Vigo is represented with diamonds, Bergen with circles, and Trondheim with triangles. Colours and labels indicate temperature.

DISCUSSION

We found only slight differences in performance of *C. maenas* larvae, in response to temperature, among populations located towards the southern (Spain) and northern (Norway) European distribution limits. Larvae from females collected in the two Norwegian populations (Bergen and Trondheim) developed slightly faster at lower temperatures (12 – 18 °C) than those from a population from Vigo (N Spain). There was only one single larva surviving to megalopa at 9 °C (belonging to Vigo population) and very few reaching megalopa at 27 °C (Vigo 1 %, Bergen 1% and Trondheim 3.6 %). On average, for both Norwegian populations, the highest survival was found at 15 °C, while the Spanish population showed a peak in survival in the range 15 - 21 °C. While the temperature that allowed highest survival for the Norwegian larvae was lower than for those from Spain, larvae from Norway did not

exhibit higher survival in low temperatures than those from Spain. However, such pattern was overridden by important variation in survival, among larvae from different females, as shown by wide error bars (Fig. 3) and highlighted by the fact that best models retained female of origin in the random structure (Table S1). This result is consistent with previous studies on invertebrate larvae (Carter et al. 2013; Applebaum et al. 2014a; Spitzner et al. 2019b). The implications of such variation, in the light of warming has been rarely considered (Bolnick et al. 2011; Violle et al. 2012) and they will depend on whether variation in survival is driven by genetic-heritable variability or parental effects. In a scenario of warming, genetic-heritable variation within the population should result in portfolio effects (Schindler et al. 2015a) whereby, populations are buffered from temperature increase through selection of warm-tolerant genotypes. The implications of the variation of environmental origin are difficult to predict as they depend strongly on the combination of two ingredients: (1) how parental environmental conditions (temperature or another stressor) drive survival when larvae experience increased temperature and (2) how parental and larval environmental conditions covary in the field. For example, if (1) increased parental temperature drives decreased larval performance (at high temperature), and (2) long warming periods affect both the parental and larval habitat, then (3) strong reductions in larval survival are expected. Parental effects are important (Parker et al. 2017; Torres et al. 2020b) but their action depends on the covariation between the environmental conditions of the parental and larval habitat. In this sense, we need further research quantifying the role of parental effects and the covariation among stressors in both the benthic and pelagic habitat.

When considering data from other populations, our results do not give evidence of a strong change in the pattern of tolerance to low temperatures towards the northern sector of *C. maenas* distribution range. For instance, the thermal tolerance in the German Bight populations (minimum temperature = 9 °C, next lower tested step was 6 °C (Dawirs 1985a, 1986) is comparable to that of the Vigo population and lower than that found for both Norwegian populations. The minimum temperature for successful larval development in the North American populations is 10 °C (deRivera et al. 2007) which compares well with our results, given that their next lower tested temperature was 7.5 °C. Following the genetic analysis by (Roman and Palumbi 2004a) the western Norwegian populations of *C. maenas* should be very similar and differ from the Spanish population: the western Norwegian population shows roughly 1/3 haplotypes from a boreal clade, and the rest from two clades that are not attributed and partially shared with the southern European populations; Northern Spain has mostly haplotypes from those two shared clades and a small fraction of haplotypes from a southern clade. There is no knowledge available on the genetic composition of the northernmost Norwegian populations. Perhaps, larval tolerance to low temperature is higher in populations located north of the Arctic circle. Further studies are needed to explore potential adaptations of the northernmost populations. Temperature has fundamental influence on marine invertebrate life history, metabolic rates, development, and dispersal (O'Connor et al. 2007) and for marine ectotherms it is generally believed

that rising temperatures will induce poleward shifts in their range boundaries (Sunday et al. 2012b). Local adaptations can help to mitigate the effects of warming. Degrees of intraspecific variation and local adaptation in thermal tolerance to latitude have been reported from different marine invertebrates e.g. fish (Fangue et al. 2006), intertidal mussels (Logan et al. 2012), intertidal snails (Kuo and Sanford 2009) or tidepool copepods (Kelly et al. 2012), and adults of *C. maenas* (Tepolt and Somero 2014). Studies of intraspecific variation in thermal tolerance also help to understand macroevolutionary patterns in the evolution of temperature tolerance and maternal investment, e.g. the variation in propagule size among temporal and spatial scales and within and among populations (Bernardo 1996).

The only evidence of some level of an adaptive response is shown in the 10 – 20 % shorter developmental time at low temperatures (12 – 15 °C) in larvae from Norway as compared to Vigo. This is consistent with that found in interspecific comparisons among closely related fish species (Yamahira and Conover 2002), sea stars (Hoegh-Guldberg and Pearse 1995), and deep-water lithodid crabs (Brown et al. 2018). We categorise this response as "adaptive" because it can be beneficial (Gotthard and Nylin 1995), without implying that it is a local adaptation (the demonstration of which requires multigenerational experiments). Reduced pelagic larval duration can be advantageous (in cold temperatures) because larvae are exposed to predators, food limitation or stressors for a shorter time (Levin and Bridges 1995; Pechenik 1999b). The ecological consequences of such reduction depend on how they result in changes in larval survival. For example, assuming constant per capita larval mortality rates (i.e. where survivors follow an exponentially decaying function of time), and taking field estimations for invertebrate marine larvae (= 0.14 d^{-1}: White et al. 2014), a reduction in 10% in duration of development (e.g. from 44 to 40 days at 12 °C) results in an increase in 75% in survival to the settling stage (from 211 to 370 survivors out of 10^5 larvae released). The effects of food limitation in high latitudes might be enhanced through mismatches between the timing of larval development and production peaks (Fortier et al. 1995; Peck et al. 2012; Toupoint et al. 2012) and local warming can exacerbate this situation (Laurel et al. 2021). Food limitation can be an important driver in survival and growth of larvae of decapod crustaceans (Olson and Olson 1989; Anger 2001), especially when the time window for successful larval release and survival is limited which is typically the case in higher latitudes (Levin and Bridges 1995). Hence, by shortening the pelagic duration, organisms might reduce the chances of a trophic mismatch. The increased level of reserves at hatching might also be interpreted as adaptive in the light of potential mismatches, and it is consistent with findings in other marine invertebrates (Marshall et al. 2012; Barneche et al. 2018; Álvarez-Noriega et al. 2020). In addition, faster developing larvae would settle earlier in the season, hence the resulting juveniles should experience a longer period of the summer temperatures enhancing post-metamorphic growth; post-metamorphic body mass and size serves as a predictor of post metamorphic performance with a positive correlation between body mass and performance (Pechenik 2006b). However, at this stage it is difficult to quantify how much advantage is given by the observed reduction in developmental time in the

Norwegian populations. This evaluation requires further field studies monitoring larval hatching, development, and settlement in combination with modelling of larval phenology (see e.g. deRivera et al. 2007; Giménez et al. 2020).

Patterns consistent with local adaptation appear in the benthic phase of *C. maenas*, perhaps because larvae disperse while adults must adapt to local conditions. Crabs from Norway (60.6 °N) showed heart collapse at lower temperatures (lower CT_{max}) and lower heart beat frequencies at low temperatures, than those from the Iberian Peninsula (38.6 °N) (Tepolt and Somero 2014). In any case, from the larval perspective, poleward expansion does not appear to be enhanced by Norwegian populations showing increased larval tolerance to low temperatures, unless this is restricted to populations located in the Arctic circle. However, range expansion is likely to occur because of the increasing temperatures, that will enable the establishment of populations through larval dispersal and the post-metamorphic survival through the increased tolerance to temperature exhibited by the benthic stages (Tepolt and Somero 2014).

Likewise, there was no evidence of increased larval tolerance to high temperatures in the Vigo populations compared to those of Norway. Survival dropped at 27 °C while growth rate dropped at temperatures > 21 °C leading to smaller body mass at metamorphosis. These results suggest limited capacity to deal with the warming at lower latitudes, unless the populations located further south shows such a trait. Some evidence of the latter is given by the fact that the temperature of highest larval survival is higher in Cádiz (24 °C) as compared to Vigo, USA and the Norwegian populations (Figs. 6 and 7). However, body mass at metamorphosis was high, peaked at 21 °C for the Cádiz population suggesting growth limitation at higher temperatures (Šargač et al. 2022). For Helgoland, two studies indicate different temperatures of highest survival (Dawirs 1985a; Šargač et al. 2022b), with the lower temperature (12.5 °C) being obtained in the early 1980s and the higher temperature (18 °C) was measured in the early 2020s. For the upper thermal limit, we did not find differences among the three tested populations, all had very low survival rates at 27 °C. In the studied populations from USA, no survival to megalopa was reported in temperatures higher than 22.5 °C (25 °C was the next higher tested temperature). This is substantially lower than the highest temperature that allowed survival Vigo, Bergen, and Trondheim and while we do not have the upper limit for Cádiz, the optimum being at 24 °C indicates that the higher limit should be considerably higher than 25 °C.

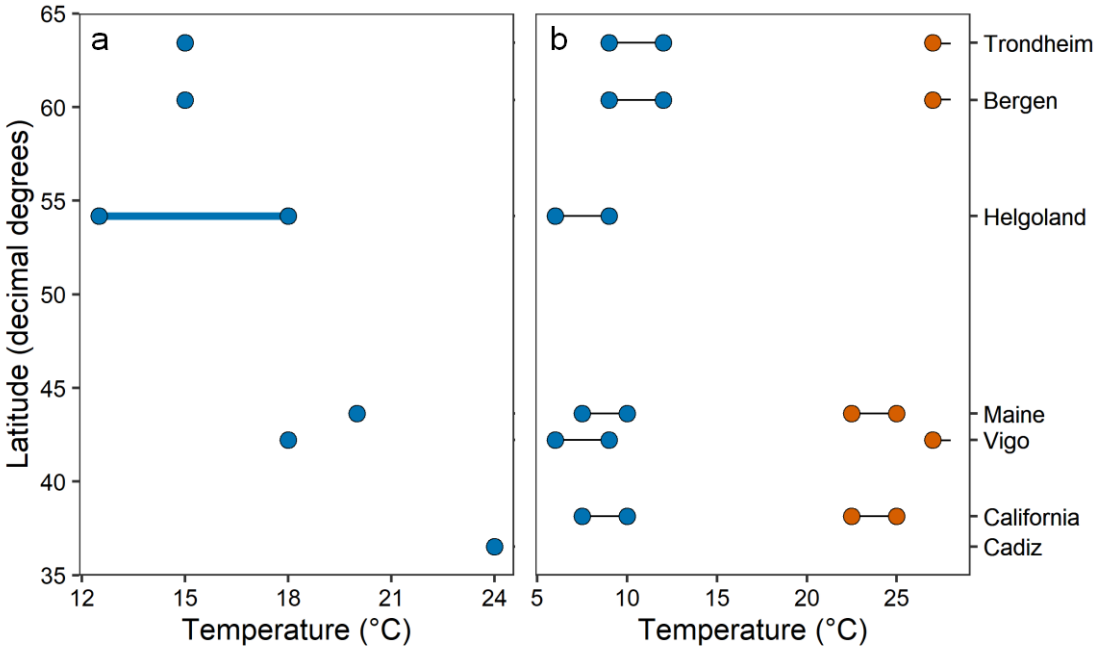

Figure 6: a) Temperature of highest survival (blue circles) by latitude (left y-axis) and population (right y-axis). Data for Cádiz, Helgoland, and Maine was obtained from literature (Dawirs 1985a, 1986; deRivera et al. 2007; Šargač et al. 2022b). For Helgoland, two studies indicate different temperatures of highest survival which is plotted here as span. b) Temperature windows for survival to megalopa, the span between the blue points indicates the lower temperature span between the lowest tested temperatures that did not and did permit survival respectively, the span between the red circles indicates the upper temperature span between the highest tested temperatures that did and did not permit survival respectively, by latitude (left y-axis) and population (right y-axis). Data for Cádiz, Helgoland, and Maine were obtained from literature (Dawirs 1985, 1986; deRivera et al. 2007; Šargač et al. 2022).

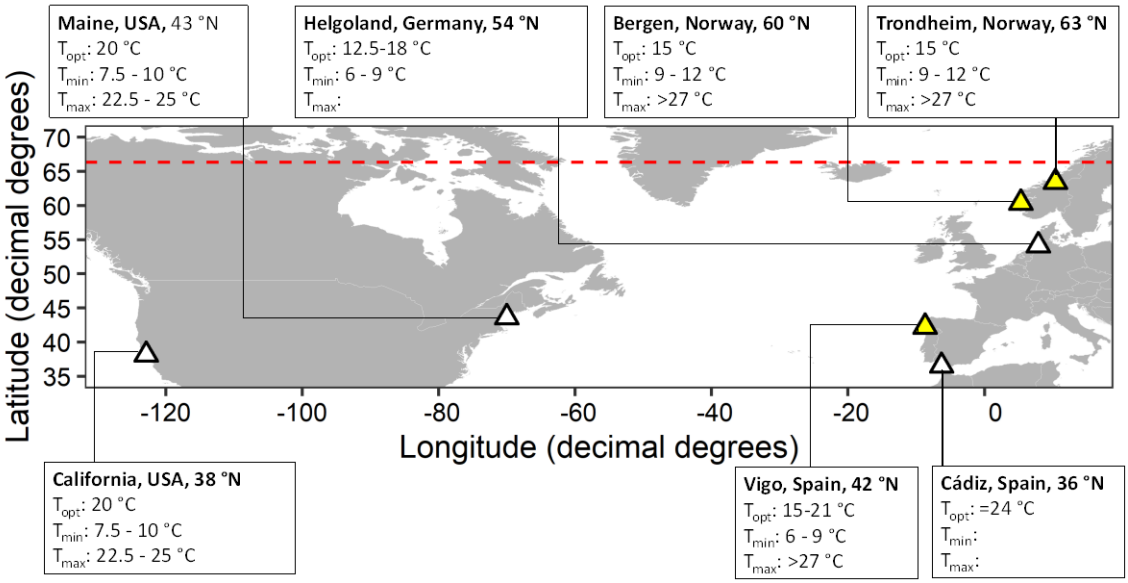

Figure 7: Map indicating the temperature of highest survival to megalopa by population. Triangles indicate site of origin, yellow for new data obtained in this study, white for data from literature (Dawirs 1985, 1986; deRivera et al. 2007; Šargač et al. 2022). The red dashed line indicates the Arctic circle.

In synthesis, both our experiment, and a comparison to literature data show that duration of development in 12 °C and 15 °C respectively decrease with latitude. In 12 °C, dry weight and carbon content of megalopa increase with latitude. In 15 °C, this trend is not as clearly visible. Larvae from Norway show slightly shorter duration of development in low temperatures which might have an adaptive value, contingent on the actual pattern of temperatures experienced during the larval phase. Populations from southern Europe show slightly increased survival at higher temperatures, but we cannot provide evidence of an increased upper limit of thermal tolerance. Further range expansions into the European Arctic are likely to be driven solely by increasing temperatures, unless populations from the Arctic show increased tolerance to low temperature. Range contraction, associated to limited larval tolerance to low temperature appears also likely unless populations located at the southern limit show increased thermal tolerance.

Acknowledgements

We would like to thank the technical staff from the ECIMAT in Vigo and anonymous Norwegian citizens that collected some of the berried females in Vigo, Bergen, and Trondheim, respectively. We are grateful to Julia Haafke (AWI) for performing the elemental analysis and to Fiona Bravo and Anne Fröhle for their help with larval rearing.

Funding

This study is part of NE-N and JPG doctoral dissertations. NE-N was supported by the Bundesministerium für Bildung und Forschung (Project MERGE; grant n° FKZ 01DN20002), Germany. JPG and AF were supported by the Deutsche Forschungsgemeinschaft (Research Training Group 2010: RESPONSE) at the University of Greifswald, in a collaboration with the Alfred-Wegener-Institute. FB was supported by a Deutsche Akademische Austausch Dienst (RISE scholarship). GT and NE-N visit to ECIMAT (Vigo, Spain) was supported through the Transnational Access ASSEMBLE+ funded by European Union's Horizon 2020 research and innovation programme under grant agreement No 730984, 8th call (Project ROSW – PID 10370)

Human or animal rights

The research presented in this paper complies with national (Germany) and international laws (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.

SUPPLEMENTARY MATERIAL

Table S1. Model selection for survival to megalopa of *Carcinus maenas* considering larvae from Vigo, Bergen, and Trondheim, reared at different temperatures. Data were analysed in the logistic and logarithmic scales. Model selection was performed using corrected Akaike information criteria (AICc). Symbols: ♀ female of origin. Highlighted in bold: the best overall model, for both the random and fixed term.

Model selection:	**Vigo- Bergen- Trondheim**	
	Raw	**Log**
Random (REML)		
♀:T:P	-476	938
♀:T	**-504**	**913**
♀:P	-335	1050
♀	-340	1046
Fixed (ML)		
P:T	-588	874
P+T	-607	888
T	**-610**	**870**
P	-538	978
Null	-542	974

Table S2. Model selection for duration of development to megalopa of *Carcinus maenas* considering larvae from Vigo, Bergen and Trondheim, reared at different temperatures. Data were analysed using two modelling approaches: a) in model 1, temperature and population were included as fixed factors and in b) model 2, temperature was included as a continuous variable. Model selection was performed using corrected Akaike information criteria (AICc). Symbols: ♀ female of origin. Highlighted in bold: the best overall model, for both the random and fixed term.

a) Model 1

Model selection:	**Vigo- Bergen- Trondheim**
	Model 1
Random (REML)	
♀:T:P	4385
♀:T	**4367**
♀:P	4371
♀	4368
Fixed (ML)	
P:T	**4379**
P+T	4398
T	4398
P	4655

b) Model 2

Model selection:	**Vigo- Bergen- Trondheim**
	Model 2
Random (REML)	
♀:T:P	-1356
♀:T	**-1134**
Fixed (ML)	
P:T	**-1375**
P+T	451

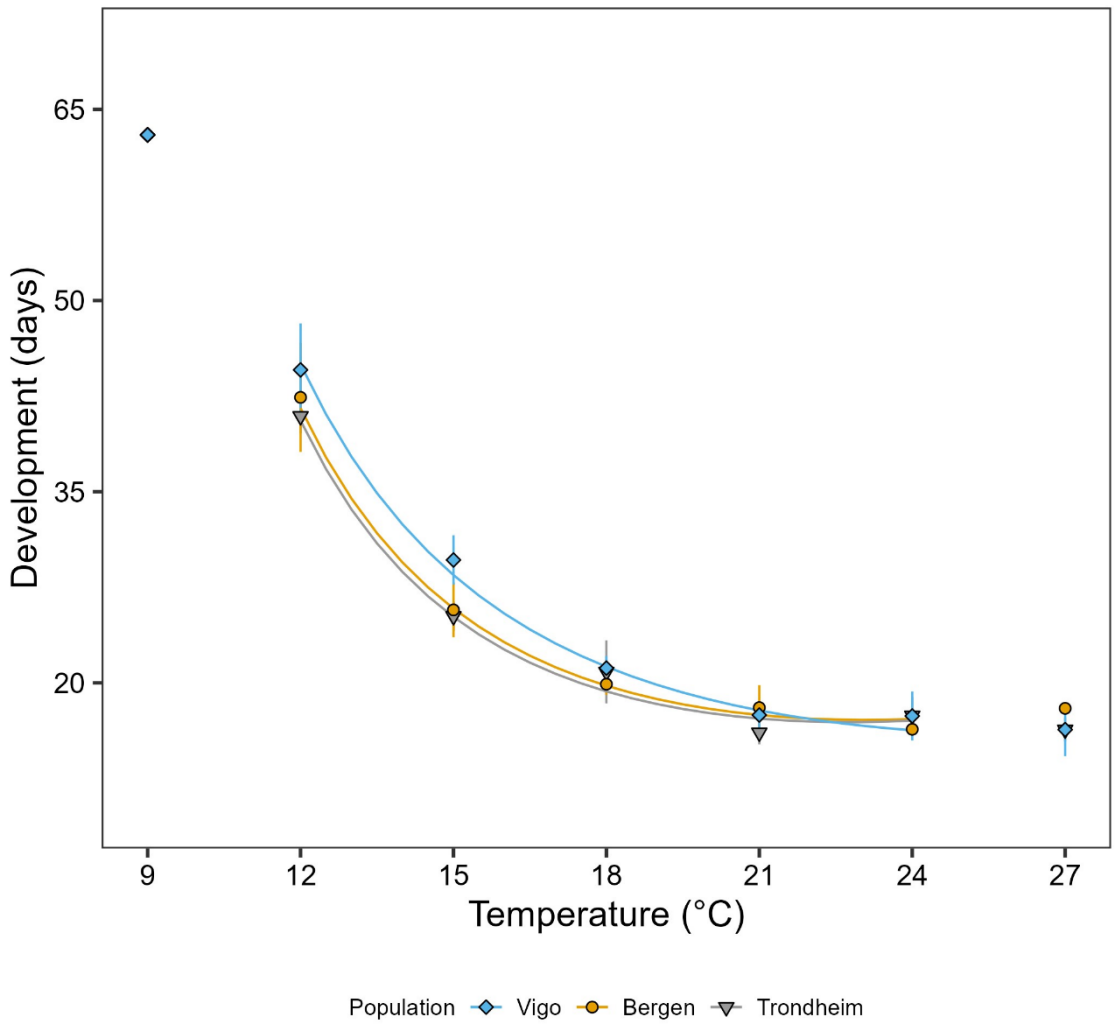

Figure S1. Average duration of development from hatching to megalopa of *Carcinus maenas* larvae reared under different temperatures, from three populations: Vigo, Bergen, and Trondheim. Data presented as mean values ± SE of larvae produced by different females of origin. Lines show predicted developmental durations based on maximum likelihood ($DD = a * T^b$) with a being constant and b being a log(T) parameter depending on population. Note that at 9 and 27 °C we did not get enough samples to add in the model. Symbols as in Fig. 1.

Table S3. Model selection for dry mass (DM), carbon (C) and nitrogen (N) content per individual and instantaneous growth rates to megalopa of *Carcinus maenas* in terms of dry mass (GrDM), carbon (GrC) and nitrogen (GrN); considering larvae from Vigo, Bergen, and Trondheim, reared at different temperatures. Model selection was performed using corrected Akaike information criteria (AICc). Symbols: ♀ female of origin. Highlighted in bold: the best overall model, for both the random and fixed term.

Random (REML)	AICc						
Term	DW	C	N	IgDW	IgC	IgN	C/N
♀:T:P	7515	5720	4451	-4906	-4850	-4392	2095
♀:T	**7499**	**5705**	**4432**	**-4925**	**-4870**	**-4411**	**2077**
♀:P	7516	5723	4506	-4920	-4862	-4362	2174
♀	7512	5782	4502	-4924	-4866	-4366	2170
Fixed (ML)							
Full factorial	7558	5737	4448	**-5068**	**-5012**	-4536	2058
P+T	**7545**	**5731**	**4443**	-5066	-5009	-4545	2051
T	7557	5741	4448	-5068	-5011	**-4546**	2051
P	7567	5744	4448	-4895	-4838	-4407	**2047**

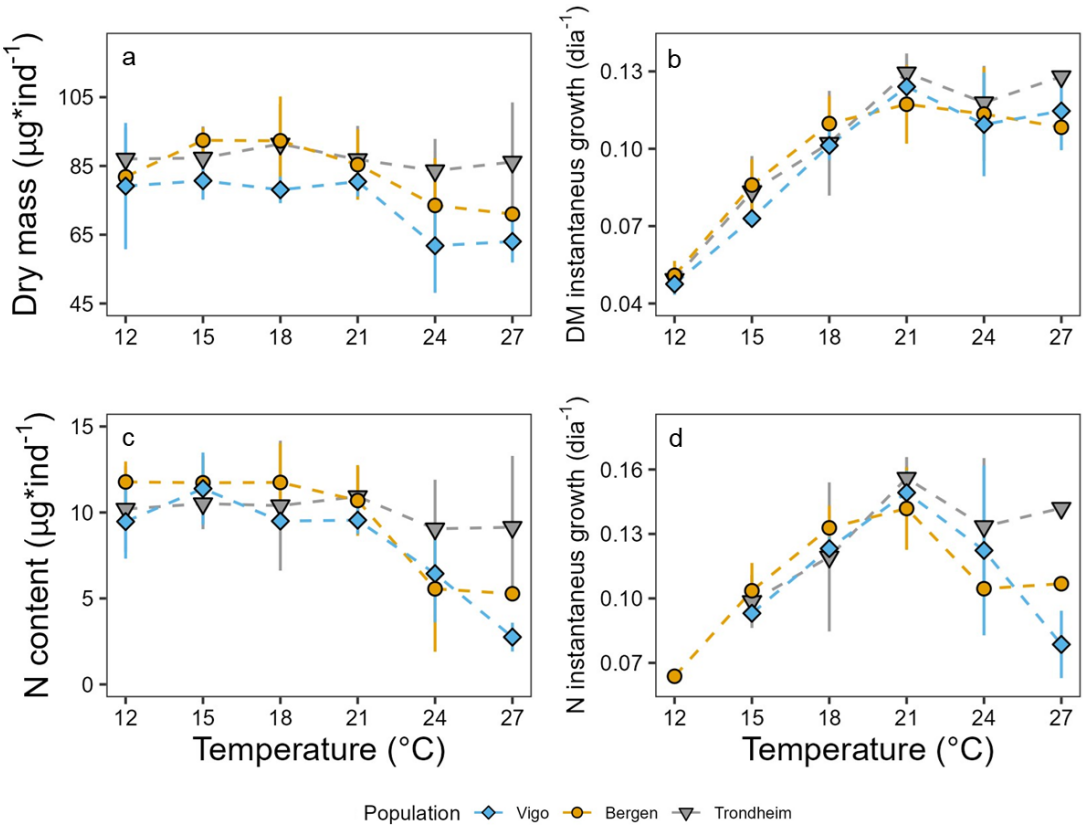

Figure S2. (a) Average dry mass. (b) Average growth rates in terms of dry mass. (c) Average nitrogen content. (d) Average growth rates in terms of nitrogen content. Data represented in the graphs correspond to larvae of *Carcinus maenas* reared from hatching to megalopa under different temperatures, from three populations: Vigo, Bergen, and Trondheim. Data presented as mean values ± SE of larvae produced by different females of origin. Symbols as in Fig. 1.

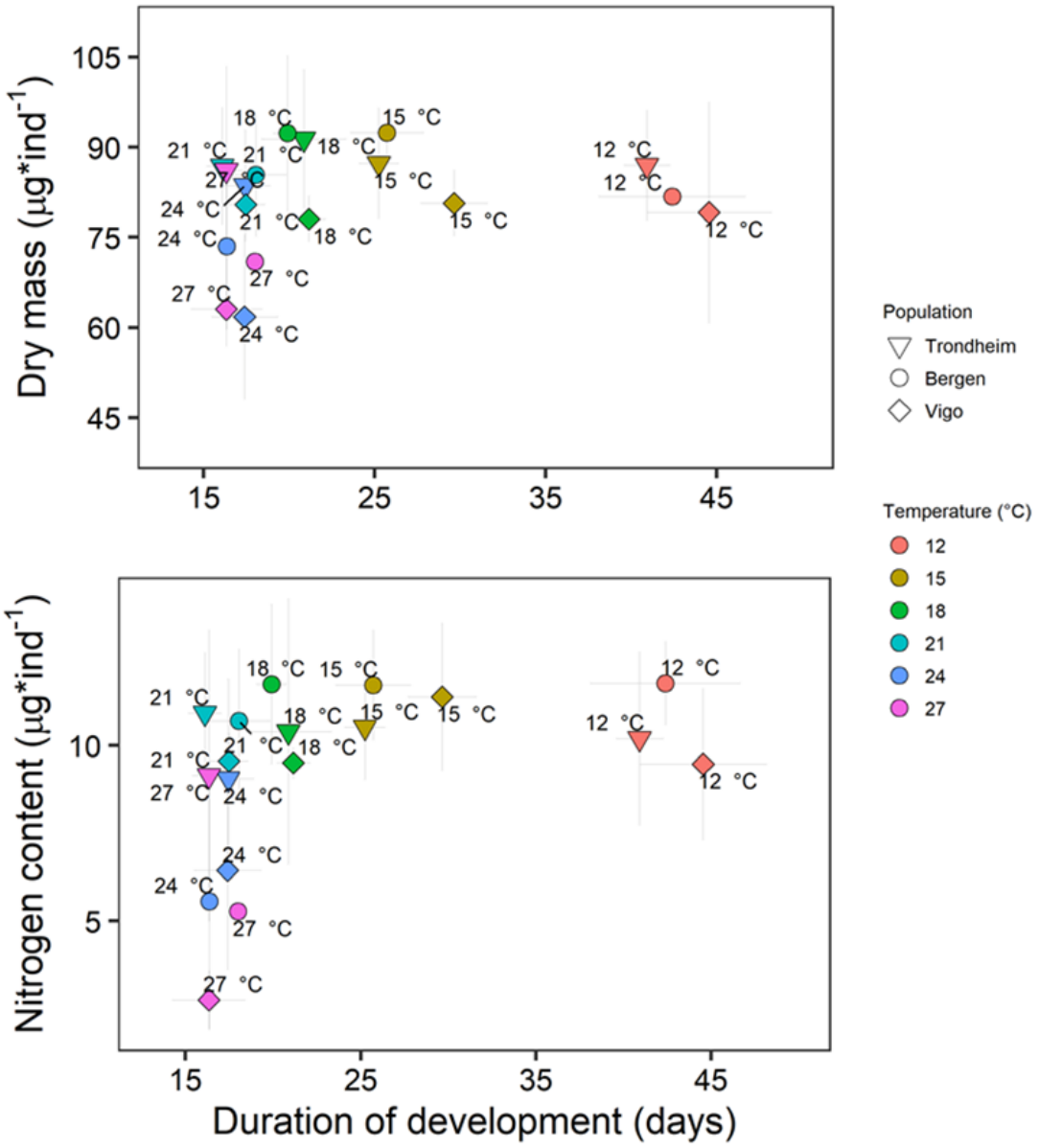

Figure S3. Integrated responses of dry mass (a) and nitrogen content (b) and duration of development of larvae of *Carcinus maenas* reared under different temperatures, form hatching to megalopa, from three populations: Vigo, Bergen, and Trondheim. Data presented as mean values ± SE of larvae produced by different females of origin. Symbols: Vigo is represented with triangles, Bergen with circles and Trondheim with diamonds.

CHAPTER 7. GENERAL DISCUSSION

The general objective of this thesis was to quantify the effects of environmental drivers in the responses of crustacean larval stages with special focus on multiple effects, variation among populations, and comparison of different species. The emphasis was on descriptors of larval performance such as survival, duration of development and growth rates; quantifying the integrated responses of development and growth rates, which in turn determine size at metamorphosis, is relevant for post - metamorphic benthic life as size drives survival and performance of juveniles.

Chapter 2 of this thesis showed for the first time for any population of *H. sanguineus*, the responses of their larval stages to the combined effects of temperature and food limitation on survival and growth of the invasive species to Europe, *Hemigrapsus sanguineus*. Chapter 2 also compares the responses of the invasive *H. sanguineus* to those of the native European green crab *Carcinus maenas*. Larvae of *H. sanguineus* were able to metamorphose to megalopa under food-limited conditions in a wide range of temperatures. One of the major outcomes of Chapter 2 is how both species responded to the combined effects of temperature in very different ways. *H. sanguineus* showed reduced survival at low temperatures, while increased temperatures allowed for higher survival rates; in the whole range of temperatures studied there was a reduction in survival and growth rates under limited-access to food. In contrast, *C. maenas* showed high survival rates in the whole range of temperatures studied and limited-access to food had negative effects at the higher temperatures studied. Evidence emphasises the fact that the invasive *H. sanguineus* may show advantages in performance over the native *C. maenas* at the high temperatures expected from ocean warming.

Chapter 3 of this thesis focused on understanding and quantifying responses to environmental drivers considering biological times, that is at a specific time in the development as for example time to metamorphosis. This chapter used a mathematical framework, based on partial differential equations to quantify the responses of organisms to environmental fluctuations with different magnitudes and considering the time scale of fluctuations (biological time and chronological time). One of the most important result from this chapter lies in the fact that choosing the correct time scale is central in the process of quantifying the responses of biological systems to environmental fluctuations, this approach should be used either to measure responses in experimental or in field conditions.

Chapter 4 quantified the responses of larval stages to the combined effects of temperature and salinity in larval stages of the invasive *H. sanguineus*, and compared them with the responses of other two non-native species *H. takanoi* and *E. sinensis* and the native *C. maenas*. Interestingly, this comparison showed that the three non-native species had higher sensitivity to low temperatures and low sensitivity to low salinity in the range tested, while the native species performed better at low temperatures and was more sensitive to low salinities. This chapter highlighted the potential of *H.*

sanguineus to invade and establish in areas of freshwater influence, especially under a warming scenario.

Chapter 5 addressed the responses of larval stages of populations of the invasive *H. takanoi* from Helgoland and Sylt in the North Sea and Kiel and Neustadt in the Baltic Sea, to the combined effects of temperature and salinity and quantified the genetic variability accounted for the degree of differentiation among the four populations studied. Populations showed high variability in the responses, but in general in all four populations survival was low at low salinities. Populations from the Baltic Sea had low survival rates at the at all tested conditions; while in the North Sea larvae showed higher survival rates, higher growth rates, and higher biomass at higher temperatures. However, we did not find evidence of a correlation between larval performance and the respective haplotypes in the different populations.

Chapter 6 looked at the thermal tolerance of larval stages of the European shore crab *Carcinus maenas*, over a wide latitudinal range from two populations from Norway (Trondheim, 60 °N and Bergen, 63 °N) and a population from Spain (Vigo, 42 °N). This chapter documented for the first time the performance of the larval stages to a wide range of temperatures (6- 27 °C, in steps of 3) for the northern range of the distribution. Surprisingly, we did not find evidence of strong pattern in the tolerance to low temperatures towards the northernmost populations studied here. The results point to a decrease in developmental duration towards increasing latitudes at 12- 15 °C, as larvae from the two Norwegian populations showed shorter developmental times to megalopa than the Spanish population, presumably as an adaptation to lower temperatures and shorter warmer seasons. Larvae from the southernmost populations showed higher survival at higher temperatures than the ones from the northern population, likely as an adaptation to warmer temperatures experienced. The intraspecific variability in the responses of larvae originating from different populations may add different physiological tolerances that may help the larvae to use windows of warmer periods to expand and establish populations in novel habitats.

Responses of native and non-native species to multiple environmental drivers

Chapters 2 and 4 of this thesis focused on the simultaneous effect of two or more environmental drivers on the performance of larvae of an invasive species and the comparison with a native co-occurrent species. This information is important to understand how species organized as open populations connected by larval dispersal become invasive. For a species to become invasive, necessary conditions are the capacity to tolerate the abiotic conditions in the new habitat but also the capacity to outperform the native competitors and avoid predators (Levine et al. 2004; deRivera et al. 2005; Crowl et al. 2008). Not all non-native species become invasive: many might colonise a new habitat develop transient populations and then go extinct, and many others may remain at low densities without

producing major modifications in the pre-existing community (Sakai et al. 2001). In the context of species with complex life cycles, larvae are relevant because they contribute to the propagule pressure (Simberloff 2009). For the model species, propagule pressure is given by larval survival and settlement which can affect the balance of the competition between juveniles/ adults (Moksnes et al. 1997; Moksnes 2002, 2004). The effect of drivers on larval performance should be context-dependent (Piggott et al. 2015a); they can act simultaneously and most often they enhance (or weaken) the action of the other (Crain et al. 2008; Orr et al. 2020). The expectation that the effect of multiple drivers vary among different species (Lenz et al. 2011) is relevant in the context of the comparison between native and non-native species. Hence, one of the most interesting findings from these two chapters is that *H. sanguineus*, *H. takanoi* and *E. sinensis* showed higher survival and performance at higher temperatures and at relatively low salinities than *C. maenas*. These physiological traits make them candidates to establish new populations in habitats where *C. maenas* may struggle, especially in a warming scenario. In principle, for *C. maenas* the negative effect of low salinity is mitigated at higher temperatures and larval performance is high at high temperatures (Spitzner et al. 2019a; Šargač et al. 2021b, 2022a). However, by contrast to *H. sanguineus* (Chapter 2), high temperatures exacerbated the negative effect of food-limited conditions (Torres and Giménez 2020a). Therefore, the mitigation effect observed at high temperatures is likely to depend on unlimited food conditions. This analysis is relevant because the North Sea has been identified as a "hotspot" undergoing cumulative impacts of many drivers (including temperature) acting in concert (Halpern et al. 2008; Bowler et al. 2020).

In the experiments in this thesis comparisons were performed in the larval phases of non-native and native species that co-occur and compete in the benthic habitat (Geburzi et al. 2018). As was already mentioned in previous chapters, larvae constitute the dispersive phase of many marine species and so successful persistence of native species as well as establishment of non-native populations depend on the survival of their larval stages (Cowen et al. 2006b; Cowen and Sponaugle 2009a). Duration of the larval phase, that is the time larvae spend in the pelagic realm, and the body mass at metamorphosis also determine post-metamorphic success, with bigger larvae performing better (Giménez 2004b; Gimenez 2006; Torres et al. 2016b). From the results obtained from the experiments in Chapter 2, one could hypothesise that in the case of *H. sanguineus*, metamorphosing to megalopa with reduced body masses but early in the season may represent a trade-off where the megalopa and juvenile stages could benefit from the warm season and grow faster to attach bigger body sizes (Fig. 3). It is important to highlight that in seasonal habitats organisms experience peaks of temperature but also variability in the resources and they respond to changes in abiotic conditions by phenological events, i.e. expressing a life history event (Post 2019). Species that live in a wide range of latitudes show the expression of their phenological events at different chronological times, in species living in higher latitude this expression occurs later in chronological time (Post et al. 2018). Higher warming has been shown in higher latitudes, as one of the consequences organisms have change their phenological events

by advancing the timing of their life history traits as a consequence of global warning (Post 2019). This pattern of advancing phenological events has been shown in an array of organisms (Root et al. 2003) and it is relevant in the context of measuring responses considering chronological and biological time.

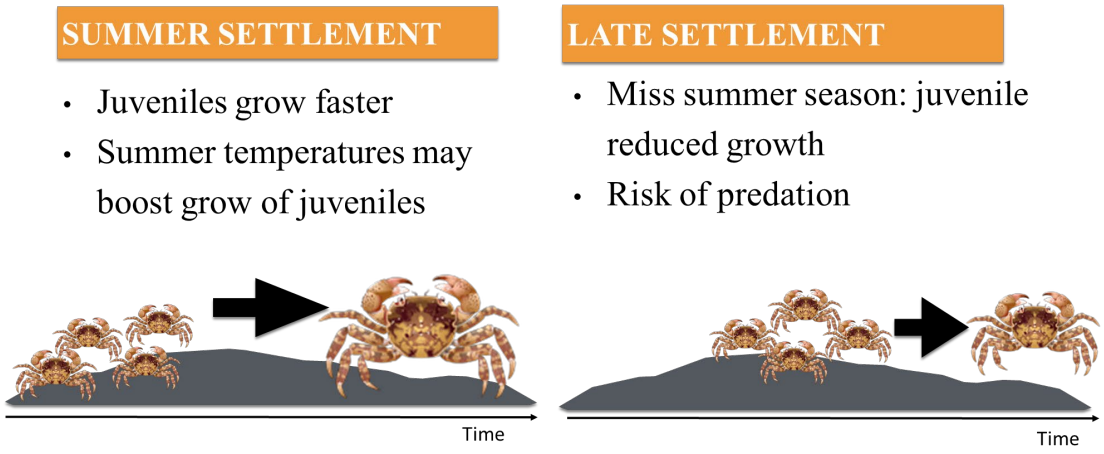

Figure 3. Graphical representation of the outcome of the results of the experiment performed in Chapter 2. The invasive *H. sanguineus* responded to food-limited conditions by metamorphosing to megalopa with reduced body mass but not extending the developmental time. Presumably, taking advantage of the summer temperatures and growing faster. Settle late in the season may represent a disadvantageous situation where juveniles miss most of the summer temperatures and growing rates may be slower achieving smaller sizes.

Results from the experiments in this thesis showed that both invasive species, *H. sanguineus* and *H. takanoi* were able to develop under a wide range of temperatures in the non-native populations studied here. Both non-native species showed strong evidence of positive effect of temperature on survival and development; higher survival, shorter developmental times and increased dry mass. As mentioned before, arriving early in the season in the benthos may have advantages in terms of growth rates. But also, metamorphosing with higher dry mass may be a predictor of post - metamorphic performance, as it has been showed that larvae with higher dry mass perform better (Gimenez 2006). These results are important in terms of invasion and establishment success, both non-native species are examples of recent invasions in the North of Europe (Epifanio 2013; Geburzi et al. 2018, 2020b) and knowledge on the tolerance of their larval stages may shed light on possible invasion expansion to areas with lower salinities as long as the temperature is high enough to allow to complete the larval cycle. Larvae of *H. sanguineus* can only complete the whole larval cycle at temperatures higher than 15 °C in its invasive range in North America and its northern limit of distribution is determined by the tolerance of their larvae to low summer temperatures (Stephenson et al. 2009). Expansion of *H. sanguineus* further north was modelled and it may be determined by the window for successful larval release and development, which depends on temperature and determines the successful dispersal of populations in

new environments. In the case of *H. sanguineus*, increasing temperatures and an increase in frequency and duration of heat waves may act as promoters of larval transport towards the north (Gimenez et al. 2020). The strong positive effect of high temperature on survival and growth of the two invasive species highlights the potential important effect that ocean warming may have in the process of invasion. In this context, and considering the results presented in this thesis, it is important to perform studies considering native and non-native species, especially during the period where the non-native is being established. For example, *H. sanguineus* and *H. takanoi* both coexist in the benthic habitat as juveniles and adults with the native European crab *C. maenas* in the North Sea (Epifanio 2013; Geburzi et al. 2018). A study in south-eastern North Sea showed that both non-native species affect recruitment of the native by predating on them, but they also showed low predation rates on each other and use cues from each other to promote their settlement in the benthos (Geburzi et al. 2018). This may represent a case of "invasional meltdown", which is defined as the process by which non-native species facilitate another non-native species invasion by increasing the probability of survival and/ or having an impact in the ecosystem that facilitate the establishment of other non-native species (Simberloff and Von Holle 1999). Invasional meltdown has been demonstrated at the level of populations and species (Grosholz 2005), but only in very rare cases a "complete meltdown" has been demonstrated with high impacts on population dynamics (O'Dowd and Green 2003). In some cases, this form of facilitation may enhance rapid invasions and aid in the process of loss of biodiversity of the invaded ecosystem (Grosholz 2005), being relevant in the context of the interaction of species.

Responses of populations to environmental drivers across gradients

Chapter 5 of this thesis showed that larval stages of the non-native species *H. takanoi* from different populations responded differently to the combined effects of multiple stressors. Larvae from the North Sea performed better (higher survival, shorter duration of development and higher body mass at metamorphosis) than larvae from the Baltic Sea. Populations from the North Sea showed evidence of higher resistance to variable environmental conditions. Contrary to what we expected, larvae from the Baltic Sea did not show any evidence of higher survival at lower salinities; in fact, larvae from females collected at the distribution limit in Neustadt showed the lowest survival rates from the four populations studied. These variability in the responses highlight again the importance of among- and within populations studies (Spitzner et al. 2019a; Šargač et al. 2021b, b). In the limit of distribution of species, it is expected that larval stages are less tolerant to variations in environmental conditions (Šargač et al. 2021b) and thus, the persistence of a population may depend on the propagule pressure enhanced by human-mediated transport or in the window of larval release (Lockwood et al. 2005; Giménez et al. 2020c), by source-sink dynamics. It is generally accepted in benthic ecology that in a local scale, benthic populations are in a source-sink dynamic connected by larval dispersal in a regional scale (Tamaki 2023). For the case of the Baltic population of *H. takanoi* this may be the factor

explaining their presence there. Contrary to the results found for the non-native *H. takanoi*, the results in Chapter 6 showed that *C. maenas* larvae did not show evidence of latitudinal thermal tolerance along a wide latitudinal gradient of the European Atlantic coast. Local adaptation in thermal tolerance to latitude have been reported for *C. maenas* adults (Tepolt and Somero 2014) and larval stages (Šargač et al. 2022a). There was evidence of some level of adaptation to low temperatures in the larvae of the females collected in Norway, larvae survived better at lower temperatures than from the larvae from Spain and duration of development was slightly shorter in larvae from the populations from the North. We still do not have data on the whole larval development of the populations located further north, in the northermost populations of *C. maenas*, it could happen that larvae from these populations show better performances at lower temperatures. Interestingly, *C. maenas* release larvae in different months of the year, depending on the temperature (Young and Elliott 2020a), so one could hypothesise that the lack of a thermal gradient may be hidden because of changes in the phenology of the organisms. But, when we consider the results found for the combined effects of temperature and salinity for both species, in the salinity gradient from the Baltic Sea, both species performed very poorly at the lowest salinity tested as there is no possibility for any of the species to change their phenology as the salinity in the Baltic Sea is always lower than in the North Sea (Šargač et al. 2021b).

In the population study of *C. maenas*, the variability in the responses to the different temperature conditions was given by the intraspecific variation in larvae produced by the different females. Intraspecific variation in the responses has been reported in invertebrate larvae (Applebaum et al. 2014b; Spitzner et al. 2019a). In the simulation in Chapter 2 (comparison of *H. sanguineus vs C. maenas*), we found that intraspecific variability in survival would be sufficient to enable metamorphic success of a proportion of the larvae of the population under conditions where the mean survival was very low. Considering all the changes happening as a consequence of global climate change, an interesting approach is to consider this variation under a warming scenario (Bolnick et al. 2011; Violle et al. 2012). Intraspecific variation in the responses of organisms is considered a response to genetic variability (Durrant et al. 2013b; Boyd et al. 2018b) or changing environmental conditions in the parental environment (Fox et al. 1997). In the context of warming, intraspecific variation in responses that result from genetic variability may drive portfolio effects (Schindler et al. 2015b; Price et al. 2021), whereby such variability buffers environmental variation by selection of warm-tolerant genotypes. The responses to environmental variations are difficult to predict, as they are modulated by the covariation of temperature and other environmental drivers and their effects could change along the life cycle of organisms (Parker et al. 2017). Considering a scenario where low larval performance under increased temperature is associated with parents also experiencing increased temperatures, then one would expect that sufficiently long warming periods should drive failures in organisms´ recruitment as a consequence of the combined action of both the parental and larval conditions experienced. This scenario could also be extended to the combination of temperature and other environmental drivers changing in concert, for

instance considering low larval performance in increased temperatures as a consequence of parents experiencing food - limited conditions. In this case, we would also expect a negative interactive effect in the field if the presence of such stressful condition covaries with the warming of the larval habitat. Considering this, in the context of changing environments, it would relevant to assess the role of the parent effects and how the responses vary under different combinations of drivers in the pelagic but also in the benthic habitat.

Another important implication of the intraspecific variation in larval performance produced by different females from the same population is given by the need to repeat experiments. Repetition of experiments becomes a relevant topic when we consider the reproducibility or replicability crisis, which has been recognised as a widespread concern in the scientific community; not only a high percentage of scientists that have tried to reproduce experiments from other scientists have failed but they have also failed to reproduce their own experiments (Baker 2016; Miyakawa 2020). Reproducibility becomes essential in the context of ecological predictions that may help us understand how biological systems work (Houlahan et al. 2017). There is a need among ecologists to produce studies whose experiments and models allow researchers to extend inferences in different species or different spatial or temporal scales (Houlahan et al. 2017; Spake et al. 2022). Determining how general biological responses are to changing climate, may allow for predictions at different scales or in different systems (Dunham and Beaupre 1998; Borer et al. 2014). For example, at the species level, responses to the combination of different environmental drivers may vary among different populations (Nasrolahi et al. 2016; Šargač et al. 2021b, 2022a), but responses also vary within populations (Spitzner et al. 2019a). Generality of responses to different combinations of drivers should be then evaluated by repetition of experiments considering different temporal or spatial scales and different levels of organisation.

Conclusions and perspectives

My work showed that larval responses of ecologically relevant invertebrate species differ among species and populations. The comparison between *H. sanguineus* and *C. maenas* allowed for a better understanding of the responses to the combined effects of multiple environmental drivers. These responses were reflected as differences in larval performance, measured as survival rates, duration of larval development, and body mass at metamorphosis. Another study comparing native and non-native species showed differences in the responses to increased temperatures and food limitation (Griffith et al. 2021b), they found the native species to be more sensitive to food limitation under increased temperatures. This kind of studies are relevant in the context of warming and species interactions, as they can give us a better understanding on the invasion processes occurring in coastal marine environments. Studying post-metamorphic responses is also relevant in the context of global warming and biological invasions, as the responses of the larvae to the environmental variables resulted in

different times to metamorphosis and different sizes at metamorphosis. In my experiments, larvae responded to different environmental conditions by shortening or lengthening their developmental times and metamorphosing with higher or lower body masses, meaning that in seasonal environments they will settle earlier or later in the season and with higher or lower body mass. It is in the benthos where intraspecific competition occur, so studying the effects of settling in the benthos earlier or later and with higher or lower body mass could have drastic consequences in the interactions among juvenile and adult stages of native and non-native benthic species accelerating or preventing invasion processes or range expansions of species as consequence of warming.

Larval responses to different environmental drivers (i.e. temperature, salinity, food limitation) showed high interspecific variation, which could be due to genetic variability or to paternal environment. It would be interesting to study this as intraspecific variability has important ecological consequences in buffering environmental variability. For instance, in order to assess whether the different responses are due to genetic variability, then a generational study is needed. For this, it would be necessary to produce F2 larvae from F1 generations; this kind of studies allow for the use of molecular markers, for instance the use of heat shock proteins. These proteins have been used as biomarkers of thermal stress in combination with other drivers (e.g. salinity and acidification) (Dunphy et al. 2013; Kim et al. 2022). In order to test if the variations come from the parental environments, experiments manipulating maternal and larval environments can be performed to test how the responses covariate. This would be very interesting in the context of increasing temperatures acting in concert with other environmental drivers to test if there are maternal adaptive effects, where mothers can use information from the environment to effectively buffer their offspring from environmental stressors (Marshall and Uller 2007).

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Acknowledgments

First of all, I would like to thank my supervisors Dr. Maarten Boersma, Dr. Gabriela Torres and Dr. Luis Giménez for their guidance throughout the PhD and for the scientific support. Special thanks to Gabi and Luis for introducing me to the larval world, for sharing their knowledge and for all the interesting discussions, especially for all the support along these years when facing the difficulties of doing a PhD and living abroad. Thank you both for making me feel at home. Big thanks to the Greifswald team for giving me the chance of joining the team. Thank you, Dr. Steffen Harzsch, for all the support and planning of experiments and trips and Jan Phillipp Geißel for all the hours doing experiments in the basement. I am also grateful to Dr. Christine Ewers and Dr. Diana Martínez-Alarcón for receiving me in their labs in Kiel and Montpellier, respectively. I thank the founders of this work: Bundesministerium für Bildung und Forschung (Project MERGE; grant n° FKZ 01DN20002).

I would also like to thank Julia and Ursula for all their support in the laboratory with the analyses but also for helping when problems appeared all along these years. Thank you, Ursula, for helping me when I needed it the most. I would also like to thank Ragna, in this short time you have also been there for helping out with the analyses and with solving problems in the laboratory. I am very grateful to all the students helping out with the experiments: Tobias, Sabrina, Pia, Fiona, Anne and Ella.

Furthermore, I would like to thank Prof. Dr. Martin Zimmer for agreeing to review this thesis and Prof. Dr. Marko Rohlfs and Dr. Christine Ewers for agreeing to join the examination commission. I would also like to thank Sheng V. Wang and Verena Wutz for being part of the student representatives.

I am grateful to POLMAR for the support and to Dr. Laura Rodríguez-Graña and Dr. Angel Urzua for being part of my committee meetings along my PhD, for the useful comments and the interesting discussions.

Thanks to all the AWI team on Helgoland. Special thanks to Ilse, Kathrin, Renate and Regina for all the administrative help, special thanks to Ilse for the support during these last months. Nico, Martin, Bärbel, Emma and everybody. It has been a real pleasure sharing these years together.

I would also like to thank all the friends I made thanks to being there: Nienke, Ella, Janne and Dieu Anh for all the adventures, hours in the laboratory or writing, for all the brunches together and so many more adventures during our time together on Helgoland. Thanks for all the time we spent together, it made this experience so much more enjoyable. Thanks Lai, Eva, Andrea, Mic and Richy for being there these last months, supporting me and for always being there when I needed a drink.

I am so grateful to my Uruguayan- German family: Miri and Mariano. Thank for all the support along these years, for sharing your friends in Bremen and for always making me feel I have a home in Bremen. Thank you, Miri, for sharing your family with me, and thank you Camilo for opening the doors to your house and for always making me feel part of your family. Thanks Santi for keeping your promise

and providing me with a refuge in the mainland with good food and plenty o´ drinks. I would also like to thank Lucas, mi refugio cultural y amigo del alma, I will never forget your time on the island. I thank all my friends in Uruguay, you have always been there sharing all my adventures through calls or messages, thank you all for support in these last crazy months.

Finalmente, quiero agradecer a mi familia. Sin ellos nada de esto habría pasado. A mis padres que son incondicionales, que siempre nos han apoyado en todas las decisiones que hemos tomado y nos acompañado. ¡Gracias! Gracias a mis herman@s y a Jazmín por también estar ahí y en especial a Ceci, la mejor amiga que la vida me pudo dar. Este trabajo se los dedico.

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