# Global ocean change: responses of crab larvae to abiotic drivers

I N A U G U R A L D I S S E R T A T I O N

zur

Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Universität Greifswald

vorgelegt von

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Greifswald, 01. Dezember 2021.

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Tag der Promotion: 03.03.2022.

## Table of Contents

# I Abstract

Under the influence of human activities, increased climate variability induces changes in multiple marine environments. Especially vulnerable are the coastal ecosystems where organisms must cope with constant extreme changes of environmental drivers, such as temperature, salinity, pH, and oxygen content. In coastal areas, brachyuran crabs are important animals that have a high impact on ecosystem functioning and serve as a link in food webs and pelagic-benthic coupling. Larval stages of crabs are crucial for population persistence and dispersal. They are generally more vulnerable to changes of environmental drivers and failure to adapt to new conditions may result in population collapse. To analyse the effects of multiple environmental drivers on larval performance and to elucidate interspecific and intraspecific difference, this project examined larval performance in the European shore crab *Carcinus maenas*. In this study, larvae of *C. maenas* from three native populations (Cádiz: Cádiz Bay, Helgoland: North Sea, Kerteminde: Baltic Sea) were reared in a factorial design consisting of different temperature (15-24 °C) and salinity treatments (20, 25, 32.5 PSU). Results demonstrated how descriptors of larval performance (growth, physiological, and developmental rates, and survival) were affected by combined environmental drivers. Larval responses to temperature and salinity showed contrasting patterns and differed among native populations originating from distant or contrasting habitats, as well as within the populations. The highest overall performance was recorded in the Cádiz population, while the Kerteminde population had the lowest performance in most of tested traits. The interactive effects of multiple drivers differed among the populations. In the Cádiz and Helgoland populations, higher temperatures mitigated the effect of lower salinity while the Kerteminde population showed a maladaptive response when exposed to lower salinity. Differences in performance showed better locally adapted populations (e.g. Cádiz) that could acclimate faster, have better adaptive mechanisms or stronger dispersive abilities. Because of their wider tolerance to increased temperature and decreased salinity, interactive effects in particular populations may favour some populations in a changing climate, especially in coastal habitats. Variation in larval performance showed complex interactions in larval performance and highlighted the necessity to quantify inter-population responses to climate-driven environmental change where responses of species should not be generalised. This study emphasizes the need for inclusion of multiple traits, drivers, and populations in experimental studies to properly characterize performance of marine coastal animals.

# II General introduction

## 1. Global ocean change

Due to major human impact over the past centuries, global climate change is often termed as "anthropogenic climate change" and can be defined as global-scale alterations, which are the product of human impact and influence many processes in the atmosphere, ocean, cryosphere, and biosphere (Duarte 2014, IPCC 2021). During the last several decades, human-induced global warming caused an increase in global average surface temperature of approximately 1 °C in comparison to the pre-industrial levels (IPCC 2018). From 2000 to 2019 annual average of the global atmospheric carbon dioxide has increased by 12 % (IPCC 2021) and disturbed a multitude of environmental properties, both in terrestrial and aquatic environments.

Oceans and seas cover ca. 70% of the Earth's surface and have the unique capability to capture $CO_2$ from the atmosphere. However, under the human influence, the $CO_2$ levels are starting to exceed the ocean's $CO_2$ buffer capabilities, leading to a rise in the temperature. Current climatic scenarios project a 2 °C increase of the global seawater over the next 100 years and average increase of global sea surface water temperature by 0.2 °C per decade (IPCC 2014, 2019). Climate warming will enhance ice cover melting which will cause more frequent salinity regime changes (IPCC 2019, Allen et al. 2018). Moreover, there is already an increase in the occurrence of extreme events, such as heat waves and floods (Oliver et al. 2018), causing the degradation of many marine environments (Frölicher et al. 2018, Ainsworth et al. 2020, Rodgers 2021). It is well known that local extirpation related to the warming is twice as common in the ocean than on land (Pinsky et al. 2019). However, while the effects of global change on terrestrial ecosystems are widely explored, marine ecosystems are largely understudied (Richardson and Poloczanska 2008, Pinsky et al. 2020).

### 1.1 Marine ecosystems

Novel climate changes, facilitated by human activities, are causing alterations in numerous aquatic habitats, with environmental variables, such as water temperature, salinity, pH, and oxygen content, changing at increased rates (reviews Poloczanska et al. 2013, Molinos et al. 2016, Boyd et al. 2018). Long term data series indicate that global ocean change already has a major impact on marine ecosystems (Wiltshire et al. 2010, Boersma et al. 2016). For instance, plankton communities are being increasingly affected by the climate change (Beaugrand et al. 2003, Burrows et al. 2011, Poloczanska et al. 2013 Molinos et al. 2016, Boyd et al. 2018). In addition, species' physiology and distribution is changing, causing shifts in community composition (Perry et al. 2005, Somero 2010, Reusch 2014). Impacts of environmental fluctuations on marine biodiversity are likely to intensify in the future. The magnitude of the changes and the corresponding animal responses may vary regionally

and locally due to different atmospheric localized events, human impact, and sensitivity of the species (Roessig et al. 2004, Harley et al. 2006, Munday et al. 2008, Delorme and Sewell 2014).

Scientists predict that regional shallow and semi-enclosed seas in the north of Europe, such as the North and Baltic Seas, will be under strong influence of climate variability (Schrum 2001, Meier et al. 2011, Gräwe et al. 2013, Hiddink et al. 2015, Robins et al. 2016, Tinker et al. 2016, Holt et al. 2016). During the next decades, oceanographic model projections for the Baltic Sea suggest warmer waters and a decreasing salinity due to reduction of the ice cover and increased freshwater supplies (Meier 2006, BACC I 2008, Neumann 2010, Johannesson et al. 2011, Meier et al. 2012, Andersson et al. 2015). The stratification of the Baltic Sea combined with a temperature and a salinity gradient decreasing from southwest to northwest (Ojaveer et al. 2010) sets a major obstacle for marine organisms. However, it may also facilitate the transition of the euryosmotic organisms into the Baltic Sea. For the North Sea, which is one of the fastest warming continental shelf seas (Burrows et al. 2011), recent climate models suggest that various animal and plant taxa will extend their range northwards (Burrows et al. 2011, Poloczanska et al. 2013) expanding into new warmer habitats suitable for life due to global warming. In contrast to northern Europe, southern parts of Europe are experiencing drought periods more often, and Atlantic coastal areas under the influence of the Mediterranean Sea are expected to show a salinity rise in the future (González-Ortegón et al. 2015, IPCC 2018). Thus, climate change raises many uncertainties in marine habitats when considering future variation of abiotic variables and their effects on organism's performance in aquatic ecosystems.

### 1.2 Approaches to study climate change in marine ecosystems

Over the last few decades, several methods have been developed to study the effects of climate change on marine ecosystems. According to Boyd et al. (2018) there are five main strategies to study the effects of environmental variables on marine biota in near-future scenarios: paleo-proxies, modern proxies, modern observations, manipulative microcosm experiments and large volume mesocosm experiment enclosures. A paleo-based approach uses the geological past to interpolate future scenarios by investigating fossils (e.g. extinctions and emergences of species; Gibbs et al. 2016) from different periods of the Earth's past. Modern proxies use present marine ecosystems with extreme conditions (e.g. deep sea vents that release $CO_2$) to project future conditions and corresponding responses on the communities. However, these methods do not provide analogues for today's changes, since the rates of climate change differ between the past and present (human increased activity), or only one variable ($CO_2$) is tested on a single community. Modern observations focus on long-lived organisms (e.g. deep-sea corals), spatial or temporal gradients, or ocean-time series (Thresher et al. 2011, Wiltshire et al. 2010, Rivero-Calle et al. 2015). This method provides estimates

of future changes in the environments and responses of organisms or "space-for-time" substitutes for long-term change (e.g. Dunne et al. 2004). Still, this approach is often confounded by the influence of natural climate variability, which exacerbates the ability to differentiate global ocean change trends over shorter periods (Edwards et al. 2013). Manipulative experiments in marine ecosystems (see review Wernberg et al. 2012) combine both small-volume microcosm experiments and larger mesocosm studies. Such methods mostly give insights into the complex relationship between variables and different responses of the studied community or organism(s) (Riebesell and Gattuso 2015). However, manipulation experiments are quite artificial systems performed over a shorter span of time (usually weeks to months) and cannot extrapolate to a longer timescale (Hutchins and Boyd 2016). Moreover, microcosm experiments cannot accurately predict ecosystem- or food web-level effects and are logistically difficult to replicate (Boyd et al. 2018). On contrary, larger mesocosm experiments can increase ecological relevance, but at the cost of not understanding individual mechanisms that govern the response (Somero 2012).

Experimentally testing various physiological responses and traits of marine organisms is important for understanding the future responses of animals. For example, studies that are testing orthologous enzymes in congeneric species or populations with different thermal gradients can help in examining the evolution of thermal tolerance (Somero 2010). By testing physiological processes (such as cardiac function or aerobic performance of organisms) in different temperature treatments, we can define the thermal limits of organisms (Pörtner et al. 2017). Acute and long-term effects of the temperature can influence the fitness due to a diminished physiological or behavioural performance at unfavourable temperatures (Pörtner 2001, Pörtner and Knust 2007, Pörtner and Farrell 2008, Somero 2010, Pörtner et al. 2017). In addition, physiological responses (e.g. survival, growth and development) can be tested during ontogeny to identify life-history bottlenecks, which can define the organisms' performance (Kroeker et al. 2013).

### 1.3 Coastal aquatic ecosystems

Environmental conditions can differ greatly between the open ocean and coastal areas. Coastal ecosystems are particularly vulnerable to enhanced climatic changes (Barry et al. 1995, Helmuth et al. 2006) and are expected to experience larger fluctuations of environmental variables (Hofmann et al. 2011, Duarte et al. 2013). Coastal, estuarine, and intertidal habitats are defined by regions of freshwater influence (i.e. ROFI, Simpson 1997) where their surface water temperature is generally expected to rise and freshen by increased river runoff (Gräwe et al. 2013, Hiddink et al. 2015, Robins et al. 2016). Such extreme and unpredictable environmental conditions can create a mosaic of abiotic conditions that can vary tremendously over spatial scales of meters or less. For example, within a single rocky intertidal ecosystem, thermal conditions can often exceed those found over wide ranges

of latitude or predicted to occur in future global warming scenarios (Helmuth et al. 2010, Denny et al. 2011). In addition, Europe's seas are subject to increased pressure from ongoing human activities, especially in the shelf and coastal areas (Halpern et al. 2008, EEA 2017, Korpinen et al. 2021)

In coastal ecosystems animals often live at the edge of their environmental limits and life there depends vastly upon fluctuations of the environmental variables. For example, organisms inhabiting the Baltic Sea often reach their physiological limits and are highly adapted to extreme conditions of salinity and oxygen availability (Feistel et al. 2008, Neumann 2010). Among the environmental variables that influence responses of organisms in coastal ecosystems, temperature and salinity are among the most essential ones, as they define many metabolic and physiological traits. In the shallow Northern European seas, fluctuations of saline and temperature regimes are main natural drivers for aquatic biota (Hänninen et al. 2000, Telesh et al. 2013) controlling the fitness of marine animals and affecting growth (e.g. biomass accumulation), metabolism (e.g. respiration rates), development, and survival of marine invertebrates (Kinne 1964, Pörtner 2001, Somero 2005, Janecki et al. 2010, Somero 2010, Ko et al. 2014). With the expected changes of salinity and temperature regime, organisms living in coastal areas and habitats with freshwater inflow will have to cope with new combinations of environmental conditions that will shape the distribution patterns and population dynamics of its inhabitants (Stillman and Somero 1996, Browne and Wanigasekera 2000, Stillman and Somero 2000, Robins et al. 2016). Hence, studies on coastal organisms can help us to better understand responses of organisms to variation of environmental variables (reviews Crain et al. 2008, Piggott et al. 2015, Gunderson et al. 2016, Boyd et al. 2018).

## 2. Environmental drivers and their interactions

Environmental variables that affect organisms are often called "stressors", because it is assumed that the effect of the variable induces negative consequences for the animal's performance and causes stress for the organism. Nowadays, such terminology is broadly avoided because many studies have shown that interactive effect of environmental variables can increase performance (e.g. Janas and Spicer 2008, Nasrolahi et al. 2012, Delorme and Sewell 2014, Spitzner et al. 2019, Torres et al. 2020). For that reason, henceforth I will use the term "driver" - an environmental factor that is tested for its effect on biological performance/biological systems (Boyd et al. 2018). This terminology is more suitable for describing the environmental factors and the nature of interactions, since the effect of a driver (or a combination of drivers) can be positive or negative depending on the organism, process, or community being considered (Boyd and Hutchins 2012).

In a changing climate, interactive effects of multiple drivers are expected to occur at an increased rate. Organisms must respond to new environmental challenges which will affect their

performance and fitness. Therefore, predicting future scenarios of species persistence and dispersal becomes difficult by investigating single drivers only. Effects of combined drivers can be independent if one driver is affecting the organisms independently of the other. Describing the nature of multiple driver interactions in such situation is simple when effects operate in the same direction. However, the interplay between two or more drivers often has many non-independent effects; one driver can enhance or diminish the effect of the other driver, or the combined influence of both drivers can overpower the variation threshold in the organisms that normally would not be reached *via* variation if either driver would act alone (Crain et al. 2008, Harley et al. 2006, Gunderson et al. 2016). Currently, our understanding of global change effects on organisms is still mostly derived from studies that manipulated single environmental drivers (Dupont and Thorndyke 2009, Byrne 2011, Todgham and Stillman 2013). Recent numerous studies and reviews (e.g. Hoegh-Guldberg and Bruno 2010, Pörtner 2012, Rocha et al. 2015, Boyd et al. 2015, Przeslawski et al. 2015, Orr et al. 2020, Tekin et al. 2020) have pointed out the disadvantages of single driver studies and highlighted the importance of incorporating multiple drivers studies in order to produce more realistic global change effects.

Depending on the type of responses they elicit, there are three main interaction types between multiple drivers: additivity, synergism, and antagonism (Fig. 1, Todgham and Stillman 2013). When responses to multiple drivers are simple and show no interaction, they can be characterized as additive or multiplicative (Boyd et al. 2018). Explaining the response direction and interaction effects is often more complex and numerous reviews offer several explanations on how to handle interplay among multiple drivers (e.g. Crain et al. 2008, Piggott et al. 2015, Orr et al. 2020, Tekin et al. 2020). For instance, Folt et al. (1999) defined synergism as a cumulative effect of multiple drivers that is greater than the sum of effects produced by the drivers acting in isolation, and antagonism as a cumulative effect that is less than additive. While identification of synergism and antagonism is generally straightforward when both drivers operate in the same direction (e.g. as in Fig. 1 where both drivers are negative and decrease the performance), for opposing individual driver's effect (e.g. one driver enhances the performance, while other causes a decrease) this classification seems problematic because what is synergistic to one driver's effect direction is antagonistic to the other and *vice versa* (for details see Piggott et al. 2015). Therefore, describing the interactions of the drivers should be based on additional parameters, such as the magnitude and response direction (positive or negative) of the cumulative effect, as well as the interaction effect (effect deviation from the additive model prediction), to resolve synergistic and antagonistic effects relative to an individual stressor (Piggott et al. 2015). The strength of the responses can be additionally quantified and presented with various tools, such as effect sizes (comparing organisms' responses to environmental drivers to the reference or control) or interaction plots (Boyd et al. 2018).

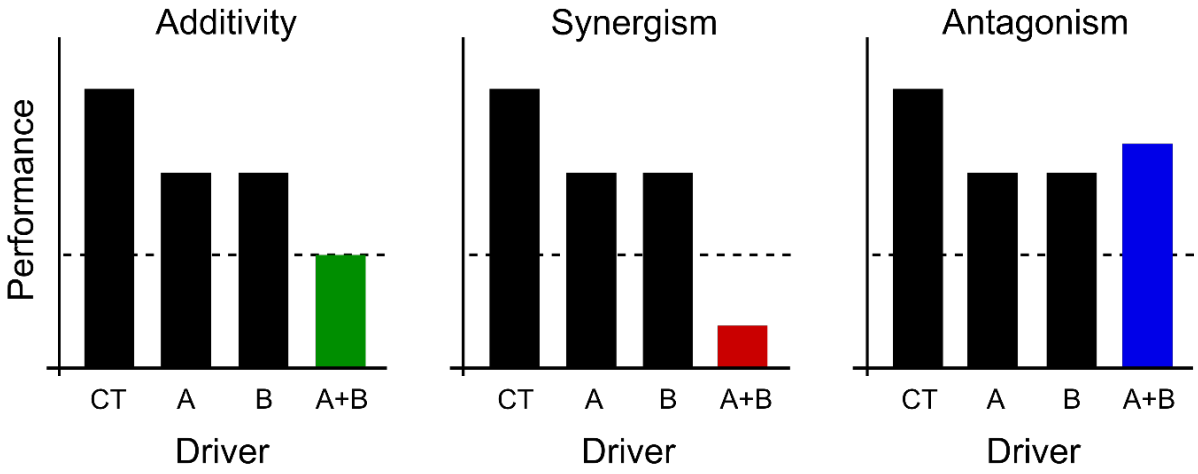


**Figure 1**. Conceptual framework for understanding possible interactive responses (A+B). In this case, the effect of two hypothetical drivers with negative effects (A and B) on performance is presented relative to the control (CT). Multiple drivers can elicit three interactive types of the response on performance: additive response (green), synergistic response (red), or antagonistic response (blue). Modified from Todgham and Stillman 2013.

### 2.1 Multiple driver studies

To decide which drivers should be included into multiple driver experiments, one may follow several recommendations: 1) the drivers must be relevant in terms of the projected change; 2) the experiment should capture the range of effects of drivers (to detect both detrimental and beneficial effects); 3) other untested drivers must be kept at the environmentally relevant levels (Boyd et al. 2018). When it comes to multiple drivers studies and projecting future state of marine ecosystems, they can be divided into mechanistic and scenario-based approaches (Boyd et al. 2018). Experimental setup in this thesis is based on a mechanistic approach, where experimental design determines the response of the organism to a selected range of environmental conditions, using a gradient of treatments, to reveal underlying mechanisms and /or to test a theory. In comparison to mechanistic, scenario-based studies are usually founded on future model projections of one or more environmental drivers (e.g. for the year 2100 by IPCC) and used in multiple climate change scenarios to create a range of treatments that represent present and past or future conditions. Manipulative experiments (see section 1.2 Approaches to study climate change in marine ecosystems) can provide mechanistic insights into how multiple drivers interact and affect marine life (Boyd et al. 2018), and, combined with other approaches, have the potential to improve a mechanistic understanding and enhance the predictive power to assess the effects of environmental change (Dupont and Pörtner 2013). Although multiple drivers designs are logistically demanding (e.g. time, effort, materials), they are essential to

distinguish and quantify the effects of drivers and to understand their biological significance (Boyd et al. 2018, Schäfer and Piggott 2018).

### 2.2 Interpopulation and intrapopulation variation

Understanding synergism and antagonism among multiple environmental drivers is essential for predicting future species' distribution (Piggott et al. 2015, Boyd et al. 2018, Galic et al. 2018, Schäfer and Piggott 2018) and may have major ecological consequences for organisms, not only on a population, but also at community level. Interactive effects of environmental drivers can affect animal's performance and differ among species, but also among populations within the same species. The importance of intraspecific trait variation in response to climate change and its variation on spatial and temporal scale have already been recognized (Moran et al. 2016, Violle et al. 2014), with numerous studies demonstrating that responses to climate drivers can vary greatly at the intraspecific level (Carter et al. 2013, Applebaum et al. 2014, Spitzner et al. 2019). It has been shown for many coastal species how thermal tolerance can vary among local populations (Iwabuchi and Gosselin 2020). For instance, in polychaetes Gosselin et al. (2019) found out that temperature and food availability produce different oocyte size in different populations (originating from habitats with contrasting temperatures and food availability). In sea urchins, early development can be affected differently by the same temperature in different genotypes (Delorme and Sewell 2016). Moreover, in Bryozoans, Pistevos et al. (2011) found intrapopulation differences in growth and reproduction when exposed to lower pH and high temperature. Survival, developmental and physiological responses to thermal and osmotic conditions may vary between populations, especially in species with broad spatial distribution. Variation in traits among different populations can change species' distribution and community structure (Bolnick et al. 2011) throughout adaptation and plasticity (Chevin et al. 2010). Unfortunately, studies that include multiple populations from the same species are still scarce.

### 2.3 Phenotypic plasticity, local adaptation, and genetic variance

In coastal areas organisms are often exposed to suboptimal conditions. Therefore, in order to cope with environmental changes, they have to develop various mechanisms that help them overcome unfavourable changes. Evolutionary adaptation and phenotypic plasticity are considered essential tools for organisms to adapt to environmental changes both in marine (Reusch 2014, Boyd et al. 2018) and terrestrial ecosystems (Chevin et al. 2010, Somero 2010). Variation among populations in response to new conditions can emerge from phenotypic plasticity and genetic adaptation (Chevin et al. 2010, Reusch 2014, Sinclair et al. 2013, Boyd et al. 2018), and indicate locally adapted populations (de Villemereuil et al. 2016). Phenotypic plasticity implies that the same genotype can generate distinct phenotypes depending on the environmental conditions under which development takes place

(Merilä and Hendry 2014, Lafuente and Beldade 2019). Variation in phenotypes (e.g. physiological or morphological changes) can provide the means to cope with environmental heterogeneity, by mitigating the potential negative effects of climate change on population persistence (Kingslover and Buckley 2017, Burggren 2018). The plastic responses can differ among populations of the same species. Some phenotypes can better tolerate the new climatic conditions and endure a wider range of environments than non-plastic ones (e.g. Przybylo et al. 2000, Réale et al. 2003, Charmantier et al. 2008, Gienapp et al. 2013) potentially leading to local adaptation in particular populations. A population is considered locally adapted to its environment if the resident genotype has a higher fitness in the native habitat than genotypes coming from more distant populations (Sanford and Kelly 2011). Phenotypes that are more adapted should therefore be favoured, making interpopulation comparisons (including multiple drivers) a focus point for future changes of species composition in coastal ecosystems.

### 2.4 Multipopulation experiments

It was long assumed that marine ecosystems are well-mixed and interconnected through high gene flow. However, recent studies have provided evidence that marine populations are less connected, with considerable differentiation among populations due to life history, larval behaviour, or oceanographic characteristics (see reviews e.g. Palumbi 2004, Levin 2006, Stanford and Kelly 2011), which enables the retention of offspring close to their natural habitat (Sherman et al. 2008, Morgan et al. 2009). Currently, a trending question in marine ecology is whether different populations distributed across a wide spatial scale will be able to cope with new environmental challenges driven by climate change. Until now, studies of biological responses to climate change and predictive models have mainly focused on the species level by investigating a single population (Currie 2001, Moritz et al. 2008, Fitzpatrick et al. 2011, Lawler et al. 2013). However, surveys including multiple populations are crucial for understanding how genetic diversity, population connectivity, and adaptive potential of particular populations in one species can shape the species' tolerance in the face of ongoing climate change (Holt 1990, Hampe and Petit 2005, Hoffmann and Sgrò 2011, Anderson et al. 2012).

Disentangling multiple driver's effects on different populations is a challenging task, but several methods have emerged over the past few decades to address this issue. A multi-population comparison is one experimental approach to indirectly address the extent of local adaptation *versus* phenotypic plasticity (de Villemereuil et al. 2016). Studies including multiple populations, and combinatorial various environmental drivers, appear to address more ecologically relevant questions than single environmental driver studies. Furthermore, there is an increased need for defining reaction norms on the local scale (in different populations) that represent natural conditions more realistically (Sultan 2017). "Space-for-time" substitution is a widely used approach in ecology that uses spatial

patterns of biodiversity as a model for temporal processes and for projecting changes through time, either into the future or into the past (Pickett 1989, Algar et al. 2009, Blois et al. 2013, Merilä and Hendry 2014, Wogan and Wang 2017). It can be applied to study long-term changes in nutrient cycling (Johnson and Miyanishi 2008), to predict the effects of climate change on biodiversity (Blois et al. 2013) or to project species' range shifts *via* niche modelling (Dobrowski et al. 2011). From the ecological perspective, it can be used to interpret the processes driving community composition and species richness, biodiversity loss, changes in behaviour and phenology, or range shifts (Blois et al. 2013, Eskildesen et al. 2013, Charmantier and Gienapp 2014, França et al. 2016). Although space-for-time substitutions are broadly used in ecology, they are not accurate. For instance, they can underperform when the environmental change is unpredictable (Johnson and Miyanishi 2008), or when the rate of change is too rapid (Blois et al. 2013). Moreover, such approaches can fail due to unrecognized effects of past events or because the testing is applied on a coarse scale and neglects spatial heterogeneity (Pickett 1989).

In "synchronous studies", populations coming from divergent habitats are compared. Two most commonly used methods are: common garden experiments (CGE) in the laboratory and reciprocal transplant experiments (RTE) in the field (e.g. Sanford and Kelly 2011, Reusch 2014, de Villemereuil et al. 2016). These methods aim to indirectly address the interpopulation differences and local adaptation (either *via* plastic responses or genetic variance) by exposing individuals from different populations to same conditions (e.g. see review Merilä and Hendry 2014). Conceptually, the main difference between these two methods is that in RTE two or more populations are introduced into each other's local habitat conditions, whereas in CGE we re-create environmental conditions of different habitats in the laboratory while controlling other factors (Kawecki and Ebert 2004). The main goal of RTE is to prove local adaptation, while CGE is designed to reveal the genetic base of complex phenotypic variation across different populations, without the confounding effects of the corresponding environment (Sanford and Kelly 2011, Merilä and Hendry 2014, de Villemereuil et al. 2016).

CGE can be used to test populations that are suspected to be locally adapted, because of an environmental gradient (such as latitude) or due to contrasting environments, such as freshwater *vs* seawater (DeFaveri and Merilä 2014). Future conditions can be mimicked using the space-for-time substitutions to compare populations of organisms located across spatial environmental gradients (related to e.g. salinity or temperature) that represent the temporal changes to be experienced by local populations in future global change scenarios. With exposure to different drivers, we can measure complex traits in various treatments, such as fitness and life-history related (e.g. survival or number of offspring produced), morphological and physiological traits, phenology or allometric relationships (Nuismer and Gandon 2008, Gonda et al. 2011, Kawakami et al. 2011, Brachi et al. 2013, de

Villemereuil et al. 2016). In a CGE, individuals from genetically distinct populations or different habitats are raised/reared/grown in the laboratory in a common environment, controlling the environment and minimizing the effect of environmental variation (Sanford and Kelly 2011, Merilä and Hendry 2014). One or more drivers (e.g. salinity and temperature) can be tested, that differ between the treatments, while other drivers are constant. Albeit CGE provide the advantage of controlling specific drivers that differ between habitats and can test the adaptive abilities among populations, there are several pitfalls of such method. One major limitation is the inability to breed the species and produce offspring in the laboratory (de Villemereuil et al. 2016). Furthermore, results can be confounded by certain genotypes that may be more adapted to the laboratory conditions (Kawecki and Ebert 2004). In addition, phenotypic plastic responses in many marine organisms, such as acclimatization or maternal effects (Marshall 2008, Marshall et al. 2008), can exacerbate detection of local adaptation (Hollander 2008, Sanford and Kelly 2011). Moreover, organisms collected in the field may retain the influence of environmental conditions prior to collection (Sanford and Kelly 2011, Merilä and Hendry 2014, de Villemereuil et al. 2016, Boyd et al. 2018) and make it difficult to separate between a phenotypic plasticity and genetic variance. Additionally, there is usually a limit for the amount of drivers that can be examined (depending on the species and research questions) due to logistic problems and overloading experimental volume. Hence, many interactive responses of other key drivers (which are present in the nature) are neglected in such a study.

Nevertheless, CGE is a useful method to test the complex interactions of multiple drivers and responses of animals under increased global change pressure, especially in marine zooplankton (Boersma et al. 1999, Dam 2013). By comparing the phenotypes generated by different genotypes under the same conditions (for instance, increased temperature), the genetic basis of a trait can be quantified and reveal locally adapted populations.

## 3. Environmental drivers in coastal habitats

There are many abiotic drivers and biotic interactions (e.g. predation or competition) at play in coastal areas. Temperature, salinity, oxygen content, and pH are some of the most important drivers with temporal and spatial variations in coastal areas. Seawater is expected to become more acidic due to the climate change, but species living in estuarine and shallow coastal areas appear to be less affected by changes of pH. For instance, in marine crustaceans, acidification can affect egg production, embryogenesis, growth, and moulting. However, crustaceans can easily cope with minor changes of pH and are expected to tolerate the predicted $CO_2$ levels in the seawater (Whiteley 2011). Thus, this thesis will focus on two key abiotic drivers: temperature and salinity.

### 3.1 Temperature

Temperature is a crucial ecological factor, playing a major role in the general metabolism of animals and influencing all developmental and other physiological processes (Costlow and Bookhout 1969, Willmer et al. 2009, Speight and Henderson 2010, McGaw and Rieber 2015, Karleskint et al. 2021). It is long known that nearly all rates of biological activity increase exponentially with temperature (Schmidt-Nielsen 1997, Brown et al. 2004, Walters and Hassall 2006, Irie and Fischer 2009, Karleskint et al. 2021), leading to various effects on rates of larval performance, as well as seasonal variation in larval plankton occurrence. The spatial and temporal variations in growth within marine species can be majorly explained by temperature (Chang et al. 2012). Animals develop faster with increasing temperatures, and the thermotolerance is closely associated with the geographic distribution of a species. For instance, decapod species inhabiting tropical and high latitude areas have stenothermal responses, while temperate climate species show eurythermal responses (Anger 2001).

In crustaceans, as well as in other zooplankton groups, higher temperatures cause increase in metabolism (Ikeda 1970), with growth being temperature-dependant, i.e. animals grow and develop faster with temperature increase, as long as they are within the margins of their thermal niche (Anger 2001). Moreover, variation in temperature can directly affect animal's performance by setting the respiratory limits in many plankton marine organisms (Ikeda 1970, Saborowski et al. 2002). A recent study demonstrated that distribution of marine animals is limited by the combined effects of high temperature and oxygen loss (Pinsky et al. 2019). While higher temperatures allow faster metabolism and respiration, higher oxygen demands caused by the heat stress cannot always be matched by an appropriate increase in oxygen supply (Pörtner 2001, 2012). According to the OCLTT (=oxygen and capacity-limited thermal tolerance) hypothesis, every organism has a thermal range which defines its demand for oxygen (Pörtner 2001). If the temperature condition is too extreme, it may exceed the animal's capacity to supply oxygen to tissues and cause a progressive decline in performance (Pörtner 2010, Deutsch et al. 2015, Giomi and Pörtner 2013). In addition, early life stages of marine invertebrates are more sensitive to temperature and have a narrower OCLTT thermal window than conspecific adults and juveniles (Pörtner and Farrell 2008). Thermal extremes outside of thermal niches can decrease physiological performance and lead to failure of metabolic mechanisms and eventually to increased mortality. Outside of metabolic and physiological adaptations, animals can also avoid unfavourable temperatures by changing their thermal behaviour, through changing their locomotor ability and swimming speed (thermokinesis) and increasing frequency of random turnings (Sulkin 1984, Lagerspetz and Vainio 2006).

### 3.2 Salinity

Open ocean areas are characterized by mostly constant salt concentrations and stable osmotic conditions. However, in shallow coastal, estuarine, and intertidal areas, salinity may fluctuate tremendously during different seasons as well as regionally and locally. Due to the irregular income of freshwater and constant changes of the osmotic regime in the coastal habitats, salinity is an essential environmental driver in plankton life, including decapod larvae, and it influences their distribution, abundance, and general physiology (Roman and Zeng 2012). Salinity affects many aspects of larval biology, such as growth, metabolism, development, morphology, behaviour, and survival (Kinne 1971, Morgan 1995, Anger 2001, Anger 2003). In addition, variability of the salinity in coastal waters is the main cause of larval mortality (Morgan 1995). To tolerate salinity fluctuations, organisms have to maintain their internal body fluids (e.g. blood or haemolymph) on a certain osmotic level, in comparison to the external medium. The relationship between the internal and external medium can be expressed *via* terms "osmoconformity" and "osmoregulation". Osmoconformers maintain their intracellular fluid isosmotic to the extracellular fluid, while osmoregulators maintain the osmotic concentration of the extracellular (body) fluids approximately constant, regardless of the salinity of the ambient medium (Péqueux 1995). The difference in the osmolality between the internal and external media is called "osmoregulatory capacity". The physiological basis of osmoregulation is essentially an effective exchange between $Na^+$ and $Cl^-$ ions. The regulation of the internal medium and maintenance of a steady state can be achieved through two mechanisms: 1) *via* processes that change the permeability properties of the borderline structures to minimize the diffusive movements of osmotic factors (e.g. by passively reducing the permeability of the integument); 2) by compensatory mechanisms that enable active movements of solutes to counterbalance the diffusive fluxes, e.g. active exchange of salts (Péqueux 1995).

Many marine invertebrates, as well as most of the crustacean species living in seawater, are osmoconformers (Péqueux 1995, Anger 2001), i.e. keep their total osmotic pressure and $Na^+$ and $C1^-$ concentrations of their haemolymph very close to those of the surrounding water. However, in brackish and estuarine environments, animals are often in unfavourable conditions exposed to low salinity and need to adapt their internal osmotic concentrations frequently. Therefore, many species living in coastal habitats are osmoregulators (Davenport 1985) and maintain their internal medium constant, irrespective to the surrounding medium. Osmoregulatory organisms can either hyporegulate (when the salt concentration of the internal medium is maintained at lower levels than those of the external medium) or hyperregulate (when the salt concentration of the internal medium is kept higher than that of the outside medium). Depending on the salinity tolerance, organisms can be divided into two groups: 1) stenohaline species that can tolerate only a very narrow range of external salinities

and 2) euryhaline species that can endure a large range of osmotic conditions. With respect to hyperosmotic regulation, we can also categorize animals into weak and strong osmoregulators, Weak regulators can only maintain the haemolymph constant in a narrow range of external media concentration, while strong regulators can control and regulate their haemolymph composition at a nearly constant level irrespective of the external salinity.

Generally, early larval stages of marine organisms have weaker osmoregulatory capabilities and are less tolerant to variations of salinity in the environment than their conspecific juveniles or adults (e.g. Schreiber 2001, Anger 2001, Anger et al. 2008, Fridman 2020). From the perspective of energy, osmoregulation is considered an energetically costly process which usually causes higher respiration rates (Dehnel 1960, Rivera-Ingraham et al. 2016), in order to fuel the catabolism of osmotically active amino acids (Gilles 1973), and active salts exchange. Notably, osmoregulatory abilities can change during ontogeny (Charmantier and Charmantier-Daures 1991, Anger et al. 1998, Charmantier and Charmantier-Daures 2001, Charmantier et al. 2002, Anger 2003, Cieluch et al. 2004) and with increasing temperature (e.g. Ostrowski et al. 2011, Torres et al. 2021b). Additionally, other drivers can affect osmotic and ionic regulation in crustaceans, such as size, age, sex, moulting cycle, temperature, season, geographical origin of the population and/or individual intrapopulation variation (Péqueux 1995, Anger 2001). Interactions of salinity with temperature can lead to different performance outcomes. For instance, in sea urchins, an increase in temperature mitigated the negative effect of low salinity in the embryonic development (Delorme and Sewell 2014), while interactive effect of high temperature and low salinity caused a decreased larval survival (Mak and Chuan 2018). In several crustacean species, such as prawns (Janas and Spicer 2008), barnacles (Nasrolahi et al. 2012, Nasrolahi et al. 2016), and crabs (Nagaraj 1993, Spitzner et al. 2019), various interactive effects of salinity and temperature were found to impact larval performance. In addition, environmental drivers can modify post-zygotic maternal effects (Wade 1998) and alter larval responses to temperature and salinity (e.g. Charmantier et al. 2002, González-Ortegón and Giménez 2014, Torres et al. 2020). Differences in larval responses to temperature and salinity conditions may affect many physiological processes and be crucial for population persistence (Pandori and Sorte 2019).

## 4. Ecological role of Crustacea and their development

In coastal ecosystems, aquatic decapod crustaceans are one of the key groups, inhabiting a wide range of environments with different salinities from freshwater, over brackish to seawater (Roman and Zeng 2012). They are known for their massive impact on coastal ecosystems through strong predation, but also as an essential link in the food web due to their complex life cycle (reviews

Anger 2001, 2006). Crustaceans are essential for healthy functioning of communities and their abundance directly affects other trophic levels. They can be important predators but also a subject of strong predation and change the structure of the habitats. Their larval stages are major contributors to the zooplankton biomass and as primary and secondary consumer in the water column they are an important link in the food webs of the oceans and seas. Many fish depend upon the availability of crustacean larvae as food, and pelagic larvae are also important in pelagic-benthic coupling *via* energy exchange and migrations (Anger 2001).

Crustaceans can occupy various habitats during ontogeny and shift their niche during the development and transition from larvae to the juvenile phase (reviews Martin et al. 2014, Anger et al. 2015, Möller et al. 2020, Olesen 2020). Larval stages are particularly sensitive in many taxa and fundamental for dispersal and population persistence (Morgan 2020). In comparison to the adults, larvae are known to be more vulnerable to fluctuations in environmental key drivers (Przeslawski et al. 2015, Pandori and Sorte 2019) and the tolerance of larval stages can vary throughout ontogeny (Harvey et al. 2013). Decapod larval stages are mostly pelagic and they either actively feed (planktotrophy) or are non-feeding and they consume internal reserves (lecitotrophy) (reviews Anger 2001, Jeffs and O'Rourke 2020). Larval development comprises several larval stages and the number of instars is species dependent. After hatching, pelagic zoeal stages develop into a megalopa (reviews Forward et al. 2001, Gebauer et al. 2020, Haug 2020), that settles in the benthic habitat. The habitat change during early stages presents a major challenge for organisms. If they fail to adapt to any of these habitats, it might cause the population to collapse, either through reproductive or recruitment failure. Thus, investigating the capacity of larvae to cope with fluctuations of environmental drivers became essential in marine ecology (reviews Anger 2006, Torres et al. 2019). Furthermore, decapod brachyuran larvae are excellent model organisms to study the effects of combined multiple environmental drivers on species persistence (reviews Torres et al. 2019, Giménez 2020, Zeng et al. 2020, Wacker and Harzsch 2021).

## 5. Thesis objectives

The objective of this thesis was to quantify variations in larval performance of a coastal decapod crustacean species at the population level in response to key environmental drivers (temperature and salinity), which could lead to adaptive responses of the specific populations. As a focus species, I chose the European shore crab and hypothesized that coastal native populations from contrasting salinity (e.g. North Sea *vs* Baltic Sea) and distant habitats overt the latitudinal gradient (e.g. South Spain *vs* North Sea) should have different larval responses when exposed to diverse combinations of environmental drivers, according to their natural habitats and possible local

adaptations. I hypothesised that the Baltic Sea population should perform better in lower salinities due to natural brackish conditions in the Baltic Sea and adaptive responses to low salinity conditions (Theede 1969). On the other hand, the southern Europe population would be expected to perform better at higher temperatures, because we know that adult *C. maenas* have seasonal changes in respiration (Breteler 1975) and can become acclimated to warmer waters (Newell 1973). Spitzner et al. (2019) have shown thermal mitigation of the negative effects of low salinity at higher temperatures on the larval performance in the North Sea (Helgoland) population. The same effect was presumed to manifest again in the North Sea population and potentially in the southern population. The combined effects of these drivers in different coastal populations were never recorded before using this species. I speculated that different populations will have different larval responses to different combinations of environmental drivers, which will affect their performance in various ways.

## 6. Model organism - *Carcinus maenas*

The European shore crab *Carcinus maenas* (Decapoda, Brachyura; Linnaeus, 1758) is a foraging omnivore inhabiting a variety of coastal habitats, such as estuaries, bays, rocky intertidal shores, muddy and sandy subtidal habitats, saltmarshes, submerged aquatic vegetation and seagrasses (Cohen et al. 1995, Jensen et al. 2002, Grosholz and Ruiz 1996, Ray 2005, Young and Elliot 2020). Adults are commonly found from the high tide level to a depth of 5-7 m (e.g. Green 1968, Klassen and Locke 2007). Adult *C. maenas* can withstand a wide osmotic range, tolerating salinities from 4 to 52 PSU (Eriksson et al. 1975, McGaw and Naylor 1992, Klassen and Locke 2007). At higher salinities crabs are osmoconformers, while at lower salinities (< 22-26 PSU) they become weak osmoregulators (Péqueux 1995, Henry et al. 2003, Cieluch et al. 2004). They are eurythermal and able to survive short-term exposure to a wide range of temperatures, ranging from 0 to 30-35 °C (Cohen et al. 1995, Hidalgo et al. 2005, Klassen and Locke 2007, Tepolt and Somero 2014). European shore crabs can live up to 4-7 years (Berrill 1982, Yamada et al. 2005, Klassen and Locke 2007) and reach a maximum size of 8-10 cm in the carapace width (e.g. Crothers 1967, Klassen and Locke 2007)

As an "ecosystem engineer" (i.e. organism that directly or indirectly change the availability of resources to other species, inducing physical changes in biotic and abiotic materials; Jones et al. 1994, 1997), *C. maenas* is an important species that can modify entire ecosystems (Crooks 2002, Klassen and Locke 2007, Garbary et al. 2014, Young and Elliot 2020). It is considered a top predator in coastal habitats (Baeta et al. 2006), active both at night and day, with different activity patterns between populations (e.g. Ropes 1968, Crothers 1967, Novak 2004). Adults feed primarily on detritus and meiofauna, with the majority of their diet consisting of molluscs, other crustaceans, and

polychaetes (Crothers 1968, Klassen and Locke 2007, Young and Elliot 2020). Their diet preference can vary due to the diversity and seasonal changes in the availability of prey (e.g. Ropes 1968, Zwarts and Wanink 1993, Baeta et al. 2006). As they age, there is also a higher incidence of cannibalism as a foraging method (Raffaelli et al. 1989, Ropes 1989), depending on the habitat complexity and prey and predator size (Baeta et al. 2006, Almeida et al. 2011). Cannibalism can cause high and strong density-dependant mortality at natural densities (Moksnes 2004), with juveniles being dominant predators on settling post-larvae and young juvenile crabs in the nursery area (Moksnes et al. 1998). *C. maenas* is crucial for marine benthic community structure and its predation can affect the abundance and distribution of commercially important bivalve species (Raffaelli et al. 1989, Grosholz et al. 2000). Their predominant predators are birds, fishes, and other larger decapods (Klassen and Locke 2007, Young and Elliot 2020). For instance, many commercial species, such as Atlantic cod, depend on the availability of the plankton species, such as *C. maenas* larvae (Beaugrand et al. 2003). Hence, European shore crabs have essential role in ecosystem functioning of coastal areas, due to their position in food web.

### 6.1 Native range and invasiveness

*C. maenas* is a European species with a large native geographical range extending across Europe, from the north of Norway to the southern-west shores of Africa (Fig. 2; Crothers 1968, Carlton and Cohen 2003, Roman and Palumbi 2004, Darling et al. 2008). However, due to its high invasive abilities, facilitated by human activities, during the 19th and 20th century it has successfully dispersed around the globe (Carlton and Cohen 2003, Young and Elliot 2020) and invaded many temperate shorelines (Roman and Palumbi 2004). It established populations on all continents except Antarctica (Leignel et al. 2014, Young and Elliot 2020) and became a dominant crab species in most of the invaded bays and estuarine areas (Kern 2002). Its strong invasive abilities have been recognized long ago (Cohen et al. 1995, Carlton and Cohen 2003, Hidalgo et al. 2005) and they are listed as one of the 100 worst invaders by IUCN (International Union for Conservation of Nature).

In Europe, a population structure analysis showed that there is a regional genetic structure between southern and northern populations, with Faroe Islands and Iceland populations being distinctively different from any other native European populations (Roman and Palumbi 2004). Moreover, it appears that the invasive potential differs among distinct native populations. For instance, Compton et al. (2010) suggested that the invasive potential depends on the population of origin and that most invasions came from southern populations. Their model for *C. maenas* predicts a further range expansion along many already invaded coastlines as well as future potential invasion of areas, such as New Zealand, China, Russia, etc. With the increasing global warming, a future northern expansion of *C. maenas* is also expected due to the northern shift of the thermal niche

(deRivera et al. 2007, Compton et al. 2010) and their broad tolerance of salinity (e.g. Klassen and Locke 2007). Thus, investigating populations of *C. maenas* from different areas across the native range might reveal new insights in the species invasiveness and ecology.

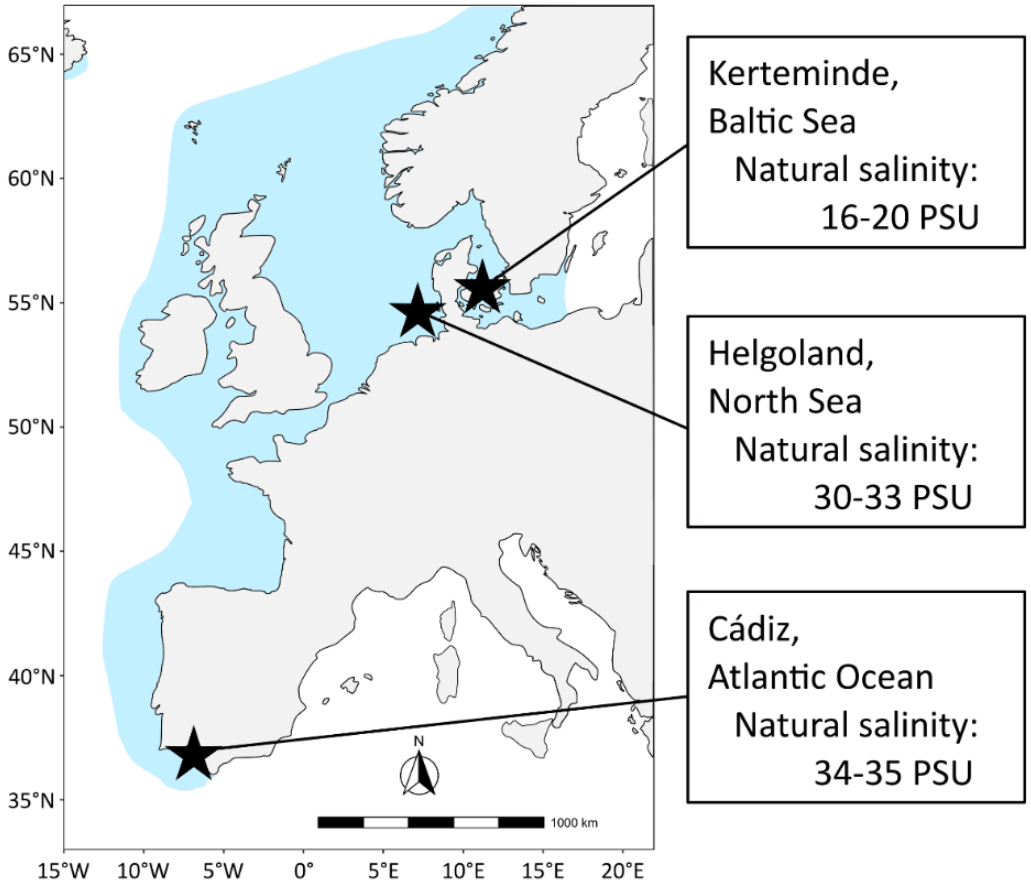


**Figure 2.** *Carcinus maenas* native European range (blue). Black stars represent three native populations investigated in this study: Kerteminde, Helgoland and Cádiz.

### 6.2 Life cycle and development

As many benthic brachyuran crabs, *C. maenas* develops through a complex biphasic life cycle and with stages that change between benthic and pelagic habitats during development (e.g. Crothers 1967, Klassen and Locke 2007). The mating season and their breeding potential differs among populations, where in European populations mating usually occurs between June and October (d'Udekem d'Acoz 1993), with a peak in summer (Broekhuysen 1936). Copulation starts after moulting of the female (Broekhuysen 1936), and once fertilized, the female carries eggs up to several months (Klassen and Locke 2007). Depending on the persistence of the colder temperature (<10°C), ovigerous females produce one or two clutches per year in all populations (Berrill 1982, Klassen and Locke 2007), with different seasonal patterns depending on the latitude (temperature) and other factors, such as available resources or size of the female (Baeta et al. 2005, Gehrels et al. 2016).

Clutch sizes can vary considerably among native (185,000-200,000 eggs: Broekhuysen 1936, Crothers 1967, Cohen et al. 1995) and invasive populations (~5000-165,000 eggs: Griffen 2014), and during embryonic development because eggs are lost during the brooding period (Audet et al. 2008). Females are berried in the intertidal/subtidal area for several months while they carry fertilized eggs until fully developed larvae hatch in spring or summer (Sprung 2001). Depending on the temperature, development of the embryos can last between 22 and 100 days (Dries and Adelung 1976). Prior to hatching, females usually migrate to higher salinities and release larvae at night into ebb tide (Queiroga 1996).

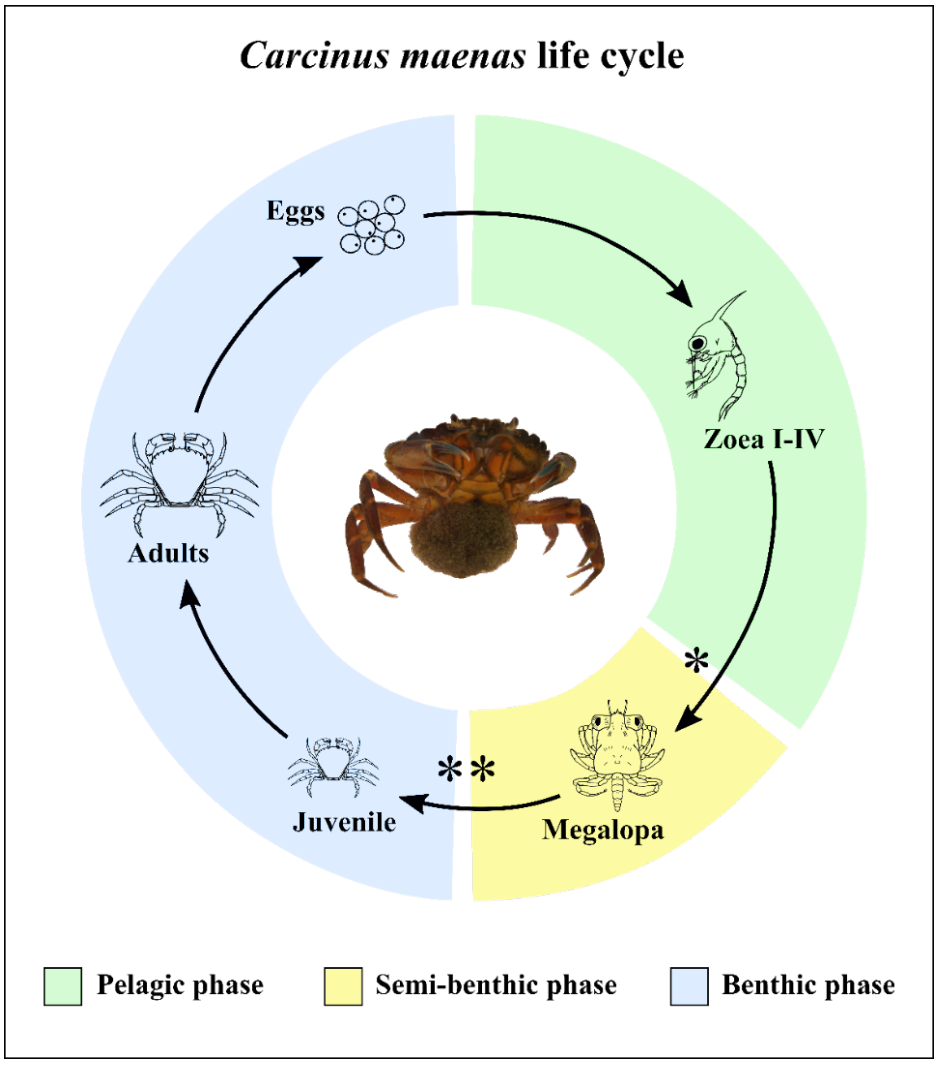


**Figure 3.** Life cycle of *Carcinus maenas* and pelagic-benthic coupling. Hatched larvae develop through four zoeal stages (Zoea I-IV) in pelagic waters (green). After a first metamorphosis (*), the last zoeal stage transforms into a megalopa, which is semi-benthic (yellow). Megalopa goes through a second metamorphosis (**), to juvenile crab that settles in the benthic habitat (blue). When crabs become mature, they eventually mate, and females carry fertilized eggs until the embryogenesis is complete. Once the embryogenesis is complete, larvae hatch and the cycle repeats. Drawings are based on Crothers 1967.

After hatching, development (Fig. 3) continues through five larval stages (e.g. Dawirs 1985, Compton et al. 2010, Epifanio and Cohen 2016): four pelagic stages (zoea I-IV) followed by a first metamorphosis to the semi-benthic larval stage megalopa (Spitzner et al. 2018). Larval stages are planktotrophic (Klassen and Locke 2007) and develop in coastal waters for upward of 50 days, to a maximum of 82 days in the laboratory (Williams 1967, deRivera et al. 2007). Through vertical migration, zoeae can enhance their export from estuary zones and reach more optimal salinities for development (Queiroga et al. 1997). Megalopae can then use the selective tidal stream transport (Queiroga 1996), return to the shore, and settle in the benthic habitat through a second metamorphosis into a juvenile crab (Fig. 3). Crabs grow and become mature after around 2 years (Crothers 1967, Moksnes et al. 1998). Embryonic and larval development of *C. maenas* is mainly controlled by temperature, with larvae being more abundant in spring than later in summer (Dawirs 1985). There are also developmental differences among populations coming from different habitats and latitudes due to different genetic structures (Roman and Palumbi 2004, Compton et al. 2010, Young and Elliot 2020).

Generally, planktonic larval stages of *C. maenas* are considered the more sensitive stages with higher risk of predation, higher sensitivity to environmental drivers and more frequent overdrift into unsuitable habitats (Young and Elliot 2020). Although larvae of *C. maena*s are physiologically more tolerant to temperature fluctuations compared to adults (Dawirs et al. 1968, deRivera et al. 2007), they need a minimum temperature of 10 °C for growth and development (Dawirs et al. 1968, Berrill 1982, Nagaraj 1993, deRivera et al. 2005), which limits their expansion into the northern areas. When it comes to salinity, larvae are less tolerant to salinity fluctuations than adults (Anger et al. 1998, Baeta et al. 2005, Klassen and Locke 2007, Leignel et al. 2014) and salinity tolerance increases with successive larval stages with euryhalinity finally achieved in juveniles (Cieluch et al. 2004). Larvae can withstand a short-term exposure to salinities from around 10 to 44 PSU (Broekhuysen 1936, Cieluch et al. 2004, Yamada et al. 2005) but cannot develop below 20 PSU (Anger et al. 1998). They also have different sensitivity during the ontogeny: zoea I has weak hyperregulatory abilities in dilute media, zoea II-IV are osmoconformers, while megalopa can hyper-regulate with fully functional gills for the first time (Cieluch et al. 2004). In addition, osmoregulatory capacity in larvae increases with rising temperature (Torres et al. 2021b). Moreover, both temperature and salinity are known to operate in combination leading to multiple driver effects on *C. maenas* larval performance (e.g. Nagaraj 1993, Spitzner et al. 2019, Torres et al. 2020). Thus, effective thermotolerance and osmotolerance of larvae is crucial in maintaining their biological mechanisms functional in the upcoming climatic challenges.

Another mechanism to cope with unfavourable conditions is through avoidance behaviour. *C. maenas* larvae display diel vertical migrations, with the majority of larvae appearing in the surface

layers at night and returning to the deeper water during the day (Queiroga 1996). Therefore, they can regulate their movements in the water column to some extent and stay in more protected areas of the coast. Such vertical migration behaviour is recorded in different populations (Zeng and Naylor 1996), meaning it is an adaptive endogenous mechanism, using selective tidal stream transport for easier estuarine retention or to help the offshore dispersal of newly released larvae (Zeng and Naylor 1996, Queiroga et al. 1997). Thus, such adaptability of larval stages during ontogeny to changes of salinity and temperature can contribute towards range expansion in the climate-warming scenario.

### 6.3 Experimental design and larval rearing

Rearing of *C. maenas* and other brachyuran larvae is a logistically demanding and time-consuming process with each species having specific set of environmental drivers within their optimum range. Natural systems differ majorly in comparison to laboratory conditions, where we can manipulate only few drivers. Therefore, according to the research question asked, best conditions for rearing must be obtained to successfully perform rearing of the larvae and get desired results. This can be acquired by having a long-term experience and knowing optimal conditions of the species, or through numerous preliminary experiments to find most suitable protocol for the rearing process (Torres et al. 2021a).

To investigate larval performance of *C. maenas*, larvae were exposed to combinations of two of the most important environmental drivers in coastal regions, namely temperature and salinity. Larval rearing of *C. maenas* was performed at the Biologische Anstalt Helgoland, Alfred-Wegener-Institut, Helmholtz-Zentrum für Polar- und Meeresforschung (AWI) and followed protocols optimized by researchers working with this species, reviewed by Torres et al. (2021a). To elucidate interspecific and intraspecific differences, larvae originating from three populations from the native range (Fig. 2) were reared using the same experimental design in a common garden experiment (Fig. 4): Cádiz (Spain), Helgoland (Germany) and Kerteminde (Denmark). Even though there are studies which investigate effects of multiple environmental drivers (e.g. Nagaraj 1993, Spitzner et al. 2019), a successful multipopulation comparison in a common garden setup for this species is performed for the first time here. Larvae from each population were reared at 12 different combinations (Fig. 4): four temperature (15, 18, 21, 24 °C) × three salinity treatments (20, 25, 32.5 PSU). Larval performance was measured via several traits, such as survival, development and respiration rates, biomass and growth rates. Interpopulation studies are essential in exploring future effects of environmental drivers in a changing world and *C. maenas* is a great model species to investigate this.

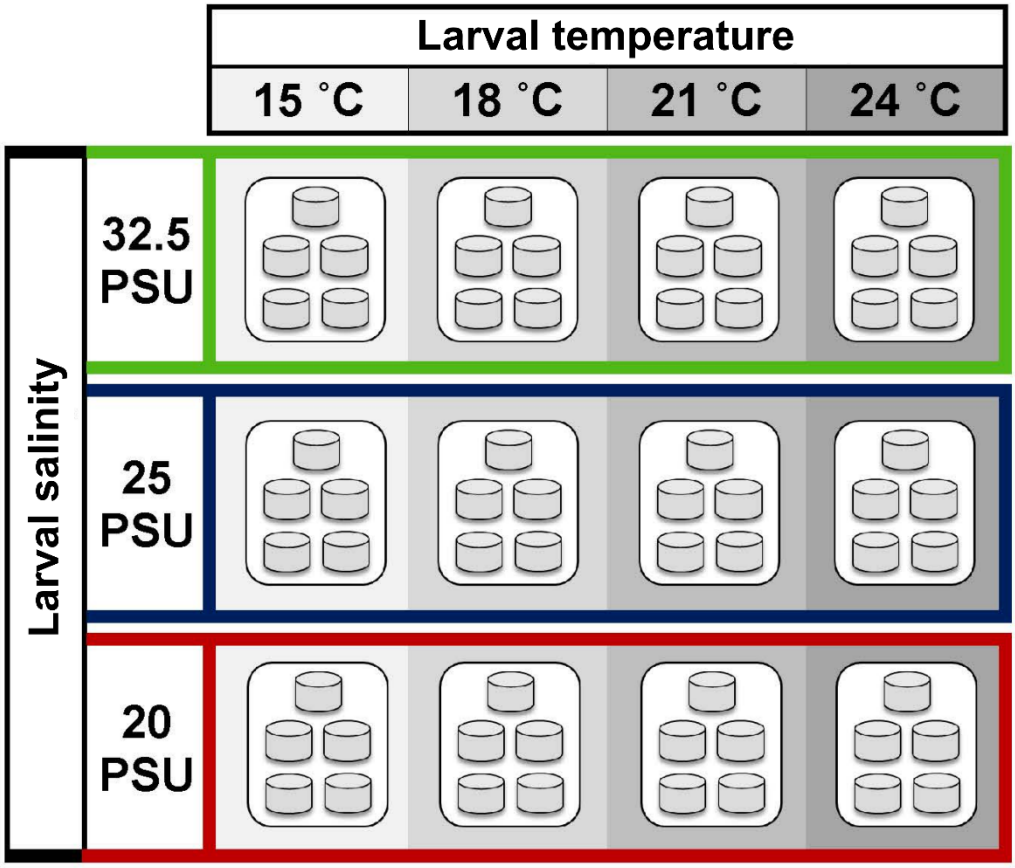


**Figure 4.** Experimental factorial design to test larval performance of *Carcinus maenas*. Larvae from each population were tested in 12 combinations of temperature (15, 18, 21, and 24 °C) and salinity (20, 25, and 32.5 PSU). For details about replicates and protocol see Šargač et al. 2021.

# III Publications

## Publication I

Published in Marine Ecology Progress Series, peer reviewed journal:
**Šargač Z**, Giménez L, Harzsch S, Krieger J, Torres G (2021)
https://doi.org/10.3354/meps13851.

# Contrasting offspring responses to variation in salinity and temperature among populations of a coastal crab: A maladaptive ecological surprise?

Vol. 677: 51–65, 2021
https://doi.org/10.3354/meps13851

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published October 28

# Contrasting offspring responses to variation in salinity and temperature among populations of a coastal crab: A maladaptive ecological surprise?

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ABSTRACT: Current understanding of species capacities to respond to climate change is limited by the amount of information available about intraspecific variation in the responses. Therefore, we quantified between- and within- population variation in larval performance (survival, development, and growth to metamorphosis) of the shore crab *Carcinus maenas* in response to key environmental drivers (temperature, salinity) in 2 populations from regions with contrasting salinities (32–33 PSU: Helgoland, North Sea; 16–20 PSU: Kerteminde, Baltic Sea). We also accounted for the effect(s) of salinity experienced during embryogenesis, which differs between populations. We found contrasting patterns between populations and embryonic salinity conditions. In the Helgoland population, we observed a strong thermal mitigation of low salinity stress (TMLS) for all performance indicators, when embryos were kept in seawater. The negative effects of low salinity on survival were mitigated at increased temperatures; only at high temperatures were larvae exposed to low salinity able to sustain high growth rates and reduced developmental time, thereby metamorphosing with comparable levels of carbon and nitrogen to those reared in seawater. By contrast, larvae from the Kerteminde population showed a detrimental effect of low salinity, consistent with a maladaptive response and a weak TMLS. Low salinity experienced during embryogenesis preempted the development of TMLS in both populations, and reduced survival for the Kerteminde population, which is exposed to low salinity. Our study emphasises the importance of evaluating species responses to variation in temperature and salinity across populations; the existence of maladaptive responses and the importance of the maternal habitat should not be underestimated.

KEY WORDS: Interpopulation variation · Post-zygotic effects · Larval performance · Environmental drivers · *Carcinus maenas*

## 1. INTRODUCTION

Fluctuations of Earth's climate and global warming have major effects on biological systems at several levels of organisation (Burrows et al. 2011, Poloczanska et al. 2013, Boersma et al. 2016, Boyd et al. 2018), and are already affecting the species' physiology and distribution (Perry et al. 2005, Somero 2010, Burrows et al. 2011, Poloczanska et al. 2013, Reusch 2014). As climate changes, organisms need to cope with varia-

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Publisher: Inter-Research · www.int-res.com

tion in many different environmental variables or stressors (hereafter called 'drivers'). The interplay between 2 or more drivers often has non-independent effects, with one enhancing (as a synergistic effect) or weakening (antagonistic effect) the effect of another driver, beyond the additive effect expected from the action of each driver considered in isolation (Folt et al. 1999, Crain et al. 2008, Todgham & Stillman 2013, Piggott et al. 2015, Orr et al. 2020, Tekin et al. 2020). Both synergistic and antagonistic effects may lead to various outcomes for particular species, including the collapse of a biotic system (Breitburg et al. 1998) and potential adaptive response to multiple environmental changes (Sinclair et al. 2013). In addition, we expect that interactions between multiple drivers are responsible for the fate of many coastal species because coastal habitats are strongly influenced by climate change (Hiddink et al. 2015, Holt et al. 2016, Robins et al. 2016, Tinker et al. 2016). For instance, temperatures in the North and Baltic Seas are expected to increase, while salinity in the Baltic is predicted to decrease (BACC Author Team 2008, Neumann 2010, Meier et al. 2012, Andersson et al. 2015), and change is already happening in those seas (Wiltshire et al. 2010, Burrows et al. 2011, Boersma et al. 2016). Salinity and temperature regimes are crucial drivers for coastal biota (Hänninen et al. 2000, Telesh et al. 2013), affecting performance and fitness (Somero 2005, 2010, Ko et al. 2014). In general, coastal, estuarine, and intertidal species have adapted to cope with large ranges of temperature and salinity, but they are often at their physiological limits (Stillman & Somero 1996, 2000, Browne & Wanigasekera 2000).

Recently, the importance of intraspecific trait variation has been highlighted in the context of species responses to climate change (Moran et al. 2016). Intraspecific trait variation can occur at several spatial and temporal scales (Violle et al. 2014) and can shape species distributions and community structure through different mechanisms (Bolnick et al. 2011), including local adaptation and plasticity (Chevin et al. 2010). However, concerning the simultaneous action of multiple drivers, we still know little about the magnitude of variation in physiological responses, in particular for coastal marine species (Carter et al. 2013, Applebaum et al. 2014, Spitzner et al. 2019). High levels of variation are likely to characterise species distributed over wide spatial scales in a heterogeneous coastal habitat. Thus, a key question is whether coastal populations experiencing contrasting environmental conditions will be able to persist in a scenario of climate-driven changes.

This is the case for the shore crab *Carcinus maenas*, which is distributed along the salinity gradient existing between the North and the Baltic Seas (salinity range — North Sea: seawater, 30–33 PSU; southern Baltic Sea: 10–30 PSU). *C. maenas* is a predator, native to Europe but a global invader elsewhere (Roman & Palumbi 2004). Shore crab larvae from populations of Helgoland in the North Sea (Spitzner et al. 2019) and North Wales in the Irish Sea (Torres et al. 2020) exhibit an antagonistic response to increased temperature and low salinity (defined by Spitzner et al. 2019 as 'Thermal Mitigation of Low Salinity stress', TMLS), by which the negative effects of low salinity on survival and developmental rates are mitigated at higher temperatures. TMLS occurs in other coastal crustaceans (e.g. Janas & Spicer 2008, Nasrolahi et al. 2012). In the local population from Helgoland, TMLS was found in larvae hatched from embryos kept in seawater, which corresponds to the natural conditions. TMLS may occur through several mechanisms. For instance, an enhanced capacity to osmoregulate is one such explanation for a number of species (Williams 1960, Hagerman & Uglow 1983, Janas & Spicer 2008). Likewise, the larval stages zoea I and megalopa of *C. maenas* from the Helgoland population exhibit increased capacity to osmoregulate when exposed to higher temperatures (Torres et al. 2021); this may reflect a higher rate of pumping ions by $Na^+$-$K^+$-ATPase in the ionocytes, located in the ion-transport tissues, as well as increased production of ATP in the mitochondria (Pörtner 2010). In addition, when performance is quantified as survival to a given stage (e.g. to megalopa), the mitigation effect may occur because, at high temperatures, larvae are exposed to suboptimal salinity for a shorter time (i.e. 'phenological effect' in Torres et al. 2021).

Larvae of *C. maenas* and other marine invertebrates are considered very sensitive to environmental changes (Przeslawski et al. 2015, Pandori & Sorte 2019), and poor larval performance due to environmental stress can lead to recruitment failure and population collapse. Hence, TMLS can enable larvae to exploit coastal habitats of moderately reduced salinity (e.g. above 20 PSU for *C. maenas*) and have a central role in population persistence under a warming scenario. TMLS may drive distribution patterns and community structure (Liu et al. 2020, Torres et al. 2021). However, while TMLS may be a trait of local populations distributed in areas influenced by seawater, we still do not know anything about the responses to temperature and salinity in populations located in habitats dominated by brackish water,

such as the Baltic Sea. We know that adults of *C. maenas* from the Baltic Sea exhibit increased capacity to osmoregulate as compared to those from the North Sea (Theede 1969), possibly providing increased tolerance to low salinity. Likewise, one would expect that larvae of *C. maenas* from populations located in the Baltic Sea should exhibit increased tolerance to low salinity for the whole life cycle. If larvae of such populations are well adapted to low salinity, one should find a shift in the optimal salinity (e.g. higher survival at low salinity, e.g. 20 PSU vs. seawater, e.g. 32 PSU) or an increase in the degree of euryhalinity (survival is little affected by low salinity as compared to seawater). From current knowledge, it is very difficult to predict how temperature may modify the response to low salinity for populations located in habitats such as the Baltic Sea, but we should not find the same antagonistic response in larvae from the North and Irish Seas.

Another important point is that, by carrying the eggs in their abdomen, female crabs determine the salinity conditions experienced during embryonic development. Such conditions can drive 'post-zygotic maternal effects' (Wade 1998) and thus modify larval responses to salinity and temperature (Giménez & Anger 2003, González-Ortegón & Giménez 2014). In some estuarine species, moderately low salinities experienced during embryonic development enhance larval performance (Charmantier et al. 2002). While, in a population of *C. maenas* from the Irish Sea, low salinity (20 PSU) impaired performance (Torres et al. 2020), this should not be the case for a population located in the Baltic Sea where embryos develop under low salinity conditions.

Here, we compared the responses to temperature and salinity in larvae of a shore crab population from the Baltic Sea (Kerteminde, Denmark) with those of a North Sea population (Helgoland, German Bight). We carried out a comprehensive study on the effects of salinity and temperature on several larval traits from hatching to metamorphosis. As proxies for larval performance, we measured survival, duration of development, dry mass, carbon and nitrogen content, and growth rates. In the same experiment, we also quantified the effect of salinity conditions experienced during embryonic development in order to determine how post-zygotic effects may vary between populations according to the salinities experienced by their embryos. In contrast to the larvae from Helgoland (North Sea), we expected that larvae from Kerteminde (Baltic Sea) would be highly tolerant to low salinity, especially after experiencing low salinity during embryogenesis.

## 2. MATERIALS AND METHODS

### 2.1. Model species

*Carcinus maenas* is a coastal–estuarine inhabitant, native to Europe but considered one of the 100 most successful invaders by the International Union for Conservation of Nature (see also Cohen et al. 1995, Carlton & Cohen 2003, Roman & Palumbi 2004, Hidalgo et al. 2005), with known established populations in Australia, America, and Asia (Leignel et al. 2014). *C. maenas* has a biphasic life cycle consisting of bottom-living (benthic) adults, 4 fully pelagic larval stages (zoea I–IV) that disperse in the water column, followed by a metamorphosis to an additional semi-benthic larval stage (megalopa) that colonises the shore habitats (Spitzner et al. 2018). Low salinities can reduce developmental and growth rates in larval stages (Anger et al. 1998, Torres et al. 2002, Spitzner et al. 2019).

### 2.2. Study design

Females with early-stage embryos were collected from the shores of 2 locations: North Sea: Helgoland, Germany (salinity = 32–33 PSU; coordinates: 54° 10′ 49.2″ N, 7° 53′ 20.2″ E) and Baltic Sea: Kerteminde, Denmark (salinity = 16–20 PSU; coordinates: 55° 26′ 59.9″ N, 10° 39′ 40.1″ E). We first exposed berried females from both populations to 2 embryonic salinities, i.e. natural salinities in which each population occurs (Fig. 1a). Once the larvae hatched, we quantified performance (survival, developmental time, dry mass, carbon and nitrogen content, and growth rates) after larvae were exposed to different combinations of temperature and salinity (Fig. 1b). Hence, we were able to evaluate the combined effect of population of origin (P), embryonic salinity ($E_S$), larval temperature ($L_T$), and larval salinity ($L_S$) on larval performance.

Animals of both populations were kept individually in 5 l aquaria. Berried females with early-stage embryos of each population were randomly assigned to 2 different $E_S$: 20 and 32.5 PSU, which resembled salinity conditions in the natural habitats of the Baltic Sea (Kerteminde) and North Sea (Helgoland), respectively (Fig. 1a). The experimental salinity 20 PSU was chosen to match the maximum salinity registered in the Danish fjord during the time of collection; and 32.5 PSU as the salinity registered for the North Sea around Helgoland. In addition, preliminary experiments with females

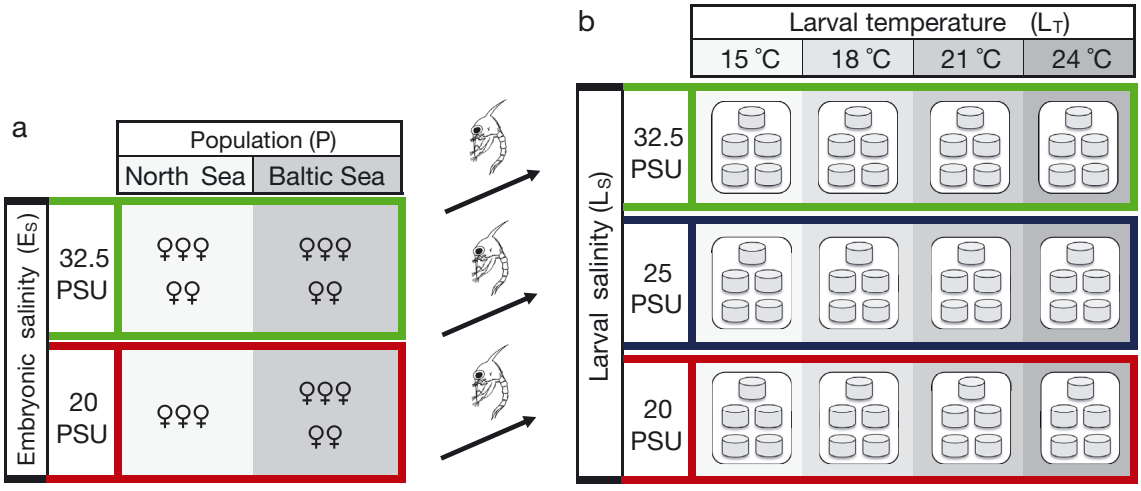


Fig. 1. Experimental design to test *Carcinus maenas* larval performance. (a) Maternal conditions: females (♀) with early-stage embryos from both populations (P) were exposed to different embryonic salinities ($E_S$) corresponding to the natural habitat salinity of each population (Baltic Sea: 20 PSU, North Sea: 32.5 PSU). Number of symbols (♀) corresponds to the number of females with successfully produced larvae for each combination of P × $E_S$. (b) Larval conditions: freshly hatched larvae from each female were tested in a factorial experimental design that consisted of 12 combinations of larval temperature ($L_T$: 15, 18, 21, and 24°C) and salinity ($L_S$: 20, 25, and 32.5 PSU) in a common garden setup to determine larval survival, development, biomass, and growth. Each treatment had 5 replicate 60 ml bowls with 10 randomly assigned larvae in each

from the North Sea population showed that females kept at salinities lower than 20 PSU could not complete embryonic development (G. Torres unpubl. data). Upon hatching, larvae were then assigned randomly to different combinations of temperature and salinity following a factorial experimental design (Fig. 1b) based on 12 combinations of 4 larval temperatures ($L_T$): 15, 18, 21, and 24°C, and 3 larval salinities ($L_S$): 20, 25, and 32.5 PSU. Parameter checks after 24 h showed that salinity changed slightly, but this change was always <1 PSU (increase at 15 and 18°C: 0.2–0.3 PSU; at 21 and 24°C: ~0.5–0.7 PSU). Larvae were assigned to the treatments in 5 replicate groups (Fig. 1b). Each replicate group consisted of 60 ml glass bowls with 10 random individuals each (i.e. the replicate units were the individual bowls).

Experiments were carried out with larvae from 18 females, i.e. 5 females per combination of population and embryonic salinity, except for those of Helgoland exposed to 20 PSU, where only 3 females produced larvae (Fig. 1a). Note that individual females are identified as Fem 1–5 for each combination of population and embryonic condition (e.g. see Figs. S1 or S3 in the Supplement at www.int-res.com/articles/suppl/m677p051_supp.pdf). Overall, the experiment started with 10 800 larvae (= 10 larvae × 5 replicates × 3 larval salinities × 4 larval temperatures × 18 females).

### 2.3. Experimental procedures

Experiments were conducted during the reproductive period of *C. maenas*. Animals from the Kerteminde population were caught using traps in the subtidal fjord next to the Marine Biological Research Centre (University of Southern Denmark, Kerteminde, Denmark). Until transport to the Marine Biological Station at the Alfred-Wegener-Institute (Helgoland, Germany), berried females were kept for a week in a flow-through system of natural water from the fjord (temperature: 10 ± 1°C, salinity: 16–20 ± 1 PSU) and gentle aeration. For transport to Germany, crabs were placed individually in plastic containers (volume: 1 l) half-filled with filtered natural seawater from the fjord and a wet towel. To ensure sufficient oxygen concentration during transport (ca. 24 h), the filtered seawater was strongly aerated before filling the containers. Afterwards, the containers with animals were placed in a Coleman® cooler box to ensure a constant temperature (10–12°C) during transport. Upon arrival at the laboratory on Helgoland, the temperature was gradually increased (1°C $d^{-1}$) to match the temperature in summer for both populations, thus reaching a common embryonic temperature (15°C). The animals from the Helgoland population were collected manually from the intertidal rocky shore and directly transported to the Marine Biological Station at the Alfred-Wegener-

Institute. For these berried females, transport stress was simulated for 24 h in the laboratory aiming to replicate the transport conditions of the animals from Kerteminde, i.e. animals were individually placed for 24 h in half-filled plastic containers with fully aerated natural seawater (natural salinity: 32.5 ± 0.5 PSU) and temperature (15 ± 0.5°C) with a wet towel. Water was changed daily and animals were fed frozen shrimp *Crangon crangon* twice per week. Salinities were adjusted gradually (salinity decrease/increase rate: 2 PSU $d^{-1}$) until the desired embryonic salinity (20 or 32.5 PSU) was achieved.

Experiments with larvae (Fig. 1b) were run in temperature-controlled rooms (±0.5°C) with a 12:12 h light:dark cycle. Filtered (filter pore size = 0.2 µm), aerated natural seawater was used for all experiments, and lower salinities were prepared by diluting natural seawater with appropriate amounts of tap water. Bowls were filled to 75% of the volume, and salinity ($E_S$ and $L_S$, ±0.5 PSU) was monitored with a salinometer (WTW Cond 3110 SET1). Water and food (ad libitum freshly hatched *Artemia* sp.; Great Salt Lake Artemia) were changed daily. During the daily water change, survival and development of the larvae were monitored; moults and dead larvae were recorded and discarded.

### 2.4. Elemental analysis

In order to determine elemental composition (C and N content) and growth, 3 replicates of freshly hatched zoea I (50 larvae each) were randomly chosen at the start of the experiment and 3 replicates of recently moulted megalopae (2 megalopae each) were randomly selected at the end of each experiment. Larvae were gently rinsed with distilled water for 10 s, blotted dry, placed into aluminium cartridges and stored at −20°C for further analyses. Prior to the elemental composition analyses, samples were freeze-dried for 48 h (Christ Alpha 1–4 freeze-drier), and dry mass was determined using a microbalance (Sartorius SC2, precision 0.0001 mg). Elemental composition was determined as carbon and nitrogen content using an elemental analyser (Vario MICRO cube CHNS analyser, Elementar Analysensysteme).

### 2.5. Data analysis

Survival to each stage was calculated as the cumulative proportion of larvae surviving from hatching to each stage. Prior to the analysis, the survival proportions (p) were re-scaled with the formula $p' = [p (N - 1) + 0.5]/N$, where N is the number of larvae per glass (= 10 larvae) at the start of the experiment. This transformation is used to avoid situations of log(0) values occurring. Survival proportions were then transformed to a logarithmic and logistic scale. Developmental time was determined as the time from hatching to reach each larval stage. Dry mass (DM) and carbon (C) and nitrogen (N) content were calculated for freshly hatched zoeae I and post-moult megalopae. Instant growth was calculated following the formula: $\log(B_{(M)}/B_{(ZI)})/DT$ where B is the biomass parameter (DW, C, or N) and DT is the developmental time from hatching ($_{ZI}$) to reach the megalopa ($_M$). Means, standard deviations and standard errors were calculated with the 'plyr' package in R (version 3.6.1) through RStudio.

The experimental design had 4 fixed and orthogonal factors: (1) population: P; (2) embryonic salinity: $E_S$; (3) larval temperature: $L_T$, and (4) larval salinity: $L_S$. In addition, there was a random factor: female of origin (F) nested in the interaction of embryonic salinity and population. The analyses were carried out through mixed models, applying backward model selection (Zuur et al. 2009) using the package 'nlme' (Pinheiro et al. 2019), and the functions 'lme' (for mixed models) and 'gls' (for fixed models).

For mixed modelling, model selection of random terms was carried out by comparing models after restricted maximum likelihood (REML). The fixed terms were then examined after refitting the model with the best random structure with maximum likelihood (ML). In all cases, model selection was based on the corrected Akaike's information criteria (AICc) and likelihood ratio tests (LRT); a Tukey HSD test was used for post hoc comparisons. When comparing multiple models, if the simplest model had the lowest AICc, it was selected for further analysis. In cases when $\Delta AICc < 3$ and a more complex model had lower AICc, we applied LRT; if models differed significantly ($p < 0.05$), the lower AICc model was selected; in the opposite situation, the model with the lower number of parameters was chosen.

The full mixed model for survival (Table 1) contained 2 components: (1) the 4 factorial fixed components combining population, embryonic salinity, larval salinity, and larval temperature ($\sim P \times E_S \times L_S \times L_T$); (2) the interaction terms reflecting different random components. The full random part of the model was initially specified with the full covariance matrix, but model fitting led to convergence issues, suggesting overfitting. By following the recommendations given by Bolker et al. (2009), we then re-specified the matrix as diagonal, coded as 'random = list (F =

Table 1. *Carcinus maenas* results of model selection (AICc values) for larval survival (logarithmic and logistic transformed data) in response to population (P), embryonic salinity ($E_S$), larval temperature ($L_T$), larval salinity ($L_S$), and female of origin (F). F is a random factor representing maternal effects, nested in the interaction P × $E_S$. The remaining 4 factors are fixed and form a 4-way factorial design. Model selection on random terms was carried out through restricted maximum likelihood (REML) fitting; in all cases, the full model performed considerably better than any alternative model. Fixed effects were tested after maximum likelihood (ML) fitting and the full fixed model was retained (P × $E_S$ × $L_S$ × $L_T$). The best model therefore contained both the random and fixed parts (marked in **bold**). ZII–ZIV: zoeal stages II–IV; M: megalopa

| Model selection | Logarithmic | | | | Logistic | | | |
|---|---|---|---|---|---|---|---|---|
| | ZII | ZIII | ZIV | M | ZII | ZIII | ZIV | M |
| **Random (REML)** | | | | | | | | |
| F × $L_T$ × $L_S$ | 1772 | 1704 | 1570 | 1685 | 2691 | 2633 | 2500 | 2462 |
| F × $L_S$ | 1841 | 1844 | 1740 | 1865 | 2771 | 2765 | 2666 | 2650 |
| F × $L_T$ | 2000 | 1945 | 1845 | 1952 | 2920 | 2864 | 2779 | 2785 |
| F | 2023 | 1980 | 1887 | 2000 | 2940 | 2886 | 2809 | 2819 |
| No random term | 2328 | 2323 | 2233 | 2237 | 3398 | 3381 | 3256 | 3095 |
| **Fixed (ML)** | | | | | | | | |
| 4-way (full model) | **1695** | **1631** | **1490** | **1604** | **2659** | **2602** | **2463** | **2415** |
| 3-way factorial | 1723 | 1663 | 1549 | 1631 | 2682 | 2629 | 2501 | 2434 |

pdDiag(~$L_S$ × $L_T$))′. For the selection of the random component, there were also simpler models specified with the full covariance matrix (e.g. as 'random = list (F = pdSymm(~$L_S$))′ or 'random = list (F = pdSymm(~$L_T$))′ or 'random = ~1|F'). For duration of development, the 4-way interaction (~P × $E_S$ × $L_S$ × $L_T$) was kept, but the lowest level of the factor larval salinity ($L_S$ = 20 PSU) was excluded from the analyses due to insufficient number of surviving larvae to estimate duration of development.

Separate analyses for developmental duration were carried out for the different stages, depending on the survival in the specific treatments. For instance, zoea II and zoea III analyses were performed for full mixed models (Table S1) with larvae reared at $L_S$ = 25 and 32.5 PSU, while duration of development until the megalopa stage was only possible for $E_S$ = 32.5 PSU with larvae developing at $L_S$ = 32.5 PSU which decreased the level of factors (Table S2). Additionally, 3-way models were fitted for the megalopae from $E_S$ = 32.5 PSU to check for significant interactions between larval salinities (25–32.5 PSU) at temperatures 18–24°C where sufficient megalopae survived.

To evaluate megalopa biomass, a mixed model for the dry mass (DW) and C and N content was performed. However, the analysis was restricted to larvae originating from seawater ($E_S$ = 32.5 PSU) due to high mortality early in the development of larvae from broods kept at $E_S$ = 20 PSU. Note that $L_S$ = 20 PSU is also excluded from the analysis due to high mortality of the larvae. Therefore, the analysis was divided into 2 parts. In the first step, we compared both populations with a 3-way fixed interaction (~P × $L_S$ × $L_T$), excluding $L_T$ = 15°C due to insufficient survivors to megalopa at $L_S$ = 25 PSU in the Baltic Sea population (Table S3). In the second part of the analysis, we included the lowest larval temperature and analysed effects of combined larval salinity and temperature (~$L_S$ × $L_T$) only for the North Sea population for all treatments (Table S4). An additional ANOVA was performed for the Baltic Sea population excluding the lowest temperature (15°C) to test the effect of larval salinity. In all steps of the biomass data analysis, female of origin (F) was kept as a random factor (coded in the model as: 'random = ~1|F').

For the instantaneous growth rates, the starting model had the same structure as the one used for DW and C and N content (Tables S3 & S4). For the biomass analyses, 2 megalopae were always merged in 1 replicate. Thus, we first calculated averages of DW and C and N content of each replicate. Then, we calculated the average DD (i.e. from freshly hatched zoea I to successfully moulted megalopa) of the 2 megalopae in each replicate, which we then used to calculate instantaneous growth rates specifically.

In addition, we studied the covariation between survival, development, and body mass of larvae as well as responses at different factor combinations. For duration of development, where we had the largest data set, we compared the output of models fitting bivariate responses (accounting for covariation between development and survival) with univariate models for duration of development. We carried out a bivariate analysis in order to account for potential viability selection or the so called invisible fraction (Hadfield 2008). Because mortality may be

trait-selective (e.g. survivors may be those characterised by higher developmental rates), animals used to estimate trait values were not sampled at random, but instead were a sample contingent on the population of trait values of the survivors. Those analyses were carried out using generalised linear mixed models based on Monte Carlo Markov chain for parameter estimation, in R, using the package 'MCMCglmm' (more details on that analysis are provided in Text S1 of the Supplement).

## 3. RESULTS

For simplicity, we use the terms 'North Sea' vs. 'Baltic Sea' corresponding to the 'Helgoland' vs. 'Kerteminde' populations, but we clarify that we do not assume that these populations are representative of each region, as we recognise that responses may well vary considerably among populations located within each particular region (see Section 4).

### 3.1. Larval survival

Larval survival varied among populations and was driven by the combination of embryonic salinity, larval salinity, and larval temperature (Fig. 2, Table 1). Overall, females that produced larvae with high survival at a given temperature and salinity combination also produced larvae with high survival at other combinations (Tables S5 & S6). For the North Sea population, and when embryos developed in seawater, we found an antagonistic response to larval temperature ($L_T$) and salinity ($L_S$) consistent with TMLS previously reported (Spitzner et al. 2019). TMLS was found in the survival to the megalopa stage (Fig. 2a) in larvae reared at moderately low salinity ($L_S$ = 25 PSU); survival dropped down significantly to less than 20% at low temperatures (15 and 18°C), but remained high at higher temperatures (>60% at 21 and 24°C) as for larvae reared in seawater. At the lowest larval salinity ($L_S$ = 20 PSU), survival to megalopa was consistently low (Fig. 2a). For this population, TMLS was initially established at the zoea II stage, in larvae from all females reared at the lowest salinity (Fig. S1). High survival rates were found at moderately low salinity and in seawater, although responses varied among larvae from different females (Fig. S1). TMLS was strong for survival to the zoea II stage: average survival was high at the lowest salinity and highest temperature (= 70%); it was more than 2 times higher than the expectations of the joint probability (0.7 > 0.28 = 0.4 × 0.7) calculated as the product of the pro-

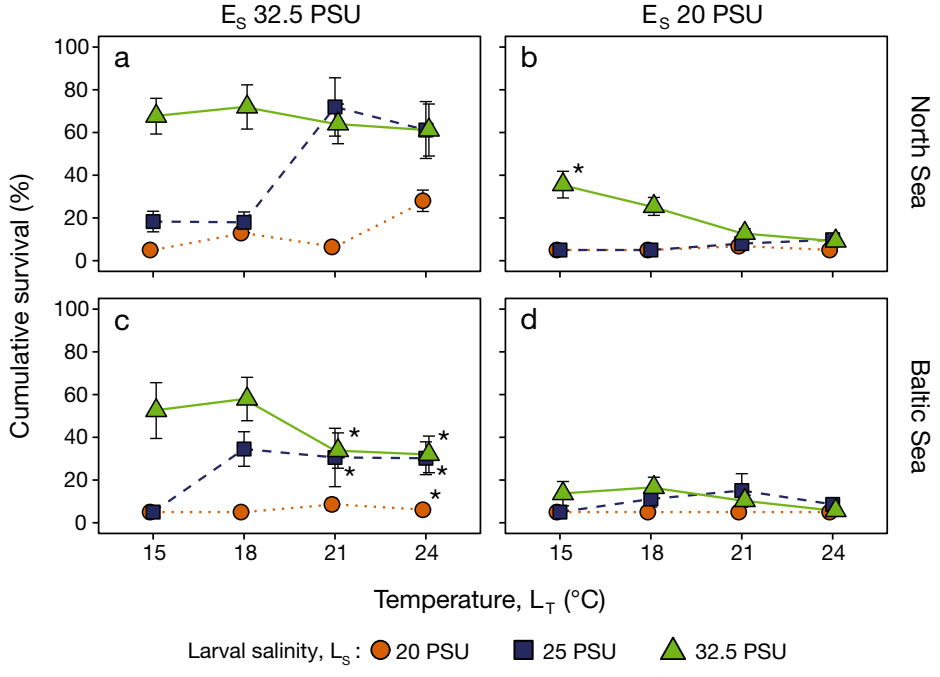


Fig. 2. *Carcinus maenas* cumulative average survival to megalopa. Comparison between populations from (a,b) the North Sea and (c,d) the Baltic Sea hatching at different embryonic salinities ($E_S$) for 12 combinations of larval temperature ($L_T$) and salinity ($L_S$). Symbols represent each combination of factors per population. Data shown as means ± SE among larvae produced by different females (n = 5 or 3). Asterisks represent significant differences between populations for each combination of $E_S \times L_S \times L_T$

portion survival at the lowest salinity (= 0.4 at optimal $L_T$ = 15°C) multiplied by that occurring under 21 or 24°C (= 0.7 at optimal $L_S$ = 32.5 PSU).

There were 4 main outcomes in the comparisons among embryonic salinity and population. First, for the North Sea population, low embryonic salinity resulted in an important and significant ($p < 0.001$) reduction in overall survival, a disruption of the TMLS, and a reduction of tolerance to high temperature (Fig. 2b; Fig. S2b). The best combination of temperature and salinity (15°C and seawater) led to only moderate survival (zoea II: 50%; megalopa: 35–40%), which was significantly lower than that of larvae hatching from embryos kept in seawater ($p < 0.01$; zoea II: >80%; megalopa: 70%). Larvae reared at low salinities had consistently low survival (Fig. 2b; Fig. S2b: no mitigation effect); in addition, increased temperature resulted in reductions in survival for larvae reared in seawater.

Second, larval survival from the Baltic Sea population was lower than that from the North Sea. Larvae reared at the lowest salinity had consistently the lowest survival to zoea II (Fig. S2c), irrespective of the female of origin (Figs. S4 & S5). There was also weak TMLS in larvae that originated from embryos reared in seawater and exposed to moderately low salinity (Fig. 2c; Fig. S2c); this weak response reflected a high variability among females in the effect of temperature on survival (Fig. S3).

Third, there were differences among populations in how embryonic salinity affected larval survival. In larvae from the Baltic Sea population, hatching from embryos exposed to low salinity, survival was consistently low irrespective of temperature, unlike the pattern observed for larvae from the North Sea (Fig. 2; Fig. S2).

Fourth, for the Baltic Sea population, larval survival did not peak at the lowest salinity expected to be experienced in the field. In addition, when embryos were kept in seawater, the highest survival occurred at the higher salinities (25 and 32.5 PSU compared to 20 PSU; for zoea II: $p < 0.001$). When embryos were kept at low salinity, survival was low irrespective of the salinity experienced by larvae (Fig. 2d; Fig. S2d). Lowest survival rates occurred at low embryonic ($E_S$ = 20 PSU) and larval salinity ($L_S$ = 20 PSU), which are the salinities experienced in the natural habitat.

### 3.2. Developmental time

Developmental time decreased at higher temperatures and increased at lower salinities, but also varied among population of origin (Figs. 3 & 4). There were 3 main responses in the developmental time to the zoea II. First, there was a clear effect of high temperature in decreasing developmental time, which varied slightly among salinities and populations (Fig. 3). Second, the effect of larval salinity (a 1–3 d increase in developmental time) was only present in larvae from the North Sea hatched from embryos kept at low salinity (Fig. 3b). Third, for both populations, low embryonic salinity resulted in longer larval development especially at low temperature (5–7 d longer at 15°C, $p < 0.001$, vs. ca. 3.5 d longer at 24°C, $p < 0.05$).

For the megalopa stage, the best models retained interactions between the 3 terms tested ($L_T \times L_S$ and $P \times L_S$; embryonic salinity, $E_S$ = 20 PSU was not considered due to low survival). Developmental time to megalopa showed a pattern consistent with TMLS (Fig. 4) especially for larvae from the North Sea population. In that population, at 25 PSU, developmental time was 5 d longer at 15°C ($p < 0.001$), but there was no significant delay in development at 21–24°C compared to larvae reared at 32.5 PSU. In addition, larvae from the North Sea had in general lower developmental times than those of the Baltic Sea population.

Bivariate models (based on 'MCMCglmm') indicated that covariances between developmental time and survival included zero in the credible interval for the random structure (Table S7), but not for the error structure (Table S8). Correlations between survival and developmental time were mostly negative, indicating that faster development was associated with higher survival (Fig. S6). In general, comparisons of results between bivariate and univariate models led to similar credible intervals (Tables S9–S11) except for a single estimate (Table S9). Overall, our interpretation is that the conclusions drawn from univariate analyses were robust to covariation between survival and developmental time.

### 3.3. Biomass and elemental composition

Dry mass (DW) and carbon (C) and nitrogen (N) content of the freshly hatched zoea I varied between populations and embryonic salinity treatments (Fig. 5). When embryos were kept in seawater, newly hatched larvae from the North Sea population had significantly higher DW and C and N content as compared to those from the Baltic Sea population (e.g. DW in the North Sea vs. Baltic Sea population: 10.7

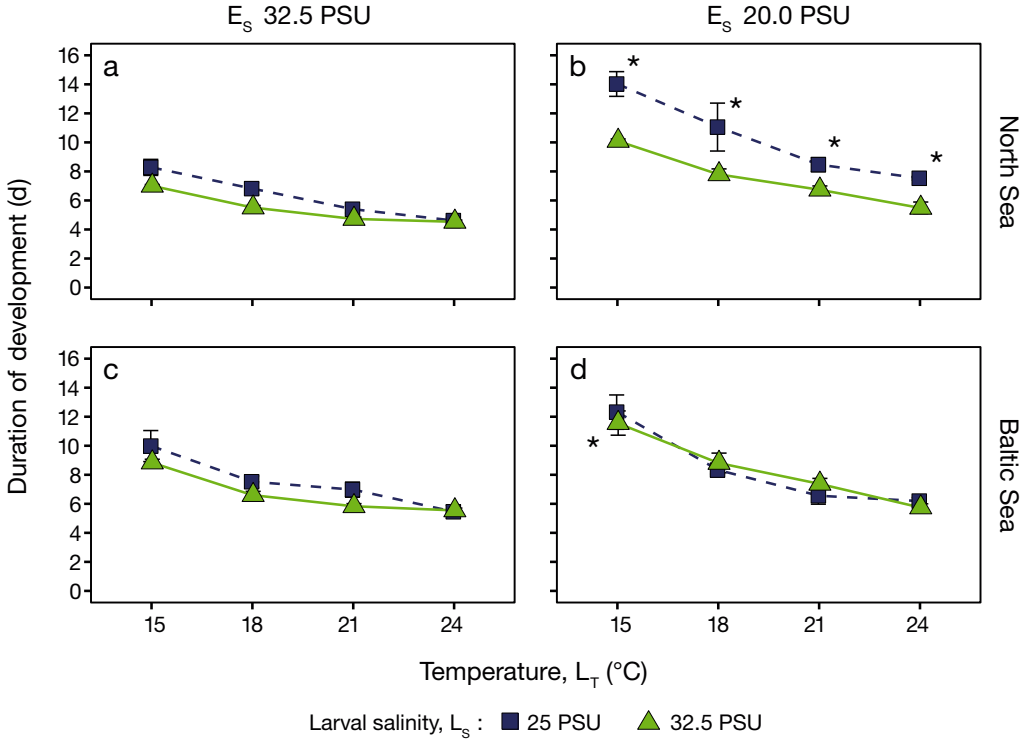


Fig. 3. Duration of *Carcinus maenas* larval development to zoea II. Comparison between populations hatching in different embryonic salinities ($E_S$) for 8 combinations of larval temperature ($L_T$) and salinity ($L_S$): (a,b) North Sea, (c,d) Baltic Sea. Other details as in Fig. 2

vs. 8.12 µg ind.$^{-1}$, respectively, i.e. ~30% lower in the Baltic Sea population). In the North Sea population, low embryonic salinity resulted in a reduction by ~20–30% of DW and C and N content ($p < 0.05$). For the Baltic Sea, embryonic salinity conditions did not affect body mass or reserves at hatching.

The dry mass and elemental composition of the megalopae varied between populations and responded to the larval conditions (Fig. 6; Fig. S7); note that all megalopae developed from larvae originated from embryos kept in seawater. The best statistical model retained the 3-way factorial interaction P × $L_S$ × $L_T$ for the inter-population comparison (Table S3), and 2-way interaction $L_S$ × $L_T$ for analysis of the North Sea population (Table S4). For the North Sea population, the combined effect of larval temperature and salinity was consistent with TMLS: low salinity resulted in a decrease in biomass at low temperatures (24–26%; Fig. 6), but such reduction was smaller at higher temperatures (<15%), especially in terms of carbon and nitrogen content (Fig. S7). By contrast, in larvae from the Baltic Sea

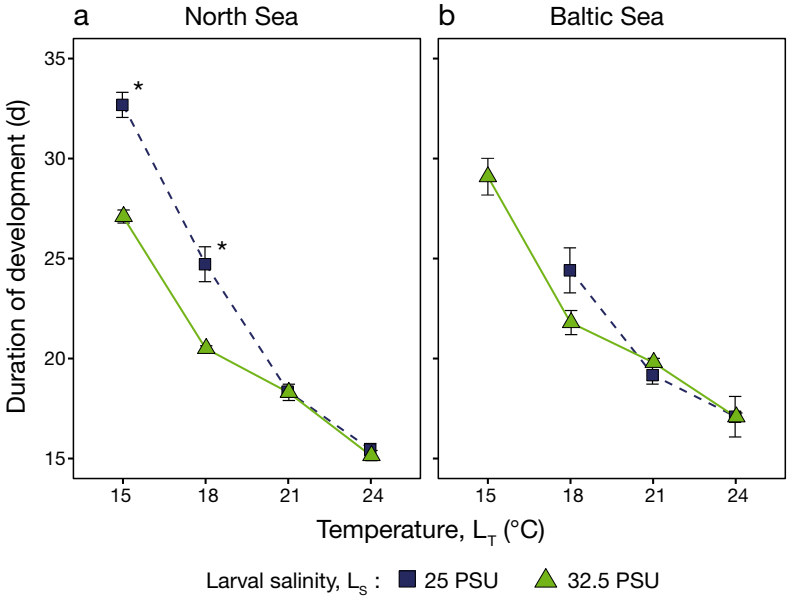


Fig. 4. Duration of *Carcinus maenas* larval development to megalopae from embryos reared at $E_S$ = 32.5 PSU. Comparison between populations from (a) the North Sea and (b) the Baltic Sea for 8 combinations of larval temperature ($L_T$) and salinity ($L_S$) (note that $L_S$ 20 PSU is excluded). Other details as in Fig. 2

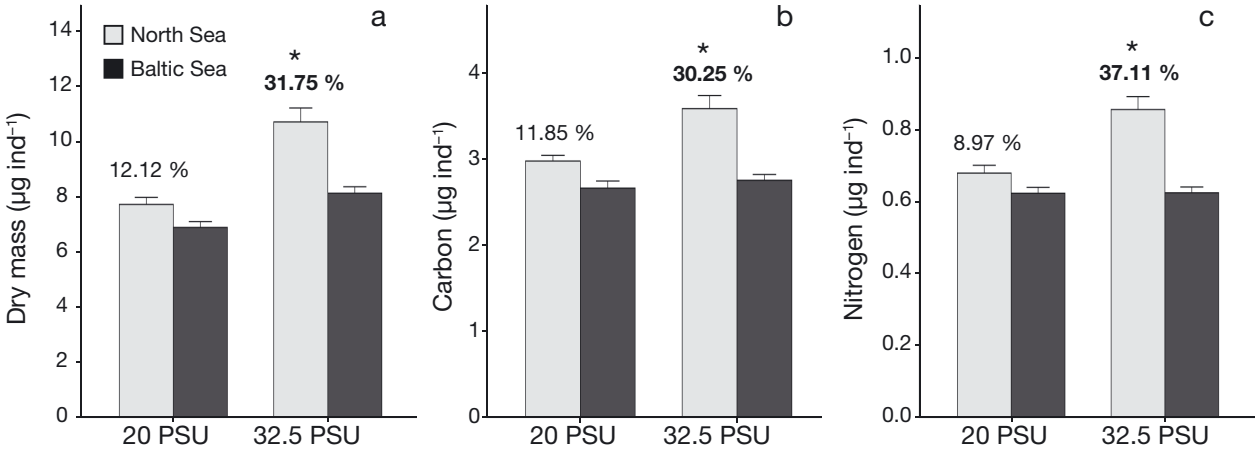


Fig. 5. (a) Dry mass, and (b) carbon and (c) nitrogen content (µg ind.$^{-1}$) of freshly hatched *Carcinus maenas* zoea I after exposure to 20 or 32.5 PSU during embryonic development (embryonic salinity, $E_S$) from 2 populations (North and Baltic Seas). Bars represent mean values for each combination of $E_S$ and population. Error bars represent ±SE among larvae produced by different females (n = 5 or 3). Percentages above bars represent significant differences between populations at each $E_S$. Asterisks represent significant differences between $E_S$ values

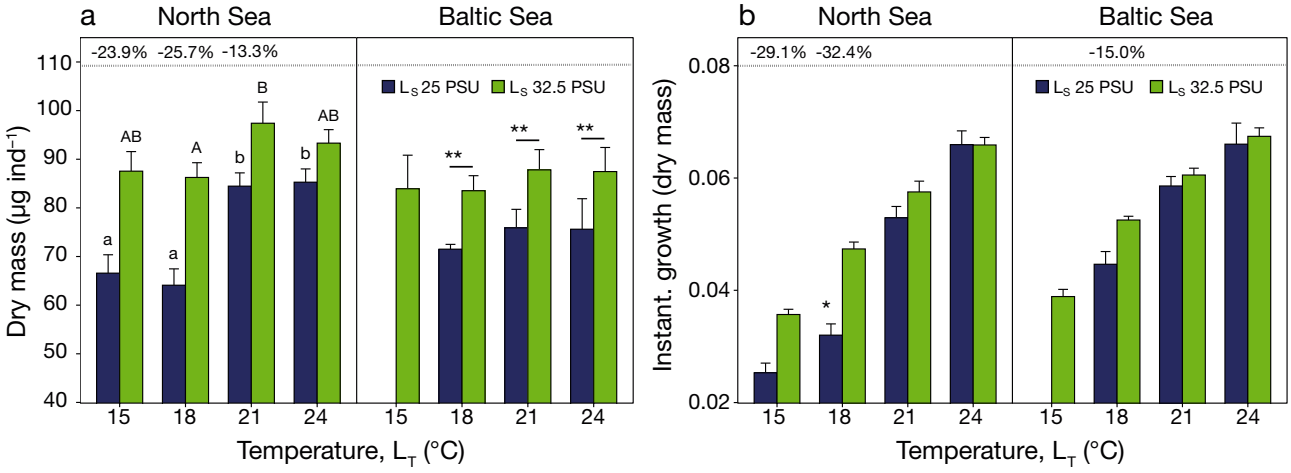


Fig. 6. (a) Dry mass of *Carcinus maenas* megalopae and (b) instantaneous growth rates (based on dry mass) from hatching to megalopa for 2 populations (North and Baltic Seas) reared at 2 larval salinities ($L_S$: 32.5 PSU, green; and 25 PSU, blue) and 4 temperatures ($L_T$: 15, 18, 21, and 24°C). Data shown as means ± SE of individual biomass (µg ind.$^{-1}$) and instantaneous growth rates (d$^{-1}$) among larvae produced by different females (n = 5 for North Sea, n = 3 for Baltic Sea). Different letters represent significant differences for total dry mass between temperature treatments (lowercase = $L_S$ 25 PSU, capitalised = $L_S$ 32.5 PSU). Percentages above panels represent significant differences between larval salinities at each temperature in each population. (*): significant differences in the instantaneous growth rates between populations for the same treatment; (**): significant differences between larval salinities in the Baltic Sea population (additional 3-way ANOVA analysis of larval salinity excluding 15°C)

population, low salinity had a consistent negative effect on dry mass and elemental composition, irrespective of temperature (Fig. 6; Fig. S7). In addition, dry mass, C and N content were higher in larvae from the North Sea than in those from the Baltic Sea, but such differences were significant only for C content (see Fig. S7) in larvae reared at low salinity and at 24°C (overall, larvae from the North Sea population had 15.9% higher carbon content). The C:N ratios (Fig. S8) did not indicate any proportional change in the C or N fractions regardless of the population of origin or in response to any treatments. Correlations between survival and dry mass were either positive or non-significant, indicating that high larval survival was associated with large body mass (Fig. S9).

Instantaneous growth rates from hatching to megalopa increased with temperature in both populations (Fig. 6; Fig. S7). For the North Sea population, growth rates showed a pattern consistent with TMLS, as in

the case of body mass and duration of development, i.e. reductions in growth rates were found only for the combination of low temperature and salinity. Larvae from the Baltic Sea population were less affected by low salinity, with a significant reduction in dry mass only at 18°C ($p < 0.05$; Fig. 6). Significant differences between populations appeared only at low salinity and at 18°C, where larvae from the North Sea population had ~25% lower instantaneous growth rates than those from the Baltic Sea (Fig. 6; Fig. S7).

The integrated responses of growth, developmental time, and body size are shown in Fig. 7 and in Fig. S10. For the North Sea, TMLS was observed as an integrated response, especially in the carbon and nitrogen content. Larvae reached an upper body mass threshold when reared in seawater, especially at the highest temperatures (e.g. at 21 and 24°C: C ~34–36 µg ind.$^{-1}$, DW ~90 µg ind.$^{-1}$); almost the same thresholds were reached when reared at low salinity and the highest tested temperatures without any increase in developmental time. By contrast, at lower salinities and temperatures, larvae metamorphosed at lower biomass thresholds in spite of extended development. For the Baltic Sea population, patterns were not consistent with TMLS; the maximum body mass (e.g. at 21 and 24°C: C ~32–34 µg ind.$^{-1}$, DW ~85 µg ind.$^{-1}$) was reached by larvae reared in seawater; those reared at low salinity were not able to reach that threshold irrespective of an extension in developmental time.

## 4. DISCUSSION

Here, we show that responses to temperature and salinity can vary considerably between populations of the same species. Using females, collected during the same reproductive season, we confirmed that most larvae hatching from females collected on Helgoland (North Sea) exhibited TMLS, which is consistent with a previous study by Spitzner et al. (2019) for the same population (females collected in consecutive previous years, 2016 and 2017). In addition, the higher survival in seawater is consistent with the fact that the larvae from the population of Helgoland are likely to develop under seawater conditions. For the Kerteminde population (Baltic Sea), we hypothesised that larvae would show a shift in the pattern of tolerance towards low salinities or an increase in the degree of euryhalinity. However, the best survival occurred in seawater instead of at the lowest salinities tested, especially in larvae that originated from females kept at low salinity; survival decreased towards lower salinity and higher temperature. The response to low salinity was surprising because embryos and larvae in Kerteminde should experience similar salinities as the adults (i.e. ~15–20 PSU).

For the Helgoland population, TMLS was exhibited in terms of the integrated effects on developmental time, body mass, and growth rate. At high temperatures, instantaneous growth and developmental rates were not affected by salinity. From an

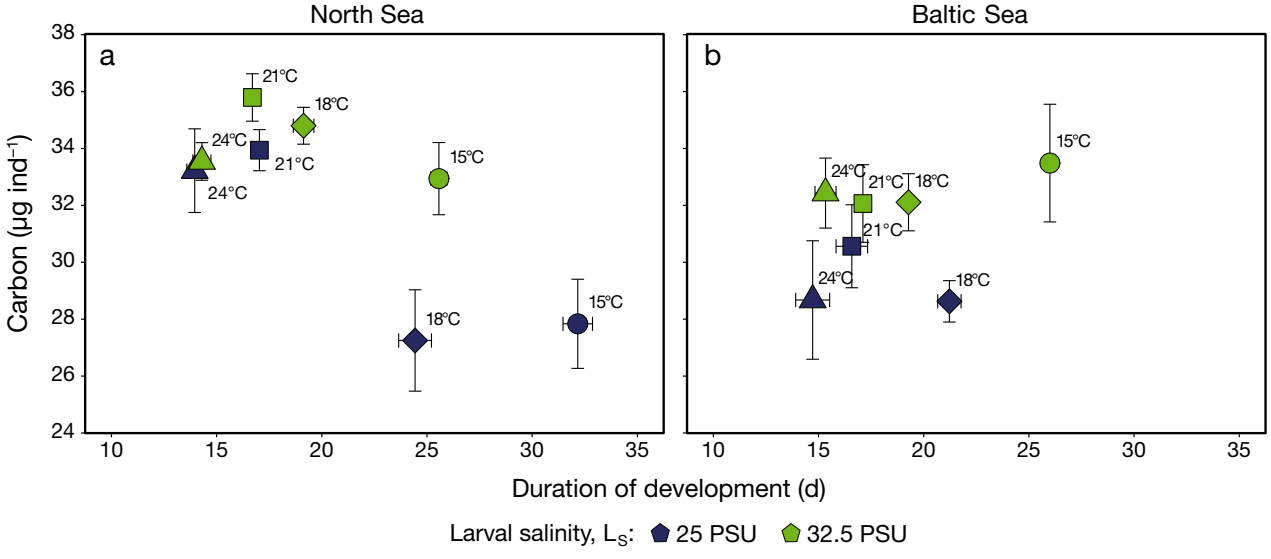


Fig. 7. Integrated responses of carbon content and developmental time for *Carcinus maenas* megalopae presented for populations from (a) the North Sea and (b) the Baltic Sea, and different combinations of larval temperatures ($L_T$) (15°C = circles, 18°C = diamonds, 21°C = squares, 24°C = triangles) and larval salinities ($L_S$) (25 PSU = blue, 32.5 PSU = green). Error bars represent ±SE among larvae produced by different females (n = 5 for the North Sea, n = 3 for the Baltic Sea)

ecological standpoint, the observed trait values of the survivors after developing at low salinities (reduced body mass at metamorphosis) may reduce post-metamorphic survival (Pechenik et al. 1998, Pechenik 2006, Torres et al. 2016); such effects would be minimised under increased temperatures, as long as larvae are not food-limited (Torres & Giménez 2020). Overall, for the Helgoland population, we report for the first time a consistent mitigation effect of high temperatures on physiology and survival that may favour the use of estuarine habitats, at least for limited time periods, under warming scenarios. By contrast, for the Kerteminde population, negative effects of low salinity on instantaneous growth dominated the response of body reserves at metamorphosis. Hence, in addition to the reductions of larval survival, one would expect ecological consequences of low salinity after metamorphosis (associated with reduced body mass) consisting of reduced post-metamorphic survival. The general low tolerance to low salinity of larvae from Kerteminde is striking and may be considered maladaptive, given that the Baltic Sea is characterised by low salinity.

Because both populations are located at sites differing in the surrounding salinities, we hypothesised that the salinity experienced during embryogenesis could modify the larval responses to temperature and salinity in a way that is adaptive to each population. We knew that such a post-zygotic maternal effect could have profound effects on salinity tolerance (Giménez & Anger 2003) by increasing osmoregulatory capacity (Charmantier et al. 2002), as osmoregulation provides buffering effects of low salinity on larval performance (Torres et al. 2011). Hence, for the Kerteminde population, we expected that low salinity experienced by embryos would enhance larval performance at low salinities, or at least not impair it. However, low embryonic salinity caused reduction of larval performance in the Kerteminde population, contrary to our expectations. Hence, the post-zygotic maternal effects of the local population of Kerteminde may be considered maladaptive.

We know that adults of *Carcinus maenas* exhibit adaptive responses to low salinity conditions in the Baltic Sea (Theede 1969). In addition, larvae of populations from the coastal waters of the North Sea vs. the Baltic Sea exhibit different behavioural traits that are adaptive for the different hydrodynamic conditions experienced in each sea (Moksnes et al. 2014: comparison of larval vertical migration patterns). So why does larval tolerance not exhibit patterns that are adaptive to the salinity conditions surrounding the Kerteminde population? We do not know if the maladaptive response of the Kerteminde population is characteristic of other Baltic Sea populations or is driven by local conditions characterising the Kerteminde Fjord (e.g. maternal nutrition, presence of additional drivers). Any other known responses for the European continent, experiencing comparable salinity conditions (Isle of Man, UK: Nagaraj 1993; North Wales, UK: Torres et al. 2020; Cádiz, Spain: unpubl. data), are similar to the one found for the Helgoland population. Maladaptive responses to temperature and salinity have, however, been found in populations of an estuarine barnacle, also occupying habitats in the Baltic Sea (Nasrolahi et al. 2016). Maladaptive responses may be maintained by gene flow (Kawecki & Stearns 1993, Bolnick & Nosil 2007, Farkas et al. 2016), sustained by larvae arriving to the Kerteminde Fjord from other local populations of the Baltic Sea or perhaps from the North Sea. Perhaps Kerteminde harbours a sink population; in theory, subsidy from sink to source populations can contribute to species distributions in areas characterised by environmental gradients (Dauphinais et al. 2018, Giménez et al. 2020).

Alternatively, larvae sustaining the population from Kerteminde develop in microhabitats characterised by increased salinity, or they develop under temperature conditions that are much lower than those tested here. Larvae may develop in deeper waters characterised by higher salinities and perhaps lower temperatures. While females in the North Sea are found in the intertidal area, those of the Baltic Sea are subtidal; perhaps larvae stay close to the bottom. However, studies of larval behaviour suggest diel vertical migrations (from bottom to surface waters) in larvae from Baltic Sea populations (Moksnes et al. 2014), which would predict that larvae should occupy near-surface waters with reduced salinity, at least for limited periods. In addition, the overall differences in larval performance would suggest increased larval mortality in those produced by the population in Kerteminde, irrespective of temperature and salinity. Overall, given the current evidence, it is difficult to envisage a scenario other than the one in which the local population of Kerteminde is subsidised from other nearby populations (taking into consideration that larvae are the main dispersal stages in this species).

A potential physiological driver for the reduced performance in the Kerteminde population could be the reduced body mass at hatching, which coincided with that observed in larvae from the Helgoland population hatching from embryos kept at low salinity. Poor performance associated with low body mass

at hatching has been found in previous studies (Giménez & Anger 2001, Marshall & Keough 2007, González-Ortegón & Giménez 2014, Oliphant et al. 2014). Reduced larval body mass, a proxy for body size, is associated with lower metabolic efficiency (Pettersen et al. 2015, Marshall et al. 2018) and it is likely to constrain the capacity to capture prey. Perhaps drivers of reduced body mass (e.g. local food availability at the time of maternal allocation of reserves to eggs) are responsible for the overall poor larval performance of the Kerteminde population.

In synthesis, irrespective of the underpinning mechanisms, our study highlights important differences among populations of the same species in the capacity to cope with various salinity and temperature combinations. Hence, when asking questions about 'winners' or 'losers' (Somero 2010), we cannot make judgements based on single population studies. For the Helgoland population, increased larval performance is found consistently under salinity conditions of the North Sea. However, the responses to low salinity found for the Kerteminde population are maladaptive for the natural conditions of the Baltic Sea and represent a form of 'ecological surprise' (Filbee-Dexter et al. 2017), highlighting the fact that adaptive responses should not be expected by default. We emphasise the importance of incorporating a multi-population approach and considering effects of the maternal environment on offspring responses in multiple stressors research. By expanding the spatial scale of observation, from local to regional, we could obtain a more complete picture of species responses to climate-driven changes in environmental variables.

*Acknowledgements.* We thank Dr. Magnus Wahlberg (University of Southern Denmark) for access to the Kerteminde population. We gratefully acknowledge Julia Haafke (AWI) for performing the elemental analysis and Siri Rohwer (University of Greifswald) for help with larval rearing. This study is part of the doctoral dissertation of Z.Š. and was supported by the Deutsche Forschungsgemeinschaft (Research Training Group 2010: RESPONSE) at the University of Greifswald, in a collaboration with the Alfred-Wegener-Institute. The research presented in this paper complies with the guidelines from the directives 2010/63/ EU of the European parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. G.T. and L.G. acknowledge support by the Open Access Publication Fund of Alfred-Wegener-Institut Helmholtz-Zentrum für Polar- und Meeresforschung.

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*Editorial responsibility: Pei-Yuan Qian, Kowloon, Hong Kong SAR*
*Reviewed by: 3 anonymous referees*

*Submitted: January 19, 2021*
*Accepted: August 2, 2021*
*Proofs received from author(s): October 21, 2021*

## Publication II

Manuscript submitted to Marine Biology for peer-review:

**Šargač Z**, Giménez L, González-Ortegón E Harzsch S, Tremblay N, Torres G (2021)

# Quantifying the portfolio of larval responses to salinity and temperature in a coastal-marine invertebrate: a cross population study along the European coast

# Quantifying the portfolio of larval responses to salinity and temperature in a coastal-marine invertebrate: a cross population study along the European coast

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## Abstract

Species' responses to climate change may vary considerably among populations. Various response patterns define the portfolio available for a species to cope and mitigate effects of climate change. Here, we quantified variation in larval survival and physiological rates of *Carcinus maenas* among populations occurring in distant or contrasting habitats (Cádiz: Spain, Helgoland: North Sea, Kerteminde: Baltic Sea). During the reproductive season, we reared larvae of these populations, in the laboratory, under a combination of temperatures (15-24 °C) and salinities (25 and 32.5 PSU). In survival, the Cádiz and Helgoland populations showed a mitigating effect of high temperatures at lower salinity, and stress effects of low salinity at low temperatures. However, Cádiz differed from Helgoland in that a strong thermal mitigation did not occur for growth and developmental rates. For all populations, respiration rates were driven only by temperature; hence, these could not explain the growth rate depression found at lower salinity. Larvae from Cádiz, reared in seawater, showed increased survival at the highest temperature, which differs from Helgoland (no clear survival pattern) and especially Kerteminde (decreased survival at high temperature). These responses from the Cádiz population correspond with the larval and parental habitat (i.e. high salinity and temperature) and may reflect local adaptation. Overall, along the European coast, *C. maenas* larvae showed a diversity of responses, which may enable specific populations to tolerate warming and subsidise more vulnerable populations. In such case, *C. maenas* would be able to cope with climate change through a spatial portfolio effect.


**Keywords:** *Carcinus maenas*, intraspecific trait variation, larval performance, multiple stressors and drivers, phenotypic physiological plasticity, thermal tolerance

## 1. Introduction

Global change already has a major impact on marine ecosystems through changes in environmental variables such as water temperature, salinity, pH and oxygen content (Wiltshire et al. 2010; Doney et al. 2012; Poloczanska et al. 2013; Boersma et al. 2016; Molinos et al. 2016; Boyd et al. 2018). However, patterns of change vary spatially depending on regional scale habitat characteristics. For instance, how anthropogenic or other types of climate change drive environmental conditions will differ between open ocean, estuarine, and nearshore habitats; coastal areas in particular are likely to experience comparatively drastic changes in environmental conditions (reviews Hofmann et al. 2011; Duarte et al. 2013). For instance, these areas are increasingly affected by rising surface temperatures and changes in river runoff (Gräwe et al. 2013; Hiddink et al. 2015; Robins et al. 2016; Reusch et al. 2018).

As in most cases with two or more climate-driven environmental variables (=drivers: Crain et al. 2008; Piggott et al. 2015; Orr et al. 2020; Tekin et al. 2020), the combination of lower salinity in a scenario of increased temperature is likely to elicit complex interactive responses. The interplay between drivers can evoke many non-independent effects, where one driver can enhance (synergistic effect) or weaken (antagonistic effect) the impact of the other driver beyond the additive effect expected from the action of each driver considered in isolation (e.g. see reviews Orr et al. 2020; Tekin et al. 2020). In coastal species, tolerance to salinity can be altered by temperature, leading to either synergistic or antagonistic effects depending on the species (e.g. Janas and Spicer 2008; Nasrolahi et al. 2012; Spitzner et al. 2019; Torres et al. 2021a).

A critical point is that survival and physiological responses to temperature and salinity may vary among populations, especially for those species with a wide spatial distribution. In situations where the magnitude of variation is important, single population studies may not be useful to characterise the responses of a given species. There is an increasing body of work showing that responses to climate drivers vary considerably at the intraspecific level (Carter et al. 2013; Durrant et al. 2013; Applebaum et al. 2014; Spitzner et al. 2019). Intraspecific variation in responses to suboptimal conditions arise from phenotypic plasticity and evolutionary adaptation (Chevin et al. 2010; Reusch 2014; Sinclair et al. 2013; Boyd et al. 2018), and local genetic adaptation is likely to be expressed as variation among populations in the responses (De Villemereuil et al. 2016). Irrespective of the mechanism driving phenotypic variation, recent studies have further pointed out the necessity to incorporate intraspecific trait

variation into models of community structure in response to climate change (Bolnick et al. 2011; Violle et al. 2014; Moran et al. 2016). Intraspecific variation in responses can drive portfolio effects (Greene et al. 2010; Schindler et al. 2015; Price et al. 2021), whereby diversity in the types of responses buffers a species from environmental variation. When such variation occurs among populations, a spatial portfolio effect should occur if adult migration or dispersion of early stages ensures sufficient connectivity among local or regional populations because populations with more resilient genotypes would subsidise the other populations (Casaucao et al. 2021). Importantly, in species of commercial interest, portfolio effects can ensure food security, but climate change and anthropogenic activities can lead to portfolio simplifications (Price et al. 2021). Along this line, quantifying intraspecific variations, but also the current portfolio, is useful to identify populations that may be more resilient or sensitive to climate change.

Within each population, developmental, growth, and other physiological rates must be maintained in a scenario of increased temperatures. These rates are key to determine fitness-related traits such as body size, and the amount of nutritional reserves needed to survive under e.g. food limitation. Salinity and temperature are known to govern physiological rates and survival in many marine organisms (Pörtner 2001; Anger 2003; Somero 2005; Sokolowa et al. 2012). Understanding how such rates are driven by temperature and salinity is important for the development of mechanistic physiological based models predicting species' responses. In most metazoans, the physiological processes associated with activities, growth, and development rely on the oxygen consumption (Hochachka and Somero 1984; Burggren and Roberts 1991), which is usually quantified as respiration rates. Physiological rates can also be viewed as a series of linked traits governing body mass at critical life history events, such as metamorphosis in species with complex life cycles (Werner 1988; Hentschel 1999; Gotthard 2001; Heyland et al. 2011; Torres and Giménez 2020). Body size, or reserves at metamorphosis, are indicators of post-metamorphic fitness because they explain variations in post-metamorphic survival (Jarrett 2003; Giménez et al. 2004; Pechenik 2006; Tremblay et al. 2007; Torres et al. 2016). Variations in body mass or reserves at metamorphosis may not only reflect passive stress responses to a given condition, but instead may constitute a part of an adaptive compensatory mechanism (Gotthard and Nylin 1995). For instance, increased developmental time in response to suboptimal food conditions may constitute a compensatory response to maintain body mass at metamorphosis despite reduced growth rates. In this example, extended development may minimise the fitness costs associated with

metamorphosing with a reduced body size, at expenses of (presumably lower) costs associated with delaying metamorphosis. We know little about such multiple trait responses to salinity and temperature, in early life history stages, where growth and development are critical. In ectotherms, increased temperatures accelerate developmental rates, but the effects of growth may vary on the patterns of thermal tolerance. Moreover, increased temperature in organisms exposed to optimal salinity results in increased respiration rates (Ikeda 1970; Anger 2001; Saborowski et al. 2002) up to a point where oxygen demands cannot be matched by appropriate increase in oxygen supply (Pörtner 2001, 2012; González-Ortegón et al. 2012). At such levels, the metabolic demands for oxygen exceed the organism's ability to distribute oxygen to tissues, and organismal performance decline (Deutsch et al. 2015; Pörtner and Knust 2007; Giomi and Pörtner 2013).

We also know that responses to decreased salinity can vary among species depending on the degree of euryhalinity, with increases in respiration rates observed in euryhaline species and decreases in stenohaline species or at salinities beyond the tolerance range for euryhaline species (Kinne 1971; Anger 2001). Osmoregulation involves ion regulation (performed by the $Na^+$-$K^+$ ATPase) at expenses of ATP, thus, high energy demands (and hence respiration rates) are expected in euryhaline species when exposed to extreme salinities. By contrast, lower salinity may reduce growth rates (Torres et al. 2011), slow development, and depress metabolism through perturbations of cellular level processes and eventually damage. We know indeed much less about how developmental, growth and respiration rates in larval stages respond to the combined action of lower salinity and increased temperature. This multiple driver scenario may result in a reduction in the critical temperature at which thermally driven metabolic depression occurs (Yagi et al. 1990) or may depress metabolic rates at all temperatures. Overall, the interactive nature of multiple driver effects and the fact that respiration rates may both increase or decrease in response to suboptimal conditions would diminish our trust in extrapolation based on current information.

Here, we quantify the effects of reduced salinity and increased temperature on the respiration, developmental, and growth rates of larvae of the shore crab *Carcinus maenas* obtained from three European populations (Cádiz: Cádiz Bay, Helgoland: German Bight, Kerteminde: Baltic Sea), located along its native range (Fig. 1a; Leignel et al. 2014; Young and Elliot 2020). The life history of *C. maenas* is characterised by a complex life cycle (Spitzner et al. 2018); females are benthic (=bottom dwelling), carry eggs and release larvae in the water column. Larvae drift in the sea and develop through four pelagic stages (zoeae),

followed by a settling stage (megalopa) that colonises the parental habitat (sheltered intertidal zones). We were interested in determining how responses of different performance rates would be shaping the integrated phenotype in terms of body mass, carbon, and nitrogen content. Additionally, we wanted to investigate if such responses were consistent among populations located as far away as the Cádiz Bay and the German Bight or the Baltic Sea, as a way to test if a general response pattern emerges. This study is based on (1) published information (Šargač et al. 2021) of survival, growth and developmental rates (Helgoland and Kerteminde populations, presented as grey sections in the figures), (2) unpublished information on similar rates from a population from Cádiz and (3) unpublished information on respiration rates for the three populations. We studied larval stages because larval supply and settlement is central for long-term persistence of marine benthic populations (Morgan 2020). Because larvae are a key driver of population connectivity (Armsworth 2002; Cowen and Sponaugle 2009), they are central to a potential portfolio effect by recovering local populations from mass mortality (Giménez et al. 2020). However, larvae are particularly vulnerable to environmental changes (Pandori and Sorte 2019) and the larval physiological quality can drive recruitment and connectivity (Tremblay et al. 2007; Shima and Swearer 2009).

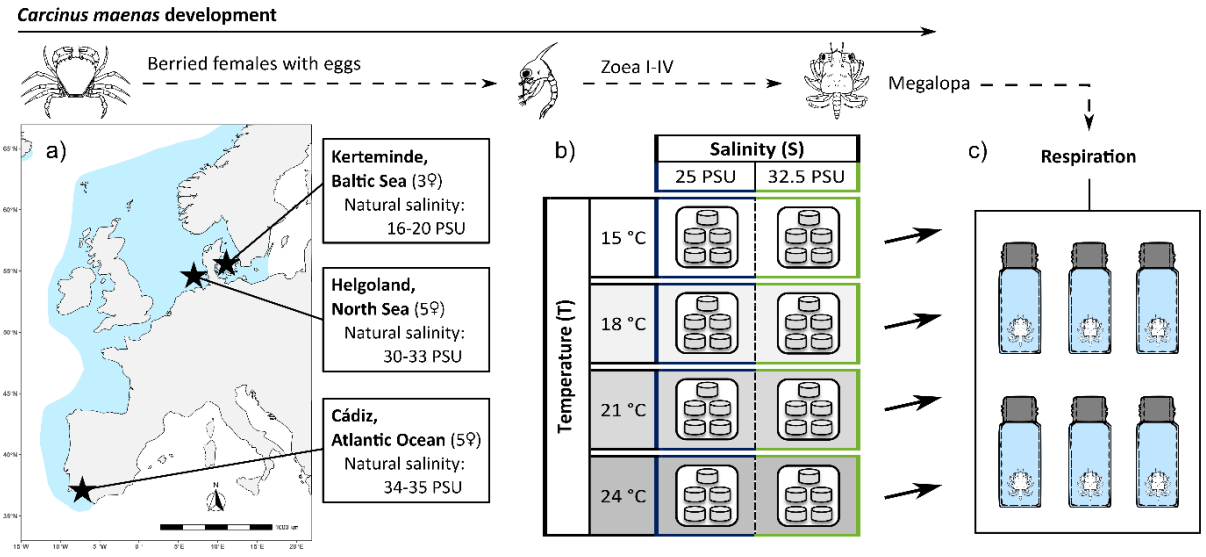



**Figure 1.** *Carcinus maenas*. Experimental setup and design. a) Native range and collection sites: berried females with eggs were collected from three different populations (black stars) across the native European range (blue area). Symbols (♀) correspond to the number of females that successfully produced larvae from each population. b) Larval rearing: freshly hatched larvae from each population and female were reared in a factorial design consisting of eight

combinations of temperature, T (15, 18, 21, and 24 °C) and salinity, S (25 and 32.5 PSU) in a common garden setup. Each combination of population × female × T × S had five replicate bowls (60 mL) with ten randomly assigned larvae in each. c) Respiration measurements design: once the larvae moulted to the megalopa stage, they were randomly taken into 4 mL vials and respiration rates were measured. Six megalopae were taken from each combination of population × female × T × S, fasted for 2 h and oxygen consumption was measured for each megalopa individually. Additionally, six replicate vials without animals were placed as a control.

We know that larvae of *C. maenas* can exhibit an antagonistic response by which the negative effect of lower salinity (range 20-32 PSU) on survival and growth is mitigated at higher temperatures (range 15-24 °C). This response has been called "Thermal Mitigation of Low Salinity stress" (abbreviated as TMLS, Spitzner et al. 2019) and has been found in other coastal crustaceans (e.g. Janas and Spicer 2008; Nasrolahi et al. 2012). We also know that for survival and development, the magnitude of TMLS can vary within and among populations (Spitzner et al. 2019; Torres et al. 2020; Šargač et al. 2021). TMLS has been found in both the North and Irish Sea, i.e. in areas that are characterised by moderately high salinities (30-35 PSU), but it is much weaker in the local population of the Baltic Sea (Kerteminde, Denmark: Šargač et al. 2021) where salinities are considerably lower (15 PSU). In the population of Kerteminde, lower salinity (20 PSU) results in the lowest survival and growth rates at all tested temperatures, although this population is exposed to low salinities (15-20 PSU) for most of the life cycle. Hence, *C. maenas* is representative of a coastal species with important intraspecific variations in the responses to lower salinity and higher temperature at the regional scale. However, the question is whether populations located as far as Cádiz show similar responses than those experiencing coastal marine waters further north (Helgoland: Spitzner et al. 2019; Irish Sea: Torres et al. 2020). Another unknown is whether respiration rates respond to salinity and temperature consistently among populations or whether we would observe variations associated to the distance between populations or to the different salinity habitats. We quantified respiration rates at the megalopa stage for which we have previous information on the effect of temperature on such rates (Dawirs 1983), but not about the combined effect of temperature and salinity nor about whether responses vary among populations. One can derive several hypothetical scenarios in responses of respiration rates with that of survival and other physiological rates. First, one may find consistent responses across all rates such that among

population differences are manifested as a global type of response. Alternatively, differences between populations in the rates may not match, which would indicate differences in the sensitivity to the drivers across different physiological processes.

## 2. Materials and methods

### Female collection, transport, and maintenance

*Carcinus maenas* berried females (with early embryos, carapace length = 2.55-5.10 cm) were collected during their reproductive period in the same year from three European locations (Fig. 1a): Helgoland (North Sea, Germany, coordinates: 54° 10' 49.176" N, 7° 53' 20.198" E, salinity = 30-33 PSU); Cádiz (Gulf of Cádiz, Spain, coordinates: 36° 33' 34.5" N 6° 12' 23.1" W; salinity = 34-35 PSU) and Kerteminde (Baltic Sea, Denmark, coordinates: 55° 26' 59.9" N, 10° 39' 40.1" E; salinity = 16-20 PSU). Females from the Cádiz population were hand-collected in February-March from the intertidal muddy shore of the San Pedro River next to the Institute of Marine Science of Andalusia (Cádiz, Spain). Females from Kerteminde were caught in April-May using traps (Torres et al. 2021b) in the subtidal fjord next to the Marine Biological Research Centre (University of Southern Denmark, Kerteminde, Denmark). Prior to transport to the Marine Biological Station at the Alfred-Wegener-Institute (Helgoland, Germany), the collected berried females were kept in aquaria with a flow through system of natural water with gentle aeration reflecting natural salinity and temperature conditions on the site (35-37 PSU and 17±3 °C in Cádiz; 16-20 PSU and 10±1 °C in Kerteminde). In preparation for transport, females were placed individually in 1 L plastic containers, half-filled with strongly aerated water from the site of origin, and a wet towel. All containers were transported in a Coleman® cooler box to ensure a constant temperature of the natural local conditions during transport (ca. 24-36 h). Females from the Helgoland population were collected manually during April-June from the intertidal rocky shore and directly transported to the Marine Biological Station. For those females, we simulated (for 24-36 h) the transport conditions experienced by the animals from the other two populations. After arrival to the laboratory (or the simulation of transport stress), females from each population were placed individually in 5 L aquaria filled with UV-treated filtered natural seawater from Helgoland (32.5 ± 1 PSU) and gentle aeration. Animals were placed at the same temperature as the time of collection which corresponds to their natural habitat of origin (i.e., 10, 15, and 16 °C for Kerteminde, Cádiz, and Helgoland, respectively), then temperature was gradually increased (1 °C per day) until the same common embryonic

temperature (18 °C) was reached for all populations. Females were fed twice a week (frozen shrimp *Crangon crangon*) and water was changed daily.

**Experimental design**

Freshly hatched larvae originated from each female were randomly assigned to eight combinations of four temperatures (15, 18, 21, and 24 °C), and two salinities (25 and 32.5 PSU) in a factorial design (Fig. 1b). Larvae were reared in groups of 10 individuals in five replicate bowls (60 mL) per treatment combination (Fig. 1b). This design was repeated with larvae of each different female separately. A total of five females from each population were used in the experiments, except for those originated from Kerteminde, where only larvae from three females were available to be included in the experiments (Fig. 1a). Note that for the Kerteminde population, measurements at the lowest temperature (15 °C) and salinity (25 PSU) were not possible due to a high sensitivity of early stages to these stressful conditions resulting in failure to develop to the megalopa in this treatment. Overall, 5,200 larvae were cultured during this experiment (i.e., 10 larvae × 5 replicates × 2 salinities × 4 temperatures × 13 females of origin). The design contains two separate three-way interaction terms (temperature × salinity × female and temperature × salinity × population) with "female" being nested within population.

Experiments were conducted in temperature-controlled rooms (± 0.5 °C) with a fixed 12-12 h light-dark cycle. Aerated natural seawater from Helgoland, previously UV-treated and filtered (filter pore size = 0.2 µm), was used in all the experiments. Diluted seawater was prepared by mixing natural seawater with appropriate amounts of tap water to reach the appropriate salinity to the nearest ± 0.1 PSU (salinometer: WTW Cond 3110 SET1) and stored in 60 L tanks at each of the experimental temperatures. Salinity was monitored, daily in the storage tanks, and before and after water changes in the experimental bowls to ensure that it remained constant during the experiments. During the daily water change, larvae were fed *ad libitum* with daily freshly hatched *Artemia sp*. (Great Salt Lake Artemia, Sanders, USA); moults and dead larvae were reported and discarded.

**Respiration measurements**

All respiration measurements were performed at the same temperatures and salinities used for larval rearing: the same four experimental temperatures in temperature-controlled

rooms (15, 18, 21 and 24 ºC) and the same salinities (25-32.5 PSU). Prior to the respiration measurements, recently moulted megalopae (<24 h after moult) from each treatment were starved for 2 hours to avoid confounding effects of excretion on the respiration measurements. Respiration measurements were conducted in 4 mL vials integrated with an optically isolated oxygen sensor type PSt5 at its bottom (PreSens, Regensburg, Germany). Each experimental vial was first filled with $O_2$-saturated seawater (25 or 32.5 PSU) at the rearing temperature (15, 18, 21 or 24 °C). Immediately after, one megalopa per replicate (six replicates for each treatment) was placed in the measurement vial, and subsequently the vial was closed underwater to avoid air bubbles (Fig. 1c). The controls for the respiration measurements consisted of six additional vials at each salinity and temperature without animals. Vials with samples and control vials were placed in a 24-well plate and placed on a SDR SensorDish® Reader (PreSens, Regensburg, Germany). This system consists of a 24-channel reader of oxygen luminescence quenching and function as the incubation chamber, providing a high-quality measurement without oxygen consumption or gas exchange between the environment and the vial (Warkentin et al. 2007). The PreSens system was calibrated with a two-point calibration (0 and 100% oxygen saturation) before the experiment for each water condition (temperature and salinity) according to the manufacturer's protocol. The 24-channel reader and vials were placed on a rocking platform shaker (IKA Rocker 2D digital, Staufen, Germany) at 40 revolutions per minute (rpm) to avoid oxygen stratification within the vials. As primary producers can increase the oxygen content in the vials, an opaque black plastic box was placed to cover the vials to block the light during measurement. Oxygen consumption was measured for ca. 4 h, and a longer period in the colder temperatures to compensate for slower respiration rates. Readings were recorded every 15 seconds as concentration of $O_2$ (mg $L^{-1}$). In cases when unfavourable conditions had led to unsuccessful development to the megalopa, measurements were made only when there were at least three replicates. After completion of respiration measurements, animals were inspected to confirm they were alive, and sampled for the biomass quantification.

**Biomass, survival and development**

In order to explain the patterns observed during respiration and its potential consequences, we included already published data for survival, duration of development, and growth of the Kerteminde and Helgoland populations (Šargač et al. 2021). In this study, we

added additional analyses including the new data of the Cádiz population together with the already published data set of the other two populations. Note that already published data is shaded in grey in figures (for significant differences between Kerteminde and Helgoland see Šargač et al. 2021), for easier comparison between the populations.

After the respiration measurements were concluded, megalopa were sampled individually for the determination of biomass (i.e., dry mass and elemental composition) following Šargač et al. (2021). Megalopae were transferred directly from the vials, gently rinsed for ten seconds with distilled water, shortly blotted dry and placed into tin cartridges and stored at -20 °C for further analyses. Afterwards, samples were freeze-dried for 48 h (Christ Alpha 1–4 freeze drier) and dry mass was determined using a microbalance (Sartorius SC2, to the nearest 0,1 µg). Carbon and nitrogen contents were determined with an Elemental analyser (vario MICRO cube CHNS analyser, Elementar Analysensysteme). Cumulative survival was calculated as proportion of larvae surviving from hatching to the megalopa according to Šargač et al. 2021. In order to avoid situations of log(0) values, we rescaled the proportions (p) with the formula p' = [p (N-1) + 0.5]/N, where N is the number of larvae per glass (= 10 larvae) at the start of the experiment. Developmental time was determined as the time required from hatching to reach the megalopa. Analyses for biomass, survival and duration of development were performed for all three populations and an additional analysis of the Cádiz population alone.

**Data analysis**

The oxygen consumption in each vial was calculated by linear regression, using the part of the curve oxygen concentration *vs.* time showing a linear trend. Individual respiration rates ($VO_2$) were calculated for each population as the difference in oxygen consumed per hour between control vials (without animals) and vials containing megalopae. Respiration rates were first calculated as averages of replicates for each treatment, female of origin and population. Then, we determined the average for all females in each treatment and population. Respiration rates were presented as µg of oxygen consumed per individual per hour ($\mu g\ O_2 \cdot ind^{-1} \cdot h^{-1}$). Dry mass (DM), carbon (C) and nitrogen (N) content were calculated for post-moult megalopae, and to calculate weight specific respiration rates ($QO_2$) we divided respiration rates by the dry mass (µg) of each individual animal that was used in the measurement. Additional statistical

334 analyses, such as means, standard deviations and standard errors, were computed in R (version
335 4.0.2) through RStudio via "plyr" package.

336 The experimental design consisted of three fixed factors (population: P; temperature:
337 T, and salinity: S) and female of origin (F) as a random factor nested in population. Mixed
338 model analyses were first conducted through backward model selection (Zuur et al. 2009) using
339 the nlme package (Pinheiro et al. 2019). For mixed models, the function lme was used, and for
340 fixed models gls. Model selection was performed in two steps: we first compared models for
341 random terms after restricted maximum likelihood (REML), then after refitting the model with
342 the best random structure, we checked the fixed terms with maximum likelihood (ML).
343 Corrected Akaike information criteria (AICc) and likelihood ratio tests (LRT) were used to
344 compare models and Tukey HSD test was used for post-hoc comparisons. The model with the
345 lowest AICc was always selected for further analysis. In cases when the difference between
346 models was $\Delta AICc < 3$ and the more complex model had lower AICc, we used LRT to test
347 which model was more suitable: (1) when models differed significantly ($p < 0.05$), the model
348 with lower AICc was chosen; (2) when there was no significant difference between the models,
349 we chose the model with the lowest number of parameters.

350 The full mixed model for respiration rates (Table 1) had three factorial fixed
351 components: population, salinity and temperature (~ P × S × T). The replicate units (n = 6)
352 were the vials used to measure respiration (measured as oxygen consumption). Replicates for
353 oxygen consumption did not fully coincide with the replicates for larval rearing because we
354 had to ensure that respiration measures were taken with larvae with the same moulting age.
355 Because some larvae inevitably die, and due to limitations in the number of parallel
356 measurements to be taken per day, it was not possible to assign larvae from different rearing
357 glasses to separate respiration vials. Importantly, because of the quick developmental changes
358 occurring within the moult cycle (Anger 2001), confounding due to larval age is more relevant
359 than potential confounding associated to the replicate glasses; hence, we focused on minimising
360 the former. In order to minimise the latter source, we focused on variation in the responses
361 among larvae from different females and kept "female of origin" (F) in the random term of all
362 selected models. Female of origin was entered in the full model in interaction with fixed factors
363 with terms coded as "random = list (F = pdDiag(~S × T))"; we also checked that the full
364 covariance matrix (pdSymm) did not result in model convergence. For more simple models,
365 the random component was also tested with the full as well as the diagonal matrices, e.g. as
366 "random = list (F = pdDiag(~S))" or "random = list (F = pdDiag(~T))". The simplest model

(for the random term) was coded as "random = ~1|F". Due to insufficient moulted megalopa from the Kerteminde population at 15 °C and 25 PSU, two analyses were performed: (1) for all three populations between all the treatments except 15 °C; (2) additional analysis between Cádiz and Helgoland containing 15 °C (Table 1).

For dry mass, carbon and nitrogen content (DM, C and N) of the megalopa, a mixed model was performed as explained for respiration rates (Table S1). We compared populations with a three-way fixed interaction (~ P × S × T). First, we analysed all three populations excluding the groups exposed to 15 °C, and then we compared Cádiz and Helgoland with all eight treatments. Female of origin was kept as a random factor throughout all the steps coded in a model as "random = ~1|F". Survival analyses were performed for all eight treatments among the three populations (Table S2). Additional ANOVAs were conducted for Cádiz population for each parameter (survival, biomass, and duration of development) to check the significant interactions between the factors (salinity × temperature), for later comparison between the three populations.

## 3. Results

For survival, development, and growth, we describe the responses for the Cádiz population and compare it with those of Helgoland and Kerteminde, already published in Šargač et al. (2021). We then report on the respiration rates for the three populations.

### Survival

Larval survival for the Cádiz population showed a pattern consistent with TMLS (detrimental effects of the low salinity treatment (= 25 PSU) were minimal at the higher tested temperatures). In addition, survival increased with temperature, with higher survival at the highest temperature (24 °C) in comparison to Kerteminde and Helgoland (Fig. 2). This made the Cádiz population slightly different from the one of Helgoland where in 32.5 PSU there was no increase of survival at 21-24 °C, as compared to 15 or 18 °C. In addition, the response of the Cádiz (and Helgoland) populations differed from that of Kerteminde where survival was depressed at 21 and 24 °C at 32.5 PSU. Best statistical models for survival retained female as a random factor (Table S1). Most of the 2-way interactions were retained (S×T and P×T for raw data; and all 2-way interactions for logarithmic data), indicating interactive effects of population, salinity and temperature. However, at the lowest temperatures, the survival was

consistently lower at the low salinity treatment ($p<0.05$ for 15 °C and 18 °C). In general, significant differences ($p<0.05$) in survival between the salinities were found in all populations in lower temperatures (15 and 18 °C).

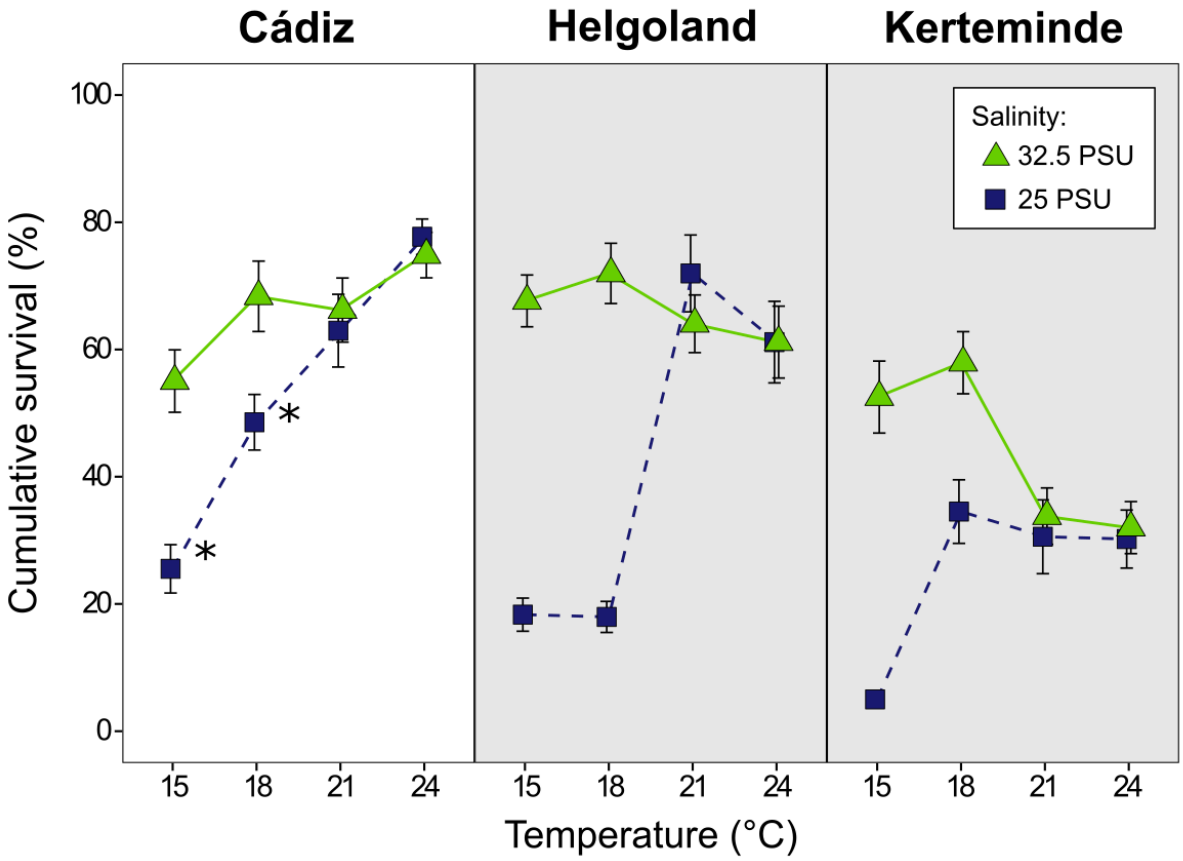



**Figure 2.** *Carcinus maenas*. Cumulative average survival to megalopa. Comparison between three populations reared at two larval salinities (25 PSU, blue and 32.5 PSU, green) and four temperatures (15, 18, 21 and 24 °C). Symbols represent each combination of factors per population. Data shown as mean values ± SE among larvae produced by different females (n = 5 or 3). Asterisks represent significant differences between salinities for larvae of the Cádiz population for each temperature. Already published data are presented in grey sections (for significant differences in those populations see Šargač et al. 2021).

## Biomass and elemental composition

The dry mass, carbon and nitrogen content of megalopae from the Cádiz population did not show clear differences among temperature treatments and were consistently lower at lower

414 salinity across the whole temperature range (Fig. 3). Contrary to what was observed for the
415 Helgoland population, there was no evidence of mitigation at high temperatures (21-24 ºC) in
416 the Cádiz population. Instead, the response pattern to the combination of temperature and
417 salinity resembled the one found for Kerteminde. The megalopa from the Cádiz population
418 showed the lowest dry mass, carbon and nitrogen content. In addition, initial dry mass of
419 freshly hatched larvae (Fig. S3) showed no significant differences between Cádiz and
420 Kerteminde (9.16 µg $ind^{-1}$ for Cádiz; 8.12 µg $ind^{-1}$ for Kerteminde), while Helgoland larvae
421 had significantly higher dry mass at hatching in comparison to the other two populations (10.7
422 µg $ind^{-1}$; $p<0.05$). In all biomass analyses, female of origin was kept as a random factor (Table
423 S2) highlighting important variations among larvae from different females. The best models
424 kept some 2-way interactions (P×T for DM; S×T for C and N). Analyses for Cádiz and
425 Helgoland between all eight treatments showed no significant interaction for DM and
426 significant 2-way interaction (S×T) for C and N. For all cases, the additive terms were
427 important and were retained in the best statistical models.

428

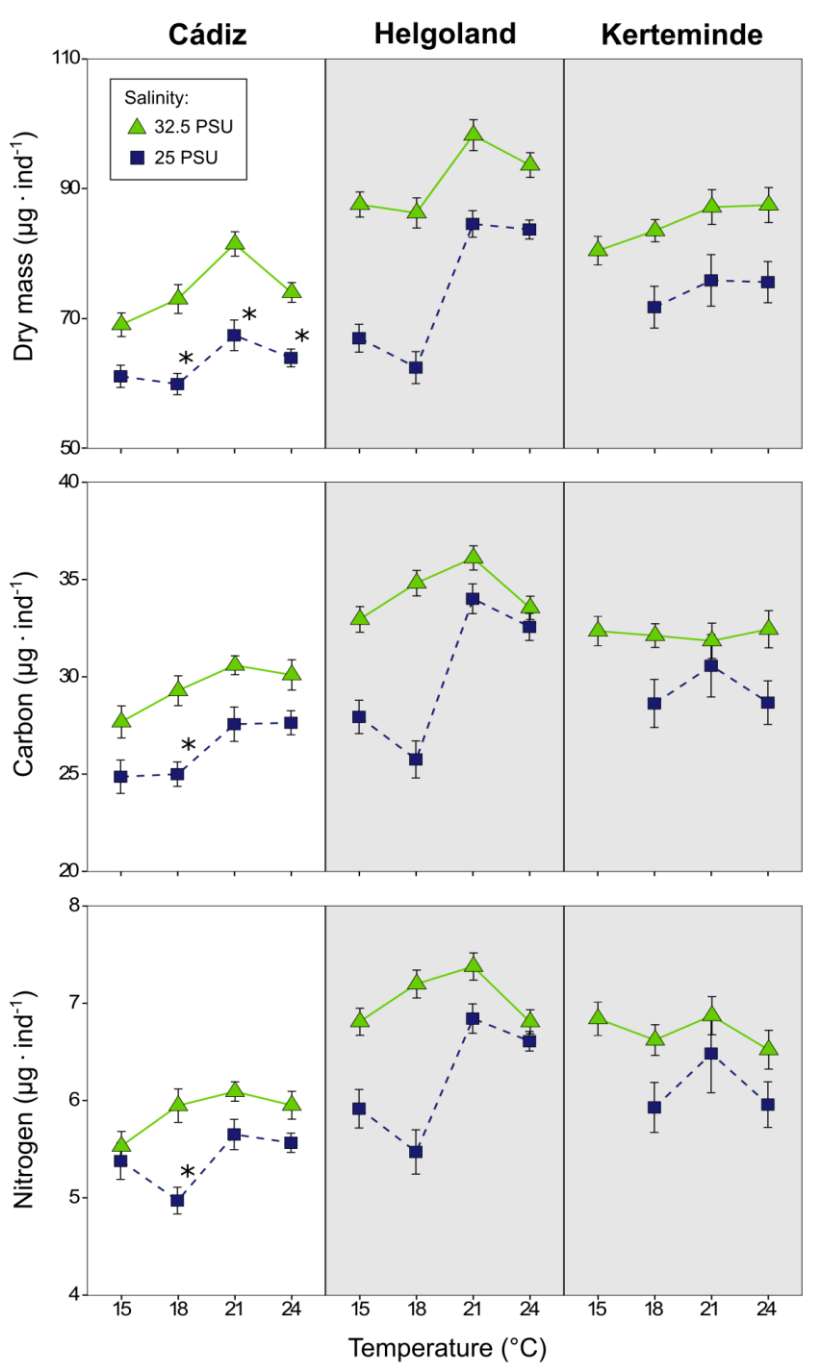



**Figure 3.** *Carcinus maenas*. Dry mass, carbon and nitrogen content (µg · ind$^{-1}$) of megalopa among three populations reared at two larval salinities (25 PSU, blue and 32.5 PSU, green) and four temperatures (15, 18, 21 and 24 °C). Symbols as in Fig. 2.

## Respiration rates

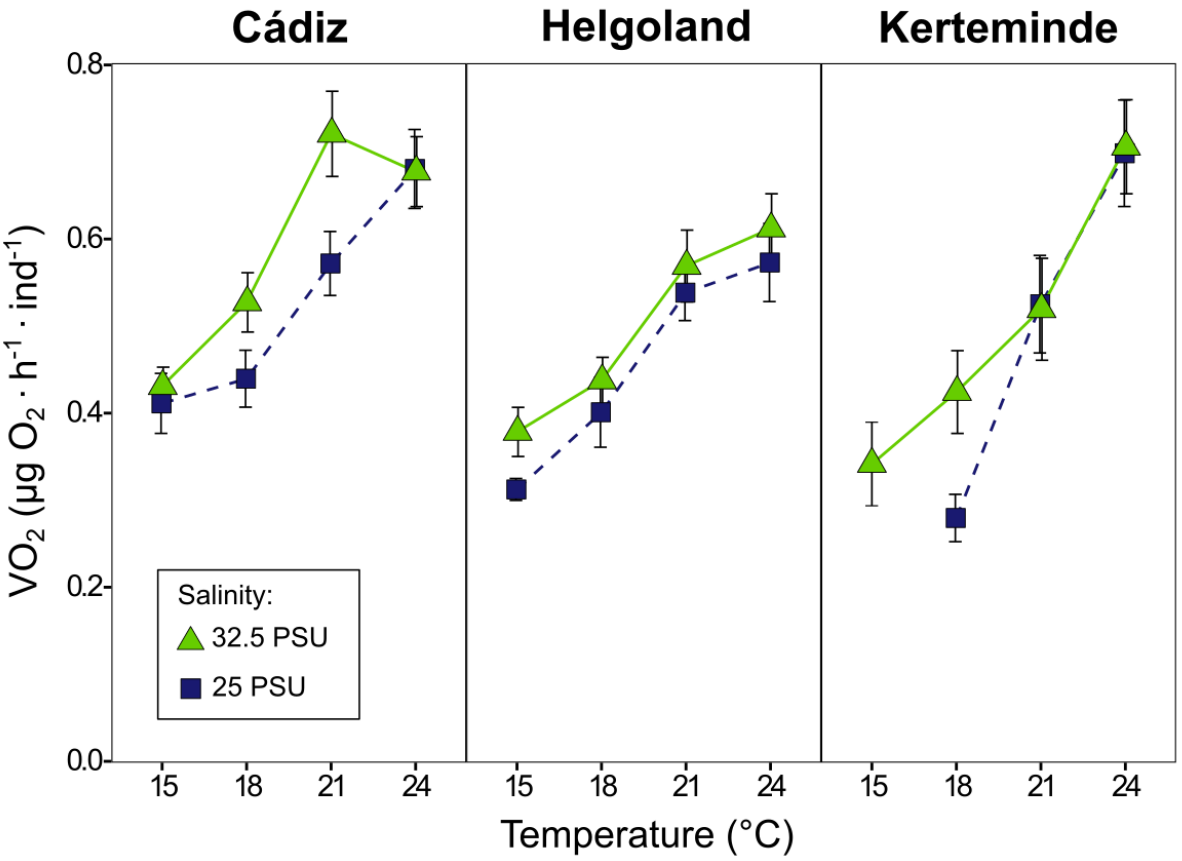
The best statistical model for respiration rates per individual ($VO_2$) did not retain any interaction between the tested terms (Table 1), while respiration rates varied between

437 populations (P) and increased with temperature (T). Temperature was the main driver for
438 respiration rates per individual, where a temperature increase generally led to higher rates in
439 all three populations. There was a tendency towards lower $VO_2$ at the low tested salinity but
440 there was also important variability in oxygen consumption within each combination of
441 temperature and salinity (Fig. 4). Slightly higher $VO_2$ oxygen consumption was recorded in the
442 Cádiz population at 21 °C when larvae were reared in seawater, while Helgoland had lowest
443 respiration rates at the highest temperature in both salinities (compared to the other
444 populations).

445

446 **Table 1.** *Carcinus maenas*. AICc values for the model selection on random terms was carried
447 out through restricted maximum likelihood (REML) fitting; in all cases, the best model was
448 the one without random term. Individual respiration rates ($VO_2$) and weight specific respiration
449 rates ($QO_2$) of megalopae in response to population (P), salinity (S), temperature (T) and female
450 of origin (F). F is a random factor representing maternal effects, nested in the population. The
451 remaining three factors are fixed and form a 3-way factorial design. Fixed effects were tested
452 after maximum likelihood (ML) fitting and the full fixed model was retained (P × T × S). The
453 best model, therefore, excluded the random part and kept some fixed parts (marked in bold).
454 Cad, Hel and Ker correspond to three different populations: Cádiz, Helgoland and Kerteminde,
455 respectively.

| **Model selection:** | | **Cad-Hel-Ker (no 15 °C)** | | **Cad-Hel (all treatments)** | |
|---|---|---|---|---|---|
| | | **$VO_2$** | **$QO_2$** | **$VO_2$** | **$QO_2$** |
| **Random (REML)** | | | | | |
| F×T×S | | 22 | -433 | 15 | -448 |
| F×S | | 11 | -448 | -3 | -467 |
| F×T | | 11 | -446 | -1 | -465 |
| F | | 7 | -452 | -7 | -471 |
| No random term | | 3 | -456 | -10 | -474 |
| **Fixed (ML)** | **Term removed** | | | | |
| 3way(full) | | -67 | -686 | -76 | -680 |
| | P×T×S | -79 | -695 | -84 | -687 |
| 2way | | -79 | -695 | -84 | -687 |
| | S×T | -84 | -701 | -92 | -695 |
| | P×T | -84 | **-696** | -93 | -692 |
| | P×S | -84 | -701 | -87 | -690 |
| Additive | | -93 | -706 | -102 | -702 |
| | T | **-60** | / | **-63** | **-680** |
| | S | -92 | -707 | -101 | -702 |
| | P | **-91** | / | **-95** | **-665** |




456

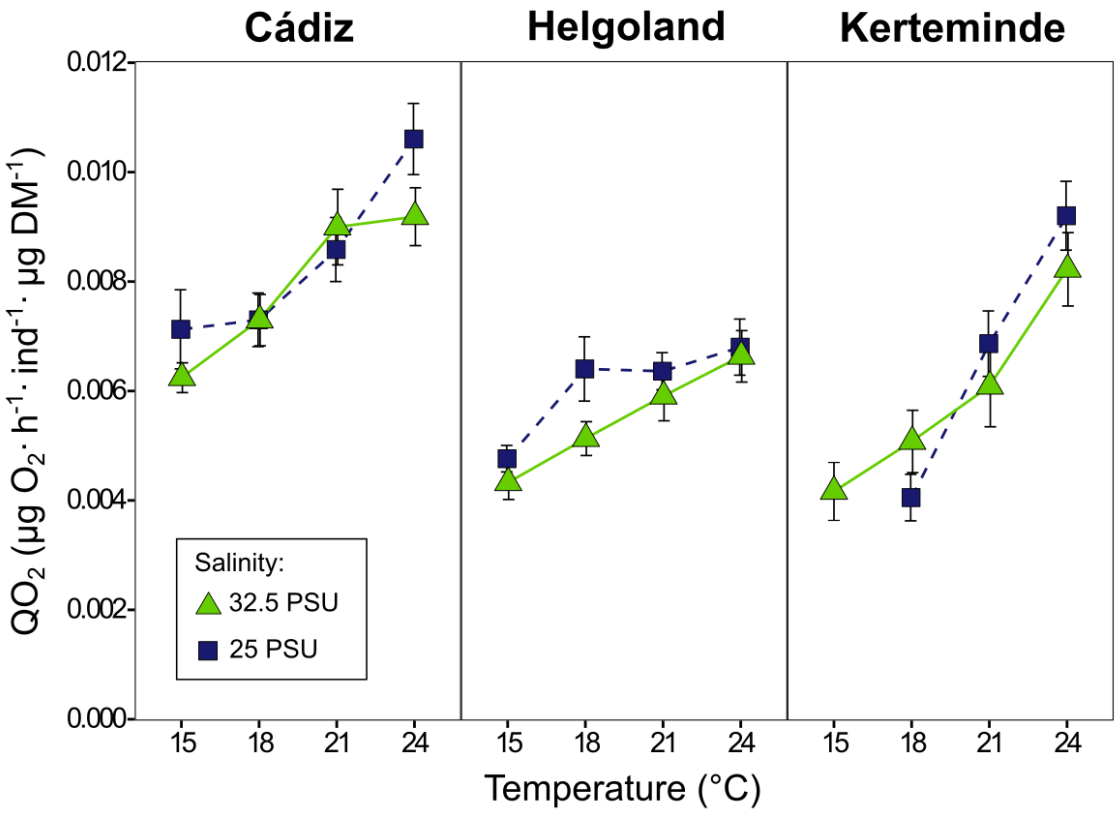
457 **Figure 4**. *Carcinus maenas*. Respiration rates per individual ($VO_2$) for megalopa. Comparison
458 between three populations measured at two larval salinities (25 PSU, blue and 32.5 PSU, green)
459 and four temperatures (15, 18, 21 and 24 °C). Symbols represent each combination of factors
460 per population. Data shown as average individual respiration rates (µg $O_2 \cdot ind^{-1} \cdot h^{-1}$) ± SE
461 among larvae produced by different females (n = 5 or 3). Asterisks represent significant
462 differences between salinities for larvae of the Cádiz population for each temperature.

463

464 Weight-specific respiration rates ($QO_2$) varied mainly between population and
465 temperatures (Fig. 5); the best statistical model retained a 2-way factorial interaction P × T
466 (Table 1). The best model for comparison of Cádiz and Helgoland for all eight treatments had
467 no interactions between populations and only additive terms were important (population and
468 temperature). At the lowest temperature (15 °C) differences between the populations where
469 small, while larvae from Cádiz generally had higher $QO_2$ at higher temperatures (21 and 24
470 °C). Moreover, there were no significant differences in any of the treatments between
471 Kerteminde and Helgoland. The main factor driving $QO_2$ in all three populations was
472 temperature, while salinity did not lead to any important change in $QO_2$ (Fig 5). Overall, an

473 increase of temperature caused increased $QO_2$ in all three populations in both salinities, with
474 slight interpopulation differences.

475




476

477 **Figure 5**. *Carcinus maenas*. Weight specific respiration rates ($QO_2$) per individual for
478 megalopa. Comparison between three populations measured at two larval salinities (25 PSU,
479 blue and 32.5 PSU, green) and four temperatures (15, 18, 21 and 24 °C). Symbols represent
480 each combination of factors per population. Data shown as average weight specific respiration
481 rates ($\mu g\ O_2 \cdot ind^{-1} \cdot h^{-1} \cdot \mu g\ DM^{-1}$) ± SE among larvae produced by different females.

482

483

484 **4. Discussion**

485 We studied larval performance of the shore crab *Carcinus maenas* from three European
486 populations and found important variations among descriptors of performance in response to
487 temperature and salinity. Mitigation effects of lower salinity at higher temperatures did not
488 occur consistently across all rates and on body mass. In addition, there were important

differences in the responses between the two populations exposed to higher coastal salinity water: Cádiz (unlike Helgoland) showed increased survival at the highest temperature and the negative effects of low salinity on growth and body mass were not mitigated at high temperature (unlike Helgoland).

For Cádiz, mitigation effects at the higher tested temperatures (21, 24 °C) were found for survival and developmental time, but not for growth rate and body mass. Hence, for Cádiz and in consistence with previous analyses (De Laender 2018; Torres and Giménez 2020), responses at a given level of organisation are not necessarily found at other levels. Because of non-linearity and compensatory effects, the patterns found in physiological responses (e.g. growth rates) do not necessarily appear in those of fitness (e.g. larval survival). Compensatory responses for the Cádiz population may be understood here as a hierarchy of processes (Knowlton 1974), operating at lower salinity but contingent on the temperature conditions. At high temperatures, survival in response to lower salinity was prioritised at expenses of growth; however, at low temperatures reductions in growth were not associated with consistently high survival. Reductions of body mass observed at any temperature may, however, have consequences for fitness at the post-metamorphic stages, which were not quantified here. For instance, reduced body size or mass has been associated with low survival rates in many invertebrates (Giménez et al. 2004; Pechenik 2006; Torres et al. 2016) and increased vulnerability to cannibalism and predation in *C. maenas* (Moksnes 2004).

We were interested in exploring links among metabolic, developmental, and growth rates because such rates are expected to drive body size at metamorphosis, which is a fitness-related trait (Werner 1988; Hentschel 1999; Gotthard 2001; Heyland et al. 2011; Torres and Giménez 2020). For Helgoland, growth and developmental rates were depressed at lower salinity only when larvae were exposed to the lowest tested temperature, but not when they were exposed to higher temperatures. One would therefore hypothesise for such population, that low salinity would lead to metabolic depression at low temperatures. However, oxygen consumption in the megalopae responded consistently to temperature, but not to salinity. In addition, the pattern found for Helgoland did not differ much from those found in Cádiz and Kerteminde, where high temperatures did not mitigate the effect of low salinity on growth and body mass. One may argue that, at least for Helgoland and Kerteminde, there was some level of reduction of $VO_2$ driven by lower salinity in larvae exposed to the lowest temperature, but that the variability in the responses was too large for an interaction between temperature and salinity to be retained in a statistical model. However, when respiration rates were expressed

on a per dry mass basis (as $QO_2$) such differences disappeared. In terms of $QO_2$, the response to temperature compared well with that reported in a previous study (Dawirs 1983). Hence, we have no evidence of a clear antagonistic effect of temperature and salinity on the respiration rates of the megalopa in any of the studied populations. A possible explanation is that, at low temperatures, moderately low salinity (25 PSU) caused reduced feeding or assimilation rates, but larvae kept spending energy in sustaining essential functions and coping with osmotic stress. Alternatively, the ability to osmoregulate of the megalopa results in that respiration rate is little affected by the tested salinities, and variation in body mass or chemical composition was not detected. Osmoregulation is considered energetically costly which usually causes higher (but not lower) respiration rates (Dehnel 1960; Rivera-Ingraham et al 2016) in order to fuel the catabolism of osmotically active amino acids (Gilles 1973) and active salt exchange. Since at 25 PSU megalopae of *C. maenas* from the Helgoland population osmoregulate, one would expect an effect of such salinity on respiration rates, as compared with 32.5 PSU where larvae do not need to regulate the haemolymph osmolality (Cieluch et al 2004). Since in addition, the osmoregulatory capacity of *C. maenas* megalopa from Helgoland increases with temperature (Torres et al. 2021a) one would expect that the effect of salinity on respiration rates would depend on temperature. Clearly, such effect should have occurred at least for the Helgoland population. Alternatively, the antagonistic effect of lower salinity and higher temperature on respiration rates may occur in the zoeal stages, which are practically osmoconformers (such as zoea II-IV). In crustaceans, the respiration sensitivity can change during ontogeny (Brown and Terwilliger 1999; Leiva et al. 2018). In zoeae I of *C. maenas* of the Helgoland population, Anger et al. (1998) found that low salinity depressed respiration rates. However, in their study, the effect of low salinity on such rates did not occur at the salinities tested by us (at T = 18 °C, $VO_2$ = 0.040 μg $O_2$ $h^{-1}$ $ind^{-}1$ at both 25 and 32 PSU in 2-day-old larvae: Table 1 in Anger et al. 1998). Instead, the drop in $VO_2$ occurred at 20 PSU where we have recorded important mortality rates during the first and subsequent larval stages (Spitzner et al. 2019; Šargač et al. 2021). Perhaps respiration rates are depressed at salinities that are lower than those tested by us.

The differences between populations in the larval thermal tolerance and in how temperature modulates the effect of lower salinity on physiological rates, highlight the necessity to quantify among population responses to climate-driven environmental change. We found that survival and developmental time of larvae from the Cádiz population showed a pattern consistent with TMLS as found for Helgoland (Spitzner et al, 2019; Šargač et al. 2021)

555 and the Irish Sea (survival to zoea II: Torres et al. 2020). At higher temperatures,
556 osmoregulatory abilities might have been enhanced *via* various mechanisms operating on the
557 molecular level (Pörtner et al. 2017) and (for the Helgoland population) those temperatures can
558 amplify the capacity of *C. maenas* zoea I and megalopae to osmoregulate (Torres et al. 2021a).
559 Most likely, the same capacity is present in the Cádiz population. However, survival for the
560 Cádiz population increased with temperature also in larvae reared in 32.5 PSU, contrary to e.g.
561 the Kerteminde population where survival decreased towards higher temperatures. Thus, it
562 appears that the populations differ in the temperature at which the metabolic demand exceeds
563 the animal`s capacity to supply oxygen to tissues and cause a progressive decline in
564 performance (Deutsch et al. 2015, Pörtner 2010). We were not expecting important differences
565 in the response of larvae to high temperatures because in Cádiz, *C. maenas* larvae are released
566 earlier than in Helgoland and Kerteminde (January-April *vs.* April-June) and hence temperature
567 differences may not be so contrasting (Cádiz: ~12-20 ºC vs Helgoland: ~9-18 ºC). Early release
568 in Cádiz is consistent with data distribution of *C. maenas* larvae in the Cádiz Bay (November-
569 May, with a peak in February: Drake et al. 1998) and in Portugal, where early zoea are present
570 in the water column already in February (Queiroga et al. 1994) and arrival of megalopa into
571 estuaries occurs already in April and May (Rey et al. 2016). However, the larval season in
572 Cádiz includes April-May, when temperatures in the inlets can be well above 20 ºC (Drake et
573 al. 1998) and those of the Cádiz Gulf reach 20 ºC (Criado-Aldenueva et al. 2006).

574 Another important difference was in the instantaneous growth rates, lower in Cádiz than
575 in Helgoland and Kerteminde (Fig. S1). Such lower growth rates explain the reduced biomass
576 of the megalopa because developmental times differed little among populations (Fig. S2).
577 Moreover, freshly hatched Cádiz larvae had lower initial biomass than Helgoland, but slightly
578 higher than Kerteminde (Fig. S3). In addition, in larvae from Cádiz, the mitigation effect of
579 high temperature on growth (and on body mass) was weaker than the one found in the
580 Helgoland population. This could be explained by the fact that larvae from the Cádiz population
581 experience salinities that are higher than the 32.5 PSU used in our experiment. Moreover, due
582 to habitat differences, some physiological process could be more sensitive to lower salinity
583 (experimental salinities) than in e.g. the Helgoland population. While larvae drifting from the
584 Helgoland population into the German Bight are likely to be exposed to moderately low
585 salinities (25-33 PSU), those of Cádiz are likely to experience salinities >35 PSU in the Gulf
586 of Cádiz (Criado-Aldenueva et al. 2006). Furthermore, crabs from the local populations of
587 Cádiz can be found in the coastal lagoons and ponds where salinity can reach values of 65 PSU

588 (Drake and Arias 1995). Hence, the continuously high survival in all the treatments, despite
589 the reduced biomass gain, shows high larval tolerance of the Cádiz population.

590 Overall, differences between the Cádiz and Helgoland populations point to either local
591 adaptation or some form of plasticity (Marshall et al. 2010; Fischer et al. 2011). Our data do
592 not let us infer whether a single or both mechanisms drive the differences. Adult *C. maenas*
593 exhibit seasonal changes in respiration rates (Klein Breteler 1975) and can become acclimated
594 in warmer waters (Newell 1973). Populations from different climatic regions can adjust
595 metabolic rates to maintain function at the prevailing temperature (Saborowski et al. 2002).
596 Plasticity, in the form of post-zygotic maternal effects, impact responses to lower salinity and
597 increased temperature in *C. maenas*: lower salinity experienced during embryogenesis pre-
598 empt the development of mitigation effects in the larval stages of a population of the Irish Sea
599 (Torres et al. 2020), Helgoland and Kerteminde (Šargač et al. 2021). Perhaps higher salinities
600 experienced during embryogenesis also pre-empt the development of mitigation responses, in
601 the Cádiz population. We also expect that genetic differences between populations underpin
602 the differences in the responses: in a genetic study covering the European coast, Roman and
603 Palumbi (2004) pointed towards slight levels of population structuring for *C. maenas*, between
604 the Atlantic and the North Sea, which may be associated with differentiation in genes driving
605 both thermal and salinity tolerance.

606 Hence, from Cádiz Bay to the Baltic Sea, *C. maenas* exhibit an important diversity in
607 larval thermal tolerance, the joint effect of salinity and temperature on physiological rates and
608 how such rates are related as linked traits. Such diversity represents part of the portfolio of
609 responses to climate-driven environmental conditions exhibited and the potential to buffer the
610 meta-population from environmental variation. The general trend for marine species, under the
611 effect of climate change, will be to shift their distribution upward or poleward related to sea
612 warming (Chust et al. 2014). For the case of dispersive larvae, the portfolio effect (Schindler
613 et al. 2015) can manifest in how individuals survive the larval phase, connect, and replenish
614 populations as temperature increases. For instance, in the light of warming, southern
615 populations may subsidise northern populations if the European coast is sufficiently connected
616 by larval dispersal and if the direction of transport is poleward. Such connection may occur
617 over the time scale of several generations as a stepping-stone process, as it is likely to have
618 occurred with exotic species invading the North Sea from the English Channel (e.g. Giménez
619 et al. 2020). However, whether such subsidy occurs, also depends on the mechanisms driving
620 the phenotypic variation in the joint effects of temperature and salinity and on the local habitat

621 characteristics. If southern populations were to be all-similar to that of Cádiz (i.e. more
622 sensitive to lower salinity than that of Helgoland), the expansion may be limited if (1) the
623 source of the variation is genetic (instead of adaptive plasticity) and (2) habitats of high latitude
624 are consistently characterised by moderately low salinities. Overall, our work emphasises the
625 need for incorporating multiple populations when quantifying the impact of multiple climate
626 drivers on marine populations.
627
628

629 **Statements and Declarations**

630 *Acknowledgements.* We are thankful to David Roque, Antonio Moreno, and Víctor Acosta for
631 their assistance in the collection and maintenance of females in Cádiz (Spain). We are grateful
632 to Dr. Magnus Wahlberg and Kasper Fjordside for their support to obtain and maintain the
633 females in Kerteminde (Denmark). We sincerely appreciate the help of Dr. Jakob Krieger for
634 transporting the females from Denmark to Germany and Siri Rohwer for her aid in larval
635 rearing. We wish to acknowledge Julia Haafke for performing the elemental analysis.

636 *Author Contributions*. Conceptual development: LG, SH, EGO and GT; study design: LG, ZŠ
637 and GT; resources: SH, EGO and GT; data acquisition: ZŠ, NT and GT; data analysis and
638 interpretation: LG, ZŠ and GT; first draft writing: ZŠ, review and editing of submitted
639 manuscript: all authors.

640 *Data availability*. The data generated and analysed during this study will be available at
641 PANGAEA ®Data Publisher (https://www.pangaea.de).

642 *Funding.* This work is a part of the doctoral dissertation of ZŠ and was supported by Deutsche
643 Forschungsgemeinschaft (Research Training Group 2010: RESPONSE) at the University of
644 Greifswald, in a partnership with the Alfred-Wegener-Institute.

645 *Conflict of interest:* The authors declare no competing interests. The funding body did not
646 influence the design of the study, collection, analysis, and interpretation of data, nor the writing
647 of the manuscript.

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

651

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## Publication III

Published in Helgoland Marine Research, peer reviewed journal:

Torres G, Melzer RR, Spitzner F, **Šargač Z**, Harzsch S, Giménez L (2021)

https://doi.org/10.1186/s10152-021-00548-x

# Methods to study organogenesis in decapod crustacean larvae. I. Larval rearing, preparation and fixation.

Torres *et al. Helgol Mar Res* (2021) 75:3
https://doi.org/10.1186/s10152-021-00548-x

Helgoland Marine Research

REVIEW

Open Access

# Methods to study organogenesis in decapod crustacean larvae. I. larval rearing, preparation, and fixation

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**Abstract**

Crustacean larvae have served as distinguished models in the field of Ecological Developmental Biology ("EcoDevo") for many decades, a discipline that examines how developmental mechanisms and their resulting phenotype depend on the environmental context. A contemporary line of research in EcoDevo aims at gaining insights into the immediate tolerance of organisms and their evolutionary potential to adapt to the changing abiotic and biotic environmental conditions created by anthropogenic climate change. Thus, an EcoDevo perspective may be critical to understand and predict the future of organisms in a changing world. Many decapod crustaceans display a complex life cycle that includes pelagic larvae and, in many subgroups, benthic juvenile–adult stages so that a niche shift occurs during the transition from the larval to the juvenile phase. Already at hatching, the larvae possess a wealth of organ systems, many of which also characterise the adult animals, necessary for autonomously surviving and developing in the plankton and suited to respond adaptively to fluctuations of environmental drivers. They also display a rich behavioural repertoire that allows for responses to environmental key factors such as light, hydrostatic pressure, tidal currents, and temperature. Cells, tissues, and organs are at the basis of larval survival, and as the larvae develop, their organs continue to grow in size and complexity. To study organ development, researchers need a suite of state-of-the-art methods adapted to the usually very small size of the larvae. This review and the companion paper set out to provide an overview of methods to study organogenesis in decapod larvae. This first section focuses on larval rearing, preparation, and fixation, whereas the second describes methods to study cells, tissues, and organs.

**Keywords:** EcoDevo, MorphoEvoDevo, Brachyura, Decapoda, Larval rearing, Ecophysiology, Organogenesis, Global ocean change

## Background

Crustaceans represent one of the most species-rich animal groups in our oceans and display a large diversity of sizes, morphologies, life-styles, and life histories (reviews e.g. [1–3]). Specifically, representatives of the crustaceans have colonized habitats extending from the deepest ocean trenches and hydrothermal vents, across the vast water bodies of the world's oceans, through intertidal and supratidal coastal habitats. Crustaceans have also representatives occurring in anchihaline caves, inland freshwater ecosystems including endorheic lakes, and terrestrial habitats such as desert saltpans, epiphytic bromeliads in mountain forests, or rocky plateaus of coastal and oceanic islands. Decapoda is a highly diverse subgroup of malacostracan crustaceans that includes well-known representatives such as

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We dedicate this review to Klaus Anger who introduced us to the incredible world of tiny crustacean larvae with utmost patience

ornamental shrimps, clawed and spiny lobsters, hermit crabs, and true crabs. Many decapods represent key species in estuarine, intertidal, and coastal areas where they can have such a high abundance that changes in their population structure may directly influence the structure of the whole ecosystem [4, 5]. Decapods inhabiting estuaries or intertidal zones are adapted to enduring tremendous variations in temperature, pH, and salinity at a daily scale as the consequence of the tides. Decapoda also embraces many species with global invasive potential signifying their ability to adapt to new and changing environments (reviews [5–9]).

Similar to many other marine benthic animals, many decapod crustaceans display a complex life cycle in that they occupy different habitats during ontogeny, and undergo niche shifts during the transition from larvae to the juvenile phase. The life cycle includes pelagic larvae and in most species benthic juvenile-adult stages (Figs. 1, 2; reviews [10–16]). The ontogenetic change in habitat represents an important link in marine food webs (bentho–pelagic coupling). The larval phase, which is essential for dispersal (review [17, 18]), occurs in the pelagic environment, and many larvae of marine crustaceans actively feed (review [19, 20]) and grow by

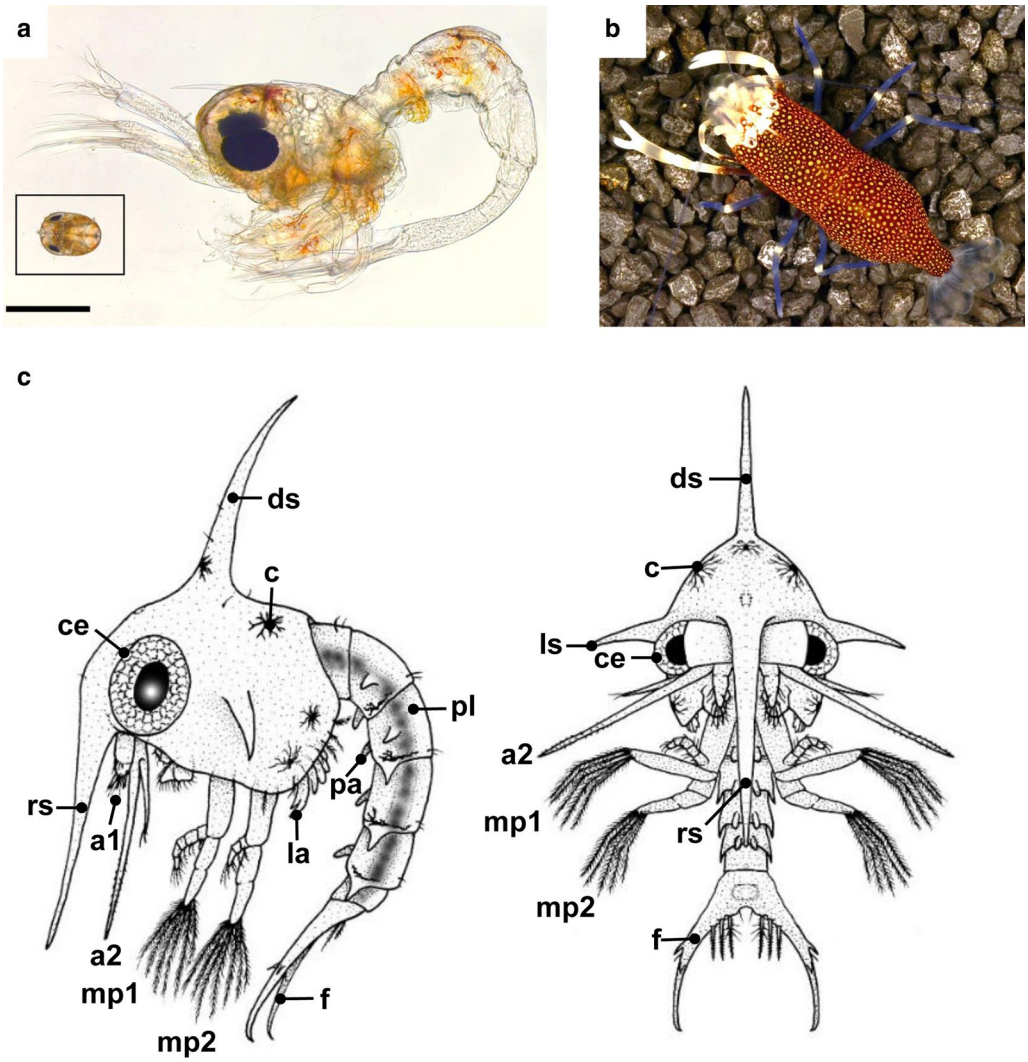


**Fig. 1** Ontogeny of decapod crustaceans. **a**, **b** Ontogenetic reconstruction of *Gnathophyllum elegans* (Risso, 1816) (Decapoda, Palaemonidae), from Zoea 1 (**a**) to adult (**b**); insert in **a** shows a ready-to-hatch embryo in eggshell (modified from [110]); scale bar 250 µm; egg diameter is ca. 750 µm. **c**: generalized brachyuran Zoea in lateral (left) and frontal (right) views (modified from [12]). a1, a2: antenna one and two; c: chromatophor; ce: compound eye; ds: dorsal spine; f: telson with furca; la: limb anlagen; ls: lateral spine; mp1, mp2: exopodites of maxillipeds one and two; pa: anlagen of pleopods; pl: pleon; rs: rostral spine

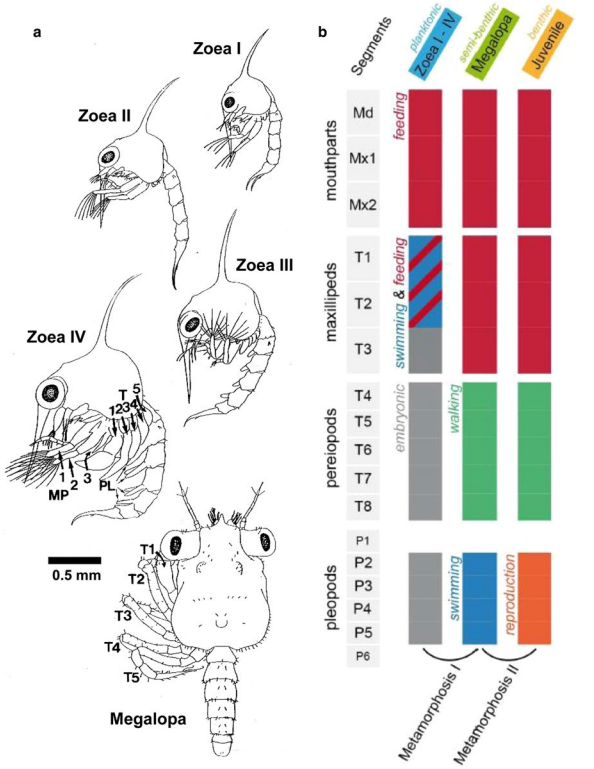


**Fig. 2** Larval development of the European shore crab *Carcinus maenas* L. (Decapoda, Brachyura, Portunidae). **a** The transition between the zoeal stages and metamorphosis I to Megalopa are characterized by moults (modified from [233]). **b** Ontogenetic change of appendage function during the double metamorphosis (see text for further details; from [35]). Md: mandibular segment, Mx1, Mx2: segments of 1st and 2nd maxilla; MP 1–3: maxillipeds 1–3 (corresponding to thoracomeres 1–3); P1–5: pereiopod one to five (corresponding to thoracomeres 4–8); PM1–6: pleomeres one to six; T1–8: thoracomeres one to eight

successive moults through a species-dependent number of larval instars that can be variable depending on environmental conditions (reviews [15, 21, 22]). In the absence of parental care, larvae need to be adapted to developing in the plankton and such morphological and behavioural adaptations are related to movement, nutrition, and sensing (reviews [10, 11]). In some decapod groups, after a metamorphic moult (review [23]) the larvae eventually settle to the benthos (review [24–26]) where juvenile growth into adulthood, reproduction, embryonic development, and larval hatching occurs (Figs. 1, 2).

The larval forms can differ drastically from their corresponding juvenile-adult stages in morphology, ecology, and behaviour (Fig. 1a, b). In the past, this has caused quite some trouble to identify larval forms as such and many of them have originally been described as separate new species. Many modern-day names for larvae refer to such names (e.g. [12]). To ontogenetically bridge the strong differences between larvae and later stages, many decapod crustaceans undergo a drastic metamorphosis during post-embryonic development (Fig. 2a; reviews [12, 16, 23]).

Decapod larvae are fascinating models to study a broad variety of biological processes, some of which we will point out in this contribution. For example, decapod larvae have attracted the attention of ethologists because they display a rich behavioural repertoire that allows studying responses to variations in environmental drivers such as light, hydrostatic pressure, tidal currents, temperature, salinity, and food concentration (reviews [11, 19, 25, 27–29]). The larval sensory systems include compound eyes as well as abundant chemo- and mechanosensory sensilla [30–35] for instance to perceive chemical cues from their conspecifics to identify suitable habitats to metamorphose and recruit (reviews [19, 26, 27, 29]). Circatidal behavioural patterns, such as active vertical migration in response to tidal currents, allow the larvae to avoid predators and to control their horizontal dispersal (reviews [4, 17, 28, 36]).

Like many marine animals, a large number of decapods develop through dispersing pelagic larvae that drive the gene flow that connects established populations and hence are important for population structure and population persistence. Furthermore, dispersing larvae play a central role in founding new populations and in species' range expansion (reviews e.g. [18, 37–41]). In species with complex life cycles characterised by habitat shifts, population persistence depends on individuals adapting to environmental changes occurring in both the larval and the adult habitats. Because failure to adapt to only one of such habitats will result in population collapse, either through reproductive or recruitment failure, decapod larvae are well-suited models to study the influence of environmental drivers on species persistence. Furthermore, we now know that larvae represent the life history phase that is highly sensitive to fluctuations of environmental parameters (e.g. [42–45]; reviews [21, 46]). Also, larval development in crustaceans encompasses risks due to higher vulnerability through predation or overdrift into unsuitable habitats for settlement ([37, 47]). Because e.g. temperature controls the dispersal potential through changes in the length of the dispersal phase (and effects on larval growth and survival), quantifying larval responses to variations in environmental drivers ("reaction norms") such as temperature, pH, salinity or food availability can provide new insights into crustacean life-history cycles (reviews [7, 11, 19]). There is a long tradition of examining the reaction norms of decapod crustacean larvae to changes in single or multiple

environmental drivers which have enriched our understanding of marine larval biology including aspects of supply-side ecology, biogeography, population connectivity, and invasion biology (reviews e.g. [11, 21, 22, 41, 46, 48]).

Analyses of decapod crustacean larvae and their development have also provided new insights into diverse aspects of animal ecophysiology such as phenotypic plasticity in developmental traits, heterochrony in developmental patterns, carry-over effects on life-history traits, and adaptive mechanisms that enhance tolerance to fluctuations in environmental abiotic and biotic factors (reviews e.g. [11, 21, 46, 49, 50]. What is more, crustacean larvae have served as distinguished models in the field of Ecological Developmental Biology or "EcoDevo" for many decades. EcoDevo examines how developmental mechanisms and their resulting phenotype depend on an environmental context as seen in "real-world environments" and explores how developmental pathways incorporate environmental cues to generate context-dependent phenotypes including resulting fitness differences [51–53]. "Environment" in the EcoDevo-context includes abiotic and biotic factors (e.g. temperature, food, conspecifics, predators), but also endocrine disruptors (i.e. environmental compounds that can disrupt normal development by changing gene expression) and teratogens (i.e. compounds causing birth defects) modifying normal development [51–53]. A contemporary line of research in the field of EcoDevo aims at gaining insights into the immediate tolerance of organisms and their evolutionary potential to adapt to the changing physical and biotic environmental conditions created by anthropogenic climate change [54, 55]. Phenotypic plasticity and evolutionary adaptation are currently discussed as essential mechanisms for organisms to adapt to environmental change in marine ecosystems [56, 57]. Such processes ultimately will determine which species will adapt to climate change so that an EcoDevo perspective may be critical to understand and predict the future of organisms in a changing world [54]. More and more, understanding synergism and antagonism among multiple environmental drivers is recognized as essential to predict future species distribution [57–59] and decapod larvae are well suited to analyse such effects of combined multiple drivers (e.g. [42, 44, 45]).

Cells, tissues, and organs are at the basis of all biological processes outlined above. Already at hatching, decapod crustacean larvae possess a wealth of organ systems, many of which also characterise the adult animals, necessary for autonomously surviving and developing in the plankton and suited to respond adaptively to fluctuations of environmental drivers. These include for example a sophisticated digestive system, osmoregulatory and respiratory organs, a well-developed neuromuscular system, and a range of sensory organs to detect environmental cues (Table 1). As the larvae develop, their organs continue to grow in size and complexity. The digestive system adapts to changes in the larval food spectrum and energetic demands as the animals grow. The central nervous system integrates new input from sensory organs on the enlarging body surface and generates adaptive behaviours as the larvae of benthic species change from life in the water column to life on the ocean floor. The respiratory and excretory organs adapt to increasing physiological demands as the animals increase their body size and metabolism. Table 1 summarises previous studies on organogenesis in reptantian crustaceans. To study organ development, researchers need a set of state-of-the-art methods, appropriate for the different demands of the different structures and for the usually very small size of the larvae. Furthermore, for descriptions of larval morphology, which is one major field of crustacean research with a very long tradition [12, 60–62], techniques for larval culture, and fixation and preparation of specimens are necessary. This review sets out to provide an overview of such methods to study the morphology and organogenesis in decapod larvae. This first section focuses on larval rearing, preparation, and fixation whereas the companion paper [63] describes the cytological and histological methods in more detail.

## Obtaining and handling larvae

### Introduction

The study of larval development and physiology (methods described in the following sections) requires the use of appropriate rearing techniques in order to reach the desired stage in sufficient numbers for a meaningful sample (Fig. 3). Furthermore, the chosen larval stages have to be correctly sampled to allow for further processing and analysis (see sampling techniques in the following sections). Here, we give a brief overview of methods for rearing decapod larvae, with emphasis on those developed and tested in the laboratory led by Klaus Anger ([64]) for nearly 40 years (see [10, 16, 19, 65, 66]) at the Biologische Anstalt Helgoland (Alfred-Wegener-Institut, Helmholtz-Zentrum für Polar- und Meeresforschung). Most of Klaus Anger's former students, including ourselves, have adopted these methods and continue to improve them [42, 45, 67–71]. Details of rearing methods have been extensively reviewed over the past 50 years [72–78] mostly for aquaculture. In this review, we focus on larval rearing for experimentation, and hence do not cover mass-rearing for aquaculture production. At the experimental level, we cover aspects of the execution (i.e. larval rearing); yet, many aspects of the experimental design depend on the question asked by the researcher and

**Table 1** Examples for studies on late embryonic and larval organogenesis in representatives of Decapoda and other selected Malacostraca including methodological approaches

| | |
|---|---|
| **General external morphology** | |
| Compendia with line drawings based on LiMi | [12, 60–62] |
| *Hippolyte inermis* | clsm, SEM [108] |
| *Carcinus maenas* | µCT, 3D [35] |
| *Portunus acuminatus* | SEM [32] |
| *Xantho poressa, Xantho pilipes* | SEM [109] |
| *Gnathophyllum elegans* | SEM [110] |
| *Periclimenes amethysteus* | SEM [111] |
| Several Anomura | EF, LiMi [112] |
| Several Stomatopoda | EF [113] |
| Several Polychelida | EF [114] |
| Hippidae | EF [115] |
| **General anatomy** | |
| *Hippolyte inermis* | clsm, SEM [108] |
| *Cancer anthonyi* | PH [116] |
| *Portunus trituberculatus* | PH [117] |
| *Carcinus maenas* | µCT, 3D [35] |
| *Maja brachydactyla* | µCT, 3D [92] |
| **Mouthparts and digestive tract** | |
| Several Anomura | EF, LiMi [112] |
| *Maja brachydactyla* | µCT, PH, SEM, TEM [118, 119] |
| *Hyas araneus* | STH, TEM [84, 120] |
| *Scylla olivacea* | PH [121] |
| *Scylla serrata* | PH [122–124] |
| *Ucides cordatus* | PH [125] |
| *Dyspanopeus sayi* | SEM, LiMi [126] |
| *Sesarma curacaoense* | PH [127] |
| Several Brachyura | SEM [128] |
| *Ranina ranina* | PH [129] |
| *Menippe mercenaria* | SEM [130] |
| *Paralithodes camtschaticus* | PH [131] |
| *Homarus americanus* | TEM, SEM, STH [97, 130, 132] |
| *Astacus astacus, Procambarus fallax* f. *virginalis* | SEM, TEM, PH [133, 134] |
| *Jasus edwardsii* | SEM [135] |
| *Macrobrachium amazonicum* | SEM [136] |
| *Palaemon elegans* | SEM, TEM [34, 136] |
| *Lysmata amboinensis* | PH, SEM [137] |
| *Porcellio scaber* | STH, SEM, TEM [138] |
| **Osmoregulatory epithelia** | |
| *Carcinus maenas* | IHC, SEM [139], LiMi [140] |
| *Eriocheir sinensis* | IHC [141] |
| *Callianassa jamaicense* | TEM [142] |
| *Astacus leptodactylus* | IHC, PP, SEM [143] |
| Several Brachyura and Anomala | LiMi [140] |
| *Homarus americanus* | IHC, TEM [144] |
| *Palaemontes argentinus* | IHC, PH [145, 146] |
| *Litopenaeus stylirostris* | IHC, STH [147] |
| *Crangon crangon* | IHC, SEM, STH [148] |
| **Antennal glands** | |
| *Astacus leptodactylus* | SEM, TEM, IHC [149, 150] |
| *Homarus gammarus* | IHC [151] |
| *Macrobrachium amazonicum* | IHC, PP [152, 153] |
| *Palaemontes argentinus* | IHC, PP [146] |
| **Integument and tegumental glands** | |
| *Hyas araneus* | STH [120] |
| *Sesarma haematocheir* | TEM [154] |
| Multiple species | STH, TEM [155] |
| **Y organ** | |
| *Cancer anthonyi* | STH [156] |
| *Hyas araneus* | STH [120] |
| **Muscle** | |
| Several decapod species | IHC [157] |
| *Homarus americanus* | IHC [158] |
| *Idothea báltica* | IHC [159] |
| **Sensory dorsal organ** | |
| Several decapod species | SEM [160] |
| **Eyestalk neuroendocrine centres** | |
| *Cancer anthonyi* | STH [156] |
| *Homarus gammarus* | STH, IHC [161, 162] |
| **Compound eyes** | |
| Several species | STH, PM, TEM [163, 164] |
| *Carcinus maenas* | STH, PM [30] |
| *Hemigrapsus sanguineus* | STH [165] |
| *Rhithropanopeus harrisii* | STH [165] |
| Various Anomala | STH [166] |
| *Panulirus longipes* | TEM [167] |
| *Procambarus clarkii* | STH, TEM [168] |
| *Alima pacifica* | 3D, osmium-ethyl gallate staining [169] |
| **Aesthetascs** | |
| *Carcinus maenas* | SEM, TEM [170] |
| *Cherax destructor* | SEM, LiMi [171] |
| **Structure of the larval CNS and neurogenesis** | Embryonic neurogenesis was recently reviewed [172–174] and will not be considered here |
| *Carcinus maenas* | STH, µCT, 3D, IHC [175–178] |
| *Hyas araneus* | IHC, PM, STH [179–183] |
| *Pachygrapsus marmoratus* | STH, 3D [184] |
| *Porcellana platycheles* | STH, 3D [184] |
| *Cherax destructor* | PM [171, 185, 186] |
| *Homarus americanus* | STH, PM, IHC [183, 186–192] |
| *Homarus gammarus* | STH, TEM, IHC [161, 162] |
| *Hippolyte inermis* | STH, 3D [184] |

clsm: confocal laser-scan microscopy; EF: epifluorescence, IHC: immunohistochemistry; LiMi: light microscopy of external morphology; µCT: X-ray microscopy; PH: paraffin histology; PM: proliferation markers; SEM: scanning electron microscopy; STH: semithin histology; TEM: transmission electron microscopy; 3D: 3D reconstruction

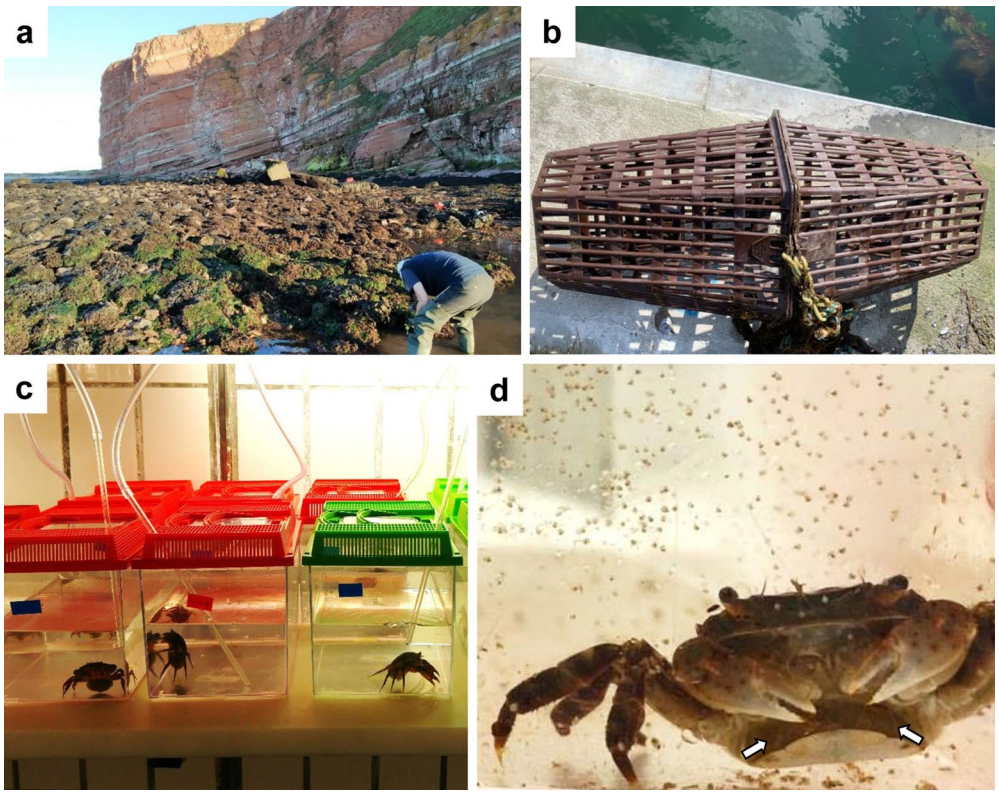


**Fig. 3** Collection and maintenance of berried crab females. **a** Collecting berried females in the rocky intertidal of the island Helgoland (German Bight, North Sea; photo S. Harzsch). **b** Baited traps (photo G. Torres). **c** Individual rearing of berried females (*Carcinus maenas*) prior to hatching (photo Z. Šargač). **d** *Hemigrapsus sanguineus,* berried female releasing larvae (photo N. Espinosa). Arrows in **d** point to the eggs with embryos being carried by berried females

should follow standard procedures of sampling theory. We refer to key references for the extensive discussion of experimental design [79–81]. Instead, we emphasise four cornerstone elements that are essential to larval rearing: *who*?, *how*?, *where*? and *how long*? Finally, yet importantly, the most essential rule: be patient (additional comments).

## Description of methods

### *Who? "Each species is a different world"*

Finding out how to rear the larvae of a "new species" (i.e. newly hosted in the lab) requires a series of preliminary experiments, the output (a meaningful result) is an optimal rearing method for the larvae in question. Literature research focussed on rearing methods for early stages (i.e. larvae) of the target or a similar species (see Table 2 for examples) is the starting point to develop a new method. In addition, such literature research is invaluable for exceptional cases (e.g. for the morphological description of a new species), when only one berried female (i.e. female carrying eggs with embryos, see arrows in Fig. 3c, d) is available. In this situation, careful consideration of the embryos' size and the amount of reserves in the embryos may point to the size and nutritional requirements of the first larval stage. For instance, large embryos will develop into large larvae, lecithotrophic larvae can be expected from embryos with a high amount of reserves (e.g. *Sesarma meridies* [82]; while small embryos with low reserve amounts will likely produce planktotrophic larvae (e.g. *Cancer pagurus* [83]. The methods used to rear larvae vary considerably among species, according to the size, shape, presence of spines, and the environmental conditions required for optimal survival (e.g. temperature, salinity, dissolved $O_2$, food availability, among others). For instance, species with long spines may require larger containers and gentle (or no permanent) bubbling as compared with species with short spines. Species with slow swimming larvae may need stronger aeration. Species producing relatively large larvae (> 10 µg dry mass) may be reared using *Artemia* sp. nauplii as the only food source, but those producing smaller larvae may require additional (or other) food sources (e.g. rotifers, microalgae). Some species are quite suitable for rearing and

have been adopted as models (*Hyas araneus*: [65, 84–87]); there are other species where success is only partial (*Cancer pagurus*: [83]).

The optimization of rearing methods is not only about trying different types of containers or environmental conditions. In order to find out what the larvae are doing inside the rearing containers and to obtain critical information, long periods of observation are essential. For instance: if larvae are cannibalistic, individual rearing is mandatory; if they interfere with each other while swimming, a lower density is vital and gentle aeration (or no aeration) is a must. If they are physically damaged by the walls or any other structure placed inside the container, another type of container is required; if they try to feed but cannot capture prey, or if they do not try to feed, another diet is needed. Furthermore, how larvae respond to light or aeration determines how to regulate the light cycle and light intensity, or strength of air bubbles.

#### *How will the culture proceed?*

There are two potential sources of larvae: either they are directly collected in the field ("wild" larvae) or they are obtained from berried females collected in the field and kept in the laboratory until hatching. Here, we focus on methods based on larvae obtained from berried females. For "wild" larvae (i.e. collected in the field), the rearing methods described here also apply as long as the species is known and specimens are handled with great care during collection (e.g. avoid individuals being entangled with the mesh of a plankton net). If the species is unknown, observations on morphology and size may help to infer the optimal rearing methods (see below).

The first step in the successful rearing of larvae is the collection and maintenance of healthy berried females. Each species will require specific collection methods (see literature cited in Table 2) depending on the habitat: for instance, the use of a dredge (e.g. *Hyas araneus* [65]) or baited traps (e.g. *Carcinus maenas* and *Homarus gammarus* [88], Fig. 3) for subtidal species, careful hand collection for intertidal and semi-terrestrial species (*Carcinus maenas* [44], *Metopaulias depressus* [89], Fig. 3). Besides, it is essential to consider the temporal range of the reproductive period, when the berried females are available. The timing of collection, considering the length of embryogenesis, will be essential for the aims of the study: i.e. early embryos will allow for manipulation during the embryonic development [45], while late embryos will minimise the acclimation to the laboratory conditions [42]. Furthermore, while the berried females are being transported, a high-quality environment is critical; this includes a constant temperature similar to the collection habitat, enough ambient humidity or access to sufficient water, $O_2$ availability, and adequate space per individual. The maintenance of the females in the laboratory must ensure that females are in an environment similar to the natural habitat (e.g. access to dry periods for intertidal species, optimal temperature, dark:light periods, access to food, Fig. 3). In addition, unnecessary manipulation should be avoided in order to minimise stressing the animals. Water quality is of utmost importance (see next section): some species will need a flow-through system, where water is renewed constantly (e.g. *Homarus gammarus* [90]; others may be kept in aquaria in still water with periodic water changes (e.g. *Armases miersii* [91]. In a flow through system, an appropriate collection system must be in place, where the larvae are gently gathered in a sieve located in the outflow of the aquaria. In aquaria, the water quality at hatching will determine a successful larval rearing; thus, it is important to provide enough water volume in the aquaria to ensure relatively low densities of the newly hatched larvae (see Fig. 3e, d).

To ensure a maximum of health, thus high survival, larvae have to be handled with much care and reared in a high-quality habitat, mimicking the natural environment as much as possible. In general, larvae are very sensitive to manipulation (e.g. water changes) and therefore, it is essential to be gentle. For most species, and in particular, for stages with large spines, pipetting (using wide-bore glass pipettes) each individual is better than using a sieve when transferring larvae from one container to a new clean one. Nevertheless, when larvae are more robust, sieving could be useful to save time in mass-rearing cultures (see below). To obtain abundant survivors, the best possible environment (regardless of experimental treatments) is an essential requisite, thus the highest water quality and high hygiene standards (e.g. researcher, rearing room, equipment) are key.

*Water quality* The water quality is critical to complete successfully the larval development. Thus, natural water, originating from an unpolluted source and with a low nutrient charge, should be treated with UV-light and filtered (0.2–1 µm filter) before use to avoid contamination with other small organisms (in some cases, even autoclaved). Moreover, the researcher has to consider also other abiotic (i.e. $O_2$ concentration, light, temperature, pH, etc.) and biotic variables (e.g. absence of other organisms, food quality, and concentration, etc.). The concentration of dissolved $O_2$ is essential, as it will provide the necessary $O_2$ required for respiration, but also prevent a decrease in pH. Ambient light is crucial to ensure development. Different species may have different requirements of e.g. photoperiod and light intensity (e.g. [92]). Most organisms have a range of temperatures where they are able to develop (e.g. [42, 43, 83, 84, 93]). Some lar-

**Table 2** A non-exhaustive table of species and references to larval rearing methods in decapod crustaceans

| Group | Species | References |
|---|---|---|
| **Lobsters** | *Homarus americanus* | [99] |
| | *Homarus gammarus* | [85, 90] |
| | *Nephrops norvegicus* | [193] |
| **Caridean shrimps** | *Crangon crangon* | [194] |
| | *Crangon allmanni* | [194] |
| | *Campylonotus vagans* | [195] |
| | *Macrobrachium amazonicum* | [196] |
| | *Macrobrachium pantanalense* | [153] |
| | *Palaemon* spp. | [197, 198] |
| | *Palaemon argentinus* | [199] |
| | *Palaemon serratus* | [200] |
| | *Palaemon varians* | [201] |
| | *Palaemonetes zariquieyi* | [68] |
| | *Pandalus montagui* | [202] |
| **Anomuran crabs** | *Galathea squamifera* | [85] |
| | *Galathea intermedia* | [203] |
| | *Lithodes maja* | [204] |
| | *Lithodes santolla* | [67] |
| | *Pagurus bernhardus* | [205] |
| | *Paralomis granulosa* | [67] |
| **Brachyuran crabs** | *Armases angustipes* | [206] |
| | *Armases miersii* | [91] |
| | *Armases roberti* | [66] |
| | *Armases ricordi* | [207] |
| | *Cancer pagurus* | [83, 85] |
| | *Carcinus maenas* | [44, 85] |
| | *Cardisoma armatum* | [208] |
| | *Chiromantes eulimene* | [209] |
| | *Chiromantes ortmanni* | [210] |
| | *Cyrtograpsus affinis* | [211] |
| | *Eriocheir sinensis* | [93, 212] |
| | *Geograpsus lividus* | [213] |
| | *Hemigrapsus sanguineus* | [43] |
| **Brachyuran crabs** | *Hexapanopeus schmitti* | [214] |
| | *Hyas araneus* | [65] |
| | *Hyas coarctatus* | [215] |
| | *Inachus dorsettensis* | [216] |
| | *Libinia emarginata* | [217] |
| | *Libinia ferreirae* | [218] |
| | *Liocarcinus holsatus* | [219, 220] |
| | *Macropodia rostrata* | [202] |
| | *Maja brachydactyla* | [221] |
| | *Maja squinado* | [202] |
| | *Menippe mercenaria* | [217] |
| | *Metasesarma rubripes* | [222] |
| | *Metopaulias depressus* | [89] |
| | *Necora puber* | [202] |
| | *Neohelice granulata* | [223] |
| | *Neopanope sayi* | [217] |
| | *Panopeus austrobesus* | [224] |
| | *Panopeus herbstii* | [217] |
| | *Perisesarma fasciatum* | [225] |
| | *Petrolisthes laevigatus* | [226] |
| | *Petrolisthes violaceus* | [227] |
| | *Pilumnus hirtellus* | [85] |
| | *Pinnotheres pisum* | [85] |
| | *Rhithropanopeus harrisii* | [217] |
| | *Sesarma curacaoense* | [228] |
| | *Sesarma cinereum* | [217] |
| | *Sesarma dolpinum* | [229] |
| | *Sesarma fossarum* | [230] |
| | *Sesarma meridies* | [82] |
| | *Sesarma windsor* | [229] |
| | *Sesarma rectum* | [231] |
| | *Uca thayeri* | [232] |

vae are lecithotrophic (i.e. they develop with the reserves invested by the mother); others need prey. There are many aspects to consider when choosing the food. For instance, the type of food will depend on whether the larvae are carnivores, herbivores, or omnivores; prey size has to be adequate to the predator's size (i.e. slightly smaller). Also, the appropriate food concentration must be considered.

To achieve the above-mentioned high water quality, water in the cultures has to be renewed on a daily basis. The main issue is finding a balance between the time invested in the daily water change and the stress caused by manipulating the sensitive larvae. The use of wide-bore glass pipettes (Fig. 4c) causes less stress during manipulation, thus it should be preferred for sensitive larvae (e.g. with long spines). When using a wide-bore glass pipette, the pipette has to be wide enough to avoid damage to the larval spines. The disadvantage of this approach is that it is time-consuming, so it would be more suitable for individual and group rearing. The use of a sieve (Fig. 4e) can save time when performing mass rearing. Here, the key issue is whether the larvae are sufficiently robust to withstand the stress caused by the sieving. When pouring the culture-water into the sieve larvae must not fall directly onto the mesh. Thus, the sieving equipment (i.e. sieve and collecting vessel, see Fig. 4e) should have the following characteristics: (1) the sieve has to be large enough (e.g. 10–15 cm high, 5–10 cm diameter; Fig. 4″) while still being manageable (e.g. to be held with one hand). (2) The mesh-size should be significantly smaller than the larval size (to avoid snagging and/or breaking the spines). (3) The

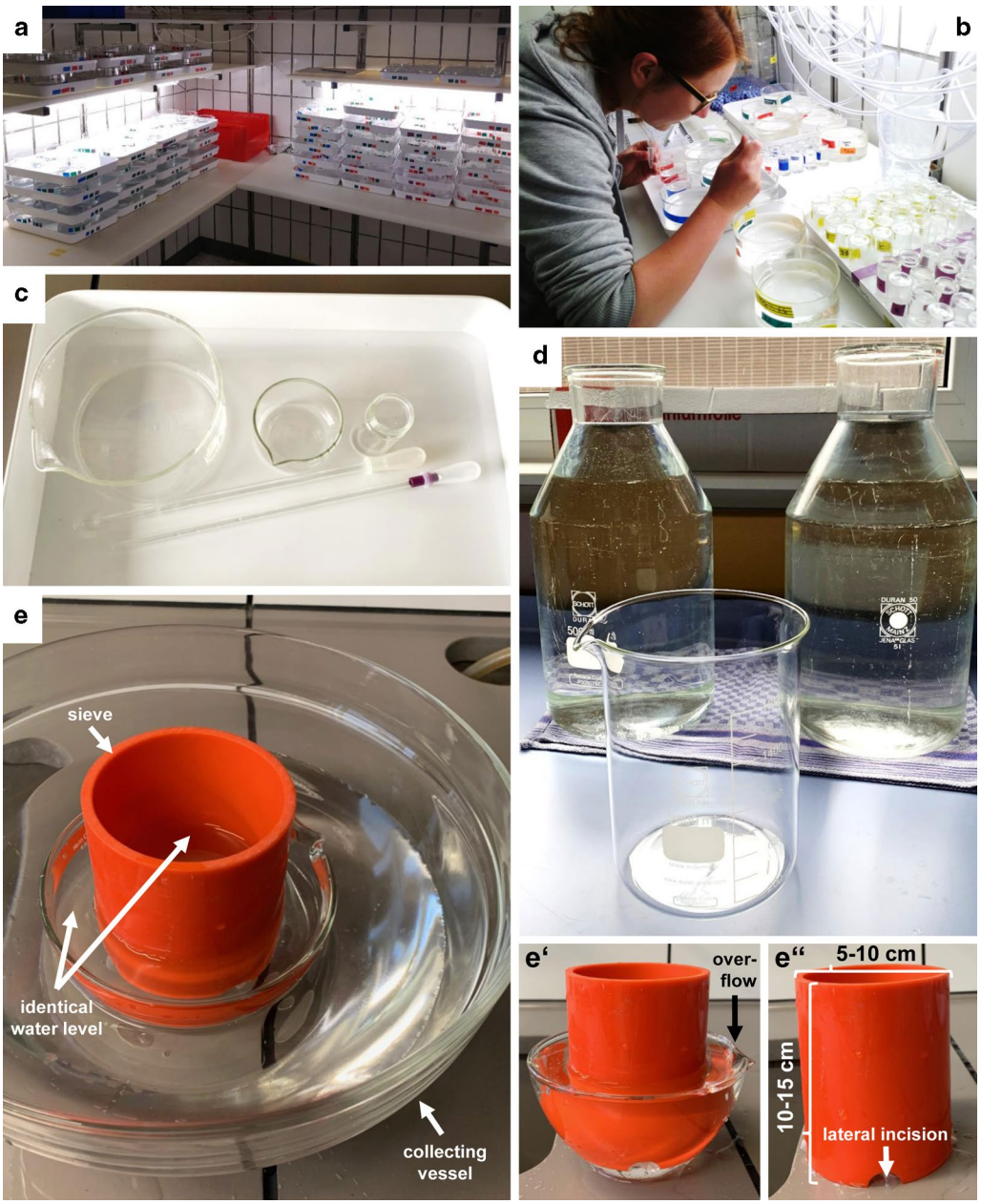


**Fig. 4** Obtaining and rearing crab larvae. **a** Climate-controlled rooms for larval rearing (photo Z. Šargač). **b** Daily water changes and feeding of larval cultures (photo S. Harzsch). **c** Rearing containers: 20 ml vials for individual rearing; 60 ml and 500 ml bowls for group rearing (from right to left); wide-bore glass pipettes (photo G. Torres). **d** Rearing containers: 2 L vessels for group rearing; 5 L bottles for mass-rearing (photo G. Torres). **e** Sieving equipment. **e'** Collecting vessel with water overflow. **e"** Sieve (ca. 10–15 cm high, 5–10 cm diameter) with lateral incisions (photos G. Torres)

sieve should be partly submersed in the collecting vessel (e.g. water level ca. 1–3 cm above the mesh). (4) The collecting vessel should allow overflowing to maintain identical water levels inside and outside the sieve (Fig. 4e, e'). (5) The sieve should have lateral incisions below the mesh to allow drainage of culture-water into the collecting vessel (Fig. 4e"). After pouring the larvae into the sieve, the concentrated larvae can be pipetted out with a wide-bore glass pipette.

*Cleanliness* *The researcher* must ensure a high level of personal cleanliness to avoid contamination of the larval cultures (for instance, with food rests, dirt, or any other

material, or with chemical residues). *Rearing rooms* (see next section for details) are usually a moist environment that favours mould growth. Therefore, rooms and working surfaces should be dried and cleaned periodically (usually at the end of the day). To avoid contamination with chemicals, it is advisable to keep chemicals (except perhaps ethanol) outside of the rearing room. When rearing occurs in *temperature-controlled incubators* (see next section) instead, these require special care: they need to be cleaned periodically and checked for ice formation (ideally, there should be a backup incubator in standby to alternate the use/rest and in-depth cleaning/de-icing of the available equipment). The walls of some incubators, as well as other furniture in rearing rooms, may be metallic. When working with seawater, those surfaces must be thoroughly rinsed with freshwater and dried to avoid corrosion due to salt deposits formed on a surface (this is also valid for stainless steel).

*Rounded glass containers* (vials, bowls, vessels, or bottles) are preferable to rear larvae, as they are dishwasher safe and autoclavable, but many types of plastic containers are usually quite useful as well (Fig. 4). Rearing containers, and any other material, should be cleaned periodically. Depending on species, cleaning may consist of e.g. rinsing containers with hot freshwater during the daily water change. Before transferring the larvae, rearing containers need to be rinsed with seawater, or the appropriate experimental salinity, to avoid changes in salinity due to freshwater residues. Additionally, a weekly thorough cleaning of the container (e.g. scrapping with a brush) may be required. The nature and length of the experiment will determine when containers will need to be dish-washed (with detergent) and/or fully sterilized.

#### *Where? Rearing containers and space*

To control the external experimental conditions of the cultures, especially a pre-determined temperature, larval rearing may be executed in climate-controlled rooms and/or incubators. The former has the advantage to allow for a larger experimental set-up with fewer treatments (ideal for mass-culture; Fig. 4). The latter allows for a smaller experimental set-up but with more treatments in a reduced space (ideal for individual rearing).

Larval rearing methods reflect a balance between the need for high habitat quality and space availability inside the laboratory or incubators. We classify larval rearing in three categories: individual, group, and mass rearing. Individual rearing is the best available method (unless the experiment involves interactions among individuals); however, it demands a high number of containers, increased handling effort (i.e. more time consuming), and usually larger spaces to allocate all the replicate containers (Fig. 4). Individual rearing is usually carried out in small glass vials (≈20–100 ml) to optimise space and handling time (Fig. 4c). Group rearing consists of culturing a group of individuals in replicate 100–500 ml bowls (10–50 larvae; Fig. 4c) or 1–3 L vessels (100–200 larvae; Fig. 4d). Finally, mass rearing may be carried out with 100 s to 1000 s of larvae in large bottles (>5 L; Fig. 4d). Keeping larval density as low as possible is the critical issue here. Unfortunately, there is no standard rule about the optimal larval density because the consumption of food and oxygen as well as the excretion of e.g. ammonia will depend on the rearing temperature, body mass, and level of activity of the species/stage being reared. Checking literature dealing with larval rearing of a similar species is the starting point to decide the rearing density. When information is not available, preliminary experiments using different rearing densities will provide the optimal density; our "rule of thumb": keep larval density: ~10 µg dry mass per 10 ml of water. However, we have managed to rear larvae of the European lobster (1 mg of dry mass) in 100 ml of water with 80–100% survival rates, albeit in individual containers [94]. In an optimal situation, aeration is provided by bubbling air into the rearing container, but bubbling may not be possible in many rearing containers. In that case, preliminary experiments should provide information on optimal levels of oxygenation. For optimal rearing, without bubbling, we recommend glasses of a wide surface to volume ratio and daily water changes.

#### *How long will the experiment take place? Beware of the moult cycle!*

In the case of crustaceans, understanding the moult cycle is essential for the appropriate larval rearing; each larval instar consists of a separate moult cycle characterized by rapid developmental changes. A key discovery in the study of the biology of decapod crustacean larvae was that the changes associated with the moult cycle are accompanied by important changes in other physiological variables [82]. Individuals can duplicate their body mass and exhibit important changes in their elemental and biochemical composition, as well as in metabolic rates ([10]); in addition, patterns of tolerance to stressors change within the moult cycle ([66, 95, 96]). There are two corollaries from those findings: (1) larval rearing procedures, aimed at quantifying physiological changes, must ensure the tracking of groups of larvae with different moulting histories, and (2) data must be reported with reference to the larval stage and the age within the moult cycle (e.g. [86]).

Tracking groups of larvae, with different moulting histories, requires that individuals moulting on different

days are monitored closely and kept in separate glasses. Depending on the objective of the experiment, the rearing may start with a single mass culture; as larvae moult to a new stage, the culture is split progressively into new rearing containers after each daily check. This procedure has to be repeated when larvae moult to a subsequent stage. To save space (rearing containers) and handling time (water change of many containers), another option is to split the culture only as soon as the instar of interest is reached.

## Additional comments

### *Be patient: the longer way is faster and boring is good*

Given the current existing pressure to publish in the scientific community, it is natural to want to develop experiments and produce publishable results as quickly as possible. However, rearing larvae, especially for the first time, requires usually 1–3 months devoted to preliminary experiments, where none of the results produced will be publishable.

*The longer way is faster*: Culturing a new species demands a series of preliminary experiments aimed to create a protocol for optimal rearing conditions: i.e. optimising the container type (size and shape), level of aeration (bubbling) and larval density required, as well as determining the combination of environmental factors that maximise survival. Furthermore, it is crucial to acquire sufficient manual skills to be able to gently pipette larvae in and out of the different rearing containers as well as identify species and/or specific larval stages. To ensure maximum larval survival, these have to be treated gently, which requires allocating an important amount of time to larval handling (counting of larvae, removal of dead individuals, and determination of moulting stages). Additional time should be allocated to preparing new water, clean the rearing containers, incubators, and rearing room. Within days and/or weeks of practice, the handling time will get shorter and eventually reach an optimum. Skipping the phase of the preliminary experiment has many risks, only justifiable when not many berried females are available. In the long run, rushing in order to "save" time may cause the experiment to fail; hence, going through the long way by running preliminary experiments will allow getting faster to the phase of obtaining reliable results.

Large experiments imply a vast demand in time availability, which requires a change of behaviour in the researchers involved. Many activities performed during the workday (i.e. computer work, breaks, teaching, administration, etc.) have to be arranged around the requirements of larval rearing. In addition, larvae only follow the moult cycle, without any regard to weekends, bank holidays, etc.; they just need their clean water and food when it is due. If a daily water change is missed, it may result in failure of the entire experiment (that may have been running for months). Therefore, a critical point for the researcher is to find the limitations regarding how much time can be allocated to larval rearing per day, to maintain an appropriate balance between work and free time but to ensure obtaining data of quality. Besides, the project leader has to evaluate the needs of each experiment in terms of handling time, in order to allocate the appropriate workforce. As in any other area of experimental sciences, there is a point when it is better not to run an experiment, rather than running a bad one. If the question addressed allows, it is better to stagger a large experiment over a long period (e.g. several months) instead of trying to run many parallel replicates within a short time (e.g. 4–5 weeks). Large experiments demand intensive work over an extensive period: in some sense, *as you cage larvae into your rearing containers, they will cage you inside the lab*.

*Boring is good*: Running a large experiment demands patience, as the same work is repeated every day over a long period. In an "uneventful" (standard) day, the researcher gets to the lab, spends long hours checking the experiments, collects data into a table (or collect samples), and goes home; some days are "eventful" because something wrong happens (e.g. food fails, water is not ready to use as it was not prepared in advance, etc.). Usually, it is during the analysis (data or sample analyses) that the researcher will regret making decisions based on impatience and going through the shortcuts. By then the situation is irreversible as the experiment is finished with insufficient individuals for sampling or bad quality data. Instead, a researcher with a patient attitude will enjoy the period of sample and data analysis and appreciate all the time and effort spent in the laboratory.

### *Conclusion: preliminary experiments are the key to success*

As mentioned above, preliminary experiments are the key to mastering rearing techniques, get self-confidence and ensure high data-quality from the main experiments. It is important to recognise that the time invested in preliminary experiments will not provide data of sufficient quality to warrant a publication, but will ensure a smooth running of the subsequent experiments. In a preliminary experiment, it is advisable to run a smaller version of the full experiment with a couple of repetitions in a temporal sequence. The temporal sequence (instead of parallel runs) enables the researcher to use the previous experience and apply the acquired knowledge to improve the work in the following sequence. Self-confidence is acquired after repetition and the confirmation that results are consistent across experimental runs and also

are consistent with those obtained in a previous study (if such study is available).

## Preparation and fixation

### Introduction

In order to ensure tissue preservation, a chemical fixation is the first step in all histological methods that we will describe in the companion paper [63]. The preparation and chemical fixation of the larvae is a key step that defines whether the subsequent histological processing will be successful. Arthropods in general, no matter how small they are, show low permeability to any fixative that has to go through their cuticle. As a rule of thumb, the higher the microscopic detail required, the more important proper fixation becomes. The low permeability of the cuticle delays the penetration of fixatives and thus may reach the organs when they are already in the process of deterioration. Therefore, a preparation that at low microscopic magnification of semi-thin sections seems acceptable may later turn out to be unsuitable to show ultrastructural details under the transmission electron microscope (TEM). In addition, the subsequent infiltration with the reagents needed to process a specimen e.g. for TEM analysis is slowed down by the low permeability of the arthropod cuticle. Note that the overwhelming majority of chemicals and reagents used in histology are highly toxic. Therefore, carefully consider the health and safety regulations in the laboratory (e.g. wear of personal protection equipment, proper storage of chemical fixatives); besides, strictly keep away those chemicals from larval cultures. In the following, we will describe basic aspects of fixation. In the companion paper [63], we will briefly repeat selected aspects of fixation related to the individual techniques described there.

### Description of method

#### *Dissection*

The two main strategies we recommend to minimize the effects of slow penetration of the fixative are (1) to dissect even small arthropods to make tissue accessible for the fixatives (and other chemicals; [84, 97]), and (2) to use cold to slow down the decay of the tissue (e.g. cold solutions and/or cooling dishes on cold packs). The first step is to anaesthetise the live organisms by chilling (i.e. exposure to a cold environment) until their movement stops. We recommend cooling the larvae for 5 min, immediately dissect them submerged in the fixatives (under a fume hood), and transfer them to a vial with fresh fixative and store them at 4 °C for at least 24 h. During dissection, the illumination source (from the top, the side or below) and choice of background (black *versus* transparent) need to be optimised. Furthermore, well-prepared tools are needed (Fig. 5). For example, fine and superfine tip forceps (e.g. Dumont® no. 5—tip dimensions: $0.05 \times 0.01$ mm; Dumont® no. 5 superfine, tip dimensions: $0.025 \times 0.005$ mm) are ideal instruments for dissection. If necessary, the forceps can be sharpened on a fine Arkansas grinding stone in a drop of immersion oil (Fig. 5; squeeze tips of forceps carefully together, grind all sides but the inner ones at a low angle to obtain square tips, then sharpen all four outer corners at an angle of 45° to obtain trapezoidal tips). By following this procedure, forceps finer than newly bought ones can be obtained. Furthermore, the use of scalpel blades with a rounded blade is useful, but only for a few cuts, because they will get dull quickly. Pieces of razor blades or micro scissors may also do the job. In order to create little perforations on the cuticle, the use of very fine minutiae or pieces of tungsten wire is recommended, without touching the surroundings of the organs of interest. To create the very pointy tips, tungsten wire can be sharpened electrolytically or on sandpaper. Following this procedure, it is possible to obtain an excellent fixation even of specimens that are larger than what is often considered a maximum tissue size (i.e., 1 $mm^3$) in textbooks on electron microscopy. Careful preparation will provide an advantage for all the subsequent steps (e.g. infiltration will enhance processes: the penetration of the fixatives, dehydration, and embedding in resin).

We recommend dissecting in glass Petri dishes with SYLGARD® 184 Elastomer outfit (black or transparent; or any silicon-based outfit of an elastomer) at the bottom to avoid bending or breaking the extremely sensitive tips of the forceps. At least a few appendages such as spines, limbs, or the pleon (those you do not intend to analyse) should be cut off under a stereo microscope to ensure access of the fixatives (e.g., *Hyas araneus* [84], *Homarus americanus* [97]. When cutting off appendages, never do this near the organs that you want to analyse, because they may be mechanically affected. Using minutiae or pieces of very fine tungsten wire to perforate the cuticle will improve fixation even of very small specimens. However, minimise touching the specimens with mechanical devices such as forceps to avoid damage or use tweezers with soft tips. Therefore, it is advisable to leave the specimens within the fixation containers (use small glass vials and not plastic tubes), where they had been transferred to after the dissection. During the subsequent steps, just change the solutions with pipettes without touching the specimens. The preparation should be performed in the cold and all solutions and glassware during the fixation process should be kept cold (e.g. on ice). For the preparation under the stereomicroscope, use a cold pack or Peltier cooling element to keep specimens and slides cold. Make sure not to freeze the specimens as ice crystals may change the integrity of the tissues.

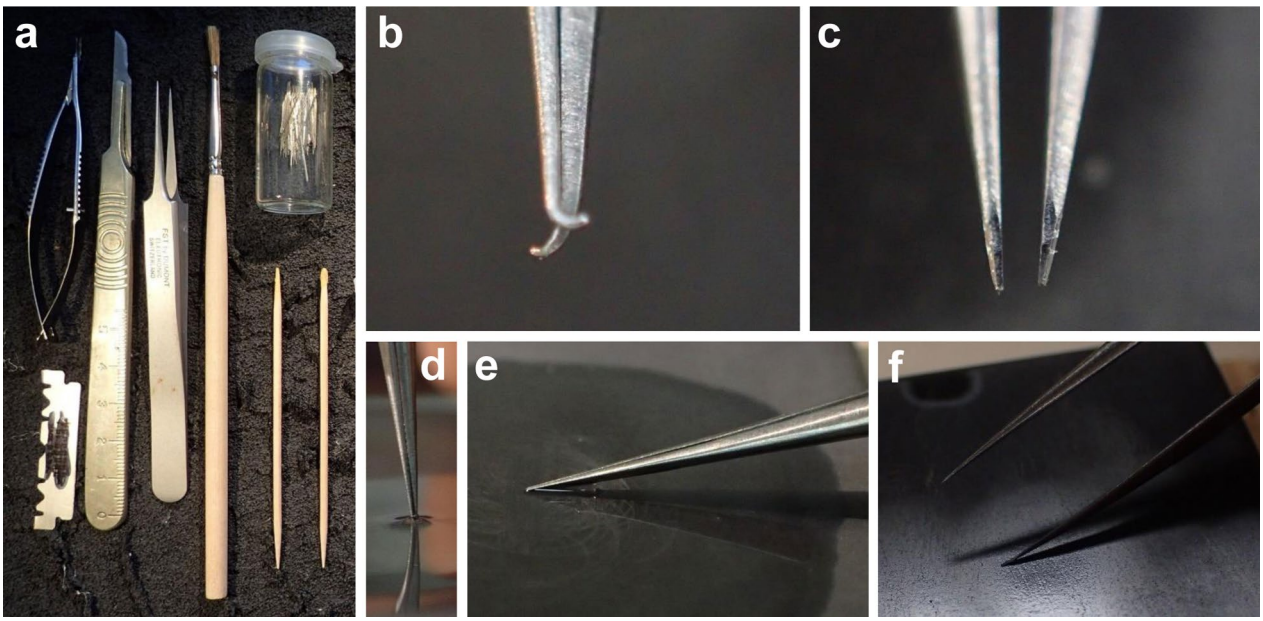


**Fig. 5** Helpful tools for microdissection of Zoeae and other minute arthropods (all photos by R. Melzer) **a** Tools for dissection, mounting, and mechanical cleansing of larvae. Micro scissors, forceps size "5", scalpel, razor blade or small pieces of razor blades, minutiae, tooth sticks with eyelashes glued onto their tips. **b**–**f** How to sharpen forceps. **b** Forceps in a not useful state. **c** Same forceps as in **b**, sharpened. **d** Sanding the tip to get equal-length arms on extra fine Arkansas grinding stone in oil. **e** Sharpening of sides of arms. **f** Tapering the upper outer edges

#### *Fixation*

During the fixation process, the specimens will be for a long time in vials subjected to various aqueous and non-aqueous solutions. Some tricks that may dramatically improve the results: (a) Specimens should never float on the surface of the fixative; they must be completely submerged in it. To ensure that the specimens are submerged, they may be carefully pushed down with a needle or by carefully squeezing a little plug of cotton wool into the vial. (b) Specimens should not dry out. A couple of drops of the old solution should always be left in the jar before adding the next solution with a pipette. The old solution will be eliminated in the subsequent washing steps. (c) Avoid the transfer of traces of the old solution into the next steps by using a fresh pipette for each step. (d) Eliminate all traces of water before the specimens are transferred to the non-aqueous solutions. A trace of water in the last dehydration steps and/or in the resin will result in badly polymerized resin blocks. (e) Follow the instructions of the manufacturers written on the package leaflets of your reagents.

In Table 3, we have summarized which fixatives in our view are optimal for the various histological methods and list which fixatives may provide acceptable results. Ideally, you have a well-equipped histology lab available to process samples from lab rearing but that may not always be the case. For example, among the available fixatives, the most commonly used for routine plankton fixation is 2–4% formaldehyde in seawater. However, this mixture is the least suitable for most histological techniques, because it causes shrinkage and various types of deformations. Nevertheless, formaldehyde or paraformaldehyde provide sufficient fixation for many immunohistochemical techniques and subsequent analysis with a fluorescent microscope (Table 3). For example, a typical immunohistochemical protocol requires immersion in 4% paraformaldehyde (PFA) in PBS (0.1 M phosphate-buffered saline pH 7.4) for 4 h at room temperature.

Furthermore, when working with marine organisms in a lab setting and controlled conditions, the osmolarity of the various fixative needs to be adjusted by adding non-electrolytes like sucrose or glucose [98, 99] to avoid volume changes of tissue. We recommend adding 2–5% glucose (or even 9%) to adjust the solution osmolarity to that of the larval body fluid. As there are considerable differences between decapod taxa, we suggest starting with 2% and if necessary, optimise by trying out different concentrations. If you can control fixation in a lab setting, adjusting the osmolarity is a *must* for all fixatives described below (see also Table 3).

If you do not depend on already fixed plankton samples but instead can control fixation yourself in the lab, we recommend using glutardialdehyde instead of formaldehyde and diluting it in phosphate or

**Table 3** Recommended fixatives discriminated by suitability for each specific method

| Histological technique | Recommended fixatives | Acceptable fixatives |
|---|---|---|
| External observation: light microscopy and epifluorescence | PFA, FASW, Karnovsky's | 75% ethanol |
| Immunohistochemistry | PFA | FASW |
| Micro CT | Bouin's, PFA | FASW, Karnovsky's, 75% ethanol |
| Scanning electron microscopy | FAE, AAF | 75% ethanol |
| Paraffin histology | Bouin's, Karnovsky's | FAE, PFA |
| Semithin sectioning | Bouin's, Karnovsky's or FAE followed by OS | |
| Transmission electron microscopy | Karnovsky's, GA or TA, followed by OS; GAOS | |

AAF: 85 ml 100% ethanol, 5 ml glacial acetic acid, 10 ml 37% formaldehyde*

Bouin's fixative: 10% formaldehyde, 5% glacial acetic acid in saturated aqueous picric acid 1.2%*

FAE: 150 ml 80% ethanol, 60 ml 37% formalin, and 15 ml glacial acetic acid*

FASW: 4% formaldehyde in water from the animal's habitat

GA: 4% glutardialdehyde in 0.1 M phosphate or cacodylate-buffered saline pH 7.1–7.3*

GAOS: 2–4% glutardialdehyde, 1–2% $OsO_4$ in 0.1 M cacodylate buffer pH 7.1–7.2*

Karnovsky's solution: 2–4% formaldehyde and glutaraldehyde in 0.1 M buffer (phosphate buffer or cacodylate buffer) pH 7.1 to 7.2*

OS: 0.5–4% osmium tetroxide ($OsO_4$) in 0.1 M buffer*

PFA: 4% paraformaldehyde in 0.1 M phosphate-buffered saline pH 7.4*

TA: 2% tannin, 2% glutardialdehyde in 0.1 M sodium cacodylate buffer pH 7.2*

*Adjust osmolarity as described in "Preparation and fixation" section

cacodylate-buffered saline (0.1 M, pH 7.1–7.3) instead of seawater for TEM fixations (Table 3). However, using glutardialdehyde is incompatible with fluorescent probes because it increases the autofluorescence of tissues. For larvae, aqueous fixatives often cause problems for ultrastructural analyses because of the impermeable cuticle even if a dissection was carried out as described above. Most non-aqueous alternatives for scanning electron microscopy (SEM) fixation are ethanol-based, and these fixatives penetrate the tissues much faster than aqueous solutions. Nevertheless, tissue fixation quality is often not good enough, and the larval surface integrity suffers from similar types of artefacts as with water-based fixatives. A long-known fixative well established in paraffin histology and semithin sectioning but also applicable for SEM fixation is alcohol–glacial acetic acid–formalin solution (AAF), a mix of 85 ml 100% ethanol, 5 ml glacial acetic acid, and 10 ml 37% formaldehyde (Table 3).

For SEM, we have tried different concentrations of ethanol [100] and the best preservation of the larval shape was achieved by using a graded ethanol series (i.e. starting with 30% ethanol for some minutes, followed by slow dehydration in 50%, 60%, and 70% ethanol), which resulted in excellent fixation of the three-dimensionality of the larvae. For paraffin histology and also semithin sectioning, frequently a formalin–alcohol–glacial acetic acid solution (FAE) is used, a mix of 150 ml 80% ethanol, 60 ml 37% formalin, and 15 ml glacial acetic acid (Table 3). For these histological methods, also Bouin's fixative is used frequently (10% formaldehyde, 5% glacial acetic acid in saturated aqueous picric acid 1.2%). For X-ray microscopy, Bouin's solution or 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) or seawater have turned out to be useful fixatives that will also allow immunohistochemical labelling after µCT-scanning (Table 3).

For TEM, a more sophisticated chemical fixation using two subsequent steps is typically used. In a first step (the "primary fixative"), tissues are immersed in a buffered aldehyde solution (glutardialdehyde or a formaldehyde/glutardialdehyde mix) to fix proteins. As the second step ("osmication" or "secondary fixation") tissues are immersed in osmium tetroxide ($OsO_4$) in a buffer to fix double bonds, e.g., lipids of cell membranes (reviews in [101, 102]). A typical TEM primary fixative is Karnovsky's solution, a mix of glutardialdehyde and paraformaldehyde in buffer (Table 3). The primary fixative usually contains about 2 to 4% aldehyde in 0.1 M buffer (Sørensen phosphate buffer or cacodylate buffer) at a slightly alkaline pH (7.1 to 7.2) and is still done at 4 °C. In most protocols, the secondary fixation (also called "osmication") is carried out after washing the specimens several times for a few minutes in 0.1 M buffer. The final osmium tetroxide concentration (0.5–4%) should be present in 0.1 M buffer solution. The incubation times are strongly dependent on the size and infiltration characteristics of the specimens. In very small specimens, 30–60 min of primary fixation is sufficient; fixation time should be prolonged to several hours for larger specimens. In addition,

washing times in buffers between the primary and secondary fixation may also be modified, depending on size. Afterwards, the specimens are incubated in the secondary fixative for, again, at least half-hour to two hours, or even longer depending on their size. A good indicator of the success of the osmication process during the secondary fixation is an increasing degree of browning/darkening of the specimen's inner parts. After the secondary fixation ("osmication"), the specimens must be washed again in the buffer to remove surplus osmium tetroxide. Specimens fixed according to this double procedure are also well suited for semithin sectioning and subsequent histological stains.

Tanning with tannin and simultaneous fixation with aldehyde and $OsO_4$ can result in excellent fixation (see Table 3). Tanning improves protein fixation and contrast and may allow microtubule subunits to become visible in TEM. To include a tanning step during fixation, prepare a stock solution by diluting ca. 2% tannin in double-distilled water for some hours (until it is well dissolved) and filtering through a microfilter. Afterwards, create a primary fixative with 2% glutardialdehyde (0.1 M sodium cacodylate buffer, pH 7.2) solution with 0.5% tannic acid (modified after [103–106]). Use this solution instead of the primary fixative described above, then wash in the buffer as usual, and afterwards do the secondary fixation (osmication) following the above-mentioned protocol.

A simultaneous fixation with glutaraldehyde and $OsO_4$ is recommended to improve the quality of fixation in small aquatic arthropods [107]. The primary fixative is 2–4% glutardialdehyde and 1–2% $OsO_4$ in 0.1 M cacodylate buffer (pH 7.1–7.2). This solution has to be mixed immediately before use at 4 °C to avoid a high percentage of the Osmium being transferred to its tetravalent (pink form) which hinders a good fixation. Afterwards, the specimens should be washed in buffer and are osmicated in the secondary fixative described above.

#### Additional comments

For all the histological methods described in the companion paper [63], the most critical step is the preparation and chemical fixation procedure. If the preparation is not adequate for a quick and thorough infiltration of the specimens with the fixatives, the tissues will degrade during the subsequent processing. However, an excessive perforating and/or dissection may mechanically destruct the specimen due to the bad preparation. When larvae are available from laboratory cultures (see "Obtaining and handling larvae" section), it is possible to perform some trials before preparation: e.g. check the best positions for perforating holes, practice the best way of dissection without damaging the target organs, which reagents offer an optimal fixation. In the once-in-a-lifetime catch from the field, however, these preliminary trials will not be possible, and the circumstances may only allow "field" fixation of suboptimal quality.

### Animal welfare and ethical issues

At the time of planning an experiment, it is important to be aware of issues associated to animals' welfare ethics, rules and regulations. Regarding ethical issues, awareness and responsibility is crucial. Due to the experimental conditions, animals may suffer discomfort, experience pain, and/or will be killed. Therefore, the researcher needs to thoroughly consider these issues when starting to plan an experiment, and decide whether to carry experiments with animals (or kill them) at all. The second step is to decide whether the specific experiment is really needed. For the planning of an experiment, there are minimal recommendations regarding the welfare of the animals that will be used. The number of animals included in the experiment or sacrificed should be optimised: sufficient animals should be used to ensure statistically robustness, but avoiding the use of excessive numbers. Both extremes are bad options: while the latter is obviously not desirable at all, too few replicates will lead to low confidence in the results and animals would have died for nothing. In addition, during the experimental procedures, the researcher should aim to be as gentle as possible and provide the best possible environmental conditions to the animals, while still addressing the experimental questions in order to gain the desired new information.

Regarding the legal issues (rules and regulations): there are national and international laws applicable for animals used with scientific purposes, and those are constantly being updated, see for instance: *European guidelines on protection of animals used for scientific purposes* (DIRECTIVE 2010/63/EU: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32010L0063&from=EN—accessed on the 11th May 2021). It is important to check the rules and regulations concerning killing and experimenting with animals by contacting the animals' welfare officer in the institution where the experiments will take place. The relevant laws and regulations usually give a definition of "animal experiment" and specify how to sacrifice animals among many other topics. Since such regulations depend strongly on the national laws of a country (they may depend on the state within a country), it is not possible to provide information of general standing here. Researchers have to be aware that regulations change frequently: in some countries, such regulations may not cover crustaceans (or their early stages) yet, but this may change in the future. Hence, it is essential to contact the animals' welfare officer well in advance of including experiments involving animals in a project proposal or performing the experiments. This first step will

ensure that the researcher will be properly instructed on the obligations and legal procedures associated to experiments involving animals. Such instructions may range from the necessity to take courses on welfare of animals used in experimentation, fill application forms to register experiments or request permissions to carry out experiments, to follow any other type of legal procedure associated to working with animals. It should be kept in mind that compliance to ethical and legal issues (e.g. permits) might be needed at the time of the submission of a project proposal or the start of an experiment. There might be a potentially lengthy period from the moment of contact with the animals' welfare officer to the moment when the project proposal can be submitted or the experiment can be started.

## Conclusions

This paper ends with having described all essential steps to rear decapod crustacean larvae from individuals to mass cultures. The companion paper [63] will describe the techniques to study cells, tissues, and organ development in these animals.

**Acknowledgements**
We dedicate this review to Klaus Anger who introduced us to the incredible world of tiny crustacean larvae with utmost patience. FS, ZS, and SH were supported by the DFG Research Training Group 2010 RESPONSE. We wish to thank Jakob Krieger, Marie Hörnig, Carolin Haug, and Joachim Haug for commenting on a draft version of this paper. We gratefully acknowledge Georg Brenneis for posing in Fig. 3a and FS in Fig. 4b. We are indebted to Noé Espinosa for contributing the figure of a berried female of *Hemigrapsus sanguineus* releasing larvae (i.e. hatching).

**Authors' contributions**
GT, RRM, SH and LG conceived and wrote this paper. SH compiled the plates. FS and ZS assisted in writing the paper and contributed images. All authors read and approved the final manuscript.

**Funding**
Open Access funding enabled and organized by Projekt DEAL. FS, ZS, and SH were supported by the DFG Research training Group 2010 RESPONSE.

**Availability of data and materials**
Not applicable, we do not report any original data here.

## Declarations

**Ethics approval and consent to participate**
Not applicable because this technical review does not report any original data and does not describe any original experiments.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare that they have no competing interests.

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Received: 4 October 2020 Accepted: 18 May 2021
Published online: 02 June 2021

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## Publisher's Note

## Publication IV

Published in Helgoland Marine Research, peer reviewed journal:

Melzer RR, Spitzner F, **Šargač Z**, Hörnig MK, Krieger J, Haug C, Haug JT, Kirchhoff T, Meth R, Torres G, Harzsch S (2021)

https://doi.org/10.1186/s10152-021-00547-y

# Methods to study organogenesis in decapod crustacean larvae II: analysing cells and tissues.

Melzer *et al. Helgol Mar Res* (2021) 75:2
https://doi.org/10.1186/s10152-021-00547-y

Helgoland Marine Research

REVIEW

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# Methods to study organogenesis in decapod crustacean larvae II: analysing cells and tissues

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## Abstract

Cells and tissues form the bewildering diversity of crustacean larval organ systems which are necessary for these organisms to autonomously survive in the plankton. For the developmental biologist, decapod crustaceans provide the fascinating opportunity to analyse how the adult organism unfolds from organ Anlagen compressed into a miniature larva in the sub-millimetre range. This publication is the second part of our survey of methods to study organogenesis in decapod crustacean larvae. In a companion paper, we have already described the techniques for culturing larvae in the laboratory and dissecting and chemically fixing their tissues for histological analyses. Here, we review various classical and more modern imaging techniques suitable for analyses of eidonomy, anatomy, and morphogenetic changes within decapod larval development, and protocols including many tips and tricks for successful research are provided. The methods cover reflected-light-based methods, autofluorescence-based imaging, scanning electron microscopy, usage of specific fluorescence markers, classical histology (paraffin, semithin and ultrathin sectioning combined with light and electron microscopy), X-ray microscopy (μCT), immunohistochemistry and usage of in vivo markers. For each method, we report our personal experience and give estimations of the method's research possibilities, the effort needed, costs and provide an outlook for future directions of research.

**Keywords:** EcoDevo, MorphoEvoDevo, Brachyura, Immunohistochemistry, Micro-computed tomography, Larval development, Electron microscopy, Organogenesis, Imaging techniques, Histology

## Background

Whereas crustacean embryos are popular study objects of developmental biologists (recent reviews, e.g., [1–7]), analyses on organogenesis in crustacean larvae somewhat lag behind (see Table 1 in companion paper [8]) although, for many decades, brachyuran larvae have served as distinguished models in the field of Ecological Developmental Biology (reviews [9, 10]). Larval development of decapod crustaceans embraces a wealth of fascinating ontogenetic processes that are well worth analysing. Many decapod crustaceans display a complex life cycle which includes pelagic larvae and in many subgroups benthic juvenile-adult stages so that these organisms undergo a distinct niche shift during the transition from larvae to the juvenile phase (reviews [9–14]). Larvae develop by successive moults through a species-dependent number of larval instars and, in the absence of parental care, are adapted to developing in the plankton concerning aspects such as movement, nutrition, and sensing (reviews [11, 12, 15]). Larvae also feature a rich behavioural repertoire which allows them to respond to variations in environmental key factors such as light, hydrostatic pressure, tidal currents, temperature, salinity, and food concentration (reviews [10, 16, 17]). In many decapod groups, after two metamorphic moults (reviews [18–21]), larvae eventually settle to the

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We dedicate this review to Mireille and Guy Charmantier, two of the pioneers in analysing organogenesis in decapod crustacean larvae

benthos where juvenile growth into adulthood, reproduction, embryonic development, and larval hatching occurs.

Cells and tissues form the bewildering diversity of larval organ systems which are necessary for autonomously surviving in the plankton ([11, 22–25]). As the larvae develop, their organs continue to grow in size and complexity. For the developmental biologist, decapod larvae provide the fascinating opportunity to observe a complete and complex organism on a single microscopic slide and the chance to analyse how the adult organism unfolds from organ Anlagen compressed into a miniature larva in the sub-millimetre range. Furthermore, several of the metamorphic processes are promising targets for future investigations by developmental biologists [22]. For example, the neuromuscular system of the pereiopods must be refined during the seemingly abrupt transition to functionality during the first metamorphic moult. Furthermore, as gas exchange switches from the branchiostegites in the Zoea to the gills in the Megalopa, the circulatory system must adapt to new routes of haemolymph flow. Contrary to holometabolous insects, for example, which in the pupal stage develop a cuticular armor to shield the developing organism against environmental impacts, such metamorphic transformations must occur "on the fly" in a planktonic larva that is constantly exposed to predation and must respond to abiotic environmental parameters.

This set of two papers sets out to summarise current techniques to analyse organogenesis in decapod crustacean larvae. In the companion paper [8], we have already described the techniques for culturing larvae in the laboratory and dissecting and chemically fixing their tissues for histological analyses. Here, we continue with describing state-of-the-art morphological and anatomical techniques that are established for various decapod larvae. We will begin our account by describing techniques to analyse the outer larval surface such as macrophotography, imaging of the cuticular autofluorescence and scanning electron microscopy. Such techniques are not only suited to analyse the ontogeny of the general larval morphology but also ontogenetic modification of specific organs such as sensory setae and the ommatidia of the compound eyes. We continue with techniques to study the anatomy of individual organs at the tissue and cellular levels such as classical histology, x-ray microscopy, immunohistochemistry and the use of cell proliferation markers.

## Methods to visualise the outer morphology

### Reflected-light-based methods: macro- and micro-photography

#### *Introduction*

Reflected-light-based methods in the context of macro- and micro-photography (Fig. 1) allow the documentation of an animal's general outer morphology, but also very fine details on the outer surface. These methods demand a relatively small amount of equipment and involve low costs (Table 1), but provide high flexibility in the setup without any destructive manipulation of the specimen. Reflected-light-based methods are essential for studying the outer morphology of an animal, especially if true colour information is desirable. Providing a well-documented general impression of the animal is also useful if the outer morphology is not the focus of the study. This allows a non-expert reader to gain an overview of the general *habitus* in high quality. One main advantage is the high flexibility of the method as it can be applied using nearly all kinds of microscopes, but also macrophotographic setups. The limitation of magnification depends on the used optics. For generating consistently sharp high-resolution images, single images in x-, y-, and z-axis can be combined with freely available or low-cost software.

#### *Description of the method*

*Specimen preparation* Fixed specimens (e.g., in ethanol) should be photographed directly in the fixative, otherwise delicate structures such as antennae or fine setae will agglutinate. These fluids of course should not contain particles that may obstruct the specimens. Small glass blocks can be used to adjust and fix the specimen in the desired orientation, and coverslips on top of the specimen (with glass blocks or plates as spacers to the bottom of the container and fixed with small weights such as screw nuts) will prevent movement. In principle, all kinds of containers such as Petri dishes or plastic containers filled with coloured silicone, e.g., in black or white are useful. If the specimen is very bright or dark, a black or white background can cause too low or too extreme contrasts. In these cases, it can be helpful to use a grey or blue background (the latter should be decolourised during image post-processing).

*Setup* Reflected-light-based imaging can be applied with nearly all types of microscopes or macro-setups. In all cases, light is one of the most important factors. Several light sources should be arranged around the specimen to avoid shadows, which may hide details or suggest false-positive details (e.g., [26]). To avoid reflections causing strong artefacts and for enhancing colour-contrast, cross-polarised light can be used. To that end, polarisation filters could be mounted in front of the light sources and in front of the objectives (e.g., [27]). If the polarisation filters are rotated about 90 degrees to each other, nearly all reflections should be obliterated.

*Micro-photography* A microscope equipped with a built-in camera and reflected light is useful (Fig. 1d), but not

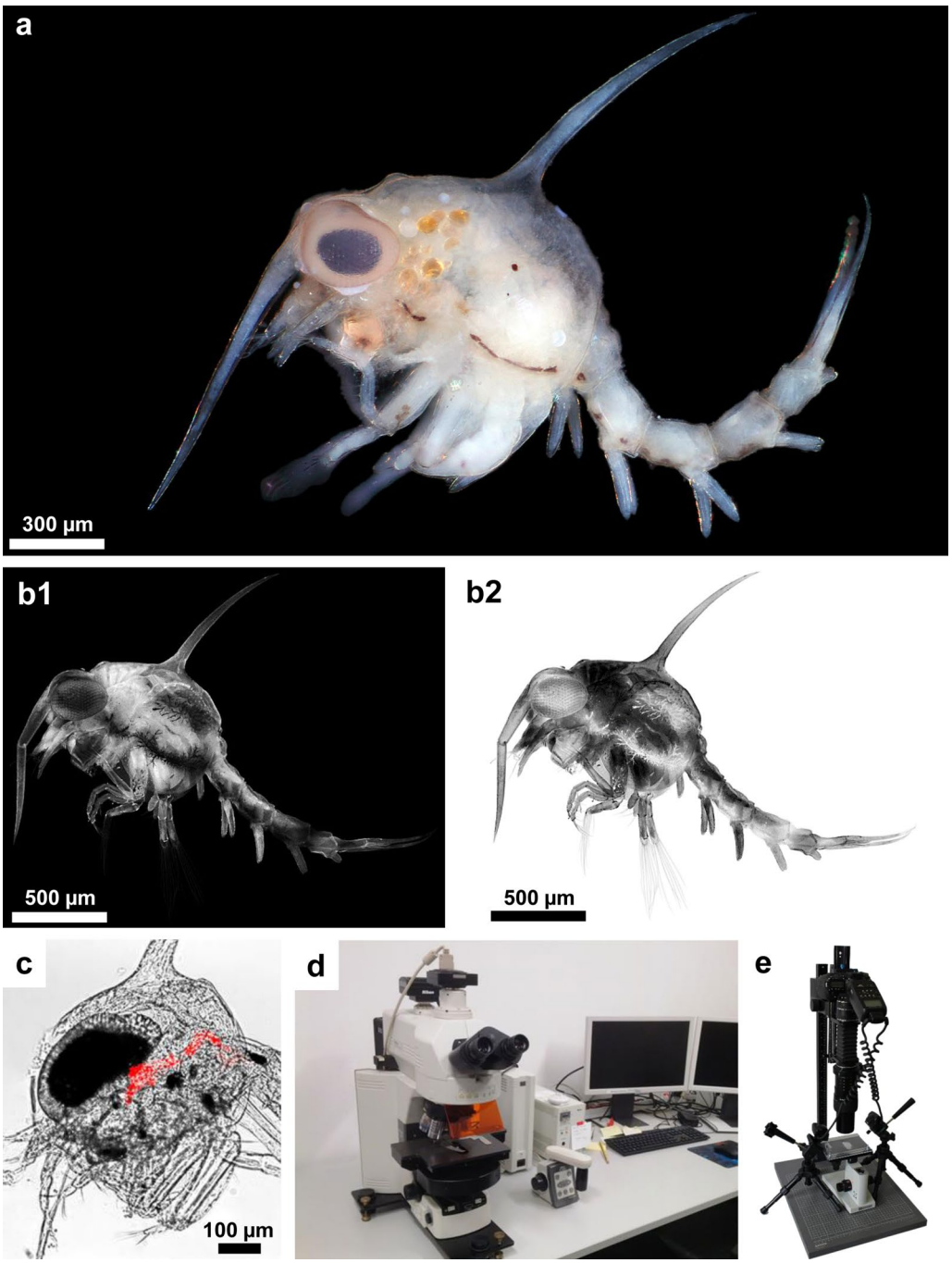



**Fig. 1** Macro- and microphotography, and autofluorescence-based imaging of decapod larvae (see section "Reflected-light-based methods: macro- and micro-photography" and "Autofluorescence-based imaging"). **a** Macrophoto of a *Carcinus maenas* Zoea IV using the camera setup shown in panel e) (M. Hörnig, unpublished). **b1**, **b2** Autofluorescence image of a *Carcinus maenas* Zoea IV mounted on a microscopic slide in ethanol and cover-slipped using modelling clay as spacers (F. Spitzner, unpublished). The image was taken using a Nikon Eclipse 90i upright microscope (excitation 359–371 nm; emission 379 nm; see panel **d** equipped with a digital camera. Image b2 was black-white inverted using image processing software. Note that more details of the setae on the exopods of the maxillipeds are visible. **c** Two-channel image of Zoea 1 of *Xantho poressa* combining brightfield image with chlorophyll autofluorescence in the intestine after feeding with diatoms (red) (modified from [33]). **d** Fully motorised upright fluorescent microscope Nikon Eclipse 90i (image S. Harzsch). **e** Macrophotographic setup (image M. Hörnig)

**Table 1** Summary of speed (from slow* to fast****) and costs (from rather cheap € to rather expensive €€€€) of the methods presented in this paper

| | Speed | Costs of reagents | Costs of lab instruments | Costs of imaging equipment |
|---|---|---|---|---|
| Macrophotography | **** | € | € | € |
| Autofluorescence microscopy | **** | € | € | €€/€€€ |
| SEM | ** | €€ | €€ | €€€€ |
| Micro CT | *** | € | €€ | €€€€ |
| Paraplast histology | ** | €€ | €€€ | €/€€€ |
| Semithin histology | ** | €€€ | €€€ | €/€€€ |
| TEM | * | €€€ | €€€€ | €€€€ |
| Immunohistochemistry | ** | €€€€ | € | €€/€€€€ |
| Proliferation markers | ** | €€€€ | € | €€/€€€€ |
| Nuclear dyes | **** | €€ | € | €€/€€€€ |

Some methods are much more time consuming than others: **** hours, *** one day, ** several days, * several weeks. The costs of reagents summarises necessary chemicals: € < 10, €€ < 100, €€€ < 500, €€€€ < 1,000. The costs of lab instrumentation summarises standard equipment such as orbital shakers, ovens and microtomes: € < 500, €€ < 5,000, €€€ < 10,000, €€€€ < 20,000. For the imaging equipment, some analysis is possible with rather simple microscopes, but more sophisticated instruments (such as a confocal laser scanning microscope) can be rather expensive: € < 2,000, €€ < 20,000, €€€ < 100,000, €€€€ over 250,000.

necessary. For usual transmitted-light microscopes, several light sources, such as gooseneck lamps or even flashlights (making the system more mobile), each equipped with polarisation filters, can be arranged around the specimen for illumination. For capturing images, a simple ocular camera can be used (such as Scopetek DCM 510). It is unnecessary to use cameras with high resolution since this factor is mainly limited by the objective lenses and single images can be stitched together (see below).

*Macro-photography* There are several ways to build up a macro-setup. Here, we refer to a custom setup widely used by the authors (Fig. 1e). The specimen in Fig. 1a was photographed with a Canon 70D reflex camera equipped with a Canon MP-E 65 mm f/2.8 1–5 × macro lens. To enhance the magnification beyond the maximum magnification of the objective, close-up rings are used (for example bridging a total of 86 mm distance in the case of Fig. 1a). Illumination is provided using a Canon MT-24EX Macro Twin Lite. Flash heads are fixed to tripods and arranged on two opposite sides close to the specimen. Flashes and objective are equipped with polarisation filters for cross-polarised light. We use linear filter foils the brand of which does not matter, and we have even used polarization foils removed from old flatscreen monitors and this works well. The camera is mounted on a repro stand with an additional macro slider/focusing rail, with the lens pointing downwards. The additional macro slider allows a more precise adjustment of the camera position and can be useful to photograph a specimen from different angles, e.g., for generating stereo anaglyph images.

*Composite images* To enhance the field of view and generate consistently sharp images in high resolution, recordings of several single images in z- and/or xy-axis can be taken and subsequently fused/stitched together ([28]; Table 2). For macro-setups, a table with fine drive in all axes (scanning stage) is highly recommended (e.g., dismounted from discarded microscopes). Z-image stacks can be fused to achieve consistently sharp images using free or low-cost software, such as ImageAnalyzer or Combine ZM/ZP. The fused images can subsequently be merged in x- and y-axis, e.g., with Photoshop (Photomerge) or Microsoft ICE (Table 2).

#### *Additional comments*

Due to the high flexibility of a custom setup, it can be adapted for many approaches without any manipulation of the specimen and to variable specimen sizes. This is especially important for very rare collection material, as well as fossil specimens ([26, 29–31]). As in principle, not much equipment is needed (Table 1), it is relatively easy to build up transportable setups for, e.g., collection material that cannot be transported. Macro-setups can be tuned to magnifications in a range close to those of light microscopes (e.g., see Fig. 1a, photographed with a macro-setup) for specimens of even less than 1 mm. Problems due to movements of specimens immersed in fluids result in strong artefacts in z-stacks. Hence, a proper mounting (without manipulation) of the specimen is necessary, but sometimes challenging. This holds also true for floating particles (dust) especially when working with high magnifications (here, filtering of the fixative can improve image quality). Even by using polarisation filters (and also diffusers), reflections cannot always be eliminated, which may cause strong artefacts, especially in fused z-stacks. This can be avoided by using fluorescence-based methods if the specimen exhibits autofluorescence or has been stained with a fluorescent dye ([27, 32]).

### Autofluorescence-based imaging

#### *Introduction*

The autofluorescence of the crustacean cuticle, without any staining, can be well used for documenting the outer morphology of a specimen (Figs. 1b1, b2, 2; [27, 32]). As compared to reflected-light-based methods, there are a number of differences, namely, that the contrast can be significantly enhanced, and that many fine details of the cuticle can be made visible (Fig. 2). In this aspect,

**Table 2** Selected examples for image processing software

| | Freely available | Operating system | Comments |
|---|---|---|---|
| **General image processing** | | | |
| Adobe Photoshop (Adobe Inc.) | | Microsoft Windows; macOS; Linux | |
| CorelDRAW (Corel Corporation) | | Microsoft Windows; macOS | |
| FIJI/ImageJ | Yes | Microsoft Windows; macOS; Linux | Open source, extremely variable in options, various plugins available [153] |
| GIMP | Yes | Microsoft Windows; macOS; Linux | Open source [157] |
| **Stacking (Z-Stacks)** | | | |
| Zerene Stacker (Zerene Systems LLC) | | Microsoft Windows; macOS; Linux | |
| Image Analyzer (MeeSoft, Michael Vinter) | Yes | Microsoft Windows | MultiFocus plugin [158] |
| Combine ZM/ZP (Alan Hadley) | Yes | Microsoft Windows | |
| **Stitching (X-/Y-AXIS)** | | | |
| Adobe Photoshop (Adobe INC.) (photomerge) | | Microsoft Windows; macOS; Linux | Photomerge plugin "Interactive Layout" only available up to CS5 |
| Microsoft Image Composite Editor (ICE) | Yes | Microsoft Windows | |
| **3D imaging/3D reconstruction (µCT, CLSM, sectional image stacks)** | | | |
| Amira (Zuse Institute Berlin; Thermo Fisher Scientific) | | Microsoft Windows; macOS; Linux | |
| Imaris (Bitplane) | | Microsoft Windows; macOS; | |
| VGSTUDIO Max (Volume Graphics) | | Microsoft Windows; macOS; Linux | |
| Drishti (Ajay Limaye) | Yes | Microsoft Windows; macOS; Linux | Open source [159] |
| SPIERS (Palaeoware; Mark Sutton, Russell Garwood, Alan R.T. Spencer) | Yes | Microsoft Windows; macOS; Linux | Open source [160] |
| Microscopy Image Browser (MIB; Electron Microscopy Unit, University of Helsinki) | Yes | Microsoft Windows; macOS; Linux | [161] |
| Blender (Blender Foundation) | Yes | Microsoft Windows; macOS; Linux | Open source; Postprocessing 3D Models, Surface visualisation [162] |
| Osirix (Pixmeo SARL) | Yes, in lite version | macOS | |

it will allow access to morphological structures that are also well recognisable in scanning electron microscopic (SEM) images, but much faster and without the necessity of the work-intense, partly destructive methods. This allows, for example, to use this method for rare and historical specimens. Additionally, also internal structures can be made visible without staining the specimen, for example, muscles, parts of the nervous system, or the digestive system. Similar to reflected-light-based methods, composite imaging (Table 2) will be necessary to overcome limitations in depth of field and field of view. Often, the same principle setups as for reflected-light based methods can be used. This allows a combination of the methods into one image. In many cases, autofluorescence, for example, is not very good for documenting projecting setae. Therefore, the same structure can be documented in addition to transmitted-light methods for gathering details of the projecting setae (e.g., differential interference contrast (DIC), polarisation contrast, or darkfield; [32], their Fig. 4f).

Not only the crustacean cuticle shows autofluorescence, but also plant cells do. For example, chlorophyll autofluorescence has been used in analyses of feeding experiments. For about 15 to 20 years, it is known that early zoea stages, which for a long time were thought to be exclusively carnivorous, also feed on phytoplankton such as diatoms. Tanković et al. [33] have detected and visualised chlorophyll in the intestine of Zoeae with the fluorescence microscope and even quantified the number of diatoms. This is possible because early Zoeae are transparent. In their paper, the authors visualised and measured chlorophyll fluorescence in unfed (control) and fed Zoea 1 stages of *Xantho poressa,* and determined the number of diatoms ingested by the animals. That way, they also visualised the intestine of living larvae (Fig. 1c).

#### *Description of the method*

*Setup* As pointed out, most setups used for reflected-light-based imaging can also be applied for autofluorescence-based methods, with nearly all types of microscopes or macrophotographic setups. The main important factor for achieving fluorescence is a reliable set of filters and a sufficient amount of light that is evenly distributed (as for reflected-light setups). For microscope setups, the

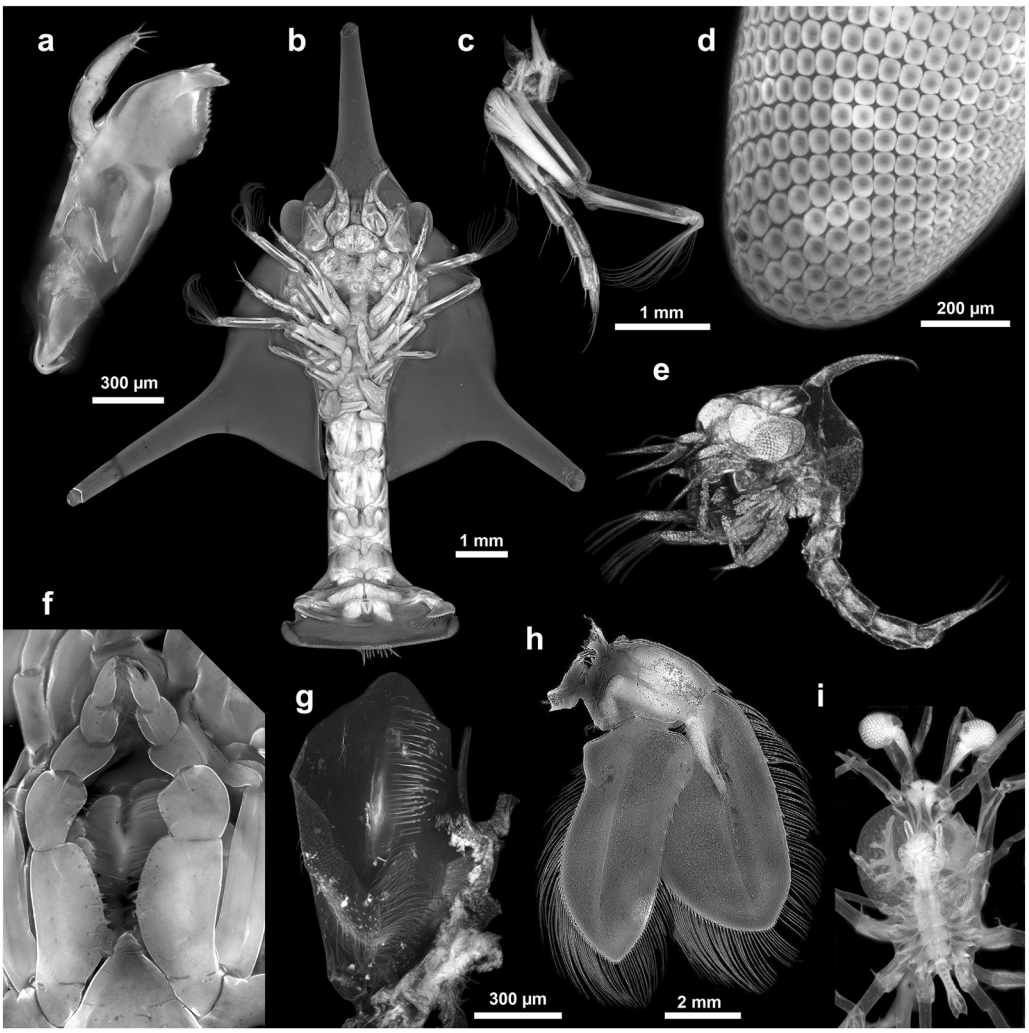



**Fig. 2** Different examples of images of decapod crustacean larvae recorded with autofluorescence as described in section "Autofluorescence-based imaging" (images not to scale); epifluorescence images except for e). **a** Isolated mandible of a late larva of *Homarus gammarus* (modified from [163]). **b** Late Zoea of a false sand crab (Hippidae), ventral view (modified from [164]). **c** Maxilliped of late Zoea of a false sand crab (Hippidae; modified from [164]); note the well apparent muscles. **d** Compound eye of late Zoea of an anomalan crustacean (modified from [165]). **e** Zoea of an undetermined brachyuran crab, lateral view, confocal laser-scan microscopy (C. Haug, J. T. Haug, unpublished). **f** Mouthparts of a Megalopa of a brachyuran crab (C. Haug, J. T. Haug, unpublished). **g** Part of gizzard of an anomalan Zoea (modified from [165]). **h** Uropod of an Eryoneicus of a polychelidan lobster (modified from [30]). **i** Central body of Phyllosoma of an achelatan lobster; note the well apparent nervous system and midgut glands (C. Haug, J. T. Haug, unpublished)

simplest illumination is coaxial (built-in) fluorescence light. Yet, for microscopes that lack such a built-in option and for macro-setups, it is possible to simply use several gooseneck lamps or focusable torches as well as LEDs. The cheapest available filters are the coloured foils of anaglyph glasses. The most readily available ones will be red-cyan foils, but it will in principle work with any type of anaglyph glasses or in general complementary filters. The filter with the colour with the shorter wavelength (e.g., cyan) has to be placed in front of the light sources; the filter with the colour with the longer wavelength (e.g., red) has to be placed in front of the lens/objective. As complementary filters are used, the light directly reflected from the specimen will be blocked, only the newly emitted (fluorescence) light will pass the filter. Autofluorescence-based imaging requires longer exposure times due to the low intensity of the emitted light. Therefore, it is especially important that the specimen is properly fixed in place and will not move during the documentation. Sometimes it is necessary to document the specimen with different exposure times as some areas exhibit strong autofluorescence while others show rather weak autofluorescence. If only one exposure

time is used, some areas will appear overexposed while others are barely visible. The images documented with different exposure times need to be combined during image processing. In addition, more complex setups with structured light can be used for recording images under autofluorescence. This includes the now more common confocal laser scanning microscopy (cLSM; [34]), but also microscopes with a spinning disc/Nipkow disc and potentially also other types such as light-sheet microscopes. The main advantage of all these setups for autofluorescence imaging is the reduction of scattered light.

*Image processing* The general processing of the recorded image stacks is the same as explained above for reflected-light-based methods. However, the resulting composite images require some further post-processing (Table 2). Images documented with autofluorescence-based methods often have a colour tinge. This may either be the result of the colour of the filter in front of the lens/objective; or it may be the result of a colour artificially added via the software of the microscope. The latter is initially intended to show which dye has been used to stain the documented specimen, e.g., in histochemical studies. However, as the specimens are not stained for autofluorescence imaging, the colour is meaningless in this context. This is in general true for all cases in which only a single channel is used, which means if only the intensity of a single dye is shown. In these cases, the artificial colour makes the image unnecessarily dark, especially when red or blue is used. It is therefore either recommended to switch off the pseudo-colour function of the microscope, or, if this is not possible, to later desaturate the image with image-processing software. The desaturated images, or the images originally documented as grey-scale images, need to be further processed to optimise their histograms, resulting in the use of the entire range.

If the images have been recorded with a true colour camera, the results might be very different. First, a single excitation wavelength can lead to several different emission wavelengths. This becomes especially apparent when you photograph under UV light ([27], their Fig. 4j; [32] their Fig. 4b–d). Furthermore, in many cases, distinct post-processing is necessary to remove the last remains of scattered light ([35]). In those cases, in which the specimen has been documented two times in the same position, but under different light settings or different exposure times, the two composite images can be further virtually combined, which provides comparative results to high dynamic range (HDR) recordings as used in modern cameras (e.g., [36]). When structured light is used for recording an image stack, a different set of programmes has to be used to process the stack. Most importantly, images will not be fused, but simply be projected into a single layer ([27]). Alternatively, such images can also be used for producing three-dimensional models or also simply stereo anaglyph images [27]. With the appropriate type of image processing, also non-structured light can be used for such approaches ([27, 37]).

#### *Additional comments*

Autofluorescence-based imaging has similar advantages and disadvantages as those mentioned above for reflected-light-based imaging (Table 1). Most critical is the light intensity and the deletion of scattered light to achieve the best possible contrast. Therefore, autofluorescence-based imaging under macro-photographic settings always demands a larger amount of light, while micro-photographic settings usually have a sufficient light intensity due to the built-in light sources. It is also necessary to provide a dark surrounding to reduce the scattered light, either by darkening the entire room or by an enclosed chamber around the microscope. In some cases, already a simple cardboard box might be helpful. As the exposure times are relatively long, no movement of the specimen should occur. Also, removing dust particles is crucial as these often show a larger fluorescence than the specimen and hence strongly disturb the image. Finally, especially the post-processing as described above is necessary for highly-contrasted and sharp images.

### Scanning electron microscopy (SEM)

#### *Introduction*

Compared to light microscopy, scanning electron microscopy (SEM) has at least one essential benefit: very high magnification at excellent resolution (overview in [38]; Figs. 3, 4). This holds for both, studying surface morphology and inner organisation. Since the objects are opaque under the SEM, an inspection of internal features, however, is not possible without dissection ([39]) or freeze fraction ([40]). In addition to the high resolution, the SEM is outstanding in its tremendous depth of focus, which particularly at low magnifications is a great advantage because, compared to time-consuming 3D image processing, researchers get it almost for free. The high resolving power of SEM has been used in larval descriptions including very small and sophisticated species-specific features that can hardly be detected under the light microscope (e.g., [41–45]). SEM is also a method of choice for analyses of morphogenetic processes during larval development, for example, the development of the external morphology of larval mandibles, their shape, armature, and setation ([46]) or the morphogenetic changes from newly hatched and adult caprellids (skeleton shrimps; [47, 48]). In this respect, correlative studies combining SEM with semithin histology or transmission electron microscopy (TEM) are very helpful,

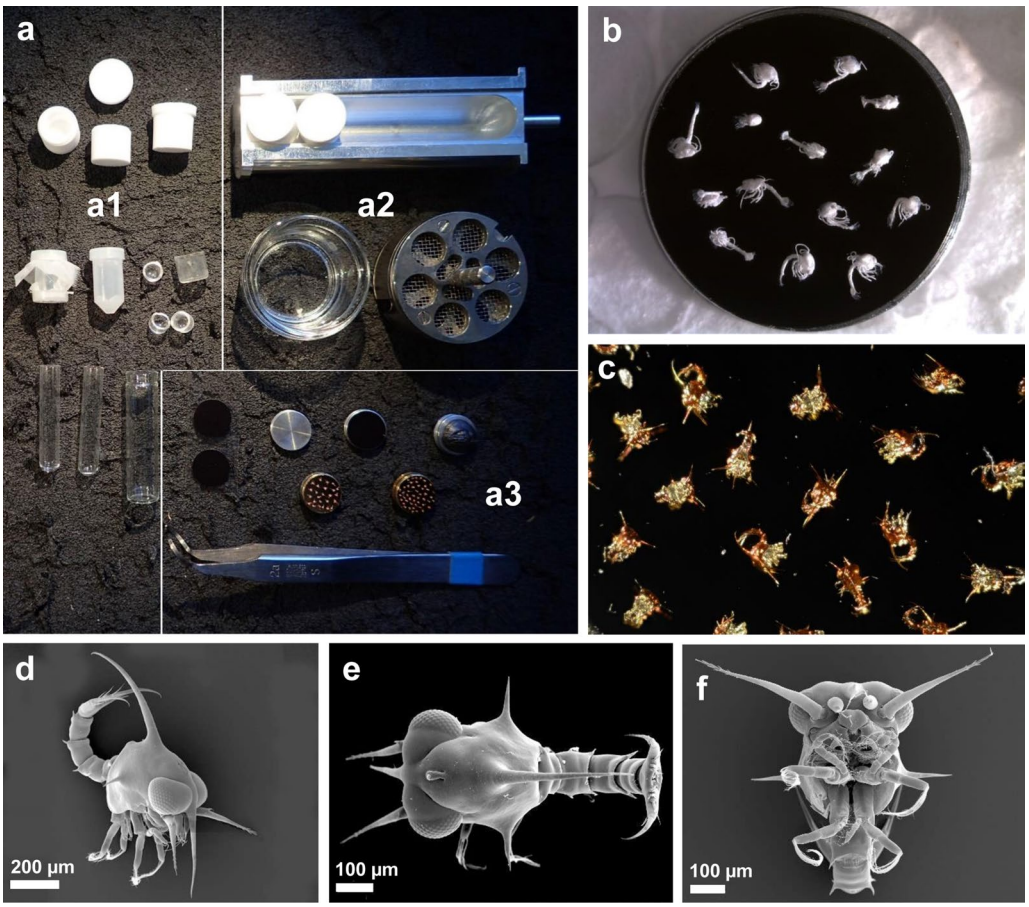



**Fig. 3** Scanning electron microscopy (see section "Scanning electron microscopy (SEM)"). **a** Accessories for critical point and chemical drying (image R. Melzer). **a1** Microporous capsules, self-made specimen holders (Eppendorf tubes with plankton mesh to ensure liquid exchange and prevent loss of larvae), small glass test tubes, and cut-off bases of test tubes to be inserted into microporous capsules. **a2** Different types of holders and vials for chemical cleaning and drying. **a3** Mounting of specimens: self-adhesive carbon stickers, empty SEM specimen stubs, specimen stub with carbon sticker, specimen stub with "Leit-C-plast", stub forceps, and two stubs with mounted and gold-sputtered specimens. **b** Higher magnification of specimen stub with cleaned, dried Zoeae of *Porcellana platycheles* before sputtering (image R. Meyer, R. Melzer). **c** "Zoea garden": Zoea 1 of *Panopeus chilensis*. **d–f** SEM pictures of Zoea 1 of *Panopeus chilensis*, viewed from anterolaterally (**d**), dorsally (**e**) and ventrally (**f**) (R. Meyer, R. Melzer, unpublished)

since thorough information about minute structures can hardly be obtained by using only one of these methods (see below). Other morphogenetic changes during larval development that can be studied with SEM are, e.g., stage-specific analyses of antennae, mouthparts, and limbs including also appendage Anlagen under the pleon (Figs. 3, 4). To use the invaluable power of the SEM to understand the three-dimensional morphology of the inner organs, the specimens to study can be dissected or deliberately broken in both, wet and dried state, to expose its inner organisation. This way, fixed, dissected and opened organs can be mounted on SEM stubs to study, e.g., gastric mills or other cuticular specialisations of the foregut and hindgut [49]. An excellent method to study soft tissues with the SEM is Tanaka-fraction [50, 51]. Here, freeze-fractured surfaces are processed with an osmium-DMSO-osmium method that allows for SEM-imaging of cracked surfaces and even intracellular structures. Scanning electron microscopy of this kind can provide important information in combined studies using various imaging techniques, whereby its resolving power is much higher than that of µCT.

Many researchers will find it challenging to prepare larvae of a total length of only 500 µm adequately for SEM studies. We, therefore, summarise here some handy hints and tricks on how to solve the task that we used successfully in the past decades. Optimal fixation procedures are essential (see companion paper [8]). For SEM studies, various preservation and preparation techniques must be applied to conserve the integrity of the form and surface

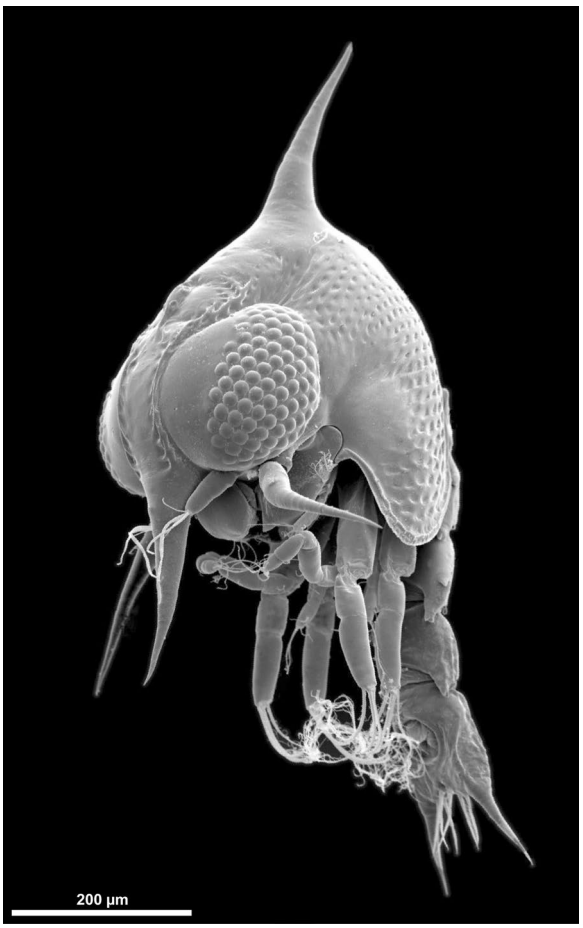



**Fig. 4** Scanning electron microscopic image of Zoea 1 of *Goniopsis pulchra*, viewed from anterolaterally (modified from [166])

of whole or partial larvae. In most cases, good preservation requires some effort, as it will depend on the fixatives, and also on the shape and stability of the study object. For example, shrimp larvae may need different manipulation than brachyuran larvae.

### *Description of the method*

*Cleaning the larvae* In our experience, a freshly hatched Zoea, or one directly after ecdysis is ideal for SEM examination. Why? Because it is clean from epibionts such as bacteria and it is undamaged. However, sometimes it will be necessary to study older larvae that have been actively feeding or have spent some time in the natural habitat. These larvae may have particles or bacteria sticking to their surface. To remove such particles there are two options the first of which is a careful mechanical cleaning (with devices such as micro brushes or eyelashes glued to toothpicks) but care needs to be taken in order not to damage the animal's delicate surface. The second option is chemical cleaning for which different methods are available, for instance, using hydrogen peroxide, $H_2O_2$, according to Boyde [52]. Incubate the larvae in ethanol in a small dish, adding one or a few drops of $H_2O_2$. Small bubbles will develop on the surface of the specimen and ascend in the fluid indicating that the cleaning is on the way. After 10 or 20 min, after a refill with a little bit of $H_2O_2$, the larvae will be cleaner.

*Drying and mounting* After fixation and maybe removing surface particles, the most challenging part of producing a good SEM specimen begins, the drying and mounting. The first method suggested here is chemical drying with hexamethyldisilazane (HMDS [53–55]; mind the toxicity of this reagent), which has also been established for crustaceans [56]. The specimen is incubated in HMDS in a small dish that is covered with a glass plate, but leaving a little space to allow HMDS evaporation overnight. Sometimes, setae or other small appendages may stick together during this process. A variation of this method is drying several larvae in HMDS mounted on an SEM stub. Note however, that the orientation of the specimens on the stubs will be random. The second method is the classical critical point drying (CPD). For larvae or small arthropods in general, this approach is tricky. For CPD, the specimens need to be placed in some kind of vial or specimen holder, and after drying, they have to be retrieved again from the vials for mounting on SEM stubs. For slightly larger larvae, this problem can be solved by using microporous capsules, available in different pore sizes for every type of CPD device. They are permeable to fluids such as acetone or liquid $CO_2$ and allow for a very good CPD. However, it is often difficult to retrieve the specimens after this process and remove them from the capsules after drying, since they might get stuck at the inner surface of the capsules. These are made of microgranules and thus have a rough instead of a smooth inner surface. An eyelash mounted on a toothpick can be used for this job. In case the samples are electrically charged, an anti-static gun (e.g., Milty Zerostat III®) can be used. Small Zoeae I or even parts of them need a more sophisticated setup. Usually, we use the lower part of test tubes as miniature containments for the specimens. For this, we cut off the tips of small glass test tubes (their diameter must be smaller than that of the microporous capsules) and place them into the capsules. Then, using a pipet, we put the specimens in drying acetone into the tubes, close the capsules with the lid, and insert the capsules as usual into the dryer. Because this procedure requires many and long liquid exchange steps, much practice is needed so that, with patience and a large supply of larvae, the success rate will improve. The amount of larvae available for achieving a perfect orientation is certainly an issue. A high number of specimens is important to increase the

chances to get some of them oriented perfectly. This is relatively easy with first stage zoeae, of which hundreds can be available from a single egg-carrying female but way more difficult with megalopae when only a few are available after longer periods of larval rearing (see companion paper [8]).

*Imaging* It is relatively easy to obtain chemically dried larvae randomly arranged on an SEM stub. However, specific orientations of the larvae or even parts of them on the SEM stubs are required to study them in detail. Chemically or CPD-dried larvae stick well to the tips of eyelashes, glued to a toothpick, and can be transferred to the SEM stubs where they can be carefully oriented on self-adhesive carbon stickers to the desired position [41–45]. For larger specimens, a special holder that allows exposing the specimens at various angles can be used [57, 58]. Afterwards, the specimens are ready for sputter coating with gold and inspection under the SEM. For more background on preparation techniques, consult references [59, 60].

#### *Additional comments*

Researchers that use camera lucida or photo-based drawings [61–64] to describe larval stages, probably will not approve of larval descriptions based on SEM alone. Larval descriptions (e.g., for taxonomy purposes) must provide a standardised presentation to show the appendages always in the same orientation in a way that corresponding features are visible and can be compared between taxa. However, if the technique is well executed, corresponding views can be obtained using both drawing and SEM approaches. This double approach needs a lot of time, effort, practice, and financial resources (Table 1). Yet, it is fascinating to apply SEM on a larva, or other small crustaceans, to use the resolution of this imaging device. With this technique, differential diagnoses can be more detailed than using light or fluorescence microscopy, and minute species-specific features can be visualised that otherwise would remain undetected. In our view, the most powerful application of the SEM is the analysis of morphogenetic changes of the larval eidonomy (externally visible structures as opposed to anatomy, internal structures) during development, since changes during development can be detected at very high resolution that are not visible with other methods (see [46] for an example).

## Methods to study the internal anatomy

### Specific markers for cell nuclei

#### *Introduction*

Observing a live, moving Zoea under a fluorescence microscope with every nucleus shining blue after a vital stain is a fascinating sight. In the following, we will show that it is rather easy to obtain a larval whole mount with cell nuclei stained, dead or alive (Fig. 5). Common nuclear markers that can be used for the technique are, e.g., DAPI® (4′,6-Diamidino-2-phenylindole), TO-PRO-3®, and SYTOX Green®, each covering a specific section of the spectrum of light (reagents available, e.g., from Sigma, Thermo Fisher). DAPI absorbs light in the UV (358 nm) and emits in the blue part of the spectrum (461 nm), SYTOX® Green has a maximum excitation wavelength at 488 nm and emits at 523 nm, and TO-PRO-3® is active at longer wavelengths (excitation at 642 nm, emission at 661 nm). These stains are universally used in many different fields of biosciences, but until now, they are not very common in studies of decapod development.

#### *Description of the method*

For vital stains, we recommend a protocol originally designed for *Chaoborus* larvae (Diptera) as described in [65], and subsequently used for Zoeae [66]. Larvae are kept in small vials in sea- or freshwater (depending on the natural habitat of the animals), and then a few drops of DAPI stain solution (ca. 1 mg DAPI in 10 ml distilled water) are added. After an incubation period of between 30 min to 1.5 h in darkness, the nuclei are stained and the larvae can be studied under the microscope. Where appropriate, 7% of magnesium chloride can be used to anaesthetise the larvae.

Chemical fixation of the larvae can be done either before or after staining. Unstained chemically fixed larvae should be incubated in the fluorescence marker shortly after fixation since otherwise, the staining reagent will penetrate less well as the tissues' biochemical composition becomes modified due to the action of the fixative. As fixatives, almost all formalin or ethanol-based mixtures like formalin-seawater or graded ethanol series can be used (see companion paper [8]). For imaging, the fixed specimens are mounted in an aqueous mounting medium suitable for fluorescence studies. Several companies such as Sigma or Thermo Fisher offer fluorescence media that already contain DAPI in an adequate concentration into which freshly fixed larvae can be embedded. After incubating larvae for ten to 30 min in these media, the nuclei of the specimen will also be stained with DAPI.

#### *Additional comments*

In cell biology and histology, fluorescent nuclear stains are commonly used as background stains for immunofluorescence labelling experiments that use specific antibodies. They help to understand the architecture of the studied organs in multichannel fluorescence analyses. Moreover, these stains can be used for nuclear and/

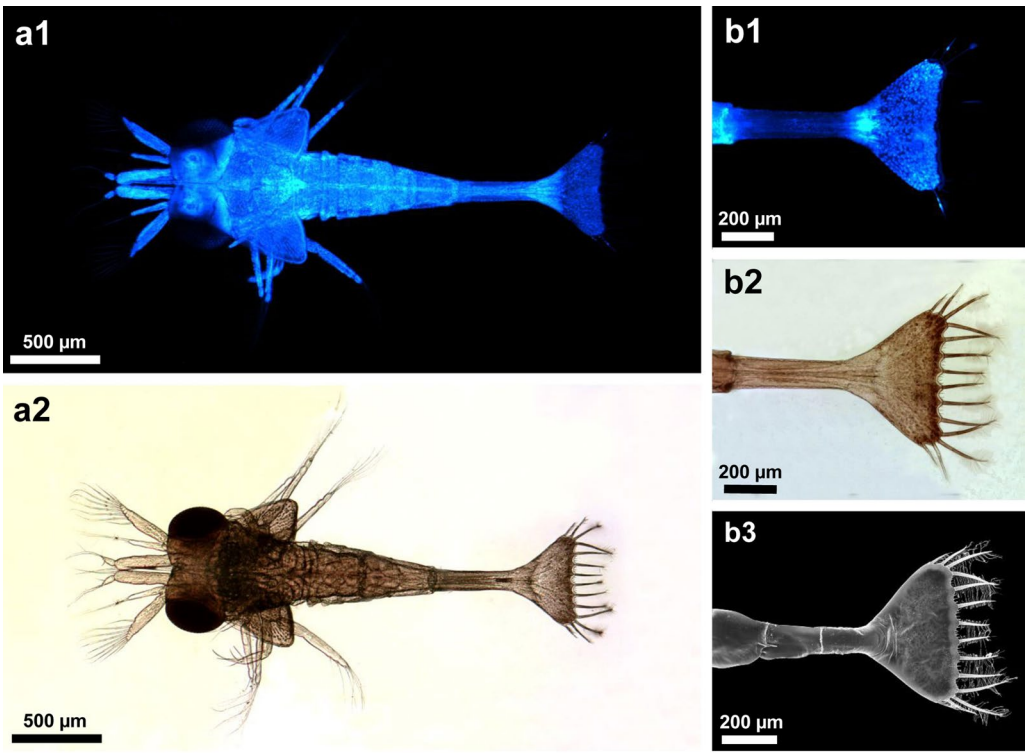



**Fig. 5** Fluorescence microscopy of specific markers for cell nuclei/DNA (modified from [66]) of Zoea 1 of *Palaemon elegans* (see section "Specific markers for cell nuclei"). **a** Two-channel analysis, dorsal view. **a1** DAPI-stained nuclei, **a2** bright field image. **b** Details of pleon and telson. **b1** DAPI-stained nuclei, **b2** bright field image, **b3** SEM. Note: all pictures, including SEM (b3), are from the same specimen

or cellular pattern analyses (e.g. [66]). We recommend employing this technique routinely as a primary tool in developmental studies of decapod larvae to study, e.g., the number and size of nuclei in certain organs or the presence or absence of clusters of densely accumulated nuclei, viz., of embryonic cell clusters. We consider it a worthwhile but scarcely used approach, e.g., to find out whether these clusters, similar to insect "imaginal discs", provide the source of morphogenetic waves of mitoses that bring about new components of the body during moults or during metamorphosis from Zoea to Megalopa. In addition to nuclear stains, there is a wealth of other fluorescent markers that allow to directly label organs that have hardly been used for larvae. For example, labelling tissue with phalloidin conjugated with a fluorescence marker was employed to study embryonic muscle development of crustaceans (review [1]) and may now be used for developmental studies on larvae.

## An oldie, but still a goldie—classical histology

### *Introduction*

Classical histology is used to study biological tissues to describe an organism's microscopic anatomy and has acquired major importance since the development of the microscope in the 15th–16th century [67]. A detailed morphological and anatomical visualisation of tissues can be achieved by this technique at the micrometre scale which is essential for functional, comparative, and developmental studies of all organisms. Furthermore, using differential staining methods allows to characterise complementary histological features of the selected organs. Despite its long history and numerous competing techniques, such as micro-computed tomography or immunohistochemistry (see other sections of this paper) to analyse tissues, classical histology may still be seen as the gold standard and a frequently used approach to analyse tissues of adult crustaceans (summarised in Table 2 in [22]; see also [68–71]). Especially for small animals, this approach allows gaining insights into anatomical and morphological structures by sectioning whole specimens where other preparation or dissection is virtually impossible. To this day, serial sectioning of entire specimens is an approach regularly used to investigate morphology, anatomy, and development of decapod crustacean larvae (summarised in Table 1 of the companion paper [8]).

We summarise here the principal steps required from a living larva to a histological image. Classical histology is generally characterised by a suite of the following

processes: (i) preparation, (ii) fixation procedure, (iii) dehydration and embedding, (iv) sectioning and staining, and (v) imaging session [72]. Steps (i) and (ii) were already described in the companion paper [8]. However, processing for transmission electron microscopy (TEM) using chemical fixation needs various additional steps, as will be detailed in the following, most of which are less important in solely light microscopic analyses. In general, microtomy (the technique of cutting an organism or single tissue into thin sections to allow for further observation under a light or electron microscope) determines the quality of tissue analysis because the thickness of the single sections and thus the resolution of the sample is defined by this step. Several modes of microtomy are classified in accordance with the section thickness, the microscopic technique, or the used microtome [72], for example: (1) histological sectioning (2–10 µm; Fig. 6); (2) semi-thin sectioning (0.25–2 µm; Figs. 7, 8, 9); (3) ultrathin sectioning (50–150 nm; Figs. 10, 11).

Histological sectioning based on paraffin wax (e.g., Paraplast®) embedded tissues presents a rapid and efficient technique to observe the overall tissue organisation. A rotary microtome is needed which is a standard piece of equipment in many histology and pathology labs. Followed by classical staining techniques, such as haematoxylin–eosin (HE) or Azan, it offers a convenient way to examine samples and differentiate larval organ systems, even with the simplest bright field light microscopes (see Table 1). Unstained histological sections can also be used in a variety of studies including in situ hybridisation or immunohistochemistry. Because classical histological sections are thicker (2–10 µm) in comparison to the other techniques, this method provides an additional aspect of "depth" in the z-direction and a better insight into the coherence of tissues. Moreover, the reagents used in histological sectioning mostly are less toxic (and expensive) and therefore safer to work with. For the analysis of the internal morphology of marine crustacean larvae and juveniles, we also obtained excellent results by using histological semi-thin sectioning (section thickness of 1–1.5 µm) of whole specimens in combination with bright field microscopy. This technique needs a higher quality microtome and profits from the use of expensive diamond knives (see Table 1). Because the specimens have to be embedded in plastic resins, it is more time consuming than the former technique. Serial section series obtained with these techniques can be used to perform 3D reconstructions.

The transmission electron microscope (TEM) is one of the mightiest (and most expensive) imaging devices in life sciences with a resolution that by far surpasses that of light microscopic methods surveyed here and, therefore, allows to analyse ultrastructure in great detail. This technique is therefore fundamental to understand the cell's ultrastructure including type, morphology, and distribution of the organelles and the cytoskeleton, and also helps to identify the cells responsible for the secretions (reviews, e.g., in [72, 73]). However, the process from the larva to the TEM image is complicated, time consuming and needs expensive chemicals and machinery (see Table 1). Hence, one should carefully consider whether the method is needed for a specific research question. For example, features such as the number of cells in an organ or analyses of the segmental organisation, or muscle fibre projections can be also unveiled with easier and faster methods than TEM. Nevertheless, whenever the development of minute structures such as sensilla, ommatidia, glands, synaptic connectivity in nervous systems and, in general, differentiation processes on the cellular level are to be studied, the TEM is the right method at hand (e.g., [74–76] for sensilla in general, and [77, 78] for crustacean larvae). Two main preparation techniques are used in TEM studies, the first is based on chemical fixation and represents the standard (see Table 1 in the companion paper [8]), whereas the second is based on cryofixation and is more sophisticated (surveys [72, 73, 79–81]). Cryotechniques circumvent some of the artefacts of chemical fixation, but are mainly useful for very small-sized specimens or objects in the 10-µm range. However, if cryofixation works, more detailed analyses, a higher resolution than with the standard protocol, and therefore even analyses of macromolecules are possible [82]. As Zoea larvae are indeed very small and include numerous minute structures, this approach should maybe be used more often, especially because high-pressure freezing extends the range of suitable specimen size into the reach of Zoea larvae or parts of them. A simple method that does not need costly equipment and can be established in any laboratory is described by Steinbrecht [83]. As the authors of this paper are not experienced in cryo-TEM, we will focus on the standard technique here.

TEM analyses using chemical fixation procedures are intricately connected with their sister technique, the semithin sectioning combined with light microscopic analysis. The latter approach originally was an aid to approach the region of interest in a specimen embedded for TEM, instead of making countless ultrathin sections. However, in times of serial 3D reconstructions, it has become a powerful tool as well (e.g., [84]). Here, the principle of reciprocity is also valid, because the quality of a 3D reconstruction based on semithin sections will improve when intermittent ultrathin sections are checked in the TEM to confirm what is "really seen" with the light microscope. That way, it is possible to access where the real borders between organs are located or what the real functional specifications of a cell or a group


**Fig. 6** Paraffin histology and Azan staining of a *Carcinus maenas* Megalopa (see section "Description of method: paraffin embedding, sectioning and staining (Azan)" for details). **a:** horizontal section of a Megalopa (Z. Šargač, unpublished). **b** Higher magnification of the compound eye (Z. Šargač, unpublished). **c** Leica RM 2145 rotary microtome for paraffin sectioning (image S. Harzsch). **d** Various tools for paraffin embedding (image Z. Šargač). **e** White embedding cassettes combined with Bio-Wraps (image Z. Šargač). Cc: crystalline cone, ce: compound eyes, cs: cardiac stomach, dg: digestive gland, la: lamina, mb: median brain, me: medulla, lo: lobula, pl: pleon, rh: rhabdom

of cells might be. However, various studies are using only one line of morphological evidence (e.g., SEM), to make statements about, for example, sensillar functions. Many studies would profit from adding TEM analyses to SEM, because the crucial structure–function relationships of sensilla can be better analysed by TEM since cellular and

dendritic ultrastructures have to be considered. There are even sensilla that are not visible at all under the SEM [77, 78].

#### *Description of the method: paraffin embedding, sectioning and staining (Azan)*

*Paraffin embedding* Prior to fixation, living larvae should be anaesthetised by a short exposure to low temperatures (see companion paper [8]). Before applying this method, animals must be immersed into the fixative and different appendages should be dissected (e.g., rostrum, dorsal spine, or limbs) for better penetration of the fixative into larval internal tissues (see paragraph on "fixation" in the companion paper [8]). We frequently used Bouin's fluid (i.e. 40% formaldehyde, 5% glacial acetic acid, 2% saturated aqueous picric acid) to preserve the animals. Animals completely immersed in the fixative should be stored at 4 °C for at least 24 h. The larvae are then removed from the fixative and different methods can be applied for easier fluid exchange, depending on the size of the larvae. Larger specimens can be transferred into labelled vials and solutions can be exchanged using pipettes. However, for small larvae such as those of *Carcinus maenas*, we recommend using white embedding cassettes (Routine VI, Loose, Leica Biosystems; Fig. 6d, e) combined with the SurgiPath® Bio-Wraps (Leica Biosystems). White embedding cassettes are great tools to save time and reagents because different samples can be marked separately with a pencil on the cassette itself and placed together in one beaker (Fig. 6d). To apply this technique, cut Bio-Wraps as squares to overlay the whole area of the embedding white cassette, with one centimetre extra on each border of the cassette (Fig. 6e). Place the larvae in the embedding cassette and close it with the lid merged with Bio-Wrap in a way that there is a surplus of the Bio-Wrap on each border (Fig. 6e). This technique presents an excellent substitute during solution exchange and washing on the orbital shaker since Bio-Wraps are permeable for solutions. Moreover, larvae do not stick to the wrap which thus prevents the larvae to wash through the pores of the cassette during the washing steps with agitation. Once the larvae are placed in the cassette, start the dehydration process of the samples by submerging them in ascending ethanol concentrations: 70%, 80%, 90%, 99% for 1–2 h in each step (we recommend four different concentrations, but additional steps with other concentrations could be added) at room temperature with moderate agitation (e.g., using a laboratory orbital shaker). Note that the first ethanol step (70%) can be repeated several times until all fixative is removed. For instance, in the case of Bouin's fluid, washing in 70% ethanol should be repeated until there is no trace of yellow colour leaching out. Especially for larger samples, washing steps can be extended to ensure successful dehydration. During all the washing steps cover the beaker with, e.g., aluminium foil or Parafilm® to minimise the evaporation of the washing solutions. After the last ethanol step, submerge samples in a mixture of Tetrahydrofuran (THF) and 99% ethanol (ratio 50:50) and leave samples at room temperature overnight with medium agitation. Tetrahydrofuran is an intermediary medium that is completely miscible with paraffin and potential residues of water in all proportions. Its use ensures the complete removal of water which results in a successful embedding in paraffin. An advantage of THF is that its vapours are much less toxic than other compounds that were widely used in the past such as dioxane (diethylene dioxide). After 24 h, exchange 50:50 THF + 99% ethanol solution and wash larvae for another 6 h on a shaker. Afterwards, place the samples in clean THF and leave overnight at room temperature with medium agitation. During this step, heat paraffin granules to 60 °C using an oven or other heated chamber to ensure good fluidity of the paraffin for the next day. We use Roti®-Plast with DMSO (Carl Roth Gmbh + Co. KG) granules but any other commercially available paraplast brands for histology can be used (note that melting temperatures might vary). Incubate samples in the mixture of THF and paraffin (ratio 50:50) in a 60 °C oven and let this mixture infiltrate overnight. After infiltration of the mixture, remove the larvae from the embedding cassettes and transfer them into a fresh change of melted paraffin. Considering the viscosity of paraffin, during this step, it is recommended to remove the larvae from the cassettes and put them directly in marked glassware filled with pure paraffin (for better mixing with paraffin and easier embedding later). In order to do so, pick up the cassettes with the larvae from 50:50 THF + paraffin (wearing gloves), open the cassettes, and pick the larvae with a plastic pipette (if paraffin is still liquid) or scrape them with forceps as the paraffin solidifies. Optimise this technique based on the size of the larvae and fluidity of the 50:50 THF + paraffin solution. It is important not to touch the specimens directly and minimise the surplus solution uptake during this step. Pure paraffin series can be exchanged several times until the smell of THF is gone to be certain that the samples are in clean paraffin. Because paraffin hardens quickly at room temperature, these exchanges need to be done in the heated chamber promptly. When the samples are in pure paraffin, they are ready for embedding, and we recommend not to keep for longer than a day in this medium. Prior to embedding, metal base moulds (Carl Roth + Co. KG, 52 × 35 × 11 mm outer part, 23 × 36 mm inner part; Fig. 6d) need to be warmed up to 40 °C. Prepare an alcohol burner and heat a dissection needle. Due to the fast hardening of paraffin, these next steps should be completed quickly and preferably by two persons (one person heating the needle and smoothening the surface

of the paraffin and the other pouring melted paraffin and positioning samples). The first step is to pour melted paraffin into the warmed-up embedding mould so that it covers most of its area. Using a pipette, place the sample into paraffin while positioning it centrally at the bottom of the mould. We recommend arranging two to three larvae close to each other without direct contact for easier sectioning afterwards. Larvae can be positioned as desired using hot metal tools such as needles with gentle movements as the paraffin is hardening. Smoothen the surface area with a hot dissection needle to ensure that the paraffin remains liquid. Remove the lid from the embedding cassette and press the cassette into the metal mould onto the melted paraffin with the bulging part facing the liquid paraffin so that it flows through the pores of the cassette. Add more melted paraffin to the top brackets of the embedding cassette. To ensure a good attachment of the paraffin to the cassette as it solidifies, it is important to remove any residual bubbles at the top area close to the pores by using a hot needle and pushing these bubbles to the surface of the paraffin. Residual bubbles in the solid paraffin can cause the sections to rupture or can result in breaking the sample during sectioning. Leave the prepared moulds to solidify at room temperature (for faster solidification, place moulds in the fridge). When the paraffin is completely solid, cut it between the metal and plastic border (e.g., with a razor blade) and carefully separate the metal embedding mould from the embedding cassette. If a bad attachment of the samples occurs, place the whole mould again in the 60 °C chamber until the wax is liquid again. Remove the old embedding cassette carefully without disturbing samples at the bottom of the mould and repeat the steps using a new plastic embedding cassette. A hot dissection needle can be used again to rearrange the samples as desired.

*Histological sectioning* Prior to sectioning using a rotary microtome (e.g., a Leica Microtome RM2145), a heating plate needs to be warmed up to 40 °C. Mark the cleaned microscope slides and warm them up on the heating plate. To decrease the water surface tension for an even distribution of water on the slide, place a droplet of glycine on the slide and spread it equally over the whole slide (e.g., using a paintbrush). Before sectioning, trim the solidified paraffin block with a razor blade in a trapezoid shape to reduce the surplus paraffin medium around your specimen. The blocks may be cooled for better trimming [85]. Make sure that the steel blade on the microtome is new and without any damage or physical scars to avoid section ruptures during sectioning. Position the block onto the microtome holder as desired and start trimming the block until the sample becomes more visible. Preservation in Bouin's fluid stains the samples with yellow colour, and since the yellow Bouin's fluid penetrates deep into the tissue (especially if some appendages are cut), the yellow stain will not disappear completely. This gives the advantage during sectioning on the microtome that the samples can be easily identified in the paraffin block. Traces of the fixative will be dissolved in the later steps after sectioning, during the washing steps on the slides. However, extremely tiny or transparent samples such as those fixed in PFA (which do not leave a colour trace of the fixative) can be stained prior to the embedding in paraffin. The most widely used solutions for this are toluidine blue, cresyl violet, or a low graded iodine solution (0.5–1% in ethanol after the final dehydration step; see previous chapter), where a drop of one of these solutions for a few seconds on the sample enhances the contrast between the sample and paraffin block. Once the sample is getting closer to the blade, switch to fine sectioning for the desired section thickness (good results can be achieved in a range of 2–10 µm). Pay special attention when the first tissue appears. Additionally, since the larvae of some species are extremely small, you can ensure the proper starting point of the sampled tissue by checking the sections under a microscope. When the first sampled tissues appear within the block, put water drops on the previously prepared heated slides and place sections onto the water surface. Since paraffin sections stick to each other, usually 3–5 sections (depending on the dimensions of the trimmed block) can be sectioned in a row, allowing for a better orientation of consecutive sections. A paintbrush, previously soaked in water, can be used to detach the sections and place them on the slides covered with water. As the heated water evaporates, the paraffin sections stretch and eventually stick to the slide. Note that transferring the sections from the blade to the slides is a tedious process and different techniques can be applied to achieve this goal es exemplified in reference [85]. Before the histological staining, the slides must be dried and can be stored at room temperature in a dark and dry place covered with aluminium foil for several months, or placed in the same way at 4 °C for even longer storage.

*Staining and mounting* Once the sections are dried on the slides, any desired histological stain can be used. Numerous dyes (acidic or basic) are available and can be used for staining, depending on the tissues or cells of interest. For an overview of the sample and revealing the general tissue anatomy, complex mixtures such as Periodic acid-Schiff reaction (PAS), Masson's trichrome, van Grieson, Azan, Mallory, etc. (reviewed in [67, 72, 85, 86]) can be used for larvae as exemplified in [23]. In principle, all stains used in analyses of adult crustacean histology such as, e.g., the popular trichromatic Masson–Goldner stain [87–90] can also be applied to sec-

tions of larval tissues (reagents are available, e.g., from MORPHISTO®). These methods complement each other and allow to analyse different cellular features of a given organ. A highly recommended compilation of staining techniques specific for crustaceans is available [85]. A differential analysis of an organ's histology using different stains can be complemented by immunohistochemistry (see below) to specifically localise cellular components such as elements of the cytoskeleton or transport proteins. In the following, we will elaborate on the Azan staining technique after Geidies [72, 86] as an example to describe steps that typically characterise a histological stain, and which we have successfully used to reveal different tissues on the paraffin sections (Fig. 6a, b). This formalin-free trichrome stain offers a great overview of the tissue, where extended washing of the samples with water does not affect the staining itself [91]. Azan staining differentiates connective and reticular tissues (blue), cell nuclei and erythrocytes (red), and muscle tissues (red–orange).

To save time and volume of exchange solutions, the slides can be placed into slide holders and stained together in one tray. The staining process starts by washing the slides with the attached sections in xylene for 10 min to remove the paraffin from the sections and to clear (i.e. make transparent) the tissues (any other clearing agent, such as the less toxic Roti-Histol®, can be used to remove the paraffin from the sections). Transfer slides into 99% iso-propanol and submerge them for 5 min to remove the xylene. Incubate slides in a descending alcohol series (we recommend a minimum of three steps: 96%, 80%, 60%) for 5 min in each concentration to rehydrate the samples. For thicker sections or bigger samples, more intermediate steps could be added, such as 90% and 70% (both for 5 min). Submerge the slides in distilled water for 5 min and afterwards incubate in a nuclear fast red solution for 30 min for selective staining. Dip the samples into distilled water and transfer them to 5% phosphotungstic acid to incubate for 5 min. Phosphotungstic acid in the dye mixture provides sufficient mordanting and also ensures the right acidity (pH: ~ 2–3) necessary for good results with the aniline blue-orange G mixture. Immerse shortly in distilled water and place slides into aniline blue-orange G solution for 10 min to stain collagen, reticular fibres and muscles. Clean the slides by shortly dipping them into distilled water, then incubate in 60% ethanol, next in 80% ethanol, and finally leave them in 96% ethanol for 5 min each step. Submerge slides in 99% isopropanol for five min and then incubate slides in xylene for at least 5 min to remove potential paraffin residues. Remove the slides from the xylene, dry them out on paper tissue and by using a piece of tissue carefully remove any residual xylene drops on the slide without touching the sample. After the xylene has entirely evaporated (after a few min), mount the slides immediately with 150 µl of Roti-Histokitt II (xylene-free polymeric mounting medium) per slide. Carefully cover samples with coverslips avoiding any bubbles and let the slides dry for at least 24 h at room temperature. Prepared dry slides can then be stored at room temperature in a dark and dry place and can last for years or even decades.

#### *Description of the method: plastic embedding, semithin sectioning, and staining with Toluidine Blue and according to Holländer and Vaaland*

*Embedding in plastic resins* We obtained good results (Fig. 7) with larvae fixed in FAE fixative or Bouin's solution (see section on fixation in the companion paper [8]). Because of the high toxicity of the fixatives and embedding media, all embedding steps need to be carried out under a fume hood. After washing the specimens in three changes (20 min each) of phosphate-buffered saline (PBS; 0.1 M, pH 7.2, 1.8% sucrose), the larvae are post-fixed for 1 h by immersion in 2% osmium tetroxide at room temperature. The specimens are then washed in three changes (20 min each) of PBS. Afterwards, the samples are transferred to 30% acetone and dehydrated through an ascending series of acetone (in concentration steps of 10%) to 100%. The dehydrated samples are transferred using micro dissecting needles in Eppendorf® plastic tubes filled with a 1:1 mixture of acetone and Araldite (Araldite® epoxy resin kit, Agar Scientific). Initially, the plastic tubes are left closed for 3 h and opened for 20 h afterwards. The completely soaked samples are then transferred into small glass dishes (embryo dishes) filled with 100% Araldite using micro dissecting needles. Also, the embedding blocks are filled with 100% Araldite. When preparing the embedding medium, the surplus of the liquid and unpolymerised 100% Araldite can be drawn into plastic syringes and stored frozen (− 20 °C) until further use. To ensure the best quality of Araldite, the syringes need to be defrosted at least 1 h before use and should be defrosted not more than three times. To avoid and remove air inclusion within the specimen and in the corners of the embedding blocks, heat the glass dishes with samples and embedding blocks to 60 °C for 15 min and subsequently expose them to a vacuum (200 mbar) for 20 min. In a final step, the samples are transferred into the pre-filled embedding blocks. Samples should be positioned at the tip of the embedding blocks using micro dissecting needles (Fig. 8a). The embedding blocks, including the samples, are then incubated for 48 h at 60 °C for final polymerisation. The viscosity of the embedding medium decreases right before polymerisation, so we recommend checking the position of the samples after 15 min and eventually

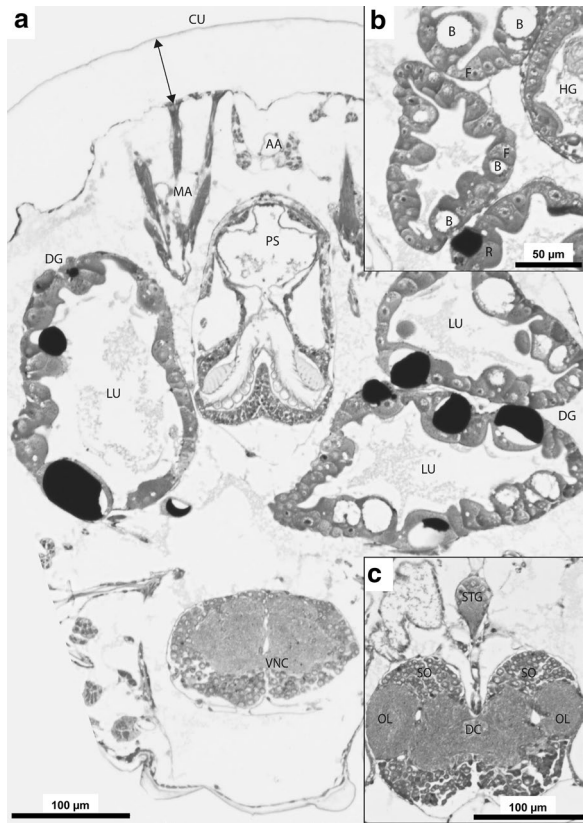



**Fig. 7** Frontal semithin sections of a Megalopa of *Carcinus maenas* (stained according to Holländer and Vaaland 1968, [22, 92]) illustrating the resolution of semi-thin sectioning (F. Spitzner, unpublished; see section "Description of method: plastic embedding, semithin sectioning, and staining with Toluidine Blue and staining according to Holländer and Vaaland" for details). Note that the cuticle (CU) and epidermis are detached (double arrow) due to poor fixation. To avoid such artefacts, adding up to 9% non-electrolytes helps to adjust the osmolarity of the fixative and therefore avoid volume changes of tissue. **a** Overview section showing the median portion of the pyloric stomach (PS) laterally flanked by the lobes of the digestive gland (DG). **b** Section through lobes of the digestive gland showing epithelium with the F-, R-, and B-cells surrounding the lumen (LU). **c** Section showing the median brain with the deutocerebrum (DC) and the dorsally positioned stomatogastric ganglion (STG). The deutocerebrum is characterised by the spherical olfactory lobes (OLs), and the neuropil of which is surrounded by neuronal somata (SO). AA: anterior aorta, B: blister-like cell, F: fibrillary cell, HG: hindgut, MA: mandibular adductor musculature, PS: pyloric stomach, R: resorptive cell, VNC: ventral nerve cord

correct the position using micro dissecting needles. The hardened embedding blocks should cool down for at least 30 min before further use.

*Sectioning* Before sectioning, thoroughly clean the microscope slides so that the sections will adhere without any folds. To that end, the adhesion microscope slides should be immersed in 96% ethanol (or a 9:1 mixture with 25% ammonia [84]) in cuvettes for 3 days, briefly rinsed with distilled water, and immersed in distilled water for at least 1 day until further use. Transfer the microscope slide onto a heating plate at 60 °C until all water has evaporated (about 30 min). Alternatively, the glass slides may also be used in a wet state directly for section uptake. The following descriptions are based on experiences with a rotary microtome (e.g., HM355 by Thermo Fisher Scientific). The hardened embedding block should be trimmed with a razor blade into a trapezoid, creating a truncated 45° pyramid with the specimen close to front face (Fig. 8b, c). For linking sections into serial bands, the upper side of the trimmed block needs to be sealed with a 1:1 mixture of superglue and xylene (for discussion of different glues see [84]). After preparation of the embedding blocks, the sectioning knife (e.g., Histo Jumbo diamond knives by DIATOME) should be carefully cleaned with ethanol (96%) and the collecting basin needs to be filled with distilled water. In a first sectioning step, the samples are trimmed roughly ("quick & thick", e.g., sectioning thickness: 15 µm; sectioning speed: 10 units in our device-specific setting), and trimming needs to be stopped before the embedded sample is reached by the knife. For semi-thin sectioning, the process needs to be slowed down (sectioning speed: 5 units), and sectioning thickness should be reduced (sectioning thickness: 1–1.5 µm). The single sections should stick together in a band due to the one-sided sealing with superglue [84]. The length of the section bands depends on the operator's experience and sample size, but should not be longer than 15 attached sections as the sections unfold (and thus expand a little) when drying on the heating plate. The section band needs to be lifted carefully from the knife and left floating within the water bath. Afterwards, the microscope slide needs to get immersed carefully into the water bath. The microscope slides should be moved carefully underneath the section bands and carefully lifted out of the water. When immersing the microscope slides underneath the section chain, it is useful to reduce the surface tension of the water bath by placing a pipette crosswise in front of the microscope slide (Fig. 6d).

*Staining and mounting* The staining after Holländer and Vaaland [92] (1% phenylenediamine in methanol-isopropanol) strongly stains nervous tissues. Moreover, it has been shown that phenylenediamine also stains nucleoli and cytoplasm of other cell types as well, and therefore allows visualisation of several types of tissues within a crustacean larva (Fig. 7). In this method, it is likely that the formation of highly stained oxidation products by phenylenediamine in combination with the reduction of tissue-bound osmium plays an important role [92]. As osmium tetroxide (from the post-fixation

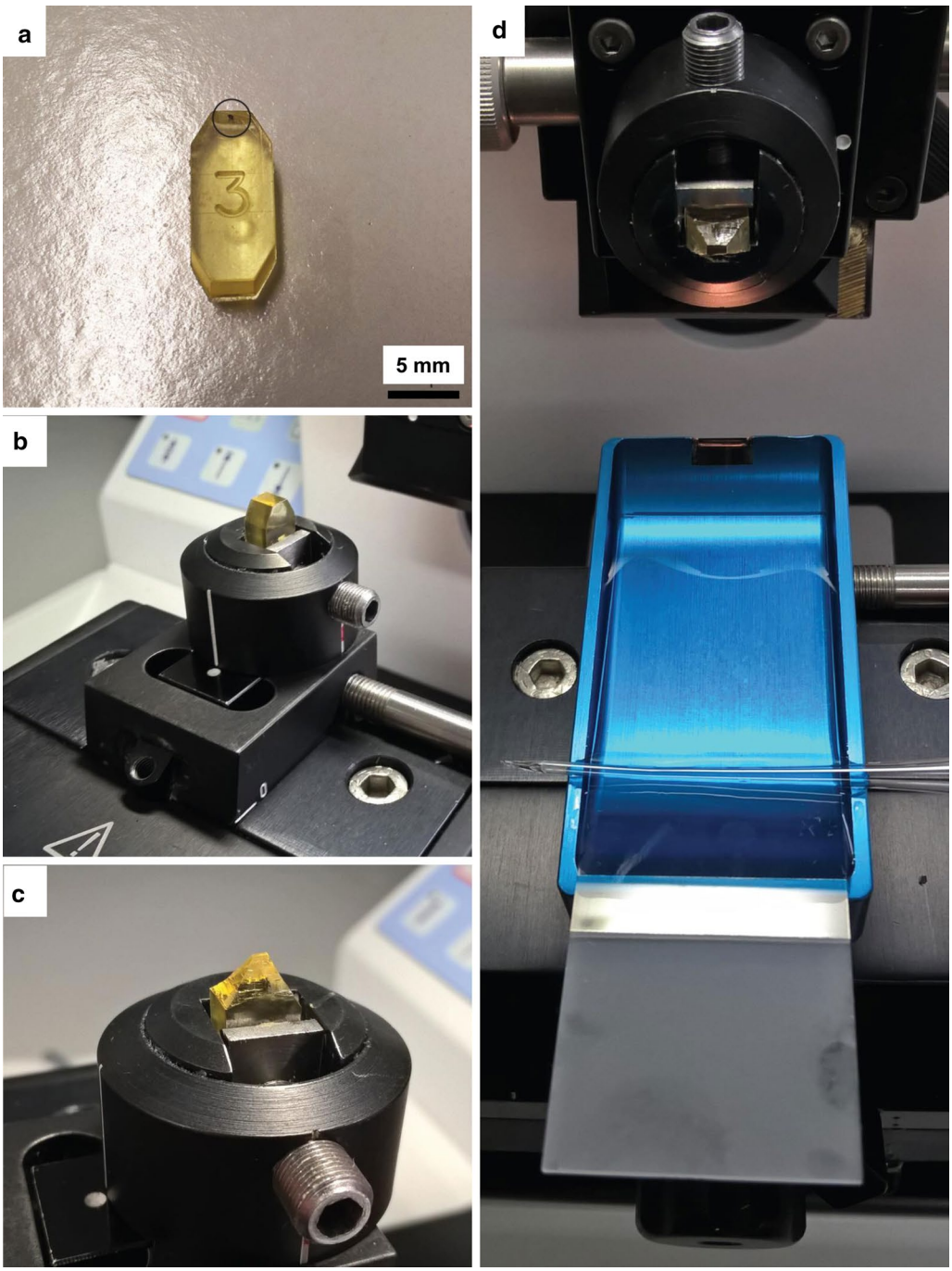



**Fig. 8** Semithin sectioning using the rotary microtome (images F. Spitzner; see section "Description of method: plastic embedding, semithin sectioning, and staining with Toluidine Blue and staining according to Holländer and Vaaland" for details). **a** Sample of a Zoea I of *Carcinus maenas* embedded in Araldite® (black circle). **b** Clamped embedding block before trimming. **c** Clamped embedding block after trimming into trapezoid shape to reduce cutting surface and duration of sectioning. **d** Lifting the section chain out of the water bath. It is useful to reduce the surface tension of the water bath by placing a pipette crosswise in front of the microscope slide when carefully immersing the microscope slide into the water bath (see section "Description of method: plastic embedding, semithin sectioning, and staining with Toluidine Blue and staining according to Holländer and Vaaland" for details)

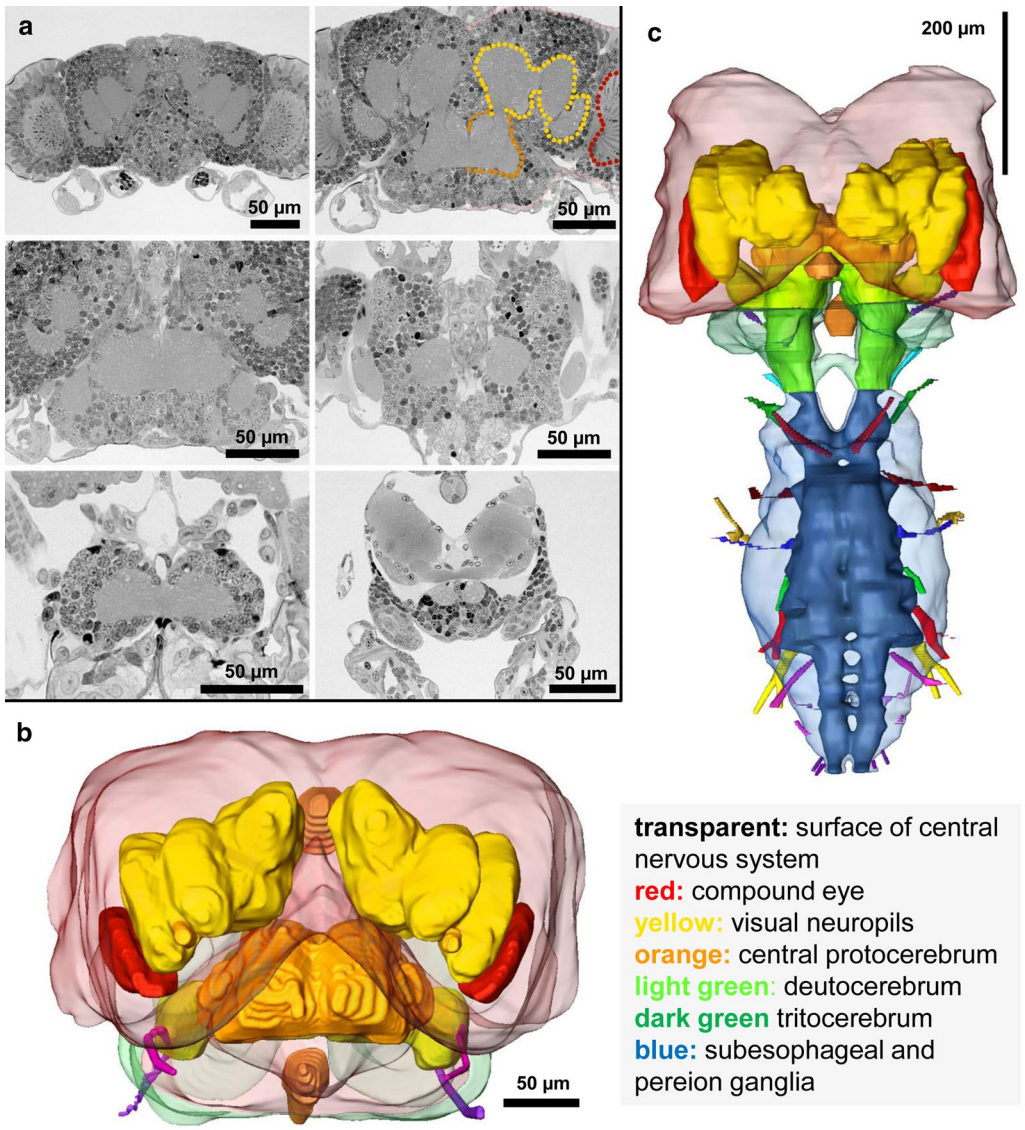



**Fig. 9** 3D-reconstruction from serial semithin sections; Zoea 1 of *Hippolyte inermis* (modified from [167]). **a** Six sections as examples from a series of transversal semithin section series. The dotted lines indicate the neuropil borders used for the 3D-reconstruction in **b**, and **c**). **b** 3D-reconstruction of median protocerebrum, visual neuropils and brain surface (transparent) in frontal view. **c** Central nervous system with neuromeres, surface (transparent), and segmental nerves. Surface renderings were made in Amira 5.2.0. **Colours:** transparent red—surface of protocerebrum, transparent green—surface of deutocerebrum and tritocerebrum, transparent blue—surface of suboesophageal and pereion ganglia, red—retina of compound eye, yellow—visual neuropils, orange—median protocerebral neuropils, light green—neuropils of deutocerebrum, dark green—neuropils of tritocerebrum, blue—neuropils of suboesophageal and pereion ganglia

process) only stains unsaturated lipids, while phenyldiamine does not stain lipids, it could be shown that the combination of both reagents results in enhanced staining of unsaturated lipids and some staining of saturated lipids [93]. The following steps should be carried out under the fume hood due to the high toxicity of the reagents. The microscope slides with the semi-thin sections should be incubated in the staining solution for ca. 14 h. Afterwards, the microscope slides need to be taken out of the staining solution and washed three times for 20 min each with iso-propanol. The microscope slides need to be rinsed for another $3 \times 5$ min with distilled water and dried afterwards on a hot plate. As an alternative to the stain presented above, good results were also achieved using Toluidine blue (1% Toluidine blue, 0.25% Pyronin G in a solution of 1% sodium tetraborate, [94]). Note however,

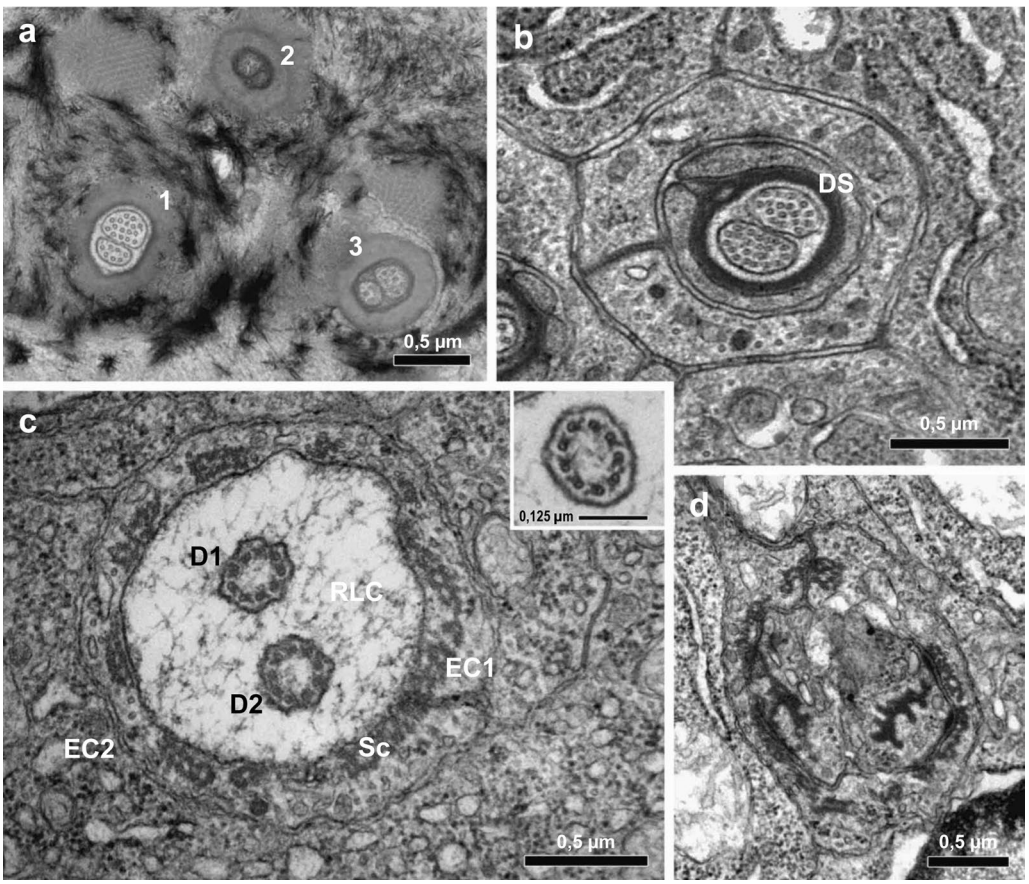



**Fig. 10** Transmission electron microscopic images of serial ultrathin transversal sections (see section "Description of method: ultrathin sectioning and transmission electron microscopy" for details) through a triplet of sensilla (1–3), each comprising two sensory cells and their dendrites (D1, D2), on the molar process of the mandible of larval *Palaemon elegans* revealing cellular ultrastructures indicative of the architecture and function of the sensilla (modified from [77]), **a:** distal dendrites near sockets of setae. **b–d** Transversal sections at different levels from distal (**b**) to proximal (**d**). **c** Dendrite with 9 × 2 & 0 pattern of microtubules and A-tubulus with arms. Abbreviations: DS: dendrite sheath, EC 1, EC2: enveloping cells 1, 2, RLC: receptor lymph cavity, Sc: scolopale

that in all available stains for plastic embedded tissues are suitable for larvae also (reviewed in [67, 72, 85]) and we describe only those stains with which we have hands-on experience. Both staining solutions described here should be prepared at least 24 h before use to ensure that all crystals are dissolved. Toluidine blue, also known as tolonium chloride, is a dye with a high affinity for acidic tissue components and organelles rich in DNA and RNA such as, e.g., the cell nucleus [95]. This stain is a fast technique since the sections need to be covered with the staining solution only for a few seconds (ca. 30 s at room temperature). For both staining techniques, it is advisable to check the results in between as these stainings are nearly irreversible and overstaining makes it difficult to analyse sections due to dark sepia or blue tones. After staining, the microscope slides should be rinsed several times with distilled water until no more dye bleeds into the washing medium, and finally dried on a heating plate (60 °C) for approximately 30 min. The final step before microscopy is mounting the coverslips on the microscope slides to protect the sections. We recommend using Roti®-Histokitt II to cover-slip the specimens. Three small droplets should be put on each microscope slide, covered slowly with coverslips (avoiding bubbles), and left to dry for 24 h at room temperature.

### *Description of the method: ultrathin sectioning and transmission electron microscopy*

*Plastic embedding* The dissection and fixation of the specimens for TEM while cooling the samples on ice are described in the companion paper [8] and briefly summarised in the introduction to Sect. 2 of this contribution. The next step, in traditional protocols, is dehydrating the specimens in a graded series of 30%, 50%, 70%, 90%, and 96% ethanol followed by 100% isopropanol, 100%

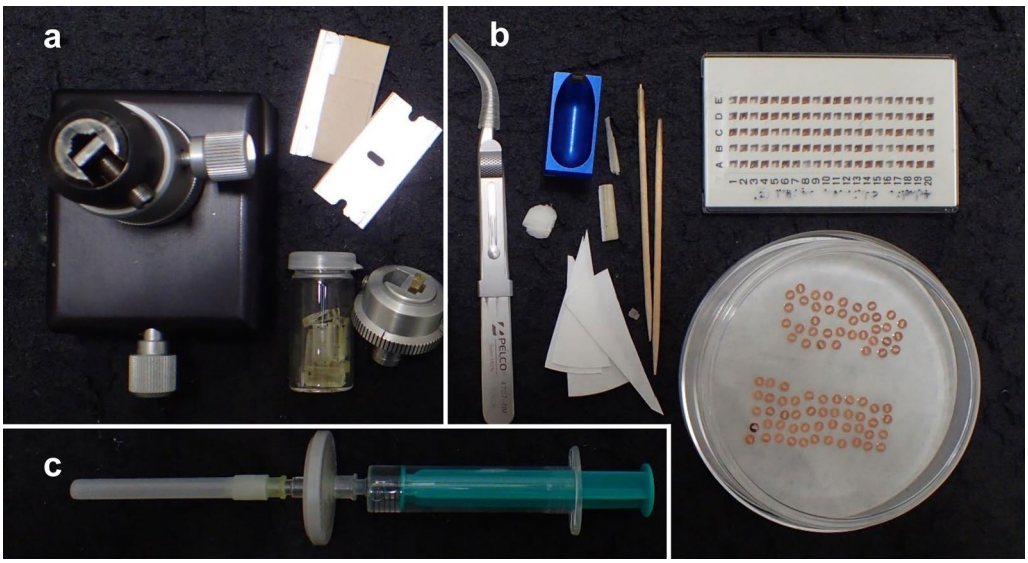



**Fig. 11** Equipment for ultrathin sectioning (image R. Melzer; see section "Description of method: ultrathin sectioning and transmission electron microscopy" for details). **a** Accessories for trimming: specimen holder socket, razor blades, vial with plastic-embedded specimens, specimen holder with trimmed specimen. **b** Tools for sectioning and fishing sections from the knife's water bath. Forceps with bent tips for grid handling, diamond knife for ultrathin sections, filter paper tips for drying grids, tooth sticks with eyelashes glued onto tips, sharpened pieces of elder marrow for cleaning of diamond knife, grid holder, Petri dish with formvar-coated copper slit grids for serial ultrathin sections. **c** Syringe with microfilter for the various steps during sectioning that require absolutely clean double-distilled water

propylene oxide, and then a mix of propylene oxide and resin [73, 96]. In many labs, however, this procedure was replaced by a graded acetone series, because acetone is a good solvent for epoxy resin. In addition, 100% acetone needed for the last dehydration steps can be kept free of water quite easily on granular calcium chloride. A feasible dehydration protocol would thus be 30%, 50%, 70%, 90%, 100% acetone for 10 min each, and then 3 times 20 min dried pure acetone. At the 70% acetone step, the specimen vial should be taken from the ice bath and exposed to room temperature, avoiding surrounding water, because otherwise there is a risk of accidental rehydration of the specimens. Afterwards, the specimens are incubated in a 1:1 mix of acetone and resin for 30 min to 2 h maximum, and then in the pure resin. This step can last overnight, but after a few hours, the lids of the vials should be removed to allow the acetone to evaporate. After that, the specimens are placed in fresh unused resin into embedding forms and oriented according to the desired sectioning angles. Some typical accessories for carrying out these and the following steps are shown in Fig. 11.

Osmium tetroxide, and cacodylate buffer, as well as aldehydes and the resin components, are very poisonous and have to be handled according to strict safety rules. Hence, the use of gloves and eye cover when preparing the solutions is essential. Always leave the solutions under a fume hood, and if possible, use a special "preparation hood" where also the dissecting microscope is placed.

Preparing the epoxy resin needs some preliminary considerations, since resins of different hardness can be obtained depending on the proportion of the resin's components. In most epoxy resins (Epon, Araldite®, Glycidether 100, etc.), there is a soft component and a harder one. Hence, it is possible to prepare resins whose hardness matches the needs of the specimens to study. After the two resin components are mixed together, the accelerator/activator is stirred in according to the manufacturer's instructions. For soft specimens, medium to soft resin is suggested, for hard ones (e.g., those containing hard arthropod cuticle), a harder resin is recommended.

Once the specimens are placed in resin into the embedding form, they are heated in an incubator or heating cabinet at 40 °C. Initially, the resin becomes more fluid due to the rise in temperature which can improve its infiltration. After about an hour, before the resin becomes waxy, there is still opportunity to improve the specimens' orientation within the embedding form, if necessary. After that, the specimens are put back into the oven and the temperature is increased to 60 °C overnight so that the resin polymerises completely. After removing the specimens from the incubator, they should be left

in the embedding form until they have cooled down to room temperature. Fresh from the incubator, they are still quite soft and flexible but at room temperature, they will become hard. An alternative to this basic technique is vacuum embedding [97], a technique which is often successful in specimens that prove difficult to be embedded using the standard protocol.

*Sectioning* Trimming, sectioning, and staining are in principle quite similar to semithin sectioning as described above (Fig. 8). Nevertheless, some specific tricks and techniques to obtain good ultrathin sections will be described in the following (see also Fig. 11). An ultramicrotome (e.g., Leica Microsystems) instead of a simple rotation microtome is needed and a diamond knife for ultrathin sections (e.g., DIATOME®) is strongly suggested instead of glass knives. With a diamond ultrathin knife, good sections can be made as a standard.

In the following, we refer here to some aspects which in our experience are crucial for a successful ultrathin sectioning and subsequent TEM analysis. As suggested in the previous section about semithin sections, the trimming (with a razor blade) should create a truncated 45° pyramid with the specimen close to the front face having the shape of a trapezoid. In small arthropods, such as larvae, it is usually better not to trim up to the specimen but leave a little resin edge around. It is important that the two parallel edges of the trapezoid, which will become the upper and the lower edge of the sections, are perfectly parallel to get straight ribbons of ultrathin sections instead of spiral-shaped ribbons. It is important to practice adjusting the block in reference to the diamond knife. It needs much experience to operate the micrometre screws for adjusting the block and the knife in combination with the upper and lower illumination devices of the microtome. This allows to precisely adjust all three angles using the "shadow line" between block and knife to allow for effective sectioning. With a well-trimmed block and a good knife, sectioning of very good ultrathin sections is not as difficult as beginners often think. For high resolution, the sections should be really thin, of grey or silver interference colour (40–70 nm). For relatively low resolution, sections of golden interference (70–90 nm) are sufficient. In ultrathin sectioning, silver sections are, therefore, more of a gold standard than gold sections. As in semithin sectioning, another essential issue is "fishing" the sections from the water bath attached to the knife and transferring them to the grids in perfect orientation. This can be done from above or below—resulting in mirror-symmetrical pictures in the TEM—with the help of formvar coated copper "slit" grids and a clean eyelash glued to a tooth stick, a basic tool of the ultramicrotomist. It can be used to split ribbons of sections into handy pieces and to orientate the sections along the grid edge. Another important trick is to stretch the sections with a little bit of chloroform vapour emitted by a piece of moist filter paper held close to the grid but not touching it. One more parameter is essential for the entire procedure, the sectioning angle, usually indicated for each diamond knife in the manufacturer's instructions. However, on some occasions, this angle should be slightly adjusted. If the section thickness is not homogenous within a ribbon of sections, increase the angle; if there are fissures or cracks within the sections, decrease the angle.

#### *Additional comments*

Despite its long history and numerous competing technical approaches, classical histology is still a commonly used method to investigate organisms' cells and tissues. While histological (paraffin) sectioning is a fast and fairly simple approach to analyse organs of interest, higher-resolution methods, such as semi-thin and eventually ultrathin sectioning provide additional insights into cells and tissues of organisms only a few millimetres in size such as crustacean larvae (Figs. 6, 7, 10). Another advantage of classical histology is the long-term stability of the finished samples. When stored correctly (dark, cool, dry), the sections (as well as the embedded blocks) can last for decades. Nevertheless, classical histology is technically challenging, and a highly invasive method as it leads to alteration and destruction of the specimen. For instance, paraffin embedding is not suitable for extremely hard samples, such as strongly calcifying organisms, where due to softer paraffin, samples usually break during the sectioning process. Furthermore, this procedure is prone to artefacts such as section loss, distortion, and staining artefacts. In addition, classical histology is time and labour consuming, as well as costly because expensive microtomes and microscopes are needed (see Table 1). Compared to light microscopy-based histology, a TEM analysis requires more work and time, but the higher resolution power from the TEM pays off when results about minute structures and cellular ultrastructures are needed.

Overall, to date (and not only limited to samples of invertebrates), the combination of histological sectioning, staining, and microscopic assessment of 2D sections, is still the gold standard for structural and compositional analysis of biological tissues [98]. Serial sections are excellent starting material for 3D reconstructions of selected organ systems such as the nervous system (Fig. 9). In a broader perspective, it has been shown that the combination of multiple analytical methods with classical histology might be the most effective approach to gain a holistic insight into the anatomy of invertebrate

organisms (e.g., [22, 23, 98, 99]) because single methods sometimes cannot fulfill all requirements. Nevertheless, the selection and combination of methods depends on the objective and certain species characteristics and therefore, should be chosen with care.

### X-ray microscopy (µCT)

#### *Introduction*

The application of X-ray microscopy (µCT) to soft tissues has now become a standard in imaging techniques (Figs. 12, 13). In the field of anatomy, µCT has been demonstrated to be a useful analytical tool applied in a rapidly growing variety of metazoan taxa, such as cnidarians [100], plathelminths [101], nematomorphs [99], nematodes [102], polychaetes [103, 104], molluscs [98, 105], echinoderms [106, 107] as well as arthropods [108–113]. Modern, lab-based µCT-scanners deliver high resolution allowing the investigation of tiny specimens with body sizes of free-living crustacean larvae ranging from 75–195 µm in Tantulocarida [114], the smallest arthropods in the world, up to 5 cm in length, e.g., in Stomatopoda [115]. Since it is a non-destructive method allowing to investigate intact specimens as a whole (reviews [109, 116]) as well as in the anatomical context (Figs.12, 13), X-ray microscopy opens up numerous opportunities for correlative microscopic workflows such as the combination with serial block-face imaging [117], scanning- or transmission electron microscopy [118], including histological sectioning for light microscopy [98, 118], or the combination with magnetic resonance tomography [119] of one and the same specimen.

#### *Description of the method*

In general, imaging of biological samples based on µCT results from different absorption coefficients of tissues and of how X-ray radiation is absorbed by these samples. Thus, X-ray absorption is dependent on the atomic number based on the tissues' biochemical composition [120]. The best results in terms of resolution, reduced scanning time, and contrast can be achieved by scanning samples that were previously fixed, contrast-enhanced and critical point dried. For a better resolution of integumentary structures, dried specimens can be additionally coated analogous to the sample preparation (sputter coating) for scanning electron microscopy [121].

Here, we briefly summarise methodological steps of the sample preparation for a workflow enabling a consecutive immunohistochemical and/or histological investigation (for further details of other possible tissue preparations see our extensive manual on µCT in decapod larvae [122]). First of all, the larvae need to be properly fixed. Note that the choice of fixative has an effect on tissue preservation as well as the choice of agents for contrast-enhancement. Bouin's solution or 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) or seawater have turned out to be useful fixatives that allow immunohistochemical labelling after µCT-scanning (see companion paper [8]). After a proper fixation for at least two hours at room temperature, the tissues need to be post-fixed in Dent's fixative (20% DMSO in methanol) and further dehydrated in two graded steps up to 100% methanol. At this stage, larvae can be stored at −20 °C. Note that fixation with methanol disrupts or denatures binding sites of some epitopes in tissues to some extent (e.g., of F-actin resulting in a limited detectability by, e.g., phalloidin conjugated with fluorescent dyes). Ethanol or isopropanol represent an alternative to methanol. However, depending on the scientific question, any of the subsequently used staining agents need to be carefully checked in advance for their compatibility with methanol-fixed tissues. After dehydration and before scanning, the tissues should be incubated in 2% iodine in methanol for contrast-enhancement for at least 24 h at room temperature. After two to three washing steps using pure methanol, the larvae can be directly scanned ideally in sealable plastic chambers filled with fresh methanol (for further details on mounting of samples, see [122]). After successful scanning, the sample needs to be rehydrated in a decreasing series of methanol in TRIS-buffer and finally be washed in several changes of PBS-TX (0.3% Triton X-100 in 0.1 M PBS) to remove any reminiscent iodine from the tissue. After washing, the tissues are ready for any further processing for histological sectioning followed by immunohistochemical or histochemical treatments.

#### *Additional comments*

The main advantage of X-ray microscopy is that it is a virtually non-destructive method allowing to investigate intact specimens as a whole. Furthermore, this technique provides a high resolution even to the cellular level in well-fixed and contrasted specimens. Finally, X-ray microscopy opens up numerous opportunities for correlative microscopic workflows of one and the same specimen. For example, we established workflows allowing for combining µCT with consecutive immunohistochemical (see next chapter) or histochemical labelling of the same specimen, and also combining µCT of Cobalt-filled axonal projections with subsequent sectioning [123]. This way, general information on the spatial relationships of the whole internal anatomy can be assembled before using other destructive techniques such as histological sectioning. In other words, the collection of µCT-based datasets can be considered as an anatomical backup and is therefore also used for taxonomy in zoological museums or collections for digital

preservation of type material being referred to as cybertypes [110, 124, 125]. For the study of type material, the undesirable brown staining of specimens due to the use of agents for contrast enhancement such as 2% iodine in ethanol is fortunately reversible. These agents can be completely removed after enduring (several days) of washing in several changes of fresh volumes of pure ethanol (Fig. 14) before transferring the samples back to 70% ethanol as often used in collections. Since µCT-imaging is a less-destructive method, it is one of the few techniques enabling scientists to have a look into the inside of, for instance, rare fossil specimens such as amber inclusions of, e.g., insect larvae (e.g., [126, 127]) or Cambrian arthropods [128]. A re-investigation by applying µCT of already described fossil crustacean larvae [129–131] would thus be feasible. Here, nature already embedded the samples millions of years ago.

Another advantage is that µCT produces isometric data which are favourably useful for morphometric approaches in contrast to anisometric imaging such as, e.g., microscopy of serial sections or confocal laser scanning microscopy where z-axially refractive mismatches can occur [111, 132]. Some possibilities to visualise these data sets are exemplified in Fig. 12. One of the most commonly used analytical processing is to generate 3D reconstructions of selected organs or tissues (Figs. 12, 13); and see Table 2). In principle, 3D reconstructions can also be generated from microscopic images based on section series (Fig. 9). However, image stacks based on µCT provide isometric datasets meaning that the pixel-size equals in all three dimensions (depending on the scanning-settings and magnification). Furthermore, artefacts only occur due to chemical fixation, drying of samples, or scanning. In contrast, image series generated from sections often suffer from more artefacts and need to be aligned before they can be used for 3D reconstruction. In addition, these datasets are anisometric which means that the pixel-size especially of the z-axis is ultimately dependent on the physical section thickness, or if generated using a confocal laser scanning microscope, the pixel sizes have to be manually corrected due to possible refractive mismatches caused by the immersion media.

Because µCT-imaging is, to put it simply, based on differences in tissue densities, it is less specific than, e.g., techniques using histological staining or antisera raised against a variety of antigens. The major disadvantage is of course that the acquisition, as well as the service and maintenance of a lab-based µCT-device are quite expensive (see Table 1). What is more, sample preparation and µCT, like most other techniques, also have specific disadvantages. For delivering good contrast, especially soft tissues need to be fixed and incubated in contrast-enhancing agents in advance which often cause tissue modifications such as shrinking (see [111, 122]). However, by using methanol as outlined above, we obtained excellent results for later immunohistochemical labelling against anti-synapsin (SYNORF1) in combination with histochemical detection of the cell proliferation marker EdU previously in vivo injected (Krieger, unpublished). The possibility of combining these techniques makes the protocol especially interesting for studying developmental stages such as larvae. If specimens are not intended to be used for additional staining experiments or immunohistochemistry, µCT of critical point dried samples delivers better contrasts as resulting from wet-scanning in, e.g., high-grade alcohols [22, 122]. Furthermore, by using dried samples, a specimen container becomes redundant so that the working distance can be decreased resulting in higher resolutions and shorter exposure and thus scanning times. However, dried material can be still later used for scanning electron microscopy (SEM).

## Methods for cellular analyses

### Immunohistochemistry

#### *Introduction*

Classical histological techniques provide insights into the general organisation of tissues and organs. By using primary antibodies that bind to specific cellular antigens, immunohistochemistry allows to selectively label a broad range of cellular components ranging from signalling molecules across elements of the cytoskeleton, across macromolecules such as channels or transport proteins to organelles such as mitochondria [133, 134]. One major strength of this technique is that in double or triple labelling experiments, primary antisera from different host species (e.g., rat, mouse, and rabbit) can be combined to label multiple antigens (Fig. 15).

Choosing suitable primary antibodies, according to the question, is an essential first step when designing an experiment. In general, the antibody toolbox of developmental biologists working with non-genetic model organisms is much smaller than that available, e.g., for the study of *D. melanogaster*, *C. elegans* or vertebrate model species. Considering aspects of both cross reactivity and specificity is an important issue when using antisera that were not raised against crustacean antigens so that identifying antisera that specifically bind in crustacean tissues may be a challenge. Therefore, suitable control experiments to test the specificity of the antisera is an essential measure to evaluate the results and often is a prerequisite for publishing the results [133, 134]. For those new to the trade, we recommend to start using antisera against antigens which are highly conserved in an evolutionary sense such as elements of the cytoskeleton. The Developmental Hybridoma Bank Iowa (DSHB) is a valuable source for such reagents. For any new antiserum you

work with, you should conduct a pilot experiment to find a suitable dilution (1:10, 1:100, 1:1,000, 1:10,000 etc.). If your signal-background ratio is unsatisfactory, decrease the concentration of the primary antisera to reduce unspecific background. Considering decapod crustacean larvae, one focus of research are ontogenetic aspects of osmoregulation (references in Table 1 of the companion paper [8]). Immunolocalisation of ion pumps such as the sodium–potassium ATPase in tissues specialised for ion transport is an essential method in these studies. Immunohistochemistry against neurotransmitters, neuromodulators and neurohormones is another research focus in decapod larvae involving immunohistochemistry. This technique has been widely used to analyse the maturation of neurons in the emerging brain in several crustacean species especially with respect to the maturation of the X-organ/sinus gland complex (references in Table 1 of the companion paper [8]). More than in larvae, immunohistochemistry has been instrumental in analyses of early embryogenesis in decapod crustaceans as summarised in a number of recent reviews [1, 2, 4–7]. The contribution by Harzsch and Viertel [3] provides a number of additional tips and tricks for immunohistochemistry in crayfish embryos which are relevant for working with other decapod larvae, too.

In immunohistochemical experiments, the penetration of large molecules such as antibodies into the tissue to reach their target antigens is an essential process. Dissected tissues from adult specimens can be cut to histological sections to achieve a good penetration by reagents, by for example using a vibratome. For decapod larvae, the manual dissection to remove individual organs such as the central nervous system or midgut gland with fine forceps may be a superior method for tissue preparation, a challenging process that needs much patience, practice, and manual skills. The dissected tissues can then be processed as whole mounts which is advantageous because the 3D organisation of the organs is preserved thus facilitating the microscopic analysis of the specimens. Nevertheless, immunohistochemistry has also successfully been used on histological sections of larvae [135, 136]. To summarise, apart from choosing suitable primary antisera, a solid strategy for tissue preparation is essential when envisaging immunohistochemical experiments.

The primary antisera are visualised by secondary antisera conjugated to certain detection systems [133, 134]. In the 1980s and 1990s, secondary antisera conjugated to enzymes were most popular. These enzymes were the key to a chromogenic reaction which catalysed the polymerisation of a dye as a marker for the primary reagents within the tissue. Such preparations could be viewed by bright field microscopy and were then open to analyses by an acute observer and drawing with a *camera lucida* apparatus [137, 138]. As an example, Fig. 16 shows drawings of neuronal elements in the central nervous system of Zoea 1 and 2 of *Hyas araneus* that were immunolabelled with an antiserum against the neuropeptide FMRFamide. This study revealed a rapid maturation of the neurons expressing this peptide from one stage to the next, especially in the ventral nerve cord [138]. For the last 15 years or so, fluorescent dyes have been the primary choice as conjugates for secondary detection reagents. Secondary antisera conjugated to fluorochromes are essential for double or triple labelling experiments (Fig. 15). However, such preparations cannot be imaged by bright field microscopy but need more expensive fluorescent microscopes for analysis. Confocal laser scanning microscopy today is the state-of-the-art method to analyse immunohistochemical preparations, an imaging technique which allows to explore the third dimension of whole mount tissues by recording Z-stacks of optical sections. The potential of light sheet microscopy to visualize the larval morphology still needs to be explored.

#### *Description of the method*

The first step in any immunohistochemical protocol is a chemical fixation of the larvae (see companion paper [8]). A typical protocol involves immersion in 4% paraformaldehyde (PFA) in PBS (0.1 M phosphate- buffered saline pH 7.4) for 4 h at room temperature. To allow a rapid penetration of the fixative, it is recommended to cut off some of the larval appendages such as spines or limbs as long as these are not the target organs (see companion paper [8]). The choice of the fixative and fixation time depends on the antiserum you plan to use and fixatives other than PFA may be more appropriate [3]. The chemical nature of the antigen that was used to immunise the host animals for generating the antiserum is an essential parameter to know to adjust the fixation protocol, a piece of information that is included in the manufacturer's product sheets that come with the antisera. Because the most essential step in immunohistochemistry is a strong and selective binding of the primary antibody to its antigen, we recommend reading some background information on the biochemical principles of fixation using available text-books to gain background knowledge of the biochemistry of this process (e.g., [133, 134]).

Wash dissected organs in a fresh dish (i.e. it has not been previously in contact with fixative) in six steps of PBS for at least 2–4 h at room temperature under gentle agitation on an orbital shaker to remove the chemical fixative. You may pause your experiment at the end of the day by leaving the specimens in PBS overnight. In this case, store the dish at 4 °C, and in the last washing step, add 0.02% sodium-azide. Sodium azide is extremely

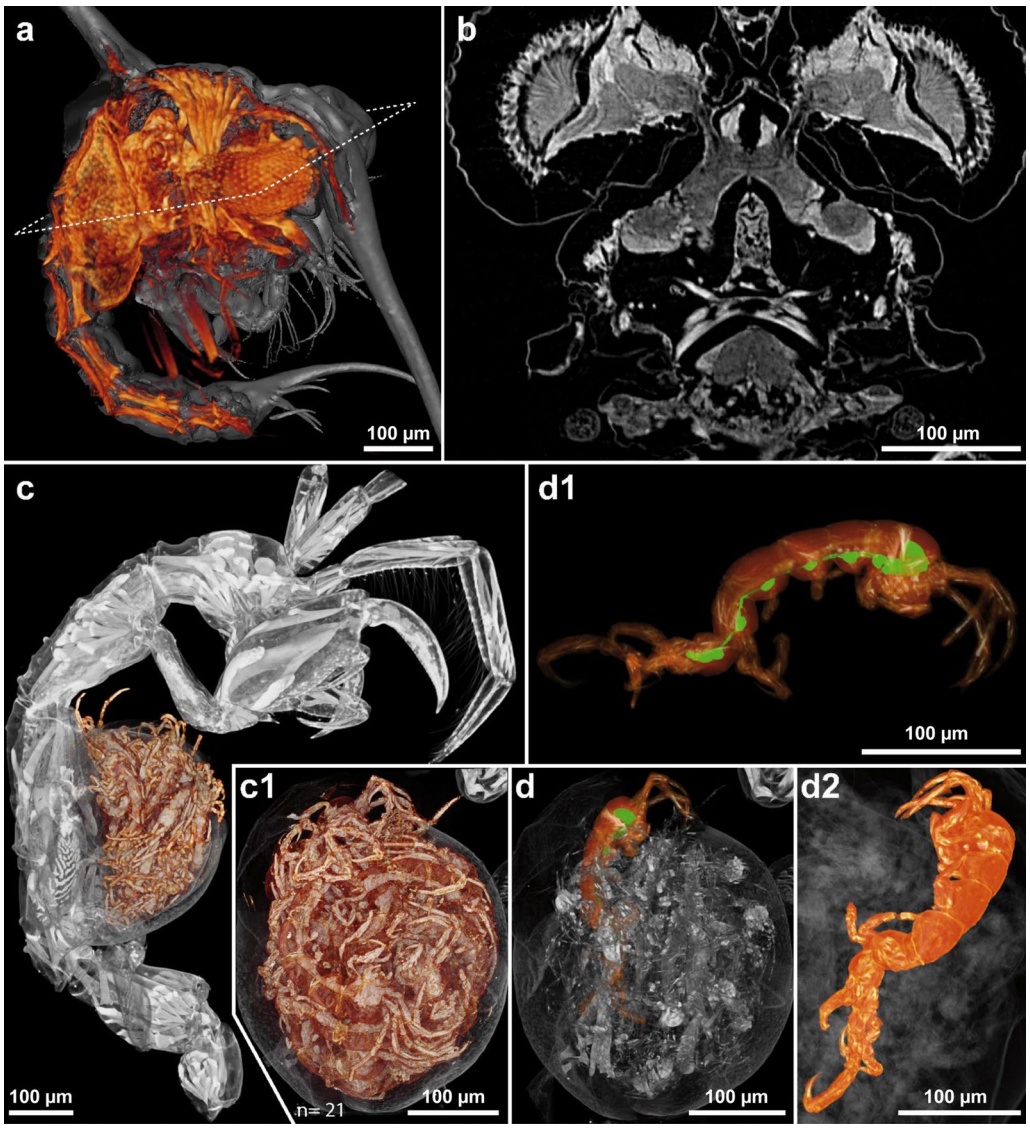



**Fig. 12** Visualisations of malacostracan developmental stages based on µCT (all images J. Krieger, unpublished; see section "X-ray microscopy (µCT)" for details). **a** Based on a µCT-dataset of a Zoea II of *Carcinus maenas*, the outer surface (here, virtually sliced along the midline in grey) can be visualised ("Isosurface"-module in Amira®; compare Table 2) in addition to a volume rendering ("Volren-module" in Amira®) showing internal elements of the musculature, stomach as well as the compound eye (orange colourmap). **b** Two-dimensional virtual slice through that larva along the dorso-ventral body-axis as indicated by the dashed line in a). Note that the space between cuticle and internal elements is an artefact resulting from tissue shrinking due to chemical fixation. Differences in the tissue densities, however, allow the discrimination for manual segmentation of desired organs based on the resulting different greyscales. **c** µCT-scanned female of the amphipod *Caprella mutica* ("Volume-rendering" in grey) and 21 post-larvae (Manca-stage) located inside the marsupium ("Volume-rendering" in orange based on the manual segmentation of the interior marsupial cavity (in higher magnification in **c1**). **d, d1, d2:** one post-larva (orange) was randomly selected and manually segmentated and also its nervous system reconstructed (green in **d** and **d1**). Using manual segmentation, a single juvenile can be either highlighted among its 20 siblings (**d**), or visualised as isolated individual for detailed anatomical analysis (**d1** and **d2**)

toxic and serves as an antibiotic to avoid contaminating bacteria growing as you process your samples. Use a dissecting microscope when changing buffers. Figure 17d shows some additional tools such as pipettes with drawn out tips and containers recommended for the following steps (see also [3]). Place the transparent dishes on black cardboard to improve visibility of the transparent tissues and use illumination from above. When exchanging the PBS, carefully replace the fluid (but not the target tissues) with a fresh volume of buffer. Cover the well plates with Parafilm® during the washing steps.

Next, preincubate tissues in PBS-TX (0.1 M PBS, 0.3% Triton X-100, 1% bovine serum albumin, 0.02% sodium azide) for one hour at room temperature with gentle agitation. Triton X-100 serves as a detergent and increases the permeability of the tissue. The bovine serum albumin in this reagent is supposed to cover the tissues with a coat of loosely bound protein. This cover is supposed to reduce the binding of unspecific antibodies in the next step (when using polyclonal antisera). After the preincubation step, immerse tissues overnight in primary antibody in PBS–TX at room temperature. For double labelling experiments, incubate in a cocktail of both primary antisera. The concentration of the primary antibody is an essential parameter and must be determined in pilot experiments. We recommend incubation overnight but longer periods are possible depending on the samples. Cover the dish with Parafilm to avoid contamination and evaporation of the liquid. Refer to [3] for more instructions on this step. Subsequently, wash the samples for at least 2 h in several changes of PBS at room temperature and gentle agitation.

Next, incubate in secondary antisera (diluted in PBS at the manufacturer`s recommendations) for 2–4 h at room temperature. Too high concentrations of the secondary antisera may increase background labelling. In double labelling experiments, incubation must be carried out in a cocktail of the secondary antisera, for example, anti-mouse Alexa Fluor 488® plus anti-rabbit Cy3 secondary antisera. As a third label, a DNA-marker to label cell nuclei may be added at this stage to visualise the general architecture of the tissues (see section "Specific markers for cell nuclei").

Wash the tissues thoroughly for at least 2 h in several changes of PBS at room temperature. Longer washing steps help to reduce background labelling. For mounting the specimens on slides and cover-slip them, commercial mounting media such as Gelmount®, Vectashield® and others are available which frequently contain "anti-fade" reagents that are supposed to reduce photo bleaching when viewing the preps. For short time preservation, plain glycerol can be used, a medium that does not polymerise. Mowiol®-Glycerol is another convenient alternative for mounting specimens.

#### *Additional comments*

Because of the availability of hundreds if not thousands of commercially available antisera, immunohistochemistry potentially is a powerful technique to study developmental processes in crustaceans. However, working with non-model systems means that specificity of the reagents is an important issue and that complex control experiments are essential to show that the chosen antisera do label what they should label. What is more, the necessary reagents are relatively expensive (see Table 1), at least in the line-up of techniques that we described here. Although the samples can be visualised with conventional fluorescence microscopy, exploring the full potential of this approach requires confocal laser scanning microscopy and hence an expensive optical instrument.

### Using an in vivo marker for cell proliferation

#### *Introduction*

Organogenesis during larval growth is characterised by a high level of mitotic cell proliferation. Markers for certain phases of the cell cycle are suited to locate mitotic cells in the tissues and to analyse the temporal dynamics of cell proliferation (Fig. 17). The thymidine analogon 5-bromo-2-deoxyuridine (BrdU) is a well-known S phase-specific marker to analyse neurogenesis in adult crustacean brains [139, 140, 140–143]. This marker was also applied to study aspects of neurogenesis in late crustacean embryos and larvae such as the development of the ventral nerve cord [144–146], the brain [147–149] and the compound eyes [150–152]. Larvae are incubated in vivo in the labelling reagent diluted in sea water for 2–10 h, during which the marker is taken up into the organisms where it gets incorporated into single-stranded DNA during S-phase in all cell nuclei which are cycling during the incubation period. Therefore, strictly speaking, this is not a mitosis marker but a replication marker. After fixation of the animals, the marker is visualised by immunohistochemistry. Because the marker gets distributed across the entire organism, it allows the localisation of cycling cell nuclei in all organs of the larvae. Depending on how long you incubate the larvae, you may study cell cycle dynamics in the target organs. In so-called "pulse-chase" experiments, larvae are transferred back to normal seawater after incubation in the marker where they continue to develop. By sacrificing cohorts of larvae at different time points after the BrdU pulse, additional aspects of cell cycle dynamics can be studied.

#### *Description of the method*

*In vivo labelling* For labelling, decapod larvae are incubated in vivo in the liquid BrdU-labelling reagent RPN 201 (Sigma-Aldrich, GE Healthcare) diluted with filtered seawater (32 PSU) to a final concentration of 0.2 mg/ml (after [149]). We determined an incubation period of 6 h at a typical rearing temperature of 12–15 °C as ideal for labelling cycling cells in the developing brain and ventral nerve cord of *Carcinus maenas* (a brachyuran crab) larvae (Fig. 17). However, studies of other target organs may

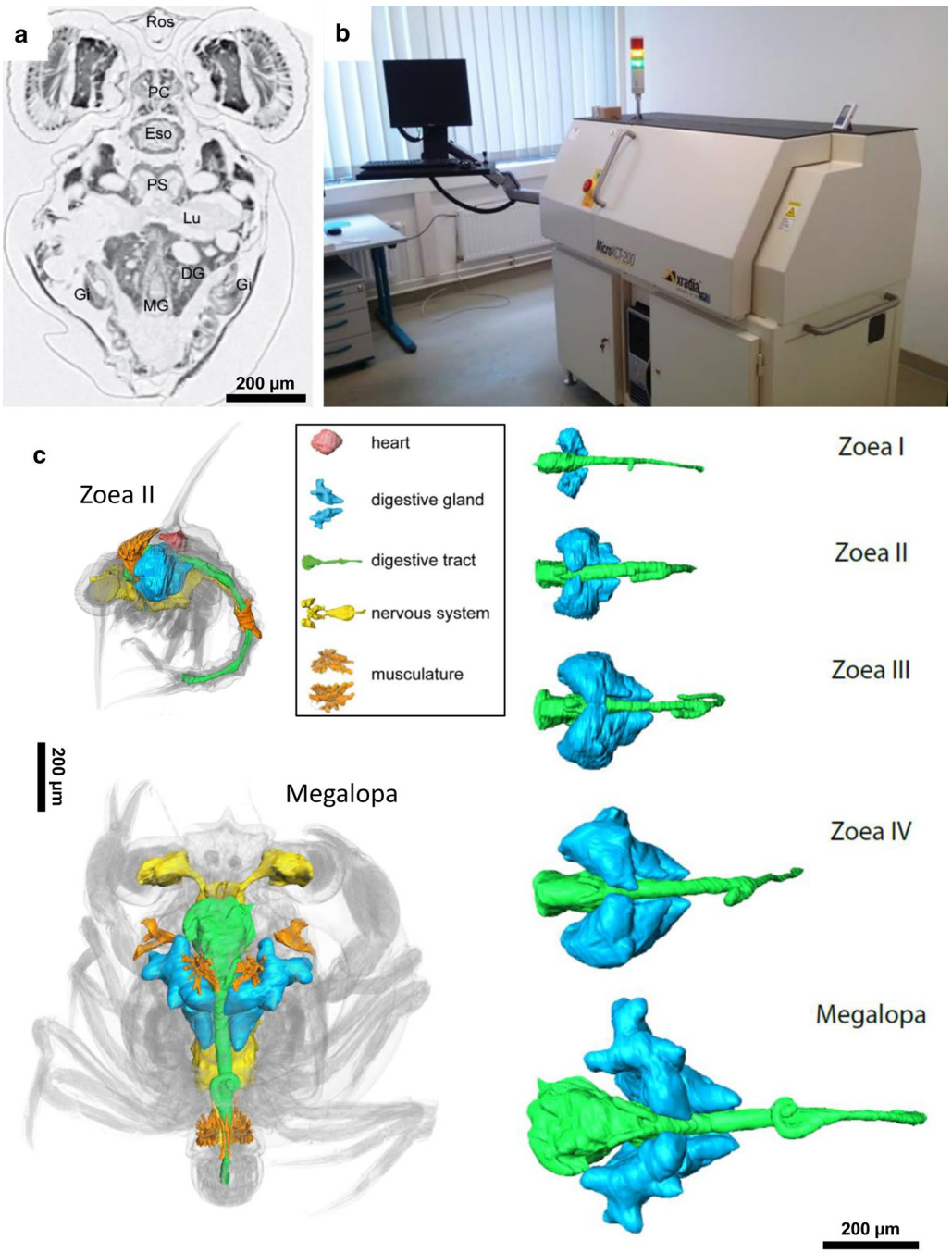



**Fig. 13** X-ray microscopy (µCT) of *Carcinus maenas* larvae (see [22, 122], and section "X-ray microscopy (µCT)" for details). **a** Zoea IV, single horizontal section from µCT analysis, black-white inverted (F. Spitzner, unpublished). **b** Micro-Computertomograph XRadia MicroXCT-200. **c** 3D reconstructions (Amira) of selected organ systems of *C. maenas* larvae based on X-ray data (modified from [22]). DG: digestive gland; Eso: (o) esophagus; Gi: gill, Lu: lumen of digestive gland; MG: midgut; PC: protocerebrum; PS: pyloric stomach; Ros: rostrum

demand a different pulse length. In order to control for circadian effect of cell cycle length in quantitative studies, the incubation should always be carried out at the same time of the day. Furthermore, we made sure that all individuals were incubated two days after moulting to avoid interference of the moult cycle. After the BrdU pulse, the larvae are fixed in 4% PFA (paraformaldehyde) in 0.1 M PBS and stored at 4 °C until further processing.

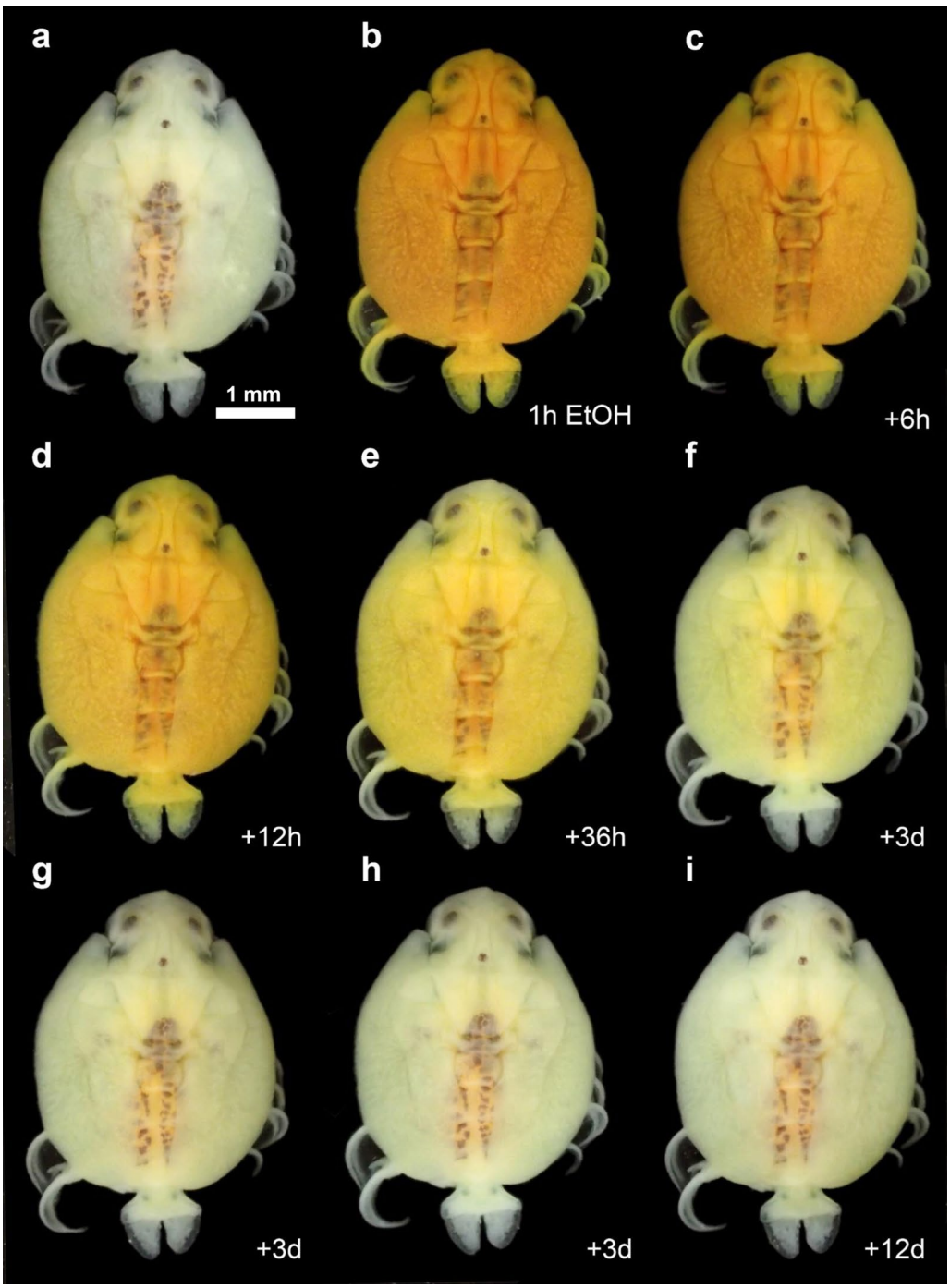



**Fig. 14** *Argulus foliacaeus* stained using a 2% iodine solution for µCT sample preparation to show the gradient of decolouration after several washing steps (Hörnig, unpublished; see section "X-ray microscopy (µCT)" for details). **a** Unstained, **b–i** Eight washing steps in 99.8% ethanol during ca. 23 days in total (cumulative timescale), **b** after 1 h, **c** after 6 h, **d** after 12 h, **e** after 36 h, **f** after 3 days, **g** after 3 days, **h** after 3 days, **i** after 12 days. All images show the same individual

*Immunohistochemical processing* Brains and ventral nerve cords of all stages are dissected using the tools and techniques laid out in the companion paper [8], and processed as whole mounts to better visualise the nuclei in s-phase in a three-dimensional context. To wash out the fixative, the tissues are incubated for at least

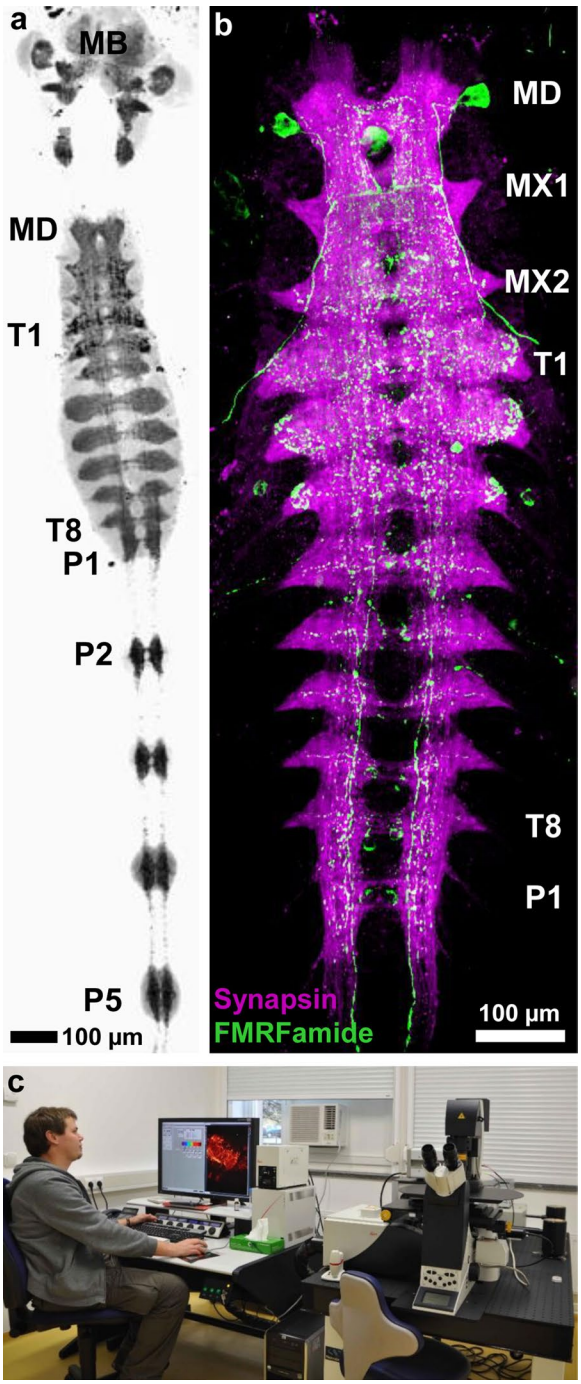



**Fig. 15** Immunohistochemical analysis of dissected whole mounts of the central nervous system from larvae of *Birgus latro* (see section "Immunohistochemistry" for details). **a** Dissected central nervous system of a Zoea 4 labelled with an antiserum against synaptic proteins to visualise the neuropil areas (T. Kirchhoff, unpublished), black–white inverted confocal laser-scan image. **b** Double labelling of a ventral nerve cord of a Zoea 2 to show areas of synaptic neuropil (magenta) and FMRFamide-like neuropeptides (green; T. Kirchhoff, unpublished). **c** Confocal laser-scan microscope Leica SP5 (image S. Harzsch). MD: neuromere of mandible, MX 1, 2: neuromeres of maxilla 1 and 2, T1, 8: thoracic neuromeres 1, 8, P1, P5: pleomeres 1, 5

$3 \times 5$ min in phosphate- buffered saline (PBS). Afterwards, the samples are incubated in 2 M HCl for 30 min to denature the DNA and improve the access of the primary antibody against BrdU. This is a harsh but essential step because commercially available nucleases will not accomplish the job. Next, the tissues are incubated for 1.5 h in PBS-TX (PBS + 0.3% Triton X-100 + 1% bovine serum albumine) to further improve antibody penetration. This step was followed by incubation in the primary antisera for 4 h. In order to visualise morphological landmarks in addition to the s-phase nuclei, double labelling experiments can be carried out. For the nervous system, immunohistochemistry against general cytoskeletal elements such as tubulin can provide a good overview of the structure of the developing brain (see [3] for more background on this topic). Therefore, we combined the incubation in the primary anti-BrdU antiserum (rat, 1:1,000) with either an anti-acetylated tubulin antibody (mouse, 1:1,000) or anti-tyrosinated tubulin antibody (mouse, 1:1,000). After the incubation, all tissues are washed in several changes of PBT for 1 h and incubated in a cocktail of secondary antibodies conjugated to Alexa Fluor 488® (goat, anti-rat, 1:500) and Cy3® (goat, anti-mouse, 1:500). As an option, the DNA marker HOECHST® 33,342 (Thermo Fisher Scientific; H3570) can be used as a nuclear marker as a third label to visualise the neuronal cell clusters during this step (see section Immunohistochemistry). Finally, the tissues are washed in several changes of PBT for 3 h. After the last wash in PBT, the brains are transferred to a 1:1 glycerol:PBS mixture for 20 min followed by a further 60 min incubation in a 4:1 glycerol-PBS mixture. Finally, the tissues are mounted in a 4:1 DABCO-glycerol:PBS mixture. For tips and tricks concerning mounting small items such as dissected brains, see[3]. Whole mount brains are scanned using a Leica TCS SP5II confocal laser scanning microscope using DPSS (561 nm), diode (405 nm) and argon (488 nm) lasers. The software Leica Application Suite Advanced Fluorescence (LASAF) is used to operate the microscope. The initial evaluation of the data can be done using the LASAF Lite software. Furthermore, the open source software FIJI [153], is recomended to generate maximum projections of substacks of some Z-stacks (see Table 2). The depth encodings shown in Fig. 17a were created using the "Z code stack" plugin for ImageJ.

#### *Additional comments*

BrdU-labelling has turned out a robust technique which can be used as a tool to analyse cell proliferation from crustacean embryos (review [1, 2]) across the larvae to adult animals [139, 140, 140–143]. However, when interpreting the results of the method presented here it has

to be borne in mind that this technique labels DNA synthesis which is not necessarily equivalent to mitosis considering that cells may replicate their DNA without cell division to become polyploid. Other markers for different phases of the cell cycle need to be established as tools to study organogenesis in crustacean larvae. Another disadvantage is that it is difficult to determine which cell type the labelled nucleus belongs to. In the nervous system for example, we know that in addition to neuronal stem cells, also the nuclei of glial cells may replicate as well as those of blood vessels in the nervous tissue. This set-back can be overcome by long-term pulse-chase experiments in which the animals are reared for days or weeks after the BrdU pulse until the labelled cells have differentiated. Double labelling experiments with cell type-specific markers may then reveal the identity of the cells which originated from the cell division while they were pulsed with BrdU.

## Conclusions and outlook

In this paper, we have provided a survey of methods useful to study morphogenesis and organogenesis in decapod larvae but so far have omitted molecular approaches such as in situ hybridisation to localise mRNA. In other words, we have given a survey of classical morphological approaches resulting in images of structures and stage-specific shapes both on cellular or organ level. Such methods must always serve a scientific question, no matter how tempting it might be just to generate attractive images. Therefore, we recommend to first develop a scientific question (some of which were summarised in the introduction) and then to select suitable methods that help to answer the questions and not the other way around. Having said that, the availability of the larvae is essential. When studying larvae reared in the lab (see companion paper [8]) more sophisticated fixation and preparation techniques can be used than in a study of rare material in the field or from museums.

Along these lines, newly established methods are not an end unto themselves. New methods should not be seen as just a tool to repeat something that has already been analysed before with a new method to eventually generate better images than the older techniques. Rather, new techniques should open new research avenues. New techniques may provide new insights into processes of morphogenesis and—by the comparative approach – the evolution of developmental processes, an approach which has been called EvoDevo or MorphoEvoDevo for many years now. As shown in Table 1, there are methods for various budgets, some of which need no more than a microscope or stereomicroscope, a staining solution – and of course larvae, some will need quite an investment before you can apply them, like SEM, TEM, and µCT.

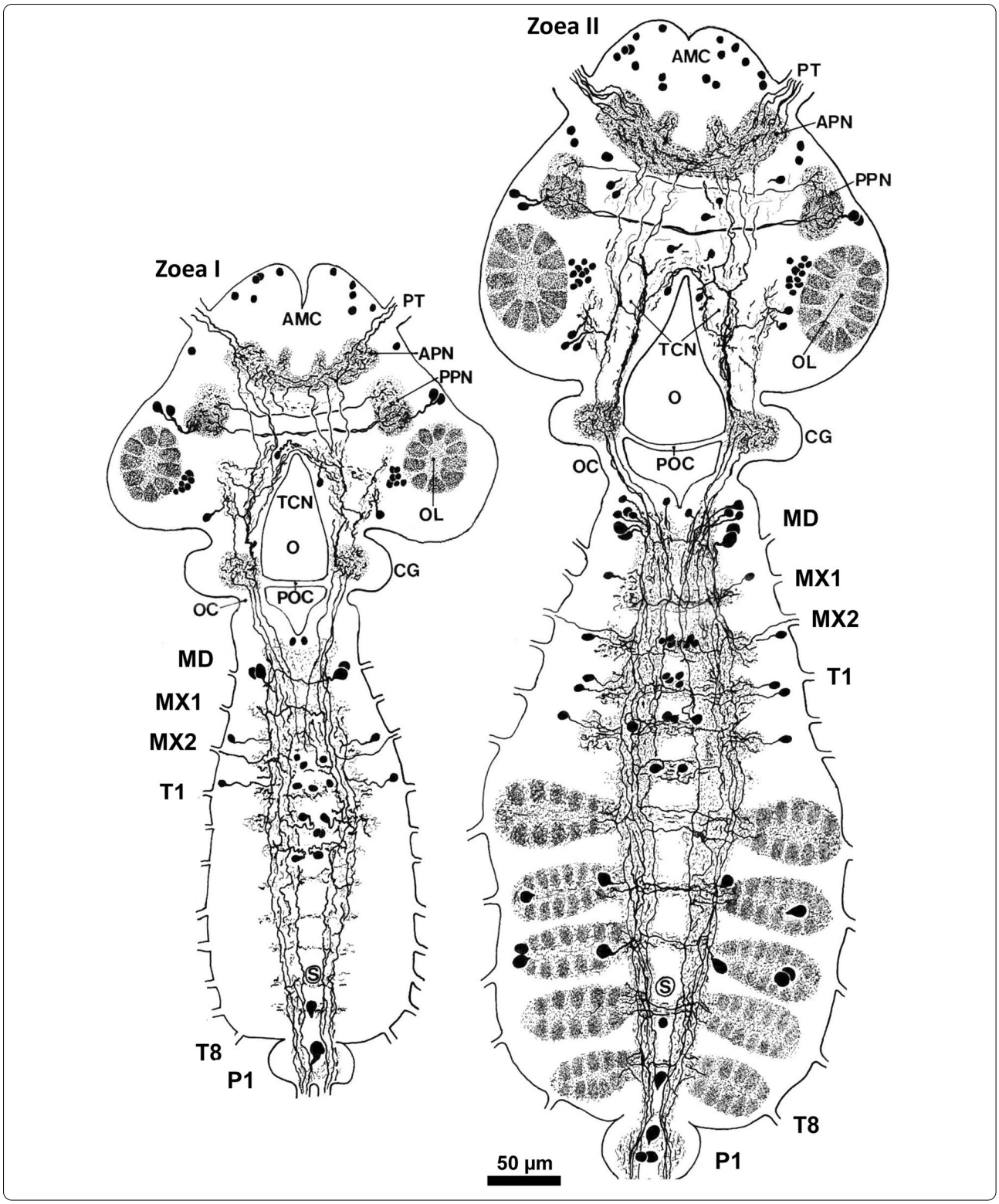
We wish to communicate one handy hint here: watch the living animals carefully before you start your experiment. Laboratory cultures as described in the companion paper [8] are an ideal starting point for such observations as are larvae caught with a plankton net. Larvae, alive, in a little aquarium or a rearing bowl, feeding on *Artemia* nauplii, are fascinating creatures. They are tiny—but they are able to do so much and so complex is their behaviour. Morphologists recommend that a study should always begin with observing the living animal, its movement back and forth, up and down, its way of feeding, its resting posture, etc. The more you know about your study object, the better the preparation and fixation will be, and only after patient observation, you will find the best way of placing the larvae under your imaging device. Use a little pocket lamp and light a corner of your aquarium. If phototactic, the larvae will immediately swim to the bright spot as they would also do in the ocean. Put some of them in a little vial and study them under the microscope before using any complicated techniques.

We have another important suggestion: play and try uncommon things. There is always a way around mediocre fixation, poor embedding, shrunken tissues—also for non-model organisms. Some of the fixations shown in the companion paper [8] have been carried out under the simplest field conditions. If necessary, a sugar bag from a coffee house at some Mediterranean harbour can be the beginning of a series of fixations of different osmolarities that produce the high-quality results you are looking for.

A substantial progress in the last two decades has been the development of 3D reconstruction methods (Figs. 9, 13), either based on images taken from serial sections or on imaging devices that produce perfectly aligned images per se, such as micro-computed X-ray tomography, confocal

(See figure on next page.)

**Fig. 16** Immunohistochemical localisation of FMRFamide-like neuropeptides in whole mounts of the central nervous system of zoeal stages of *Hyas araneus* (modified from [138]; see section "Immunohistochemistry" for details). The primary antiserum was visualised with an enzyme-based detection system and the preparations analysed with a bright field microscope (Polyvar, Reichert Jung) and drawn using an attached camera-lucida apparatus to thoroughly analyse the maturation of single neuronal elements AMC: anterior median cell cluster; APN: anterior protocerebral neuropil; CG: commissural ganglion; MD: neuromere of mandible; MX 1, 2: neuromeres of maxilla 1 and 2; T1, 8: thoracic neuromeres 1, 8; P1, P5: pleomeres 1, 5; PPN: posterior protocerebral neuropil; O: oesophageal foramen; OC: oesophageal connective; OL: olfactory lobe; POC: postoesophageal commissure; TCN: tritocerebral neuropil


Zoea II
AMC
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APN
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T1
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P1
50 µm
Zoea I
AMC
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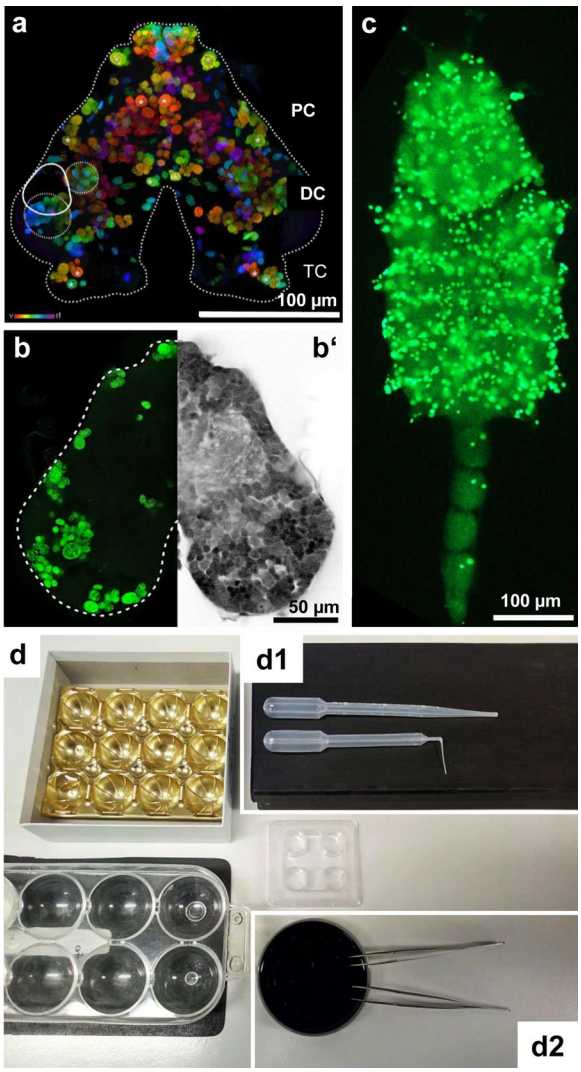


**Fig. 17** BrdU labelling of cell nuclei in s-phase in dissected whole mounts of the median brain and ventral nerve cord of *C. maenas* larvae scanned using a Leica TCS SP5II confocal laser scanning microscope (see section "Using an in vivo marker for cell proliferation" for details). **a** Median brain, coloured depth coding using the "Z code stack" plugin for ImageJ (R. Meth, unpublished). **b** Dorsal surface view of a Zoea III median brain (R. Meth, unpublished). **b'** Autofluorescence of the right brain hemisphere visualised by UV excitation (R. Meth, unpublished). **c** Ventral nerve cord of a Megalopa imaged with a Nikon Eclipse 90i upright fluorescence microscope (S. Harzsch, unpublished). **d** Various containers for immunohistochemistry (image S. Harzsch). **d1** Plastic pipettes with drawn-out tips (image S. Harzsch). **d2** Petri dish with black Elastosil® coating and micro-forceps. PC: protocerebrum; DC: deutocerebrum; TC: tritocerebrum

laser scanning microscopy, light sheet microscopy, and also a "Heidelberger Hobel" (serial block-face SEM) or "focused ion beam" (FIB-SEM) device in combination with a field emission SEM. This has allowed us to study morphologies in their full three-dimensionality as they really are, and sometimes also the changes of morphology with time, with developmental stages, allowing for 4D analyses. Moreover, we are sure that we are not at the end of staining tricks for all kinds of tissues and cells. We have shown some, but there are many more, and also some we don't know yet. Hence, try something new, but also use old tricks that otherwise would be forgotten. Try, for example, methysalicylate, oil of wintergreen, to clear larvae under the microscope, and maybe see things you have never been able to see before [72, 154]. Then, switch on the epifluorescence—wintergreen oil increases the autofluorescence of the cuticle in a quite interesting way [155]. Furthermore, the latest aqueous tissue clearing methods will surely open new avenues to the study of larval morphology [156]. And there is much more to try, much more to dig out of almost forgotten publications about classical preservation and histological methods, and of publications about other taxa that have never been tried with decapod larvae. Good luck!

**Acknowledgements**
We dedicate this review to Mireille and Guy Charmantier, two of the pioneers in analysing organogenesis in decapod crustacean larvae. SH gratefully acknowledges the hospitality of the Biologische Anstalt Helgoland, Alfred-Wegener-Institut, Helmholtz-Zentrum für Polar- und Meeresforschung, during the final phase of writing this manuscript. We wish to thank Gabriele Uhl and Shou-Wang Lin (Greifswald) for the collaboration in the experiments shown in Fig. 14. Grant numbers: DFG INST 292/119-1 FUGG, DFG INST 292/120-1 FUGG. FS, ZS, and SH were supported by DFG Research training Group 2010 RESPONSE JTH is currently funded by the Volkswagen Foundation with a Lichtenberg professorship.

**Authors' contributions**
RM and SH conceived and wrote this paper. SH compiled the plates. FS, ZS, MKH, JK, CH, JH, TK, RM and GT all contributed major parts of the text, assisted in writing the paper and contributed images. All authors read and approved the final manuscript.

**Funding**
Open Access funding enabled and organised by Projekt DEAL. FS, ZS, and SH were supported by the DFG Research training Group 2010 RESPONSE. Additional funding: DFG INST 292/119–1 FUGG, DFG INST 292/120–1 FUGG.

**Availability of data and materials**
Not applicable because we do not report any original data here.

## Declarations

**Ethics approval and consent to participate**
Not applicable because this technical review does not report any original experiments.

**Consent for publication**
Not applicable.

**Competing interests**
none.

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**Received: 4 October 2020 Accepted: 10 May 2021**
Published online: 02 June 2021

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## Publisher's Note

# IV General conclusions and outlook

This study investigated larval performance of the green shore crab (*Carcinus maenas*) among populations occurring in distant or contrasting habitats (South Spain *vs* North Sea *vs* Baltic Sea) manipulating two environmental drivers, temperature and salinity. Larval performance (physiological, developmental and growths rates, and survival) was affected by the combined drivers and contrasting patterns in responses were found between populations. Some physiological rates (such as respiration rates) were not affected by salinity changes and showed similar patterns across populations. For some physiological rates (e.g. growth or development) temperature modulated the effect of lower salinity. In populations that naturally inhabit higher salinities (30-35 PSU; Cadiz and Helgoland), a mitigating effect of higher temperatures (21-24 °C) to negative effects of low salinity (20-25 PSU) was recorded (TMLS) in the majority of the traits with some slight interpopulation differences (Šargač et al. 2021, Šargač et al. under review). In future climate change conditions, populations such as Cadiz and Helgoland might have an advantage in coping with new conditions, due to the presence of interactive effects, such as TMLS. Because of their better tolerance to increased temperature and decreased salinities they could invade or adapt to estuarine habitats with higher success. By contrast, in Kerteminde (population that naturally inhabits lower salinities) lower salinity generated the weakest performance in the majority of the tested traits and an absence of TMSL, suggesting the presence of maladaptive traits (Šargač et al. 2021). The highest overall performance of the Cadiz population at higher temperatures and salinities corresponds with their natural habitat and may reflect local adaptation or some form of plasticity (Marshall et al. 2008; Fischer et al. 2011).

Interpopulation differences can play an important role in future scenarios, and the case of *C. maenas* shows that we cannot make predictions for marine organisms based on a single population study. For *C. maenas*, as for many marine species, the shift in distribution northward is expected due to the warming of the sea (Chust et al. 2014). Variation among populations can pinpoint locally adapted populations that acclimate faster, have better adaptive mechanisms or stronger dispersive abilities. Larvae are vital for species' persistence and dispersal in most of marine invertebrates. Hence, the ability of larvae to adapt and/or overcome future environmental conditions will define the "winners" and "losers" of climate change (Somero 2010) as well as help in the future management of coastal habitats in the upcoming change. Southern populations (like Cadiz) may expand their range poleward if the source of the variation is adaptive plasticity and the habitats have high enough salinity. Therefore, different populations may come in contact with each other and increased gene flow among populations may provide better tolerance to environmental stress in the changing environment. Moreover, Baltic populations may harbour sink populations from higher salinities which would contribute to species distribution in areas characterized by environmental gradients (Dauphinais et al.

2018, Giménez et al. 2020). Thus, involving more drivers and including several populations from contrasting habitats can give a more comprehensive picture about species dynamics when it comes to the outcome of climate change on marine areas.

Studies like this emphasize the need for incorporating multiple drivers and populations. However, expanding the scope of the study could provide additional and even more realistic results. For example, the addition of more drivers (e.g. UV radiation, pH, pollution), widening the range of the tested drivers, inclusion of all life cycle stages or expansion the spatial scale of observation from global to regional and local (e.g. see Boyd et al. 2018). Expanding the experimental scope requires good knowledge of the ecology of the species and their optimal rearing conditions in the laboratory. During this research I aimed to refine an existing protocol for larval rearing of *C. maenas* (review by Torres et al. 2021a) which represents a great starting point for larval rearing of other marine decapod species but can also serve as a base protocol for other marine taxa with similar ecology.

Although fundamental processes governing larval responses are still unknown, identifying differences among populations from contrasting habitats is essential for better management of coastal ecosystems. If the larval response to multiple drivers is not universal among the populations, additional analysis is required to unravel the underlying mechanisms that govern response. Numerous methods and techniques on the cellular and tissue level may help to interpret observed changes. For instance, proteins that are important for thermotolerance and osmotolerance, such as heat shock proteins or ion pumps, could be quantified (e.g. Western blot) or localized (e.g. immunohistochemistry). In addition, organogenesis in response to changes in drivers or the structure of the osmotically active ion-transporting organs and tissues could be observed using morphological methods (Melzer et al. 2021) and compared among treatments and populations which may reveal interpopulation differences. For instance, during this research I optimized paraffin embedding technique for larvae of *C. maenas* that can be used for histological staining or even for immunohistochemical analysis of the tissue (for details see Melzer et al. 2021). Many of these methods are species-dependent and require a long and exhaustive examination, especially for fragile larval stages, in order to develop successful protocol. However, once developed, measurements using such methods can additionally explain effects of environmental drivers and reveal locally adapted populations, making them crucial in multipopulation experiments in marine ecology. Coastal marine ecosystems (such as estuarine, intertidal and brackish zones) still require detailed insight into environmental drivers and their effects that govern responses of marine invertebrates, on both spatial and temporal scale. Successful larval responses to climate-driven change and more adapted populations will shape future distributions and ecosystem functioning of marine coastal habitats. Thus, multi-population experiments, combined with various techniques, present an excellent starting

point to help determine interactive effects of multiple drivers (Boyd et al. 2018) and identify interpopulation differences in larval responses.

## V Disclosure of shares in the cumulative dissertation

1) **Šargač Z**, Giménez L, Harzsch S, Krieger J, Torres G (2021) Contrasting offspring responses to variation in salinity and temperature among coastal populations: a maladaptive ecological surprise? Marine Ecology Progress Series 677:51-65.

| | Data collection | Data analysis | Manuscript writing |
|---|---|---|---|
| ZŠ | 80 % | 70 % | 60 % |
| LG | 0 % | 10 % | 10 % |
| SH | 0 % | 0 % | 10 % |
| JK | 10 % | 0 % | 5 % |
| GT | 10 % | 20 % | 15 % |

2) **Šargač Z**, Giménez L, González-Ortegón E, Harzsch S, Tremblay N, Torres G (submitted) Quantifying the portfolio of larval responses to salinity and temperature in a coastal-marine invertebrate: a cross population study along the European coast. Marine Biology

| | Data collection | Data analysis | Manuscript writing |
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| ZŠ | 80 % | 70 % | 60 % |
| LG | 0 % | 10 % | 10 % |
| EGO | 5 % | 0 % | 5 % |
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| NT | 5 % | 5 % | 5 % |
| GT | 10 % | 15 % | 15 % |

3) Torres G, Melzer RR, Spitzner F, **Šargač Z**, Harzsch S, Giménez L (2021) Methods to study organogenesis in decapod crustacean larvae. I. larval rearing, preparation, and fixation. Helgoland Marine Research 75(3): https://doi.org/10.1186/s10152-021-00548-x

| | Data collection | Data analysis | Manuscript writing |
|---|---|---|---|
| GT | 30 % | 30 % | 40 % |
| RRM | 20 % | 20 % | 25 % |
| FS | 15 % | 15 % | 5 % |
| ZŠ | 15 % | 15 % | 5 % |
| SH | 10 % | 10 % | 10 % |
| LG | 10 % | 10 % | 15 % |

4) Melzer RR, Spitzner F, **Šargač Z**, Hörnig MK, Krieger J, Haug C, Haug JT, Kirchhoff T, Meth R, Torres G, Harzsch S (2021) Methods to study organogenesis in decapod crustacean larvae. II: analysing cells and tissues. Helgoland Marine Research 75(2): https://doi.org/10.1186/s10152-021-00547-y

| | Data collection | Data analysis | Manuscript writing |
|---|---|---|---|
| RRM | 25 % | 25 % | 25 % |
| FS | 10 % | 10 % | 10 % |
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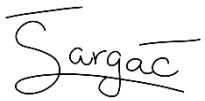
Signature of the supervisor



Signature of the doctoral candidate

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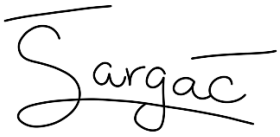
## VII Eigenständigkeitserklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.



Zoran Šargač

**VIII Curriculum Vitae**

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## Education

**DOCTORAL STUDY (2018-2022)**

Biology (Marine ecology), University of Greifswald, Zoological Institute and Museum, Department of Cytology and Evolutionary Biology, Germany

**MSc. DEGREE (2015–2017)**

Experimental biology (Zoology), University of Zagreb, Faculty of Science, Department of Biology, Croatia

**BSc. DEGREE (2012-2015)**

Environmental sciences, University of Zagreb, Faculty of Science, Department of Biology, Croatia

## Research experience and training

**01/2020, 10/2020 – LABORATORY WORK**

Swedish University of Agricultural Sciences, Uppsala, Sweden

**03/2018-08/2018, 02/2019-08/2019 – EXTERNAL PhD COLLABORATION**

Alfred Wegener Institute for Polar and Marine Research, Germany

**03/2017-07/2017 – ERASMUS+ TRAINEESHIP**

University of Bergen, Faculty of Mathematics and Natural Sciences, Norway

**02/2016-06/2016 – MULTISEK PROJECT**

University of Zagreb, Faculty of Science, Croatia

**01/2016-06/2016 – LABORATORY SKILL TRAINING**

Institute Ruđer Bošković, Division for Marine and Environmental Research,
Laboratory for ecotoxicology, Croatia

## Skills

**Laboratory:** biomass analysis (C, N, dry mass), immunolocalization and semi-quantification (Western blot) of proteins, oxygen consumption and oxidative stress quantification, histochemical methods, AZAN staining, electrophoresis, PCR, isolation and determination of invertebrates, experience with laboratory animals (larval rearing)

**Microscopy:** confocal, fluorescence and scanning electron microscopy

**Computer:** R (mixed modelling in ecology), MS Office, ArcGIS (basic), Inkscape, GIMP, Adobe

## Publications

**Šargač Z**, Giménez L, Harzsch S, Krieger J, Fjordside K, Torres G (2021) Contrasting offspring responses to variation in salinity and temperature among coastal populations: a maladaptive ecological surprise? Marine Ecology Progress Series 677: 51-65.

Torres G, Melzer R, Spitzner F, **Šargač Z**, Harzsch S, Giménez L (2021) Methods to study organogenesis in decapod crustacean larvae I: larval rearing, preparation and fixation. Helgoland Marine Research 75:3

Melzer RR, Spitzner F, **Šargač Z**, Hörnig M, Krieger J, Haug C, Haug JT, Kirchhoff T, Meth R, Torres G, Harzsch S (2021) Methods to study organogenesis in decapod crustacean larvae II: analysing cells and tissues. Helgoland Marine Research 75:2

## Conferences

111th Annual Meeting of the German Zoological Society - 2018, Greifswald, Germany (poster)

20th Symposium of Biology Student in Europe - 2017, Lund, Sweden (presentation)

2nd Central European Symposium for Aquatic Microinvertebrate Research CESAMIR

- 2016, Pésc, Hungary (poster)

## Languages

CROATIAN – native; ENGLISH – C1; GERMAN – A2

## IX Acknowledgements

First and foremost, I would like to express my gratitude to my supervisor Prof. Dr. Steffen Harzsch, without whom completion of this study could not have been possible. Thank you for the opportunity, endless support, many advices and guidance through this exciting and challenging journey.

I am extremely grateful to my other supervisors, Dr. Gabriella Torres and Dr. Giménez Luis. Thank you, Gabi, for introducing me to the larval rearing and for immense support, reviews, and supervision during the whole doctoral study. I want to thank you, Luis, for showing me the wonders of statistics and helping me enhance my statistical skills, for numerous reviews and patience throughout long R sessions. Moreover, I wish to thank Prof. Dr. Maarten Boersma and his working group at the Biological Station Helgoland (Alfred-Wegener-Institute) for the support and help during intense experimental periods. Also, I want to thank everyone from Kerteminde and Cádiz for access to populations and assistance with females.

I am grateful to DFG for funding the whole RESPONSE project and everyone that made this research possible and gave me the opportunity to work on these interesting crustaceans.

I would also like to thank my co-supervisor Dr. Jakob Krieger, 'Cytology and evolutionary biology' working group and RESPONSE colleagues for many recommendations and critical scientific discussions during the journey, for introducing me to many lab techniques, as well as for friendly work atmosphere. Moreover, I want to thank Prof. Dr. Jan-Peter Hildebrandt and his department for all the help and guidance during many Western blot experiments.

Special thanks to Siri Rohwer for her immense help with the larvae and for making me sane and cheerful during many exhaustive days and nights in the basement. Furthermore, I would like to acknowledge Alexander Kolb, Henriette Rossa and all the other student assistants that helped me with experiments.

Big thank to all my old and new friends for keeping me going in tough moments and lifting my spirit. Thank you, Manu, for all the love and support.

Last, but not the least, I want to thank my lovely family, my parents and sisters, for believing in me, pushing me to chase my dreams and always supporting me in every decision throughout my life.

Special thanks to my favourite "9-years older twin turkey" who is the reason I fell in love with biology. Thank you for all the amazing moments during this doctorate and for always being there for me in every step of my life. Čkkkkkkkk!