

**Stress ecology: interactive effect of temperature and salinity on
early life stages of barnacle, *Amphibalanus improvisus***

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Der Dekan

Dedicated to my parents and my wife

Table of Contents

Veröffentlichungen	I
Summary	II
Zusammenfassung	IV
General introduction	1
<i>Marine invertebrate with complex life cycle</i>	<i>1</i>
<i>Early life stages</i>	<i>2</i>
<i>Concept of stress</i>	<i>2</i>
<i>Salinity and temperature stress</i>	<i>3</i>
<i>Barnacle life cycle</i>	<i>4</i>
<i>Cyprid exploration</i>	<i>5</i>
<i>Biofilms</i>	<i>6</i>
<i>Study organism</i>	<i>7</i>
<i>Study area</i>	<i>8</i>
<i>Thesis objectives</i>	<i>9</i>
<i>References</i>	<i>11</i>
Chapter I: <i>Being young in a changing world: how temperature and salinity changes interactively modify the performance of larval stages of the barnacle <i>Amphibalanus improvisus</i></i>	19
Chapter II: <i>Temperature and salinity effects on larval performance of <i>Amphibalanus improvisus</i>: a maladaptation?</i>	43
Chapter III: <i>Temperature and salinity interactively impact early juvenile development – a bottleneck in barnacle ontogeny</i>	61
Chapter IV: <i>A protective coat of microbes on macroalgae: inhibitory effects of bacterial biofilms and epibiotic microbial assemblages on barnacle attachment</i>	83
General discussion	114
<i>Biological and ecological roles of the various ontogenetic phases</i>	<i>116</i>

<i>Temperature and salinity effects on barnacle performance</i>	117
<i>Findings and interpretation of different early life stages</i>	118
<i>Significance of cumulative impacts of ontogenetic stages</i>	122
<i>Overall impact of stress combinations</i>	123
<i>Possible responses of the barnacles to climate change</i>	123
<i>Consequences for the ecosystem</i>	125
Conclusion	127
Looking ahead	127
<i>References</i>	129
Acknowledgements	139
CURRICULUM VITAE	141
Erklärung	142

Veröffentlichungen

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Summary

Summary

It is well accepted by now that in the course of global change, several potential stressors will appear and intensify simultaneously and may exhibit antagonistic, additive, or synergistic effects, which make predictions based on the assessment of single stressors unreliable. Intertidal barnacles, as conspicuous, widely distributed, and ecologically important species have served to study the effects of environmental factors for decades. The interactive effect of putative stresses such as supra- or suboptimal temperature and salinity on early stage performance and survival of barnacles has, however, been subject of only a few studies. Early life stages seem to be particularly sensitive to environmental changes and alteration of their fitness might drastically affect subsequent life stages and subsequently the whole population. Responses toward multiple stressors of *Amphibalanus improvisus*, the dominant barnacle species within the Western Baltic Sea, have not been investigated so far, neither in larval stages nor in juveniles.

In this study, I first focused on the combined effects of temperature (12, 20 and 28°C) and salinity (5, 15 and 30 psu) on pelagic phase (naupliar and cypris stages) of *A. improvisus*. Also, there is an urgent need for comparative assessment of separate populations from one species to enhance our ability to extrapolate local results to regional or global scale. Therefore, I evaluated the performance of early stages of two different populations of the Baltic Sea (one at Kiel Fjord, Germany and another one at Tjärnö, Sweden) under different temperature and salinity combinations. Afterwards, I concentrated on early benthic phase (juvenile) of this species and had a closer look on the critical and relatively neglected transitional period between the cyprid and the adult stage (settlement and early juvenile). Because of the extremely important function of biofilms in attachment processes of barnacles and the potential interaction with their macroalgal host, I furthermore, investigated the effect of biofilms composed of macroalgal epibiotic bacteria, on the number of attached cyprids in field assays.

Naupliar duration of *A. improvisus* from Kiel population increased with decreasing temperature) and, nauplii survival decreased with increasing salinity. Cyprid metamorphosis success, in contrast, was interactively impacted by temperature and salinity. Highest settlement rates occurred at the intermediate temperature and salinity combination, i.e., 20°C and 15 psu. Settlement success of “naive” cyprids, i.e., when nauplii were raised at 20°C/15 psu, was less

Summary

impacted by stressful temperature/salinity combinations than that of cyprids with a stress history. The proportion of nauplii that transformed to attached juveniles was highest under “home” conditions (local conditions during peak settlement i.e., 20°C/15 psu). In contrast to Kiel population that performed relatively better at home conditions of temperature and salinity, barnacles from Tjärnö population showed a better performance at lower salinities and higher temperatures than that of typical for Sweden west coast during the season of peak settlement. Juvenile mortality was high (42 - 63%) during the first week following attachment under all temperature and salinity treatments. Highest mortality and lowest growth of juveniles occurred at lowest temperature (12°C) and salinity (5 psu). Juvenile barnacles constructed more shell material compared to soft body mass at low temperature and low salinity while the reverse was observed at high temperature and high salinity. Barnacle attachment is reduced by monospecies bacterial biofilms and natural microbial assemblages (originally isolated from *Fucus*) by 20% to 67%. However, the repellent effects of bacterial biofilms were no longer observed when settlement pressure was high. The composition and abundance of biofilms on macroalgae (*Fucus vesiculosus*), a potential host for barnacles, were affected by temperature which led to a reduction of their repellent effects by 60% at high temperature.

In summary this thesis shows that stressors may enhance the high natural mortality when occurring at crucial ontogenetic phases (larval and early juvenile stages). The responses of different populations to these stresses may differ as observed for Kiel and Tjärnö population of *A. improvisus*. Low performance of Tjärnö barnacle larvae at its local conditions implies that this population is apparently maladapted to its habitat. For the juveniles, environmental changes can alter patterns of survivorship and growth. Warming and desalination as predicted for the Baltic Sea in the course of climate change may, however, act antagonistically and compensate each other's isolated effect on juvenile barnacles. Indirectly, barnacle settlement may be impacted by stress-driven shifts in epibiotic biofilms. However, high settlement pressure may outrun any effects of biofilm.

Zusammenfassung

Allgemein anerkannt, treten Umweltstressoren im Zuge des globalen Wandels nicht isoliert voneinander sondern gemeinsam auf und können somit antagonistische, additive oder auch synergistische Effekte auf marine Organismen haben. Das natürliche Auftreten mehrerer Stressoren in sich verändernden Habitaten macht es somit schwer, Zukunftsprognosen aus Experimenten mit Einzelstressoren abzuleiten. Seepocken aus dem Gezeitenbereich werden, als eine auffallend, sehr weit verbreitete und ökologisch bedeutende Gruppe mariner Organismen, schon seit vielen Jahren in Studien zur Stressökologie genutzt. Die gemeinsamen Effekte von Temperatur- sowie Salinitätsschwankungen auf frühontogenetische Lebensstadien von Seepocken wurden jedoch bisher nur in wenigen Studien untersucht. Diese frühontogenetischen Stadien sind im Gegensatz zu ausgewachsenen Tieren sehr anfällig gegenüber Umweltveränderungen und eine Beeinträchtigung ihrer Fitness könnte sich auch auf nachfolgende Stadien und somit auf die gesamte Population nachhaltig auswirken. Die Effekte multipler Stressoren auf *Amphibalanus improvisus*, der in der westlichen Ostsee dominierenden Seepockenart, wurden bisher jedoch weder an Larvalstadien noch an juvenilen Individuen untersucht.

In meiner Arbeit habe ich mich anfänglich mit den Auswirkungen von Temperatur (12, 20 und 28 °C) und Salinität (5, 15 und 30) auf die pelagischen Larvalphasen (Nauplius- und Cyprislarven) von *A. improvisus* beschäftigt. Um generalisierte Aussagen über die Zukunft von Populationen bis hin zu Arten machen zu können ist es weiterhin wichtig, sich auch verschiedene Populationen anzuschauen und deren Stresstoleranz miteinander zu vergleichen. Aus diesem Grund habe ich die Auswirkungen der genannten Stressoren nicht nur auf Larven der Seepocken Population der Kieler Förde sondern auch auf die der Tjärnö Bucht, Schweden untersucht. Im Anschluss daran habe ich mich mit den Auswirkungen von Temperatur- und Salinitätsstress auf juvenile Stadien von *A. improvisus* beschäftigt und mich hierbei auf die bisher sehr wenig untersuchte aber wichtige Phase der Metamorphose von der Cyprislarve zur gesiedelten juvenilen Seepocke konzentriert. Aufgrund der Bedeutung von Biofilmen bei der Siedlung der Seepocken und der Bedeutung der Interaktion auch mit Makroalgen als Siedlungssubstrat, habe ich mich zusätzlich noch mit den Effekten von Algen Biofilmen auf die Siedlung von Seepocken in Freilandversuchen beschäftigt.

Zusammenfassung

In der Kieler Seepockenpopulation war die Larvalphase (Naupliusphase) mit Erniedrigung der Temperatur verlängert, wobei Überlebensraten der Naupliuslarven mit steigender Salinität herabgesetzt waren. Der Metamorphoseerfolg der Cyprislarven war hingegen wechselwirkend durch Temperatur und Salinität beeinflusst. Die höchsten Siedlungsraten konnten bei mittleren Temperatur- und Salinitätswerten (15 °C und 15 PSU), also den Bedingungen wie sie im Mittel in der Kieler Förde zur natürlichen Hauptsiedlungszeit der Seepocken vorkommen, beobachtet werden. Der Siedlungserfolg der Larven, welche zuvor in 20°C und 15 PSU Wasser herangezogen wurden, war weniger durch stressvolle Temperatur- und Salinitätsbedingungen beeinflusst als derer von Cyprislarven mit einer Vorgeschichte unter Stressbedingungen. Der Anteil an Naupliuslarven, der am Ende bis zur gesiedelten juvenilen Seepocke überlebte, war unter 20°C und 15 PSU (Sommerbedingungen der Kieler Förde) am größten. Im Gegensatz zur Kieler Population, in der sich Larven unter natürlichen Siedlungsbedingungen (15 °C und 15 PSU) am besten entwickelten, zeigte die Tjärnö Population beste Entwicklungsraten unter höheren Temperaturen und niedrigeren Salinitäten als den natürlich im Hauptsiedlungszeitraum vorkommenden.

Die Mortalität juveniler Seepocken war mit 42-63% in der ersten Woche nach der Metamorphose von der Cyprislarve zur gesiedelten Seepocke in allen Temperatur- und Salinitätsbedingungen sehr hoch. Die höchste Mortalität und das geringste Wachstum wurden hierbei jedoch unter 12°C und 5 PSU beobachtet. Im Vergleich zu den höheren Temperatur- und Salinitätsbehandlungen haben die juvenilen Seepocken hierbei unter niedrigen Temperatur- und Salinitätswerten mehr Schalenmaterial im Verhältnis zum Körper gebildet.

Das Siedlungsverhalten von Seepocken war im Freiland durch den Biofilm von Monospezies-Bakterien sowie durch den Biofilm aus natürlichen Bakterienkompositionen von *Fucus vesiculosus* zu 20 bis 67 % herabgesetzt. Bei sehr starkem Siedlungsdruck zu Zeiten der höchsten jährlichen Siedlungsraten von *A. improvisus*, konnten diese Effekte jedoch nicht beobachtet werden. Die Zusammensetzung des Biofilms sowie die Abundanz einzelner Bakterien innerhalb dieses Biofilms wurde weiterhin durch hohe Temperaturen beeinflusst, wodurch deren verteidigender Effekt gegenüber siedelnden Organismen (in diesem Fall *A. improvisus*) ebenfalls bis zu 60% herabgesetzt war.

Zusammenfassung

Zusammenfassend zeigt die vorliegende Arbeit, dass bestimmte Stressoren wie Salinitäts- und Temperaturmaxi und Minima, die natürlich vorkommende Mortalität, gerade in Verbindung mit anfälligen frühontogenetischen Lebensstadien (Larval- und frühe juvenile Lebensstadien), zusätzlich stark erhöhen können. Die Reaktionen verschiedener Populationen können, wie in der vorliegenden Arbeit gezeigt, sehr verschieden sein, wobei eine schlechte Fitness der Larven der Tjärnö Population unter dort vorkommenden natürlichen Bedingungen zur Hauptreproduktionszeit auf eine Maladaptation in diesem Habitat hindeutet. Umweltveränderungen wie sie hier untersucht wurden, können das Überleben und das Wachstum juveniler Seepocken nachhaltig beeinflussen. Erwärmung sowie die Aussüßung der Ostsee im Rahmen des globalen Wandels werden sich auf Seepocken jedoch antagonistisch auswirken und somit im gewissen Grad auch gegenseitig kompensieren. Indirekte Effekte wie eine Veränderung des Biofilms auf natürlichen Substraten wie der Alge *Fucus vesiculosus* und eine somit veränderte Verteidigung der Alge gegen Aufwuchsorganismen könnten den Siedlungserfolg von Seepocken jedoch zusätzlich beeinträchtigen. Hierbei kann ein hoher Siedlungsdruck durch Seepocken solche Effekte jedoch möglicherweise kompensieren.

General introduction

Marine invertebrate with complex life cycle

Many marine invertebrate species have complex life cycles including one or more microscopic dispersive free-living developmental stages (larvae) that eventually reach to a juvenile stage through a conspicuous metamorphosis. This juvenile is morphologically and often ecologically and physiologically different with previous stages (Nielsen 1998; Emler & Sadro 2006). Among marine invertebrates, complex life cycles are widely distributed such as in diverse animals like sponges; turbellarian and trematode flatworms; gastropod and bivalved mollusks; polychaete worms; lobsters, crabs, barnacles, and other crustaceans; bryozoans; and echinoderms (Thorson 1950). Marine invertebrate larvae may feed on phytoplankton or subsist entirely on yolk or other nutrients provided by the mother. They may spend as little as a few minutes or as long as several months in the plankton before metamorphosing to adult form (Pechenik 1990; Sanford & Kelly 2011).

Having larval stages during life cycle of many marine benthic invertebrates has both advantages and disadvantages. The presumed advantages of such larvae include the avoidance of competition for resources with adults, temporary reduction of benthic mortality while in the plankton, decreased likelihood of inbreeding with the next generation, and increased ability to withstand local extinction (reviewed by Pechenik 1999). Possible disadvantages include dispersal away from favorable habitat, mismatches between larval and juvenile physiological tolerances, greater susceptibility to environmental stresses, greater susceptibility to predation, and various costs that may be associated with metamorphosing in response to specific chemical cues and postponing metamorphosis in the absence of those cues (reviewed by Pechenik 1999).

For marine benthic invertebrates with complex life cycles, such as barnacles, pre- and post-metamorphic factors varying at spatial and temporal scales determine the successful addition of recruits to a population (e.g., Raimondi 1990; Miron et al. 1999; Olivier et al. 2000; Underwood and Keough 2001).

General introduction

Early life stages

Larval stage and early juvenile (early life stages) are very important in determining population dynamics of adult organisms (Pechenik 2006). During their long period of drifting (from a few hours to months), the larvae are frequently exposed to environmental fluctuations such as temperature, salinity, and likely pH drifts in the water column (McEdward, 1995). These pose significant physiological stresses to the larvae and it is estimated that mortality during the planktonic dispersal phase is well-over 90% in the natural ecosystem setting (Gosselin and Qian, 1996). Larval survival and fitness is highly dependent on the environment (Thiyagarajan et al. 2005). Essentially, the health of early life phases commonly determines whether new individuals successfully recruit into a population (e.g., Miron et al., 2000; Emler and Sadro, 2006). Likewise, larval experience, such as exposure to environmental stress, could have latent effects on the subsequent fitness and performance of the juveniles (Pechenik et al., 1998). When these larvae metamorphose into juvenile (early juvenile), they are subject to high rates of mortality (e. g. Thiyagarajan et al. 2005). Causes of mortality include delay of metamorphosis, biological disturbance, physical disturbance and hydrodynamics, physiological stress, predation, competition, developmental abnormalities, insufficient energy reserves, disease and parasitism (Hunt and Scheibling 1997). Since early developmental stages are often more sensitive to environmental stress than late juveniles and adults (Ricketts et al., 1992; Fichet and Miramand, 1998; Kurihara, 2008), and that juveniles are most vulnerable during first few days following metamorphosis (Qiu and Qian 1998), these factors can thus ultimately impact recruitment through cumulative mortality at different stages (Jarrett 2003). Thus, the progressively increasing climate change stressors are expected to exert pronounced potential effects on larval and juvenile physiology, which in turn, would compromise their fitness, thereby increasing the likelihood of mortality in early life stages as well as reducing the supply of recruits to the adult population.

Concept of stress

Stress is the impact of abiotic and/or biotic factors that reduces individual performance (survival, growth and/or reproduction) and ultimately impairs population growth rate (Grime 1989; Vinebrooke et al. 2004). Since these abiotic and biotic factors do not interact in isolation therefore, to understand the amount of impact on organisms, it is necessary to determine if these

General introduction

stresses act additively, antagonistically or synergistically (Wahl et al. 2011). Abiotic environmental stresses are one of the strongest selective forces shaping the behavior, physiology and morphology of organisms (Hoffman and Hercus 2000). Fluctuations in the coastal environment, such as daily and seasonal variations in temperature and salinity, have significant impact on intertidal organisms with respect to their population dynamics, physiology, morphology and development (Anil et al. 1995; Thompson et al. 2002). For these habitats which are relatively shallow and currently under stress because of human population growth and coastal developments, global climate change may enhance adverse effects on the population and introduce several stressors at the same time, leading to multiple stresses (Przeslawski et al. 2005). Some species however, may avoid or limit their exposure to stressor through behavioral modifications (e.g., closing the operculum when exposing to low salinity or desiccations, Foster 1970). If exposure cannot be avoided, organisms may develop tolerance to a stressor via acclimation, either through rapid physiological changes in phenotype within an organism's lifetime (Price et al., 2003) or via non-genetic maternal effects (Simpson and Miller, 2007). Alternatively, populations can develop tolerance through adaptation, where resistance genotypes are passed on to future generations.

Salinity and temperature stress

Among the various abiotic factors affecting the survival of marine invertebrates in coastal and estuarine regions, salinity and temperature are of primary importance (Tomanek and Helmuth 2002). Sessile animals are potentially vulnerable because, after settlement, they have no way of escaping the heat of the sun or the precipitation of fresh water (Bhatnagar and Crisp 1965).

Although relatively constant in open seas, salinity varies considerably in intertidal zones, estuaries and other biotopes. The ability to exist at varying salinity i.e., euryhalinity, depends on different adaptations. The most effective one is osmoregulation based on active ion transport mechanisms. Many osmoconformers, lacking the ability to regulate the osmotic pressure of the internal medium, nevertheless demonstrate considerable euryhalinity (reviewed by Berger and Kharazova 1997). Most barnacles are osmoconformers, the osmotic pressure of the haemolymph following that of surrounding water (Foster 1970). They are able to acclimate to reduced salinity over few days by closing the opercular valves preventing the entry of low salinity water into the

General introduction

mantel cavity and also by having tissues with increased tolerance to reduced osmotic pressure (Foster 1970). The euryhalinity of the estuarine barnacle, *Amphibalanus improvisus* is however thought to be dependent partly on hyperosmotic regulation of the haemolymph and partly on cell volume regulation (Fyhn 1976). Tolerance of barnacle to salinity is highly dependent on its interaction with temperature. When the temperature deviates from that of prevailing during the breeding season of the species, the tolerance to salinity change becomes impaired (Bhatnagar and Crisp 1965; Irwin et al. 2007). Temperature is another important factor which influences the larval development of barnacles (Anil and Kurian 1996; Qiu and Qian 1999; Thiyagarajan et al. 2003; Nasrolahi et al. 2012) controlling metabolism and thus influencing larval growth and development as well as utilization rates of reserves (Desai and Anil 2004).

Barnacle life cycle

The barnacle life cycle progresses through six planktotrophic nauplius stages- during which they accumulate energy reserves- and the final, non-feeding cyprid stage. The cyprid stage is specialized for benthic habitat selection and in the presence of suitable substratum and environmental stimuli, cyprids attach and metamorphose into sessile juveniles, grow and become adults (Thiyagarajan and Qian, 2008) (Fig. 1).

The transition between the cyprid and the adult stage (settlement, metamorphosis and early juvenile growth) is recognized as a critical period in the life cycle of barnacles, yet the linkage between these stages has received little attention (see Jarrett 2003, Phillips 2004; Thiyagarajan et al 2005). Similarly, although the importance of juvenile performance for subsequent recruitment success has been recognised in many marine invertebrates (reviewed by Gosselin & Qian 1996, Moran 1999, Pechenik 1999), several aspects of juvenile performance in barnacles, e.g. their in situ growth response to intrinsic and extrinsic factors, have not been well documented (see Thiyagarajan et al 2005; Thiyagarajan et al 2007).

General introduction

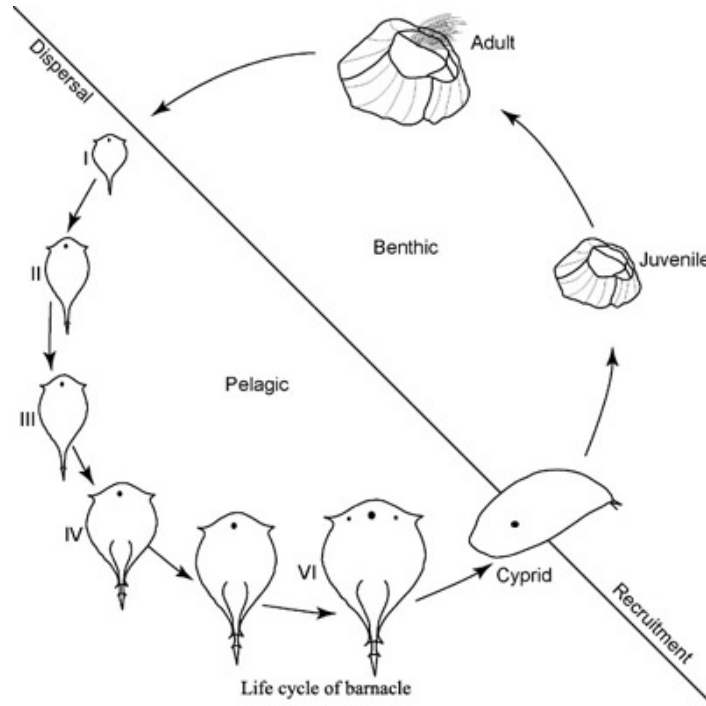


Fig. 1 Life cycle of barnacle (http://www.nio.org/index?option=com_nomenu/task/show/tid/85/sid/92/id/73)

Cyprid exploration

Recruitment of larvae of fouling organisms and their metamorphosis is the most important step in the fouling process. A cyprid larva does a substratum search before undergoing metamorphosis. Cyprids use their antennae to “walk” along a substratum until a suitable location is found to settle. The third antennular segment with its attachment disc is the most important point of contact between the cyprid and the substratum during the search. Flicking of the fourth antennular segment is also evident while a cyprid explores a substratum (Clare et al. 1994; Maruzzo et al 2011). Faimali et al. (2004) found that barnacle cyprids may perceive signals about the substratum surface or its biofilm, which plays a role in their settlement preference. Studies by Hills et al. (1998), Kawahara et al. (1999) and Thiyagarajan (2010) are examples of how cyprids respond to chemical cues and the hormone arthropodin from adult barnacles (Clare et al. 1995). Barnacles produce a pheromone like substance and neurotransmitters such as serotonin and dopamine, which are thought to act as settlement inducers (Clare and Matsumura

General introduction

2000). They also respond to physical cues from the surface of a substrate (Roberts et al. 1991) and can distinguish between habitats based on the community structure of initially settled bacterial biofilm (Maki et al. 1992; Olivier et al 2000; Lau et al. 2005).

Biofilms

A biofilm is a complex assemblage of microorganisms, including multiple bacterial species, (initial biological colonizers of new surfaces in the sea), diatoms, fungi, protozoa, and other small organisms (Hadfield 2011). Typically there is a large amount of secreted extracellular polymeric substance in which the cells of the component organisms are buried (Dobretsov 2009; Hadfield 2011). It has long been recognized that larvae of a broad spectrum of marine invertebrate taxa, are selective regarding where to settle from the planktonic realm, choosing sites favorable for both survival and reproduction (Hadfield and Paul 2001). To do so, the larvae must be able to detect, via external chemoreceptors, environmental cues (mostly from biofilms) that indicate “right spots”, transduce the cues into internal processes with neural and/or hormonal elements, and proceed through the behavioral changes that bring about settlement and the developmental changes that culminate in transformation into a juvenile form, that is, metamorphosis (Hadfield 2011).

Biofilms can have inhibitory (e.g., Maki et al. 1990; Olivier et al. 2000; Lau et al. 2003; Dobretsov and Qian 2006; Rao et al. 2007, Ganesan et al. 2010), inductive or neutral effects (e.g., Wieczorek et al. 1995; Harder et al. 2002; Ganesan et al. 2010) on larval settlement. The inhibitory effect of biofilms has been mainly attributed to their bacterial components (Maki et al., 1988; Holmström et al., 1992; Avelin Mary et al., 1993; Anil & Khandeparker, 1998; Lau and Qian, 2000; Khandeparker et al., 2002, 2003, Dobretsov and Qian, 2006, Dobretsov 2009). Previous studies have shown that bacterial surface chemistry, micro-topography, and a range of bacterial products from small-molecule metabolites to high-molecular weight extracellular polymers mediate cyprid settlement (reviewed by Qian et al., 2007). In view of their focal role for recruitment, it is important to recognize that biofilms are complex and dynamic and that the interactions of microorganisms within them produce changes in their species composition and relative abundance in space and time (e.g., Shikuma and Hadfield 2006, 2010).

General introduction

In marine habitats nearly all submerged natural and artificial surfaces become colonized by bacteria, fungi, diatoms, protozoa, and larvae of marine invertebrates (Wahl 1989; Abarzua et al. 1999). They can substantially change the physical and chemical properties of the substratum (Characklis and Cooksey, 1983) making it suitable or unsuitable for colonization by invertebrate larvae (reviewed by Qian et al. 2007). However, the blade surfaces of many seaweeds are often rarely fouled. This phenomenon is attributed to known and putative antifouling compounds produced by the macroalgae (see Clare, 1996; Dobretsov et al. 2006). These compounds may deter the survival and settlement of larvae directly or indirectly via the control of biofilm formation and composition (Schmitt et al, 1995; Steinberg et al, 1998; Lachnit et al. 2010). Bacterial biofilms have pronounced effects on invertebrate larval settlement and may protect their macroalgal hosts from epibionts (Avelin et al. 1993; Lau and Qian 1997).

Study organism

Amphibalanus (= *Balanus*) *improvisus* (Darwin, 1854) Pitombo 2004 (Fig. 2) is a sessile crustacean that has a smooth conical shell made of white to grayish plates (Weidema 2000). The opening of the shell is diamond-shaped and toothed and the base of the shell is radially calcareous (base of the similar barnacle, *B. crenatus*, lacks this star-like ornamentation), flat and thin and permeated with pores (Zaiko 2005). It occurs in brackish water, bays and estuaries to shallow marine habitats with hard substrata (stones, rocky shores and man-made constructions such as breakwaters and ships). The species is also found as epibiont on macroalgae, crustaceans and molluscs. It filter feeds on detritus and phytoplankton. Like all sessile barnacles the bay barnacle is hermaphroditic. It is also a facultative self-fertilizer, which may be a favorable trait when establishing and maintaining new populations (Weidema 2000). This species is widely distributed from warm temperate to tropical regions (Leppäkoski 1999). *A. improvisus* is an euryhaline and eurythermal species (Leppäkoski and Olenin 2000). Furthermore it is tolerant of low oxygen concentrations, eutrophication and pollution (Leppäkoski and Olenin 2000; Ovsyannikova 2008). *A. improvisus* can dominate the community by competing for space and food. It is a strong competitor of blue mussel *Mytilus edulis* and these two together have a negative synergistic effect on community structure in the Baltic (Dürr & Wahl, 2004). Shore crab

General introduction

Carcinus maenas and the starfish *Asterias rubens* are the main predators of this species in the Western Baltic (Dürr and Wahl 2004).



Fig. 2. Adult barnacle, *Amphibalanus improvisus*

Study area

The study was performed mainly in the Kiel Fjord, Western Baltic (N: 54°22', E: 10°09') Germany. The Kiel Fjord is a narrow, N–S extending, 10–20 m deep (mean depth of about 13 m) inlet of the Kiel Bight in the Belt Sea (western Baltic Sea). Its hydrography is characterized by often rapid changes in water salinity and a low-salinity surface water which is separated by a halocline from the more saline Kattegat Water (Schweizer et al. 2011). Higher saline water masses from the Kattegat and brackish waters from the central Baltic Sea make a transition zone (The Belt Sea) which under the influence of the wind moves back and forth. Salinity changes in

General introduction

the Kiel Bight directly influence the salinity in the Kiel Fjord by baroclinic exchange. Under strong wind conditions the Kiel Fjord can be completely flushed within a few days (Javidpour et al. 2009). In Kiel Fjord, salinity fluctuates between 10 and 20 psu at the surface and between 16 and 23 psu at the bottom (Madhupratap 1996, Nasrolahi et al. 2012). Rivers such as the Schwentine as well as other drainage have only a minor effect on the salinity of Kiel Fjord (Gocke 1975) but can drastically change the salinity locally down to 6 psu (personal measurements). Sea surface temperature (SST) can easily reach from 0°C in winter to up to 23°C during late summer (Baumann 2007). In coastal lagoons and in shallow waters the temperature can even be several degrees higher as shown in 1 m depth at the Schlei Fjord (Kiel Bight, Western Baltic) with a maximum temperature of 27°C (K. Maczassek unpubl. data). Kiel Fjord is also characterized by strong fluctuations in water pCO₂ and pH due to heterotrophic degradation and river discharges (Rabalais et al. 2002; Diaz and Rosenberg 2008; Salisbury et al. 2008) with even temporary acidified conditions in shallow habitats (Thomsen et al. 2010).

A part of the study was performed at Tjärnö Marine Biological Laboratory on the Swedish west coast (N: 58°52', E: 11°09'). The area has a tidal range of 0.3 m and consists of rocky shores and islands, frequently interrupted by shallow, soft-bottom bays and fjords (Jonsson et al. 2004). In this area, *A. improvisus* reproduces during the summer and the planktonic cyprid larvae settle during June–October (Berntsson et al. 2000). Temperature during the peak settlement season of barnacles in this area is between 20 and 25°C and salinity is between 22 and 30 psu (Kvarnemo 1996).

Thesis objectives

The fate of key species, such as the barnacle *Amphibalanus improvisus*, in the course of global change is of particular interest since any change in their abundance and/or performance may entail community-wide effects (Dürr and Wahl 2004). Since early life stages of intertidal invertebrates may be more vulnerable to physiological extremes than adults (Gosselin and Qian 1997; Przeslawski et al. 2005; Gosselin and Jones 2010), the impact of predicted future relative to present stress regimes on these early stages are of particular concern. Yet, these life history phases are notoriously understudied especially with regard to the impact of combined stressors. Thus, in this study, the combined effects of temperature and salinity on early life stages

General introduction

(nauplius, cypris and juvenile stages) of the barnacle *A. improvisus* were examined. Biofilms (especially bacterial biofilms) have an immensely important function in the attachment process of barnacles. Thus, the effect of biofilms composed of epibiotic bacteria on macroalgae on the number of attached *A. improvisus* cyprids in field assays were further investigated.

This thesis covers different life stages during the planktonic and early benthic phase of barnacle. In the first part of this study (***Chapter I***), the focus was on three understudied aspects of stress ecology: the interaction of stressors, their impact on early life stages, and the influence of stress history on subsequent life stages. For this, larval duration, metamorphosis success from nauplius to cyprid, survival and settlement of *A. improvisus* from Kiel population were investigated at a fully crossed design of temperature and salinity. Likewise, settlement success and survival were examined under the same stress regime for the naive cyprids. However, no such comparative assessment of separate populations from one species was made until the beginning of my study which would enhance our ability to extrapolate local results to regional and global scales. Taking this into account and also concerning the possible different local adaptations (Sanford and Kelly 2011) and sensitivity towards stresses between populations of the same species, I conducted a similar assay on the Swedish west coast population of *A. improvisus* at Tjärnö Marine Biological Laboratory (***Chapter II***). Moreover, the combined effects of temperature and salinity on juveniles of *A. improvisus* was investigated to elucidate how various combinations of these factors interactively impact juvenile phase in terms of early and cumulative mortality as well as growth (***Chapter III***). Lastly, the effect of biofilms on barnacle attachment was assessed (***Chapter IV***) aiming to test the effect of monospecies bacterial biofilms and natural biofilms originally isolated from macroalgal hosts on attachment of barnacle larvae. All the results are then discussed in ***Chapter V***.

General introduction

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Being young in a changing world: how temperature and salinity changes interactively modify the performance of larval stages of the barnacle

Amphibalanus improvisus

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Abstract

The fate of key species, such as the barnacle *Amphibalanus improvisus*, in the course of global change is of particular interest since any change in their abundance and/or performance may entail community-wide effects. In the fluctuating Western Baltic, species typically experience a broad range of environmental conditions, which may pre-select them to better cope with climate change. In this study, we examined the sensitivity of two crucial ontogenetic phases (naupliar, cypris) of the barnacle toward a range of temperature (12, 20, and 28°C) and salinity (5, 15, and 30 psu) combinations. Under all salinity treatments, nauplii developed faster at intermediate and high temperatures. Cyprid metamorphosis success, in contrast, was interactively impacted by temperature and salinity. Survival of nauplii decreased with increasing salinity under all temperature treatments. Highest settlement rates occurred at the intermediate temperature and salinity combination, i.e., 20°C and 15 psu. Settlement success of “naive” cyprids, i.e., when nauplii were raised in the absence of stress (20°C/15 psu), was less impacted by stressful temperature/ salinity combinations than that of cyprids with a stress history. Here, settlement success was highest at 30 psu particularly at low and high temperatures. Surprisingly, larval survival was not highest under the conditions typical for the Kiel Fjord at the season of peak settlement (20°C/15 psu). The proportion of nauplii that ultimately transformed to attached juveniles was, however, “home” conditions. Overall, only highest under particularly these stressful combinations of temperature and salinity substantially reduced larval performance and development. Given more time for adaptation, the relatively smooth climate shifts predicted will probably not dramatically affect this species.

Chapter I

Key words:

Amphibalanus improvisus, temperature, salinity, interactive stress effects, larval performance, settlement, cyprid

Introduction

Intertidal barnacles as conspicuous, widely distributed, and ecologically important species have served to study the effects of environmental factors, e.g., temperature, salinity, food type and concentration, light (e.g., Crisp and Ritz 1973; Harms 1984; Holm 1990; Konya and Miki 1994; Anil et al. 1995, 2001; Anil and Kurian 1996; Qiu and Qian 1997, 1999; Hentschel and Emlet 2000; Thiyagarajan et al. 2002a; Nasrolahi et al. 2007), and more recently global warming and ocean acidification (Findlay et al. 2008; Hung et al. 2008; Findlay et al. 2009; McDonald et al. 2009; Wong et al., in press) on larval development and settlement success. Much of this progress on what is frequently considered the most sensitive life history phase is facilitated because barnacle larvae can be easily reared under controlled conditions (Thiyagarajan et al. 2000). Thus, numerous studies of stress impacts have been conducted using the widespread barnacle *A. amphitrite* (e.g., Anil et al. 2001; Desai and Anil 2002, 2004; Desai et al. 2006; McDonald et al. 2009) and other locally common barnacles species, e.g., *Balanus trigonus* (Thiyagarajan et al. 2003a), *Balanus glandula* (Berger 2009), *A. improvisus* (Nasrolahi et al. 2006), and *Semibalanus balanoides* (Pneda et al. 2002; Findlay et al. 2010). It is well accepted by now that in the course of global change, several potential stressors will appear and intensify simultaneously and may exhibit antagonistic, additive, or synergistic effects, which make predictions based on the assessment of single stressors unreliable (e.g., Wahl et al. 2011). The interactive effect of putative stresses such as high temperature and low salinity on larval performance and survival of barnacles has, however, been subject of only a few studies (Harms 1986; Anil et al. 1995; Qiu and Qian 1999; Thiyagarajan et al. 2003b). Responses toward multiple stressors of *A. improvisus*, the dominant barnacle species within the Western Baltic Sea, have been investigated so far, neither in adult nor in larval stages. The energy content of cyprids depends on the previous planktotrophic naupliar development (Pechenik 1987; Anil et al. 2001; Thiyagarajan et al. 2002b, 2003b) and thus, not only stressors may interact, but also their impact on successive developmental stages. This development is determined by various environmental factors such as

Chapter I

temperature, salinity, and availability of food (Anil et al. 1995, 2001; Anil and Kurian 1996; Desai et al. 2006). Consequently, the stress acting during early larval development might impact the performance and stress sensitivity of later stages, another aspect relatively neglected so far (but see Qiu and Qian 1999).

When stress impacts a foundational species, it may percolate through the community easily (e.g., Wahl et al. 2011). *Amphibalanus improvisus* is such a common, locally dominant, and ecologically important fouling organism in the Western Baltic (Dürr and Wahl 2004). While its recruitment peaks in the summer (June–September), lower numbers of recruits can be encountered throughout the year (personal observation). Due to small-scale spatial and seasonal environmental variability, larvae within the Western Baltic may experience a broad range of salinities and temperatures during different months of their occurrence, at different locations and even at different depths. In Kiel Fjord, the site of the investigated barnacle population, salinity fluctuates between 5 and 20 psu at the surface and between 16 and 23 psu at the bottom (Madhupratap et al. 1996, personal observation). Sea surface temperature (SST) can easily reach from 0°C in winter to up to 23°C during late summer (Baumann et al. 2007). In coastal lagoons and in shallow waters, the temperature can even be several degrees higher as shown in 1 m depth at the Schlei Fjord (Kiel Bight, Western Baltic) with a maximum temperature of 27°C (K. Maczassek unpublished data). During *A. improvisus* peak recruitment from July to August (Thomsen et al. 2010), SST in Kiel Fjord typically is around 20°C and surface salinity is about 15 psu (own unpublished logger data). These conditions, if not optimal, can at least be considered benign. Although the rearing conditions may influence the performance of subsequent stages such as cyprids or post-larvae (Pechenik et al. 1993; Qiu and Qian 1999; Pechenik 2006; McDonald et al. 2009), earlier studies have mainly examined the settlement success of cyprids without a stress history (e.g., Dineen and Hines 1992; Thiyagarajan et al. 2003a; Dahlstrom et al. 2004; McDonald et al. 2009). In this study, we focus on three understudied aspects of stress ecology: the interaction of stressors, their impact on early life stages, and the influence of stress history on subsequent life stages.

Materials and methods

Rearing of barnacle larvae

Chapter I

Pebbles and stones bearing adult *A. improvisus* were collected from Kiel Fjord (Western Baltic) in summer 2009 and transferred to the laboratory. After rinsing with seawater, they were placed in seawater-filled trays at a constant temperature of 20°C under a 12:12 h day/night light cycle and provided with a flow through of filtered fjord water. Barnacles were fed daily on a diet of freshly hatched brine shrimp (*Artemia salina*). Adults released nauplii after few days, which were collected in sieves (90 µm) from the tray overflow. These nauplii were at stage I and developed into stage II within few hours. Mixed batches of stage II larvae from multiple parents were used for the two experiments. The life cycle of barnacles includes six planktotrophic naupliar larval stages followed by a non-feeding, planktonic cypris (Honglei et al. 2010).

Experiment I

The influences of the combination of temperature and salinity on various aspects of larval performance were assessed during the 4-week span until larvae metamorphosed and settled. Response variables were (1) total larval duration (days from hatching until 50% of the surviving nauplii had developed into cyprids), (2) survival (percent surviving nauplii or cyprids relative to the respective initial numbers), (3) percentage of cyprids (percent of the initially introduced 20 nauplii per well, which had turned into cyprids by the given time of the experiment), and (4) settlement (percent of settled juvenile barnacles relative to initial number of nauplii or relative to the number of surviving cyprids). We used three different salinities (5, 15, and 30 psu) and three different temperatures (12, 20, and 28°C) in a fully mixed experimental design. The different salinities used in the experiment were obtained by diluting filtered (0.2 µm) artificial seawater (30 psu) with double distilled water. Although diluting seawater with distilled water may create unnaturally low alkalinities at low salinity values, this is a common method used in salinity assays on barnacle larvae (e.g., Qiu and Qian 1999; Thiagarajan et al. 2003a; Tindle et al. 2004). Personal measurement in our study, however, showed that alkalinity decreased with diluting seawater in a natural range as measured by Beldowski et al. (2010) for the Baltic Sea at different salinity values. Each treatment combination consisted of eight replicates (one thermo bath for each treatment) with 20 larvae per replicate (one individual well per replicate, see below). Cultures were kept in thermo bathes (Thermostat HAAKE DC10, Karlsruhe, Germany) adjusted to the target temperatures ($\pm 0.1^\circ\text{C}$). Larvae were transferred into six-well plates (CELL

Chapter I

STAR # 657160) at the respective salinities (defined per well) and temperatures (defined per plate in a given thermo bath). Marine diatoms *Chaetoceros calcitrans* and *Skeletonema costatum* were grown in f/2 medium (15°C and 15 psu) and used as larval food. For each treatment, 20 larvae were incubated in 10 ml of filtered (0.2 µm) seawater (FSW) and were fed daily with 50:50 mixture of both diatom species at 2×10^5 cells ml⁻¹. Larval cultures were kept under continuous light (16 µmol m⁻² s⁻¹) to minimize mortality and precipitation of algal cells (Thiyagarajan et al. 2003a, 2003b). Every 24 h, larvae were observed using a binocular microscope (WILD M3C Heerbrugg, Switzerland) and the number of survivors, percentage of cyprids, and settlement were recorded for each replicate. Since the counting was fast, the water temperature in each treatment did not change noticeably during observation under the binocular. Hereafter, all cyprids reared in this experiment (Exp. I) are called “pre-stressed” regardless of the fact that some of them were reared in the ambient, i.e., no-stress conditions (20°C/15 psu). The water and food in the wells were replaced every second day by fresh seawater with the respective temperature and salinity. To keep the temperature constant for each treatment while changing the water in the wells, preconditioned water was used from 2 l water bottles that were kept in thermo bathes with the respective temperature.

Experiment II

Nauplius II larvae (new batch) were obtained as in Experiment I and kept at a density of 1 larva per 2 ml filtered (0.2 µm) seawater in 20-l buckets at 15 psu. Each bucket was provided with a mixture of *C. calcitrans* and *S. costatum* as described for Experiment I. The buckets were kept in a water bath equipped with circulation and an immersion heater at 20°C. The cultures in the buckets were gently aerated, while seawater was replaced and microalgae were provided every third day. When cyprids appeared in the cultures after 7–8 days, the cultures were sieved (200 µm) and cyprids were removed and subsequently used for settlement bioassays. As in Kiel Fjord, settlement peak usually occurs in July and August (mean temperatures of about 20°C and the mean salinities of 15 psu); these cyprids were reared under conditions as prevail in Kiel Fjord during peak settlement time (habitat condition) (Thomsen et al. 2010) and thus, presumably, without temperature and salinity stress. The levels of temperature and salinity tested were the same as in Experiment I. 10 cyprids and 5 ml of FSW were transferred into sterile six-well plates with different salinities and incubated at the different temperatures under continuous light. The

Chapter I

transition from starting to target conditions was done gradually (1 psu/h, 1°C/2 h) to allow for acclimatization. Settlement and survival of cyprids were monitored daily over 25 days. Every second day, seawater in experimental containers was replaced. Four replicates were used for each treatment.

Statistical analysis

The majority of data sets were not normal due to the frequent observation of zeros. This is an inherent property of the response variables we collected: no cyprid metamorphosis/settlement during early phases of the experiment resulted in strongly skewed frequency distributions, which could not be transformed to normality. Therefore, we refrained from applying parametric statistical procedures and employed permutational multivariate analysis of variance (PERMANOVA) instead. Analyses were undertaken using the PERMANOVA+ add-on for PRIMER 6.0 (Anderson et al. 2008) for elucidating the effects of temperature and salinity on the different response variables we measured. This test procedure provides values of p from permutations (4,999 in all our tests), which we based on binomial deviance dissimilarities. This measure is appropriate for this kind of empirical distributions (Anderson and Millar 2004), and we derived it from the non-transformed data. Our PERMANOVA design had two fixed factors (“Temperature,” 3 levels; “Salinity,” 3 levels) and the interaction term between these two. Since we sampled our experimental units repeatedly, we viewed the different sampling days as dependent variables and thus employed the multivariate approach. PERMANOVA does not assume any underlying data distribution, but is sensitive to differences in dispersion across samples. We tested for this using the PERMDISP routine in PERMANOVA+ and found it given for the majority of our tests. However, since PERMANOVA is robust toward violations of the homogeneity of dispersion, we continued with the analysis even in those cases when dispersion among groups was not homogenous (Anderson et al. 2008). The effects of temperature and salinity on total larval duration and final settlement were evaluated using parametric two-way ANOVAs (Table 1). For this, we focused on the last day of the experiment and ignored all previous samplings. Furthermore, we ran a t test to test for a difference among the 2 larval batches used in the two subsequent experiments regarding mean settlement rates under no-stress conditions (15 psu, 20°C). We verified normality of data with Shapiro–Wilk’s W test and

explored homogeneity of variances with Levene's test. In case of deviations from the assumptions, we applied appropriate transformations prior to the analyses.

Results

Experiment I

The time required for larvae to develop from nauplius II to cypris stage (Exp. I) was almost twice as long at 12°C than at 20°C and 28°C, but did not differ substantially between the two latter temperatures (Fig. 1). The effect of temperature on total larval duration was significant (ANOVA, $F = 97.02$, $df = 2$, $p < 0.001$), while the effect of salinity (ANOVA, $F = 2.93$, $df = 2$, $p = 0.06$) and its interaction with temperature (ANOVA, $F = 1.11$, $df = 4$, $p = 0.35$) was not statistically significant.

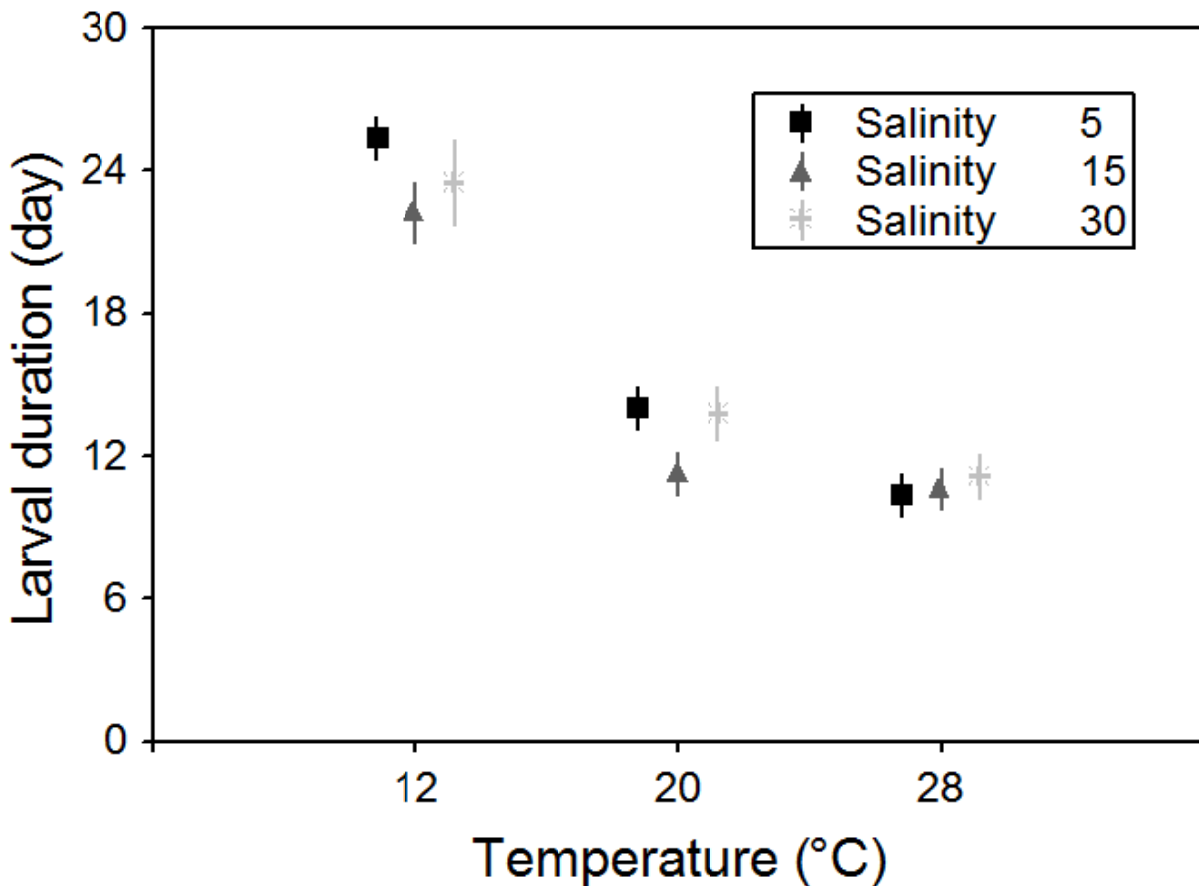


Fig. 1 *Amphibalanus improvisus*. Effect of temperature and salinity on total larval duration. Each bar represents the mean (\pm SE) of eight replicates. Each replicate consisted of 20 nauplius larvae

Chapter I

The rate of successful metamorphosis of nauplii to cyprids (Exp. I) was interactively impacted by temperature and salinity (Table 1a). At 5 psu, the percentage of successful metamorphosis was highest at all temperature levels most conspicuously so at 28°C (Fig. 2). Metamorphosis was particularly low at 12°C where only under 5 psu it reached 20% by the end of the experiment (Fig. 2).

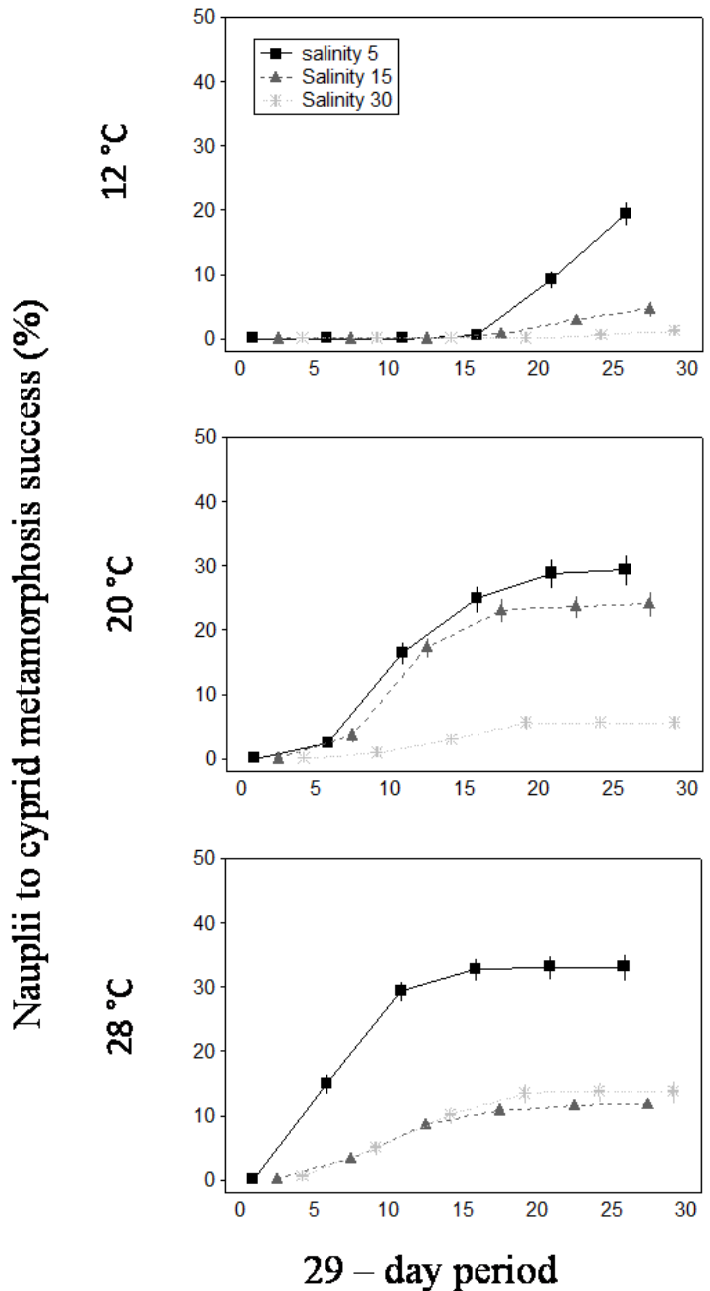
Table 1. Exp. I (with pre-treated larvae): effects of temperature and salinity on nauplii to cyprid metamorphosis success (a), survival (b) and cyprid settlement from nauplii (c) in *Amphibalanus improvisus*. Results from multifactorial PERMANOVA.

(a) nauplii to cyprid metamorphosis success						
Source	df	SS	MS	Pseudo-F	P(perm)	perms
Te	2	1765.1	882.54	58.356	0.0002	4988
Sa	2	344.12	172.06	11.377	0.0002	4989
TexSa	4	144.93	36.232	2.3958	0.0254	4979
Res	63	952.77	15.123			
Total	71	3206.9				
(b) survival						
Source	df	SS	MS	Pseudo-F	P(perm)	perms
Te	2	45.521	22.76	1.5214	0.2088	4986
Sa	2	390.64	195.32	13.056	0.0002	4989
TexSa	4	74.54	18.635	1.2456	0.2872	4984
Res	63	942.52	14.961			
Total	71	1453.2				
(c) settlement from 20 nauplii						
Source	df	SS	MS	Pseudo-F	P(perm)	perms
Te	2	672.52	336.26	18.091	0.0002	4988
Sa	2	183.38	91.688	4.933	0.0084	4990
TexSa	4	144.95	36.237	1.9496	0.1006	4983
Res	63	1171	18.587			
Total	71	2171.8				

PERMANOVAs were based on the binomial dissimilarity measure. *P*-values were obtained using 4999 permutations of residuals under a reduced model.

Survival of nauplii and cyprids (Exp. I) was affected by salinity but not by temperature, while there was no interaction between the two factors (Table 1b). Survival of nauplii decreased with increasing salinity under all temperature regimes with nauplii mortality ranging between 70% at 28°C/5 psu and almost 100% at 12°C/30 psu (Fig. 3). In contrast, cyprid mortality tended to be highest at 5 psu (except at the lowest temperature) and was low under all other treatment combinations (Fig. 3). The sum of nauplii and cyprid mortality always exceeded 70% and was particularly high under the combination of lowest temperature and highest salinity (Fig. 4a).

Fig. 2 *Amphibalanus improvisus*. Success of nauplii metamorphosed to cyprids under different temperature and salinity regimes over a 29-day assay period. Each bar represents the mean (\pm SE) of eight replicates. Each replicate consisted of 20 nauplius larvae



Chapter I

Among the survivors at the cyprid stage, settlement success (percent of the initial 20 nauplii settled) (Exp. I) was influenced by the main effects of temperature and salinity (Table 1c). Highest settlement occurred at the intermediate temperature and salinity combination, i.e., 20°C and 15 psu, while the lowest settlement was seen at 12°C regardless of the salinity level (Fig. 4b). Final cyprid settlement success (percent of settlers relative to the number of surviving cyprids, Exp. I) tended to be lower at 12°C than in the two warmer treatments regardless of salinity (Fig. 5).

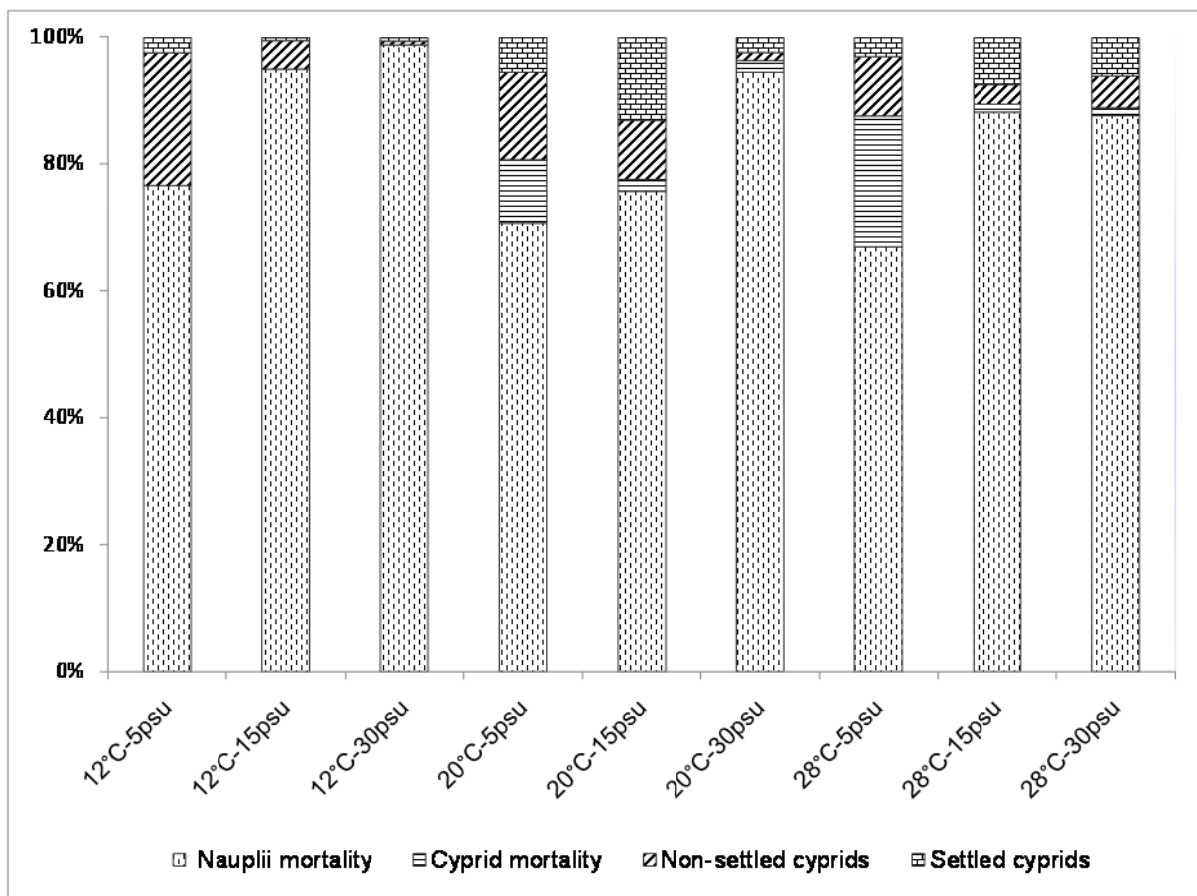


Fig. 3 *Amphibalanus improvisus*. Performance of barnacle larvae after 4 weeks under different combinations of temperature and salinity (with prestressed larvae).

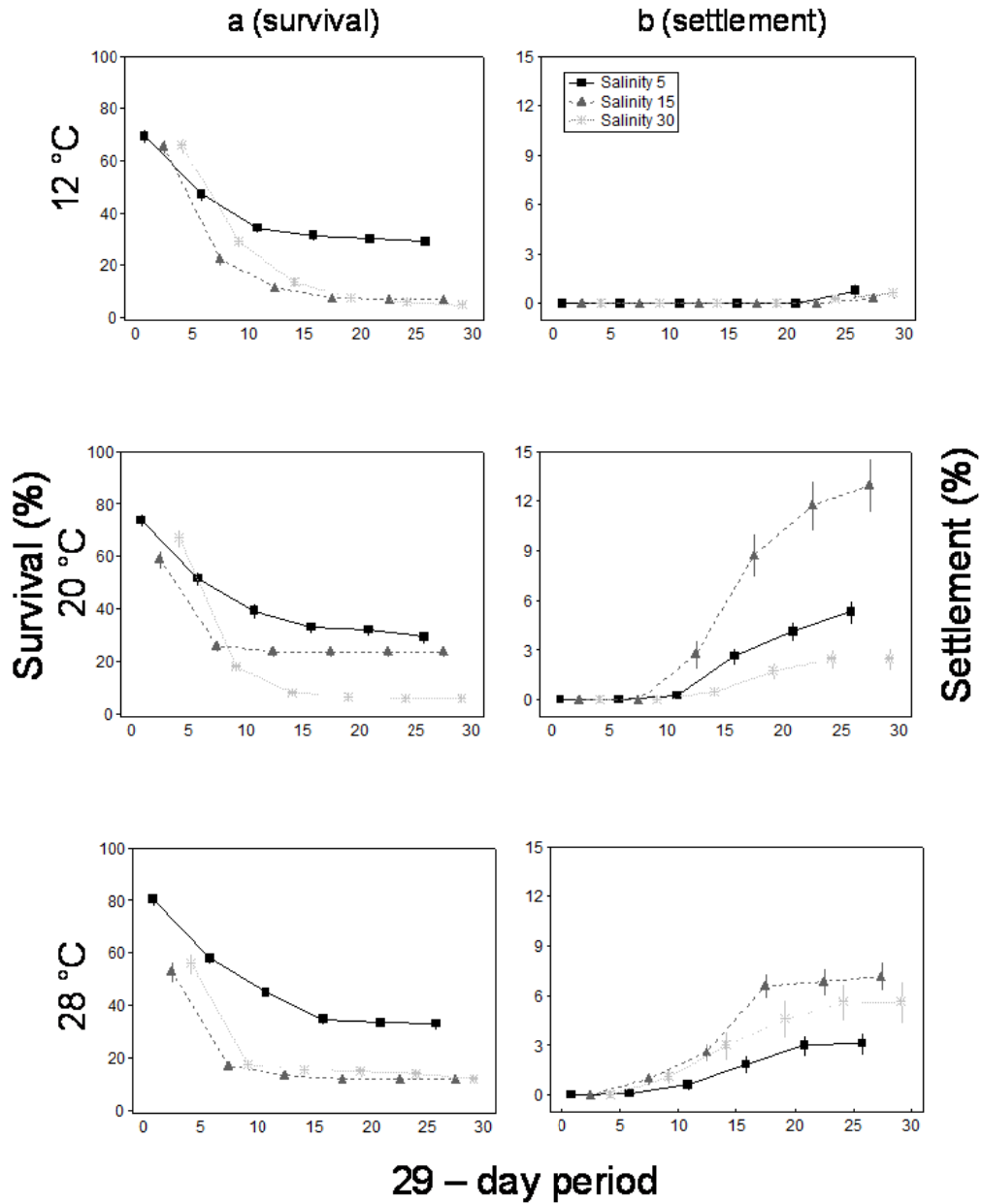


Fig. 4. *Amphibalanus improvisus*. Effect of temperature and salinity on cyprid survival (a) and settlement (b) in Exp. I (with pre-stressed larvae). Cyprid settlement was calculated as the percentage of all larvae in a replicate (20 nauplii) that settled. Survival has been accounted 24 h after the incubation of nauplii (day 1). Each bar represents the mean (\pm SE) of eight replicates. Each replicate consisted of 20 nauplius larvae.

Experiment II

Settlement of cyprids without stress history (“naive” cyprids, Exp. II) was significantly affected by temperature and salinity, while no interaction between the two factors emerged (Table 2). Settlement success was high at 30 psu when combined with the low (~90%) and the high (~75%) temperature regime and was low when the intermediate (~65%) temperature was applied (Fig. 6a). High and low temperatures decreased settlement success at 5 and 15 but not at 30 psu. Survival of the naive cyprids was high (>90%) under most factor combinations and only slightly reduced (~80%) at 28°C and 5 psu (Fig. 6b). The interaction between temperature and salinity on final cyprid survival was significant (ANOVA, $F = 4.25$, $df = 4$, $p < 0.05$).

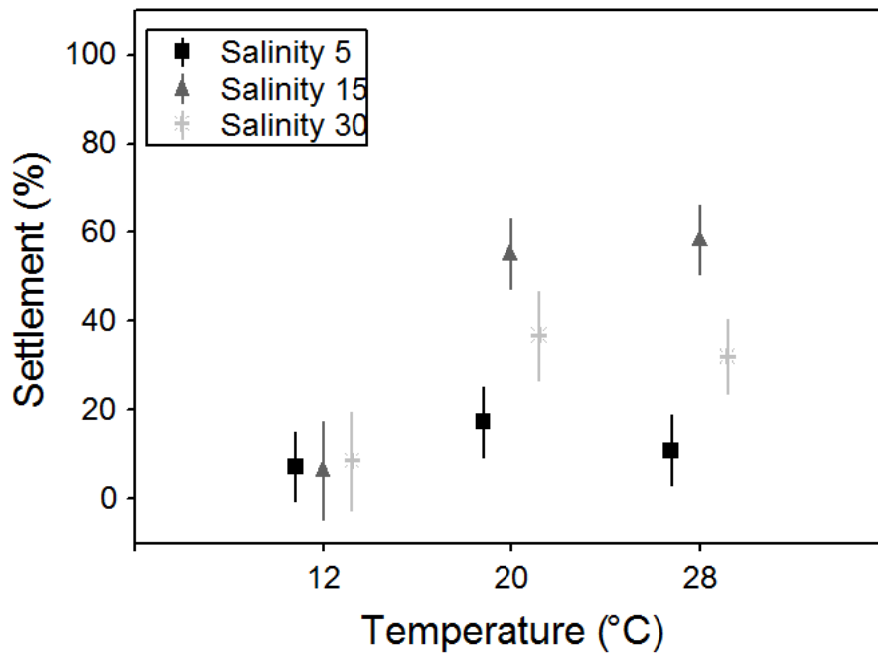


Fig. 5. *Amphibalanus improvisus*. Effect of temperature and salinity on final cyprid settlement in Exp. I (with pre-stressed larvae). Cyprid settlement includes percentage of settled cyprids from surviving cyprids. Each bar represents the mean (\pm SE) of eight replicates.

The two batches of cyprids used in Exp. I and II did not differ in their settlement success at no-stress conditions (20°C, 15 psu, Fig. 7, t test: $t = 0.78$, $df = 6$, $p = 0.46$). In contrast, prestressed (at the nauplii stages) and naive cyprids differed in their settlement at other stress conditions (Fig. 7).

Chapter I

Table 2. *Amphibalanus improvisus*. Exp. II (naive cyprids) PERMANOVA testing of temperature and salinity on cyprid settlement

Source	df	SS	MS	Pseudo-F	P(perm)	perms
Te	2	38.357	19.179	3.9185	0.0170	4980
Sa	2	60.064	30.032	6.1361	0.0026	4989
TexSa	4	30.97	7.7425	1.5819	0.1778	4983
Res	27	132.15	4.8944			
Total	35	261.54				

PERMANOVAs were based on the binomial dissimilarity measure. P-values were obtained using 4999 permutations of residuals under a reduced model.

Fig. 6. *Amphibalanus improvisus*. Effect of temperature and salinity on cyprid survival (a) and settlement (b) over a 25-day period in Exp. II (with naive cyprids). Day 0 shows settlement and survival after 24 h of incubation. Each bar represents the mean (\pm SE) of four replicates. Each replicate consisted of 10 cyprids.

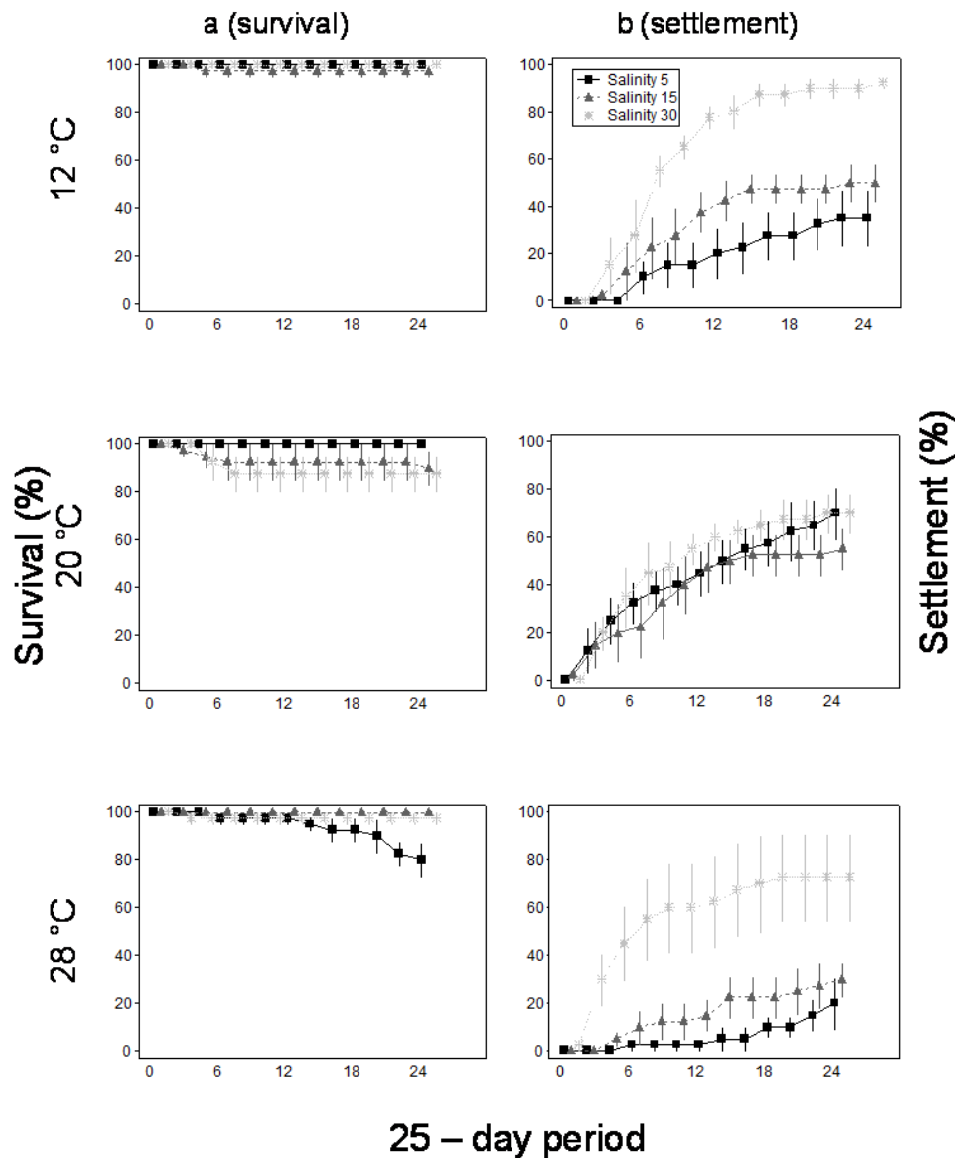
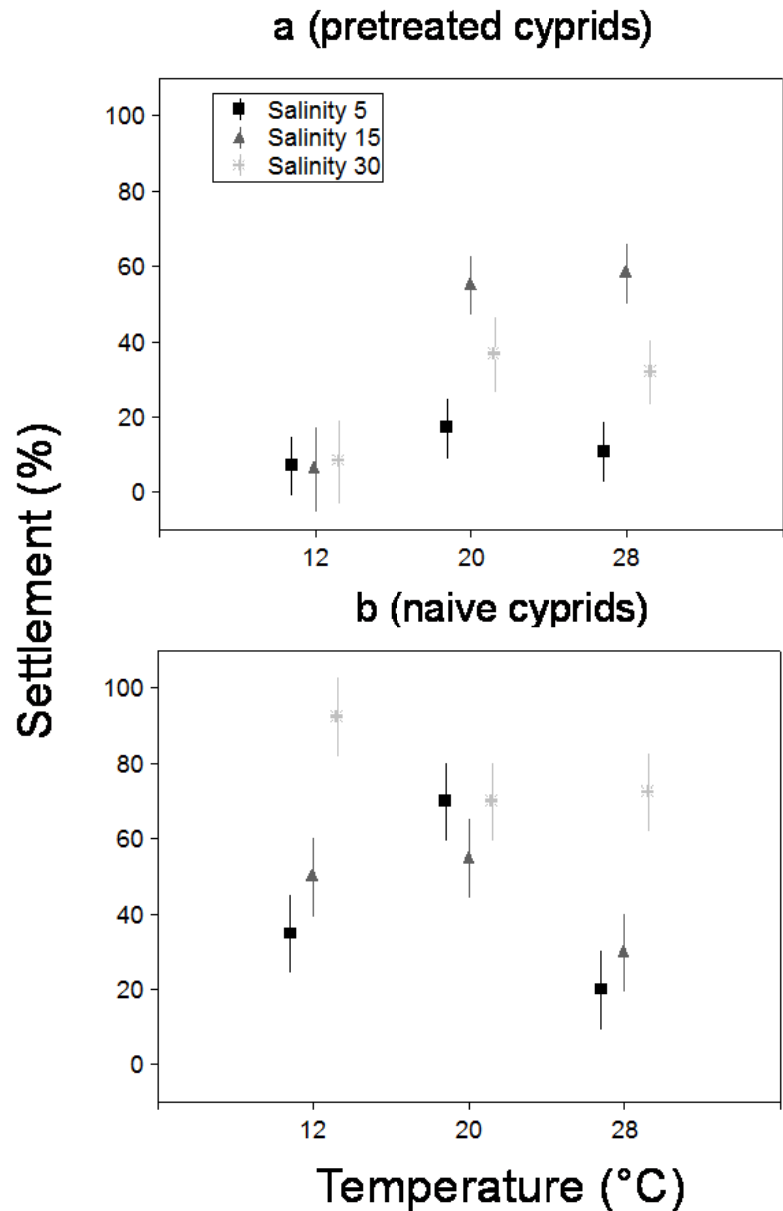


Fig. 7 *Amphibalanus improvisus*. Final settlement under different temperature and salinity regimes in Exp. I (settled cyprids from surviving cyprids) (a) and in Exp. II (b). In Exp. I cyprids were pre-stressed during their naupliar stage, while in Exp. II cyprids were reared under habitat conditions. Each bar represents the mean (\pm SE) of eight replicates (Exp. I) or four replicates (Exp. II), respectively.



Discussion

Our results show that temperature and salinity have a strong potential to impact successful larval development of the barnacle *A. improvisus*. In our study, in several instances, the two factors interacted, i.e., the effect of one stressor depended on what level of the other stressor was present. Some factor combinations in our study were intentionally chosen to constitute suboptimal conditions, i.e., environmental stress, and we previously defined this as any setting of an environmental variable or several variables that reduces an organism's performance (Wahl et

Chapter I

al. 2011). At the same time, we assumed that the environmental conditions during peak recruitment represent a scenario to which the population we investigated is adapted to, i.e., these conditions are optimal. Surprisingly, this was not always the case. In some instances, we observed that stress sensitivity of a given larval stage depended on the stress history experienced by earlier stages.

Total larval duration was shorter at higher temperatures. Similar results have previously been observed for *Amphibalanus amphitrite* (Anil et al. 1995, 2001; Qiu and Qian 1999), *B. trigonus* (Thiyagarajan et al. 2003a, b), *A. eburneus* (Scheltema and Williams 1982), *Elminius modestus* (Harms 1984, 1986), and *Semibalanus balanoides* (Harms 1984). Independently of salinity, larval duration was accelerated by 1.2 days per °C of warming ($R^2 = 0.75$ and 0.96 for 15 and 5 psu, resp.). Salinity, in the range applied in our study, did not influence larval duration significantly. Inconsistently, Nasrolahi et al. (2006) found that increasing salinity led to a slight deceleration of larval development in *A. improvisus* in the Southern Caspian Sea population, where barnacles occur at an average salinity of 13 psu. In contrast, Anil and Kurian (1996) observed that total larval duration of *A. amphitrite*, which has a more marine distribution than *A. improvisus*, decreased with increasing salinity. The relatively low sensitivity toward salinity changes we observed in this species may arise from the fact that euryhaline barnacle species often have osmotic regulation by active ion transportation (Fyhn 1976; Gohad et al. 2009). *Amphibalanus improvisus* is widely distributed, inhabits warm temperate seas, and can be often found in areas of low (as the Baltic or Caspian Sea) or widely fluctuating salinities (Furman and Yule 1990).

The chance of survival of non-feeding cyprids depends upon the stored energy during the naupliar developmental history (Gaonkar and Anil 2010). Temperature and salinity stress may have an important effect on the amount of energy stored in nauplii by influencing the feeding pattern (Anil and Kurian 1996) and/or by increasing their metabolic activity (Fraser 1989). This, as a consequence, influences the survival rate of cyprids. In this study, in contrast to larval duration, the rate of survival to the cyprid stage at all temperatures was highest at 5 psu. These results are similar to those reported by Nasrolahi et al. (2006) for the same species in the Caspian

Chapter I

Sea. Possibly, low salinity compensates to some extent for negative effects of temperature stress on metamorphosis success in our experiments.

We found settlement to be almost zero at the lowest temperature (12°C), regardless of salinity, while survival at this temperature was between 5 and 40% depending on salinity. However, we know that in the Western Baltic, nauplii, to some extent, hatch and survive at this and even lower temperatures as shown by Javidpour et al. (2010), who found barnacle nauplii in gut samples of *Mnemiopsis leidyi* in Kiel Fjord during most of winter. Surprisingly, though nauplii are obviously present in the water column at this time of the year, successful settlement is at an almost undetectable level between November and April (Wahl and Schütt, unpublished data; Thomsen et al. 2010). Various reasons for settlement failure of barnacle larvae during winter have been documented as, e.g., food constraint (Barnes and Barnes 1958; Thiyagarajan et al. 2002a), low temperature (Anil et al. 1995), predation, starvation and misrouting (Crisp 1984), starvation in early naupliar stages (Lang and Marcy 1982), and finally the quality of released nauplii (Anil et al. 1995).

If the barnacle population we investigated was optimally adapted to the local conditions, we would have expected best performance (e.g., survival) among those individuals that experienced conditions prevailing during peak settlement in July and August (Thomsen et al. 2010), i.e., at about 20°C and 15 psu. However, larval duration was shortest at 28°C (regardless of salinity), metamorphosis success and overall survival (from start to end of Experiment I) was best at 5 psu (regardless of temperature). Only when we look at the proportion of settled cyprids, i.e., nauplii that completed their entire larval development and successfully transformed into settled, juvenile barnacles, then the “home” conditions of 20°C/15 psu proved the most beneficial (Exp. I, Figs. 3 and 4b). This is in line with Leppäkoski (1999) who showed that larval settlement in *A. improvisus* peaks at salinities around 15 psu.

Both increased temperature, via unfavorable de- or acceleration of metabolic activity (Pörtner and Farrell 2008; Neuheimer et al. 2011), and decreased salinity, due to physiological investment into osmoregulation (Pechenik et al. 2000), are potential stressors and therefore can reduce the amount of energy reserves available to non-feeding cyprids. In our experiment, in naive cyprids (without stress history or carryover effects), settlement was lowest at a high

Chapter I

temperature (28°C) and low salinity (5 psu) and highest at 30 psu, regardless of temperature. These results corroborate the findings of Thiyagarajan et al. (2003a) on *B. trigonus* in Hong Kong waters.

In our study, naive cyprids performed better under most salinity–temperature combinations than cyprids with a stress history (Fig. 7). The two experiments were performed on two separate batches of larvae, and therefore, differences between them might be due to differences between batches rather than differences in responses to the treatments. However, it is unlikely that these differences originate from using different larval batches of the same Kiel Fjord barnacle population in the two experiments. Comparing the two experiments, we could not detect any difference in settlement between those cyprids of the two batches the nauplii of which were raised under no-stress conditions (20°C/15 psu). On average, 20% more of naive cyprids settled than of pretreated cyprids (all nauplii treatments pooled). The combined effect of nauplii treatment (Exp. I) and cyprid treatment (Exp. II) is, however, complex because different larval stages exhibit different sensitivity patterns. For instance, naive cyprids settled best at 30 psu and least at 5 psu especially at intermediate and high temperatures (12 and 28°C), whereas the highest metamorphosis success of nauplii was found at 5 psu under all temperatures. When settlement success was expressed as percent of initial cyprid numbers in both experiments, only at the lowest temperature, carryover effects (i.e., higher sensitivity in prestressed as compared to naive cyprids) were detectable. Here, cyprids with a stress history at the naupliar stage reacted more sensitively to a given stress treatment than cyprids whose nauplii had been raised under no-stress conditions.

Osmotic stress experienced as found by Qiu and Qian (1999), in one life stage of a barnacle can be passed over to the next life stage. Corresponding to this, we saw that settlement of cyprids with a stress history (at the nauplius stage) was less than 15% of the initial number of nauplii, while in naive larvae (Exp. II), settlement and survival were much higher (relative to the initial number of cyprids, not nauplii). Such carryover effects could potentially work in two directions: either stress-induced selective mortality at the naupliar stages results in a preselection for stress resistance in the (surviving) cyprids population (composed of the more resistance genotypes) or stress conditions throughout the naupliar stages lead to unfit cyprids, which are

Chapter I

low in energy, have a retarded development and exhibit higher mortality. Since we did not observe strong carryover effect, we assume that the two opposing effects compensated each other (except at the lowest temperature).

A temperature increase of more than 0.7°C during the last century has been documented for the Baltic Sea and a further warming by up to 6°C is expected until 2100 (BACC 2008). Simultaneously, a decrease in salinity due to higher precipitation is predicted (BACC 2008). For Kiel Bight in the Western Baltic and during the season of peak barnacle settlement, this would correspond to a warming from 20 to 26°C and a desalination from 15 to a still unknown value between 14 and 8 psu (“up to -45%,” BACC 2008). If *A. improvisus* cannot adapt to these changes, both factors would additively or synergistically accelerate larval development (if food is not limiting), which could reduce larval mortality due to external factors such as food limitation or predation. Survival might slightly increase as suggested by the rates found for 20°C/15 psu (22%) and 28°C/5 psu (37%), respectively. Furthermore, our results suggest that settlement success of cyprids might decrease ~55% at 20°C/15 psu and ~10% at 28°C/5 psu. Thus, the beneficial effects of climate change on larval development and survival may be partially compensated by the detrimental effect on settlement success.

This scenario disregards the fact that *A. improvisus* undoubtedly has the potential to adapt to these changes to some, yet unexplored, extent, and it ignores further potential interactions of future salinity and temperature regimes with other changing environmental variables. Therefore, any prediction about climate change effects on species performance remains uncertain as long as we do not know about the compound effect of the whole suite of shifting environmental factors (temperature, salinity, nutrients, pCO₂, stratification, predation, parasitism, etc.) and the evolutive potential, at all of their ontogenetic stages, of the affected species to adapt to these shifts (e.g., Reusch and Wood 2007; Wahl et al. 2011). Future studies on marine invertebrates under climate change scenarios should therefore focus more on the interactive effects of different environmental stressors considering all ontogenetic stages.

Chapter I

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Temperature and salinity effects on larval performance of *Amphibalanus improvisus*: a maladaptation?

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Abstract

The larval stage of the marine invertebrate life cycle is often held to be the most susceptible to environmental stress, and therefore may be the first stage at which the effects of near-future climate change are manifested. In this study, we examined the sensitivity of two larval phases (naupliar, cypris) of the barnacle *Amphibalanus improvisus* to a range of temperature (12, 20, and 28°C) and salinity (5, 15, and 30 psu) combinations in a population from Tjärnö (Sweden, Kattegatt). The longest and shortest larval duration occurred at 12 and 28°C (21 and 6 days, respectively) regardless of salinity. Nauplii to cyprid metamorphosis success, larval survival, and larval settlement were highest at the highest temperature and intermediate salinity, i.e., 28°C and 15 psu. Settlement was almost zero at 12°C regardless of salinity. In a separate experiment, when cyprids reared at 26°C/30 psu were exposed to the same temperature and salinity combinations, almost all cyprids survived (>95%). Cyprid settlement, in contrast, was impacted by salinity and was lowest at 30 psu regardless of temperature. Surprisingly, larval performance was not highest under the conditions typical for Sweden west coast at the season of peak settlement. This implies that this population is probably in suboptimal conditions and maladapted to its habitat. Both warming and desalination as predicted for the Baltic Sea in the course of climate change may however, favor *A. improvisus* and additively or synergistically increase the performance of this population.

Introduction

Many marine invertebrates with complex life cycles produce planktonic larvae that experience environmental conditions different from those encountered by adults. These larvae play a specialized role in determining population dynamics of adult organisms (Pechenik 2006). Furthermore, these rapidly developing stages are more susceptible to environmental stresses than their adult counterparts that are relatively protected from

Chapter II

physiological extremes. For example, it has been observed that larvae have greater sensitivity to heavy metal toxicants (Fichet and Miramand 1998) and physical and biological factors such as temperature and salinity (Ricketts et al. 1992) than adult marine invertebrates. Since, survival and fitness of these larvae is highly dependent on the environment, the health of the larvae commonly determines whether new individuals successfully recruit into a population (e.g. Miron et al. 2000; Emler and Sadro 2006). Therefore, any set of environmental factors that impact the larval period, can affect the number of recruits to the new population and subsequently entail community-wide effects (Jonsson et al. 2004; Jenkins 2005).

Barnacles are common subjects for investigations of the larval ecology of intertidal benthic invertebrates (e.g., Anil et al. 1995; Gosselin and Qian 1996; Qiu and Qian 1999; Holm et al. 2000; Thiyagarajan et al. 2003; Nasrolahi et al. 2006, 2007; Gaonkar and Anil 2010; Nasrolahi et al. 2012). Barnacle larvae begin life in the first of several nauplius stages, which feed in the plankton before metamorphosing to the, non-feeding cyprid stage specialized for settlement (attachment and metamorphosis; Thiyagarajan and Qian 2008). The energy reserves acquired during the nauplius stages are used to fuel the costly metamorphic processes (Thiyagarajan et al. 2005; Thiyagarajan, 2010).

In the course of global climate change both the average and the variability of key abiotic variables such as temperature and salinity are shifting (BACC 2008). As a result, organisms may be simultaneously exposed to new combinations of physiologically stressful conditions (Andrady et al., 2004). For the intertidal environments which are already physiologically stressful habitats (Gosselin and Chia 1995; Tomanek and Helmuth 2002), global climate change factors may act in synergy to introduce multiple stresses (Przeslawski et al. 2005). These stresses can have detrimental effects on the barnacle larval quantity (i.e., survival, Thiyagarajan et al. 2003; Desai and Anil 2004) and/or larval quality (i.e., food intake and energy reserves, Thiyagarajan et al. 2002; Thiyagarajan et al. 2005) and determine recruitment of barnacle via these crucial early stages of their life.

In a recent study, we examined the sensitivity of the two larval phases (nauplius and cypris) of the barnacle *Amphibalanus improvisus* from Kiel Fjord (Western Baltic) to a range of temperature and salinity combinations (Nasrolahi et al. 2012). However, given the global distribution of this species (Weidema 2000) and the likelihood of local adaptation (Sanford & Kelly 2011) – especially in different salinity regimes – there remains an urgent need for repeated assessment of global change impacts on multiple separate populations of the same species. Only then can local results be extrapolated to regional or global scales. To address

Chapter II

this need we assessed the sensitivity to temperature and salinity of barnacle larvae (*Amphibalanus improvisus*) from a population on the Swedish west coast, at Tjärnö Marine Biological Laboratory.

Materials and methods

Barnacle larvae

Nauplii and cyprids were obtained from the barnacle hatchery at Tjärnö Marine Biological Laboratory on the west coast of Sweden in 2009. Adult *Amphibalanus improvisus* that had been collected June 2009 (ambient temperature of 20-25°C and ambient salinity of 22-30 psu) were kept in flow-through seawater at 20°C and 30 psu for 2-3 months and fed daily on a diet of freshly hatched *Artemia* sp. Released nauplii were collected on sieves (60 µm) through an overflow (seawater salinity ~30 psu). The nauplii were at stage I and developed into stage II within few hours. Batches of stage II larvae from different subsets of multiple parents were used for each of two experiments.

Experiment I

The experimental design was as described in our earlier study (Nasrolahi et al. 2012). Briefly, we used three different salinities (5, 15, and 30 psu) and three different temperatures (12, 20, and 28°C) in a fully crossed experimental design. The different salinities used in the experiment were obtained by diluting filtered (0.2 µm) seawater (30 psu) with distilled water. Each treatment combination consisted of six replicates (one thermo bath for each treatment) with 20 larvae per replicate. Each replicate comprised one well of a six-well plate (CELL STAR # 657160) at the respective salinity (per well) and temperature (per plate in a given thermo bath). Cultures were kept in water baths equipped with circulation and an immersion heater adjusted to the target temperatures ($\pm 0.5^\circ\text{C}$). Six-well plates were randomly redistributed within the baths every day. For each treatment, 20 larvae were incubated in 10 ml of filtered (0.2 µm) seawater (FSW) and were fed daily with 50:50 mixture of the diatom species *Chaetoceros calcitrans* and *Skeletonema costatum* at 2×10^5 cells ml⁻¹ under continuous light (Thiyagarajan et al. 2003). Every 24 h, each replicate was observed using a dissecting microscope (Olympus SZX12) and the number of survivors, cyprids, and settled juveniles were recorded. The rapidity of these observations caused no discernible change in the water temperature in each treatment during observation. The water and food in the wells

Chapter II

were replaced every second day by fresh seawater with the respective temperature and salinity.

Response variables measured were (1) naupliar duration (days from hatching until 50% of the surviving nauplii had developed into cyprids), (2) survival (percent surviving nauplii or cyprids relative to the initial numbers), (3) cyprid metamorphosis success (percent of the initial number of nauplii that metamorphosed into cyprids by the end of the experiment), and (4) settlement (percent of settled juvenile barnacles relative to initial number of nauplii or to the number of surviving cyprids).

Experiment II

Newly released nauplius larvae were held at a density of 0.5 larvae.ml⁻¹ in filtered (0.2 µm) seawater in 20-l buckets at 26°C and 30 psu. Each bucket was provided with a 50:50 mixture of *Thalassiosira pseudonana* and *S. costatum* at 2×10^5 cells ml⁻¹. The cultures in the buckets were gently aerated, and clean seawater and fresh microalgae were provided every third day. When cyprids appeared in the cultures (usually after 6–7 days), the cultures were sieved (200 µm) and cyprids were removed. 10 cyprids were subsequently transferred into sterile six-well plates using the salinity and temperature combinations outlined for Experiment I. Plates were held under continuous light. The transition from initial to target salinity and temperature conditions was done gradually (2 psu/h, 1°C/1 h) to allow for acclimatization. Settlement and survival of cyprids were monitored daily over 26 days. Every second day, seawater in experimental containers was replaced. Eight replicates were used for each treatment.

Statistical analysis

Data were checked for normality and homogeneity of variances by visual inspection of Q-Q plots and box plots (Quinn & Keough, 2002). In case of deviations from the assumptions, we applied appropriate transformations prior to the analyses. All percentage data were arcsine-transformed before analysis. The effects of temperature and salinity on total larval duration, survival, cyprid metamorphosis success and settlement of cyprids at the last day of experiments were evaluated using a two-way ANOVA. Test on total larval duration was followed by a Tukey's test ($P \leq 0.05$).

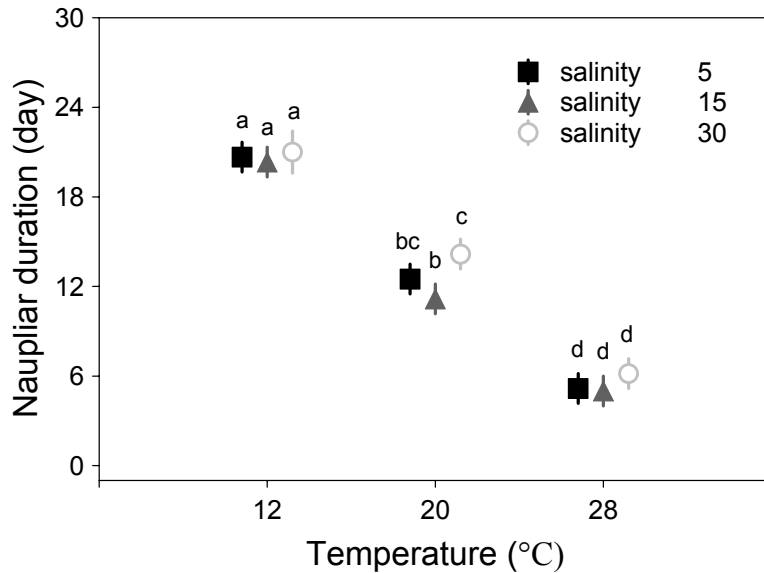
Results

Experiment I

Chapter II

Both temperature and salinity had significant direct effects (no interaction) on naupliar duration (nauplius II to cypris stage) of *Amphibalanus improvisus* (Table 1). The longest and shortest naupliar duration occurred at 12 and 28°C regardless of salinity levels (21 and 6 days, respectively, Fig. 1). Differences in salinity showed no significant effect on naupliar duration at all temperature levels except at 20°C in which nauplii required a slightly longer time to reach cypris stage at 30 psu than at 15 psu (Fig. 1).

Fig. 1. *Amphibalanus improvisus*. Effect of temperature and salinity on naupliar duration. Each bar represents the mean ($\pm 95\%$ CI) of six replicates. Each replicate consisted of 20 nauplius larvae.



Temperature and salinity also had direct (non-interactive) effects on nauplii to cyprids metamorphosis success (Table 2a). Metamorphosis success increased with increasing temperature and was highest at 15 psu and lowest at 30 psu at all temperature levels (Fig. 2). Despite the fact that the onset of metamorphosis was later at 12°C, metamorphosis was particularly low at this temperature where only under 15 psu it reached 20% by the end of the experiment (Fig. 2).

Table 1
Amphibalanus improvisus. Experiment I: two-ways ANOVA showing the combined effect of temperature and salinity on the total naupliar duration. Values of $p \leq 0.05$ in bold (N=6)

Source	SS	df	MS	F	P
Temperature (T)	1792.258	2	896.129	611.990	0.000
Salinity (S)	20.096	2	10.048	6.862	0.002
T × S	8.209	4	2.052	1.402	0.250
Error	61.500	42	1.464		

Chapter II

Table 2

Amphibalanus improvisus. Experiment I: two-ways ANOVA showing the combined effect of temperature and salinity on the nauplii to cyprid metamorphosis success (a), survival (b), and cyprid settlement from nauplii (c). Values of $p \leq 0.05$ in bold (N=6)

(a) Nauplii to cyprid metamorphosis success					
Source	SS	df	MS	F	P
Temperature (T)	0.876	2	0.438	6.993	0.002
Salinity (S)	0.519	2	0.259	4.141	0.022
T × S	0.025	4	0.006	0.102	0.981
Error	2.819	45	0.062		

(b) Survival					
Source	SS	df	MS	F	P
Temperature (T)	0.847	2	0.423	7.015	0.002
Salinity (S)	0.460	2	0.230	3.807	0.029
T × S	0.031	4	0.007	0.131	0.969
Error	2.719	45	0.060		

(c) Settlement from 20 nauplii					
Source	SS	df	MS	F	P
Temperature (T)	1.161	2	0.580	8.809	0.0005
Salinity (S)	0.370	2	0.185	2.813	0.0705
T × S	0.117	4	0.029	0.447	0.7738
Error	2.966	45	0.065		

Survival of nauplii and cyprids was affected directly by both salinity and temperature (no significant interaction; Table 2b). Survival of nauplii decreased with decreasing temperature under all salinity regimes with nauplii mortality ranging between 34% at 28°C/15 psu and 95% at 12°C/30 psu (Fig. 3). Survival at all temperature levels was always highest at 15 psu and lowest at 30 psu (Fig. 4a). Settlement success (percent of the initial 20 nauplii that settled) was significantly influenced only by temperature (Table 2c). Highest settlement occurred at the highest temperature and intermediate salinity, i.e., 28°C and 15 psu, while settlement was almost zero at 12°C regardless of the salinity level (Fig. 4b).

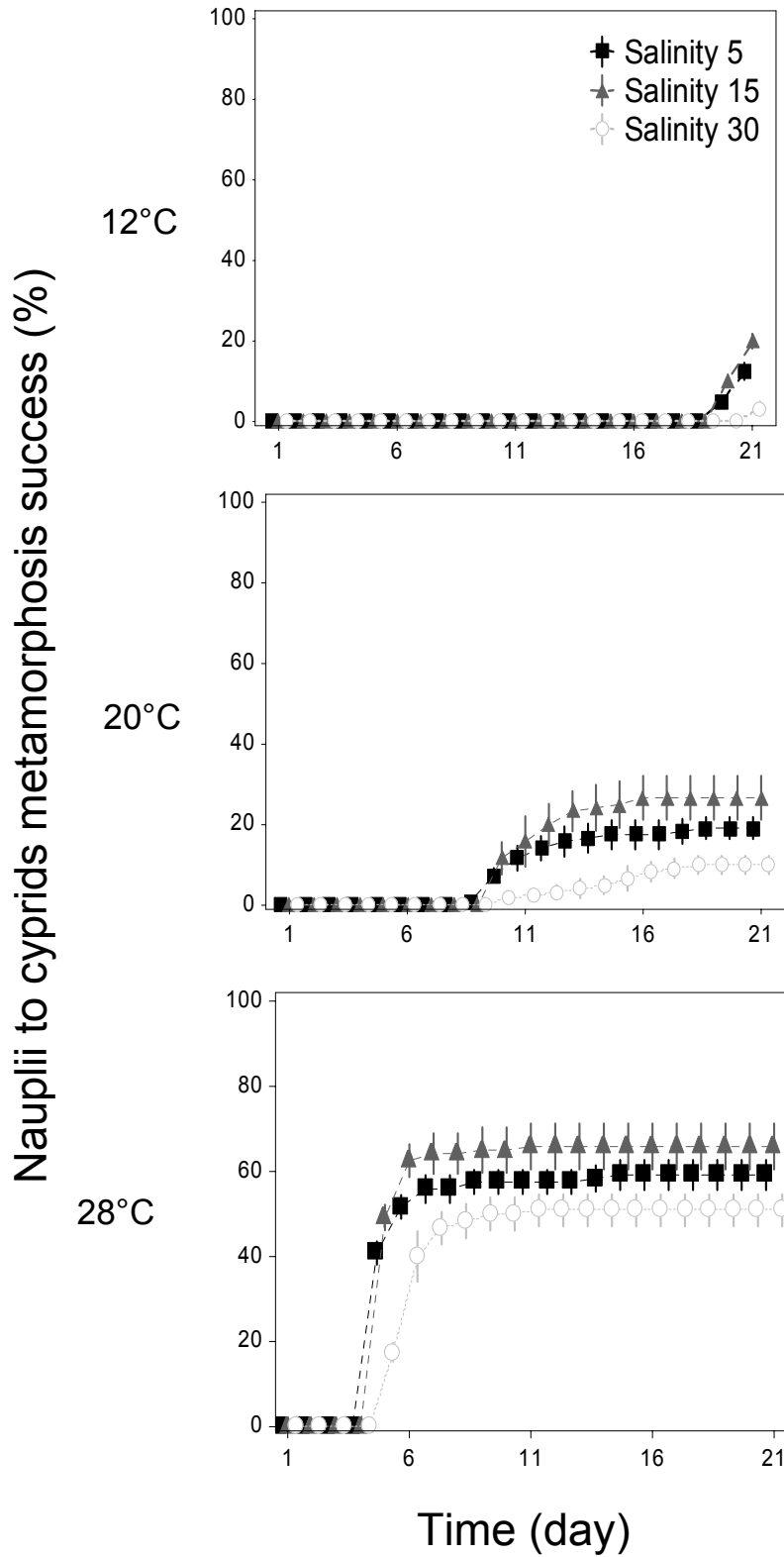


Fig. 2. *Amphibalanus improvisus*. Nauplii to cyprids metamorphosis success (%) under different temperature and salinity regimes over a 21-day assay period. Each bar represents the mean ($\pm 95\%CI$) of six replicates. Each replicate consisted of 20 nauplius larvae.

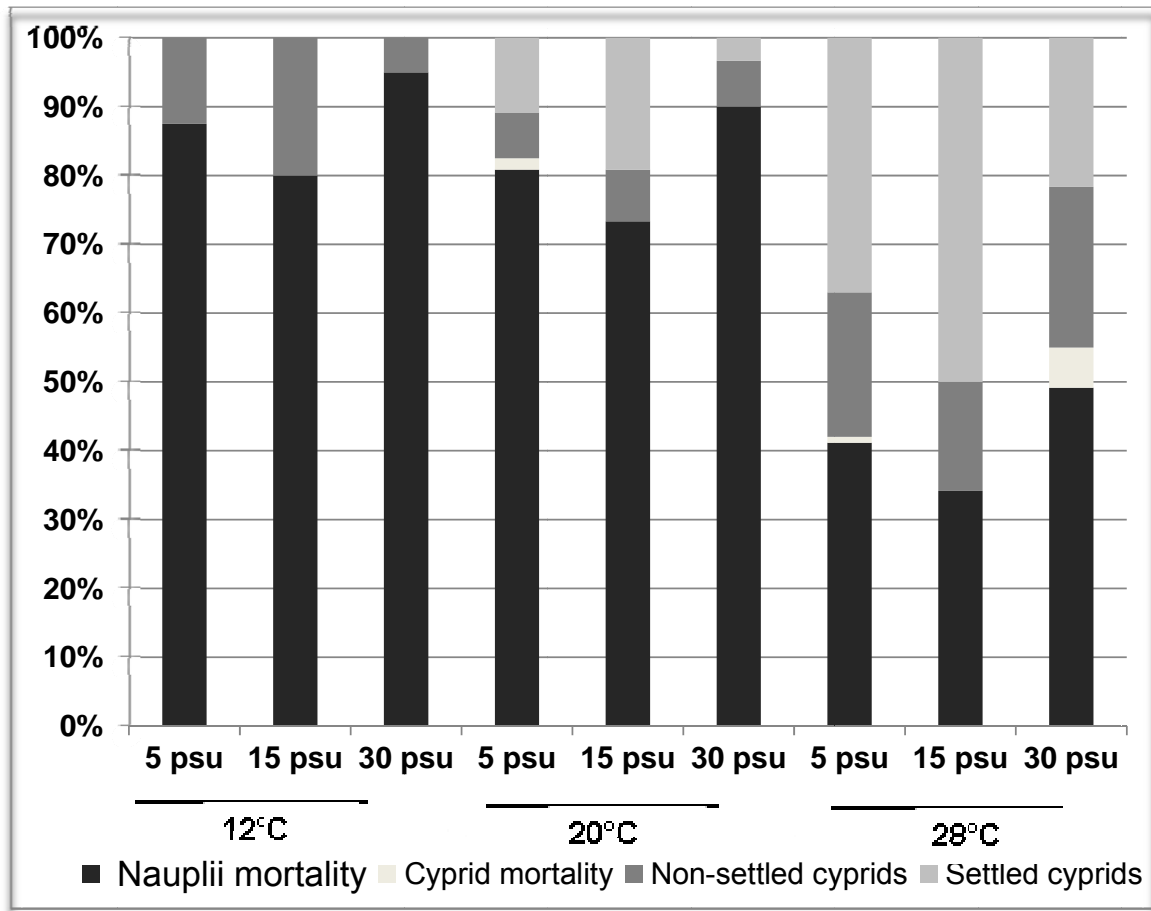


Fig. 3. *Amphibalanus improvisus*. Performance of barnacle larvae after 21 days under different combinations of temperature and salinity (experiment I).

Final cyprid settlement success (percent of settlers relative to the number of surviving cyprids) was also highest at 15 psu (about 75%) at both 20 and 28°C (Fig. 5).

Experiment II

Almost all cyprids (>95%) survived over the experiment period regardless of treatment (Fig. 6a). Settlement of cyprids was significantly affected only by salinity (Table 3). Surprisingly, settlement was lowest at 30 psu (the rearing salinity) regardless of temperature levels. Settlement was in general, low in this experiment (Fig. 6).

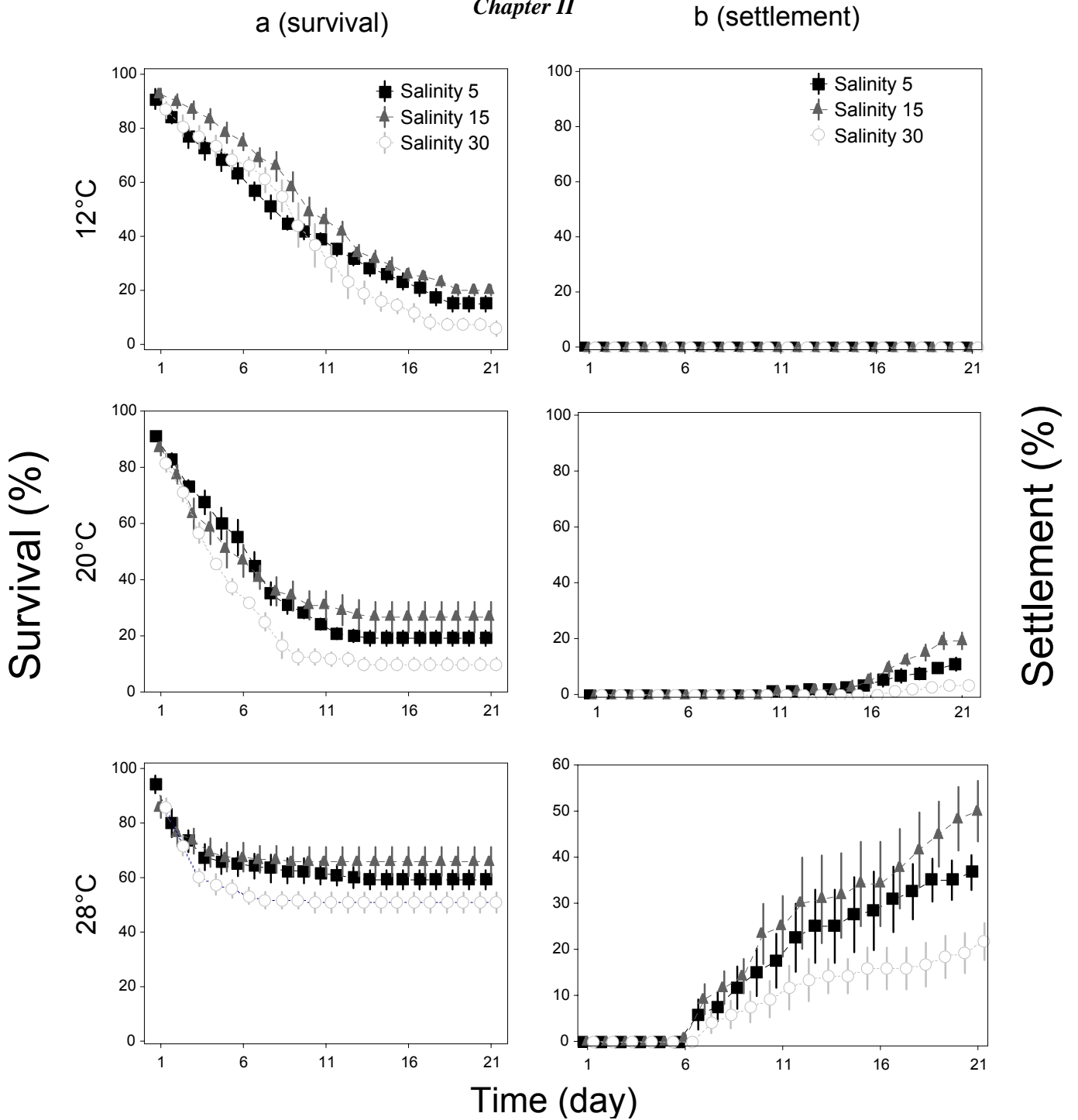


Fig. 4. *Amphibalanus improvisus*. Effect of temperature and salinity on cyprid survival (a) and settlement (b) (experiment I). Cyprid settlement was calculated as the percentage of all larvae in a replicate (20 nauplii) that settled. Survival has been accounted 24 h after the incubation of nauplii (day 1). Each bar represents the mean ($\pm 95\%$ CI) of six replicates. Each replicate consisted of 20 nauplius larvae.

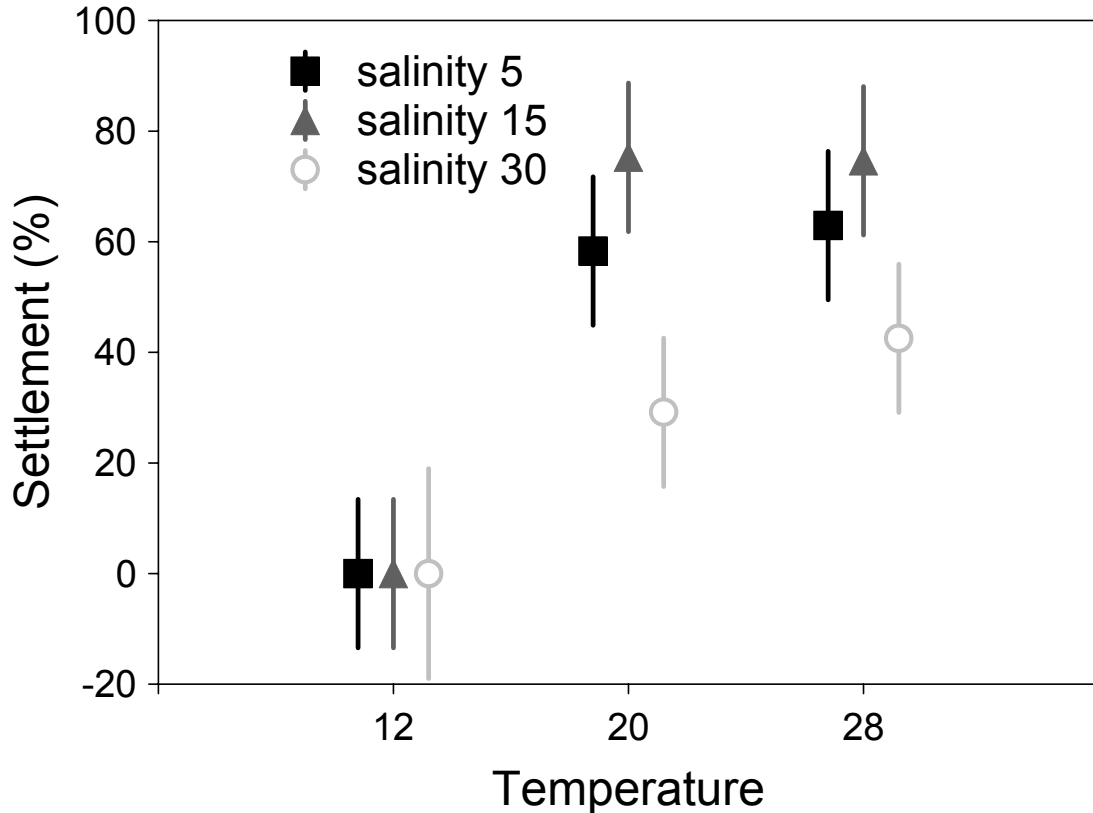


Fig. 5. *Amphibalanus improvisus*. Final settlement under different temperature and salinity (settled cyprids from surviving cyprids, experiment I). Each bar represents the mean ($\pm 95\%$ CI) of six replicates.

Discussion

Our results on larvae of *Amphibalanus improvisus* showed that temperature and salinity responses of this population were different to other populations of this species. Larval performance (nauplii to cyprid metamorphosis success, survival and settlement) was always lowest at low temperature and high salinity (12°C/30 psu). Nauplii performed following a consistent pattern of temperature and salinity (i.e., temperature: 28 > 20 > 12°C; salinity: 5 < 15 > 30 psu). Although the species is considered eurythermal and euryhaline, previous studies on this species from other populations showed that it performs best in intermediate temperature and salinity (i.e., about 20°C and 15 psu: Dineen and Hines 1992; Leppäkoski 1999; Nasrolahi et al. 2006, 2012). This implies that the Tjärnö population studied here lives under suboptimal conditions and is apparently maladapted to its habitat.

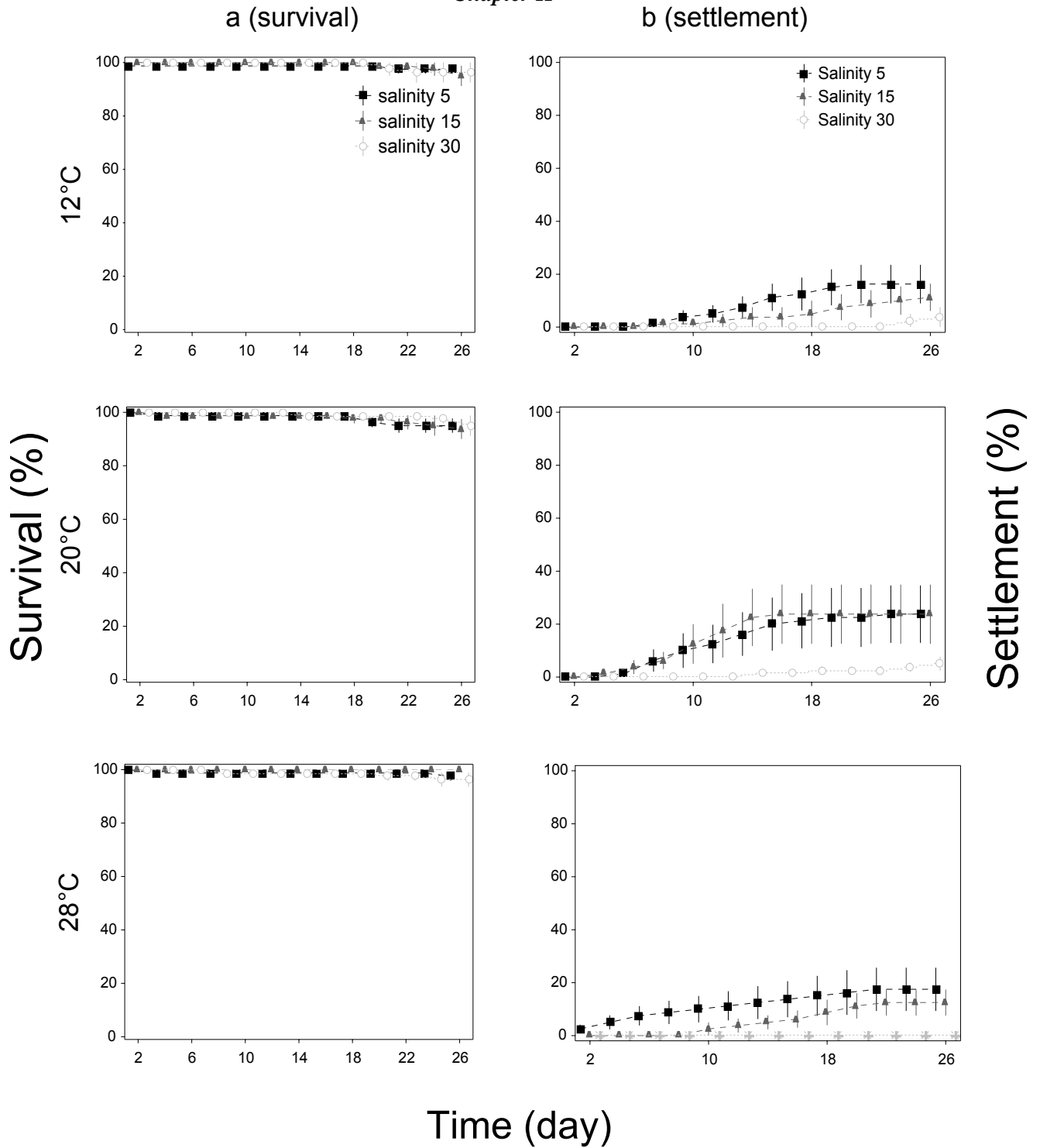


Fig. 6. *Amphibalanus improvisus*. Effect of temperature and salinity on cyprid survival (a) and settlement (b) over a 26-day period (experiment II). Day 0 shows settlement and survival after 24 h of incubation. Each bar represents the mean ($\pm 95\%$ CI) of eight replicates. Each replicate consisted of 10 cyprids.

Chapter II

Low temperature is known to reduce the feeding efficiency (Scheltema and Williams 1982) and the filtration rate via increasing the water viscosity (Riisgård and Larsen 2007; Melzner et al., 2011) in barnacles and other marine suspension feeders. In the present study, low temperature also seemed to reduce food consumption (non quantified observation on the clearance rates) resulting in less energy intake. Consequently, a low energy budget may have contributed to the low rate of transformation from nauplius to cyprid under cold conditions. Similarly, Anil et al. (1995) reported that during the winter *A. amphitrite* larvae were present in the water but failed to develop successfully because of lower water temperature and food supply. High salinity (30 psu) resulted in lowest rate of metamorphosis at all temperature levels whereas intermediate salinity (15 psu) caused highest successful metamorphosis. This was unexpected because both the in situ habitat conditions and the laboratory culture conditions of the parent population were at high salinity (about 30 psu). *A. improvisus* larvae from a population further into the Baltic Sea (Kiel Bight) show a similar pattern i.e., larvae metamorphosed more efficiently at a lower salinity (5 psu) than at their habitat salinity (15 psu) (Nasrolahi et al. 2012).

While 95% of larvae died at 12°C/30 psu, mortality was only 34% at 28°C/15 psu (Fig. 3). In contrast, nauplii mortality of the Kiel population was salinity dependent and ranged between 70% at 28°C/5 psu and almost 100% at 12°C/30 psu (Nasrolahi et al. 2012). After the nauplii have metamorphosed to cyprids survival was very high, i.e., almost all mortality occurred during the naupliar stage (Fig 4). This early stage is very important in determining population dynamics of adult organisms (Pechenik, 2006). Essentially, the health of early life phases commonly determines whether new individuals successfully recruit into a population (e.g. Miron et al., 2000; Emllet and Sadro, 2006) and the most common factor to determine recruitment success is nauplius mortality (Desai and Anil 2004). During their long period of drifting (from few hours to months), the larvae are frequently exposed to environmental fluctuation in the water column, such as temperature, salinity, and pH drifts (McEdward, 1995). These pose significant physiological stresses to the larvae and it is estimated that mortality during the planktonic dispersal phase of many marine species exceeds 90% in the natural ecosystem setting (Gosselin and Qian, 1996).

At 12°C, even the 5-20% survivors (that reached later to cyprids) did not settle until day 21. This has been previously observed for *A. improvisus* cyprids in Kiel population as well (Nasrolahi et al. 2012). The attachment success of cyprids largely depends on the amount of stored energy reserves (Lucas et al. 1979; West and Costlow, 1988; Thiagarajan

Chapter II

et al., 2002, 2005). Cyprids that were grown at low temperature probably fed less during the naupliar period and thus, presumably, stored too little energy for settlement. In the marine realm, during the winter predation, misrouting and low quality of released nauplii can add to the negative effects of low temperature and cause settlement failure of barnacle larvae in this season (Crisp 1984; Anil et al. 1995). Cyprid settlement at higher temperatures followed the same pattern of salinity-dependency (most at 15 and least at 30 psu). This response pattern was still present when we calculated the settlement as the percent of settled barnacles relative to the number of surviving cyprids (Fig. 5). Since the ambient salinity for the parents of these larvae is between 22 and 30 psu, they appear to be maladapted to this local condition.

Cyprid mortality in both experiments was negligible implying that nauplii are more susceptible than cyprids. Unlike the cyprid survival, cyprid settlement in experiment II was impacted by salinity i.e., the lowest settlement occurred at the local habitat salinity for this species in its habitat (30 psu).

Naupliar duration was shorter at higher temperatures. An increase of temperature by 8°C (from 12 to 20°C) at 5 and 15 psu shortened the larval duration by about 42%. At 30 psu this decrease in larval duration was about 34% only. Naupliar duration further reduced by approximately 50% by a warming from 20 to 28°C at all salinities. Reduced larval duration by increased temperature has been observed for this species in our previous study (Nasrolahi et al. 2012) and for other species (e.g., *Amphibalanus amphitrite*: Anil et al. 1995, 2001; Qiu and Qian 1999, *B. trigonus*: Thiyagarajan et al. 2003, *A. eburneus*: Scheltema and Williams 1982, *Elminius modestus*: Harms 1984, 1986, and *Semibalanus balanoides*: Harms 1984). This may be a direct consequence of the increase in metabolic rate (to a certain amount of increased temperature, Neuheimer et al. 2011) and better energy assimilation at higher temperatures (Anil et al. 2001). Larval duration was independent of the salinity level (except at 20°C/30 psu) in this and in the Kiel population (this study, Nasrolahi et al. 2012) which contrasts with a study on the same species in the Southern Caspian Sea population showing a slight deceleration of larval development with increasing salinity (Nasrolahi et al. 2006). *A. improvisus* is a wide-spread species in warm temperate seas, and can be often found in areas of low (as the Baltic or Caspian Sea) or widely fluctuating salinities (Furman and Yule 1990). Low sensitivity of this species in larval duration to salinity may reflect its euryhalinity and the ability of partial osmoregulation in different salinities (Fyhn 1976; Gohad et al. 2009).

For the water body of the Baltic Sea, a temperature increase of 3°C to 5°C has been projected until 2100 (BACC 2008). Simultaneously, a decrease in salinity due to higher

Chapter II

precipitation is predicted (BACC 2008). For the Swedish west coast during the peak settlement season (July–September, Jonsson et al. 2004) this would correspond to a warming from 20–25°C to 23–29°C and a desalination from 22–30 psu to a still unknown value between 13 and 18 psu (“up to -45%”, BACC 2008; Meier et al. 2011). Both warming and desalination as predicted for the Baltic Sea would favor *A. improvisus* by decreasing mortality and increasing developmental rate of the nauplii. Whether *A. improvisus* can respond to this improvement by larger populations will depend how its competitors, consumers or parasites are impacted by these aspects of climate change. The interactions of desalination and warming with other environmental variables expected to shift (pCO₂, pH, UV, nutrients, etc) should, however, be considered before any prediction about climate change effects on species performance can be made.

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Temperature and salinity interactively impact early juvenile development – a bottleneck in barnacle ontogeny

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Abstract

When juvenile mortality or juvenile growth is impacted by temperature and salinity, these factors have a substantial effect on recruitment success and population dynamics in benthic ecosystems. Using freshly settled cyprids of *Amphibalanus improvisus*, we investigated the combined effects of temperature (12, 20 and 28°C) and salinity (5, 15 and 30 psu) on early juvenile stage performance. Mortality as well as growth (basal diameter, dry weight and ash-free dry weight) were monitored for a period of 40 days. Mortality was high (42 - 63%) during the first week following attachment, regardless of the temperature and salinity treatments. Subsequently, mortality and growth were interactively influenced by temperature and salinity. Highest mortality and lowest growth of juveniles occurred at lowest temperature (12°C) and salinity (5 psu). Apparently, low temperature (12°C) narrowed the barnacles' salinity tolerance. Juvenile barnacles constructed more shell material compared to body mass at low temperature and low salinity while a reverse situation was observed at high temperature and high salinity. Our results demonstrate that environmental changes can alter patterns of survivorship and growth. Warming and desalination as predicted for the Baltic Sea in the course of climate change may, however, act antagonistically and compensate each other's isolated effect on barnacles.

Key words: Juvenile tolerance, climate change, barnacles, *Amphibalanus improvisus*, temperature stress, salinity stress

Introduction

Barnacle ontogeny shows a complex life cycle in which the nauplius larva molts six times before transforming into the cypris stage. The competent cypris explores the potential settlement substratum and eventually chooses a suitable place to attach and metamorphose into the juvenile stage (Thiyagarajan 2010). Substantial changes in morphology, physiology and habitat

Chapter III

characteristics make the attachment and metamorphosis of a cypris to the post-larval stage the most critical steps in the life history of a barnacle (Thiyagarajan et al. 2002). Furthermore, the transition from pelagic to the benthic life style exposes the post-larva to an entirely different set of environmental conditions within a very short period of time (Gosselin and Qian 1996). Heavy mortality during the onset of juvenile life of benthic marine invertebrates has been reported previously varying between 10 and 80% for different barnacle species (reviewed by Gosselin and Qian 1997; Jarrett 2003; Thiyagarajan et al. 2005; Shanks 2009; Gosselin and Jones 2010; Lathlean et al. 2010). This observed high mortality is due to various combinations of ecological (e.g., predation and competition) and, environmental stresses (e.g., desiccation or variations in salinity and temperature, biological and physical disturbances, mechanical damage or deposition of sediments (Rumrill 1990; Gosselin and Qian 1996; Gosselin and Qian 1997; Hunt and Scheibling 1997; Qiu and Qian 1998; Moran 1999; Osman and Whitlatch 2004; Chan et al. 2006). Stress driven mortality at the juvenile stage ultimately affects the distribution, abundance and population dynamics of barnacles (reviewed by Barnes 1999; Chan et al. 2006).

Growth rate is a critical biological trait determining population demography, evolution, and community interactions (Urban 2007). Rapid juvenile growth after successful settlement and metamorphosis is a particularly important determinant of the recruitment success (Thiyagarajan et al. 2007). Growth in many crustaceans such as barnacles, however largely depends on physiological conditions, energy availability, and environmental characteristics (Hartnoll 2001) such as temperature (e.g., Sanford and Menge 2001; Skinner et al., 2007), salinity (Simpson and Hurlbert 1998) or food supply (e.g., Thiyagarajan et al. 2003; Thiyagarajan et al. 2005). Any factor reducing juvenile survival or rates of growth will likely affect overall recruitment success in barnacles (e.g., Larsson and Jonsson 2006).

Larval settlement is especially critical for sessile organisms living in fluctuating environments (Dineen and Hines 1994). In the western Baltic Sea, species typically experience a broad range of environmental conditions such as temperature, salinity and pH. *Amphibalanus improvisus* is a common and locally dominant fouling organism in the Baltic (BACC 2008). In Kiel Fjord (the collection site), salinity fluctuates between 5 and 23 psu over the year (Madhupratap 1996; Nasrolahi et al. 2012) and may reach to salinities of few degrees higher in

Chapter III

small tide pools. Sea surface temperature (SST) ranges from 0°C in winter to 23°C during late summer (Baumann 2007). Temperature within small tide pools in the Schlei Fjord (Kiel Bight, Western Baltic) can even reach temperatures of 27°C during summer (K. Maczassek unpubl. data). Peak recruitment for *A. improvisus* in Kiel Fjord usually occurs in July and August at typical temperatures of about 20°C and salinities around 15 psu (Thomsen et al. 2010; Nasrolahi et al. 2012).

In the context of climate change, of particular concern are the impact of predicted future relative to present stress regimes on early life stages of intertidal invertebrates, as these life stages may be more vulnerable to physiological extremes than adults (Gosselin and Qian 1997; Przeslawski et al. 2005; Gosselin and Jones 2010). Yet, these life history phases are notoriously understudied especially with regard to the impact of combined stressors. Thus, in this study we examined the combined effects of temperature and salinity on juveniles of the barnacle *Amphibalanus improvisus*. The main objectives of this study were to determine (1) how various combinations of temperature and salinity interactively impact post-larval mortality, (2) to elucidate how juvenile growth is impacted by these two factors and finally, to see (3) whether temperature and salinity can interactively change the body mass/shell material ratio.

Materials and methods

144 transparent PVC panels (9 × 9 cm) were exposed to settlement in a water depth of 1 meter in the Kiel Fjord (54°19.5'N, 10°09.0'E) during peak settlement of *Amphibalanus improvisus* in July 2010. Panels were orientated horizontally with the upwards facing side covered with grey PVC panels to prevent settlement on one side of the transparent panel. One set of settlement panels were collected after 24 h, another one after 2 weeks of in situ exposure and gently transferred to the laboratory. Since panels were deployed during peak settlement, all panels had more than 40 settled barnacles on them. Surplus barnacles were removed leaving 40 individuals per panel. The retained barnacles were evenly spaced to avoid crowding. Culling was done under a loop microscope while panels were kept in water and the largest individuals remained. As culling was fast, panels did not experience noticeable change in water temperature during observation under the binocular. The 24 h old freshly attached cyprids (hereafter refer to as post-

Chapter III

larvae) of *Amphibalanus improvisus* were used to investigate their performance under different temperature and salinity regimes during early attachment and metamorphosis („post-larva” experiment). Post-settlement mortality had been shown to be highest during the first few days after metamorphosis due to fragile shells and complex processes involved in the process of metamorphosis (Thiyagarajan et al. 2005). Thus, to include this period, we measured the mortality rate after one week of the onset of experiment. To exclude this first part of mortality, we used 2-week old barnacles (hereafter refer to as early juvenile) to investigate their mortality under different temperature and salinity regimes. For this, after two weeks the biggest individuals were remained on the panels assuming that they settled at the first day after deployment. These two experiments allowed comparing stress sensitivity of post-larvae and early juveniles of this regionally important barnacle species.

We applied three salinity (5, 15 and 30 psu) and three temperatures (12, 20 and 28°C) treatments in a fully crossed experimental design. All experimental scenarios can be naturally encountered by this species of barnacles within the Western Baltic (Wahl et al. 2011; Nasrolahi et al. 2012). To avoid unnatural alkalinities due to the use of artificial seawater, we obtained the different salinity values by diluting filtered (0.2 µm) North Sea water (30 psu) with double distilled water. Each treatment combination consisted of aerated 1-liter plastic beaker containing 800 ml of filtered seawater and a panel with 40 post-larvae or early juveniles. We used eight replicate per treatment combination. Beakers were placed in thermo bathes (Thermostat HAAKE DC10, Karlsruhe, Germany) adjusted to the target temperatures ($\pm 0.1^\circ\text{C}$). The transition from starting to target temperature/salinity was done gradually (1 psu/h, $1^\circ\text{C}/2\text{ h}$) to allow for acclimatization. Marine diatoms *Chaetoceros calcitrans* and *Skeletonema costatum* were grown in f/2 medium (15°C and 15 psu). A 50:50 mixture of both diatom species was fed daily to the post-larvae and early juveniles at a concentration of 2×10^5 cells ml^{-1} . The experiments were run under continuous light ($16\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$) to minimize mortality and precipitation of algal cells (Thiyagarajan et al. 2003). The water and food in the beaker were replaced every other day by fresh filtered seawater and food with the respective temperature and salinity. In order to keep the temperature constant for each treatment while changing the water in the beaker, preconditioned water was used from 2 l water bottles, which were kept in thermo bathes with the respective temperature.

Chapter III

The post-larvae or early juveniles were kept in the experimental units in the laboratory for 40 days and 28 days, respectively. Post-larval mortality was assessed after 7 and 40 days in the post-larvae experiment and after 21 and 28 days in the early juvenile experiment using a binocular microscope (WILD M3C Heerbrugg, Switzerland). For the post-larvae experiment, pictures of the basal plates of the remained developing barnacles were taken through the transparent panels in the end of experiment using a digital camera (CANON EOS 20D, EFS 18-55 mm). The basal diameter (rostro-carinal) was measured using NIH ImageJ (version 1.43u). Besides post-larval basal diameter, dry weight (DW) and ash-free dry weight (AFDW) were used to assess juvenile growth. At day 40, the remaining juveniles (post-larvae experiment) were carefully removed from their panels and rinsed briefly with distilled water to remove adhering salts. Juveniles were grouped in pre-weighed foil pans (juveniles on each panel were pooled into one sample), dried at 80°C for 24 h and weighed to the nearest 0.001 mg (DW). The pans were then placed in a muffle furnace at 500°C for 24 h and reweighed (ash weight, AW). The AFDW is the total weight lost during combustion, i.e. subtracting the AW from the DW. DW and AFDW were then divided by the number of juveniles in the pan. The organic to inorganic material ratio was calculated by dividing the AFDW by the AW. In order to test whether the basal diameter was a reliable predictor of body tissue growth, the relationship between biomass (dry weight and ash-free dry weight) and basal diameter of juveniles at the end of experiment was determined.

Table 1. Mortality of *Amphibalanus improvisus* - repeated measures ANOVA showing the effect of temperature, salinity, duration of the assay and their interactions on post-larval mortality

Source of variation	df	MS	F
Temperature (A)	2	951.1	15.14 **
Salinity (B)	2	2881	45.87 **
Time (C)	1	1125.8	276.32 **
A × B	4	399.8	6.36 **
A × C	2	57.7	14.16 **
B × C	2	7.1	1.73 ns
A × B × C	4	15.9	3.89 *
Error	63	4.1	

The assumption of sphericity was tested and in the case that sphericity is violated, we used the Greenhouse-Geisser correction for the p level.

*p < 0.05.

**p < 0.001.

ns: not significant

Chapter III

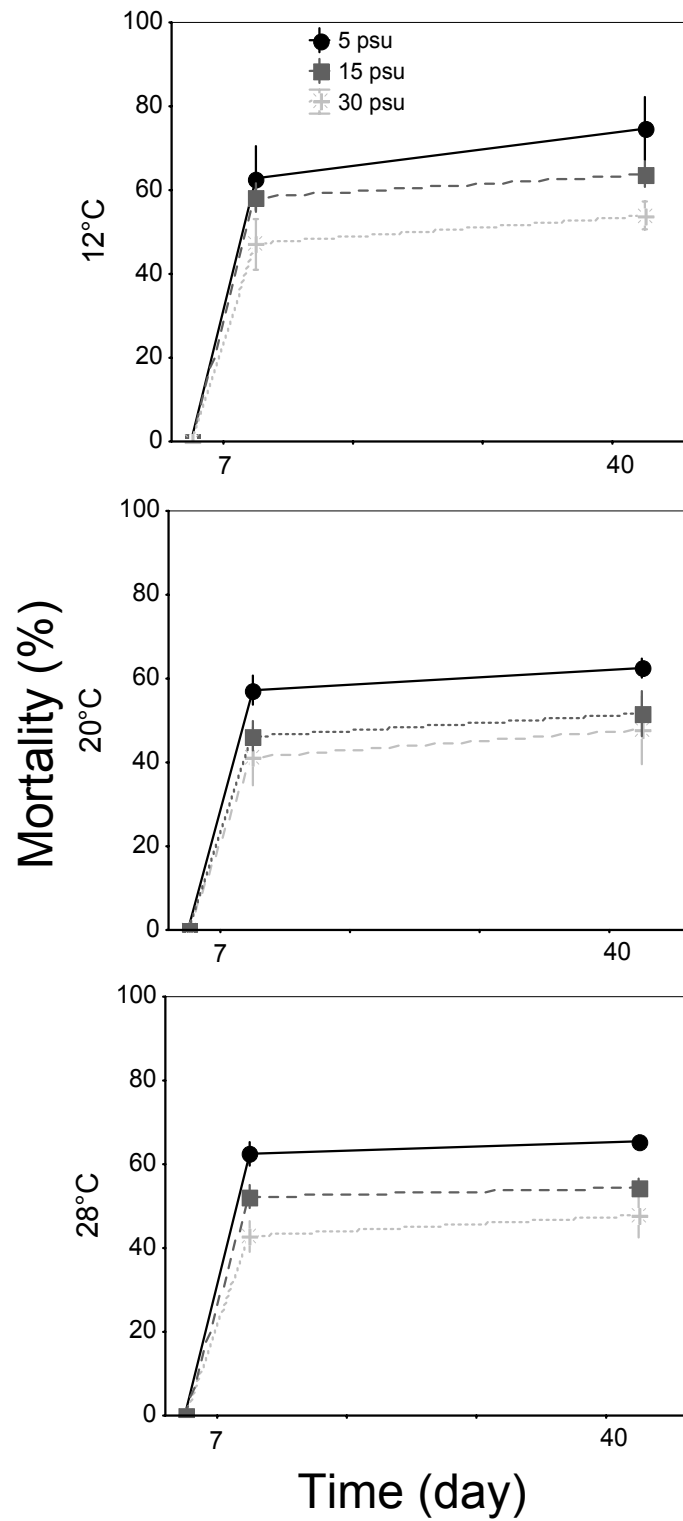


Fig. 1. *Amphibalanus improvisus* – effect of temperature and salinity on the mortality of 24 h old post-larvae. Each bar represents the mean (\pm CI) of eight replicates.

Chapter III

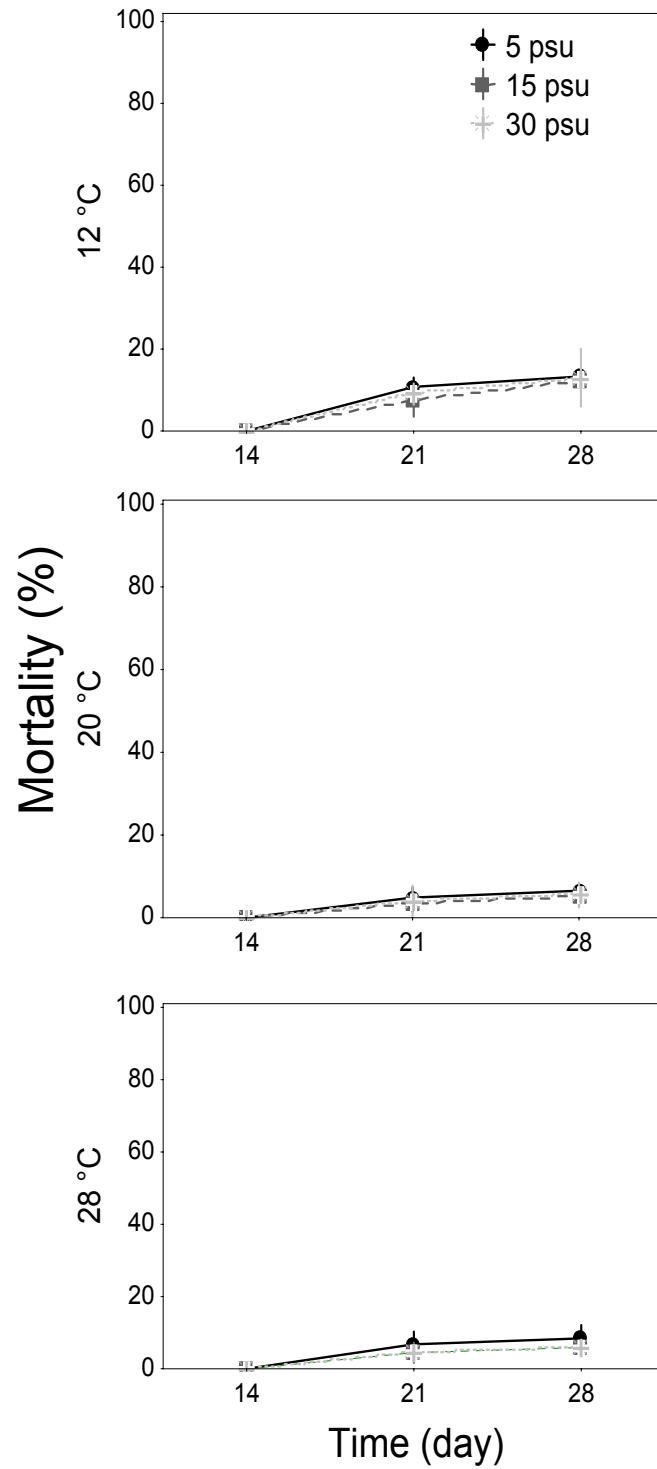


Fig. 2. *Amphibalanus improvisus* - effect of temperature and salinity on the mortality of 2-week old early juveniles. Each bar represents the mean (\pm CI) of eight replicates.

Statistical analysis

The assumptions of normality and homogeneity of variances were assessed with Shapiro-Wilk's W-test and Levene's test, respectively. In case of deviations from the assumptions we applied appropriate transformations prior to the analyses. A repeated measures ANOVA was used to examine the effect of temperature and salinity on post-larval mortality over the experiment time. The assumption of sphericity was also tested and in the case that sphericity is violated, we used the Greenhouse-Geisser correction for the p level. The effects of temperature and salinity on basal diameter, DW and AFDW were evaluated using parametric two-way ANOVA. A two-way ANOVA was also used on arcsine transformed data to test the effect of temperature and salinity on organic to inorganic material ratio (OIR) of the juveniles.

Results:

Mortality of *Amphibalanus improvisus* post-larvae during the first week following attachment was high in all temperature and salinity treatments and ranged from 42 to 63%. The observed mortality following this initial period was substantially lower in all treatment combinations leading to a maximum mortality of 74% at the end of experiment, i.e. day 40 (Fig. 1).

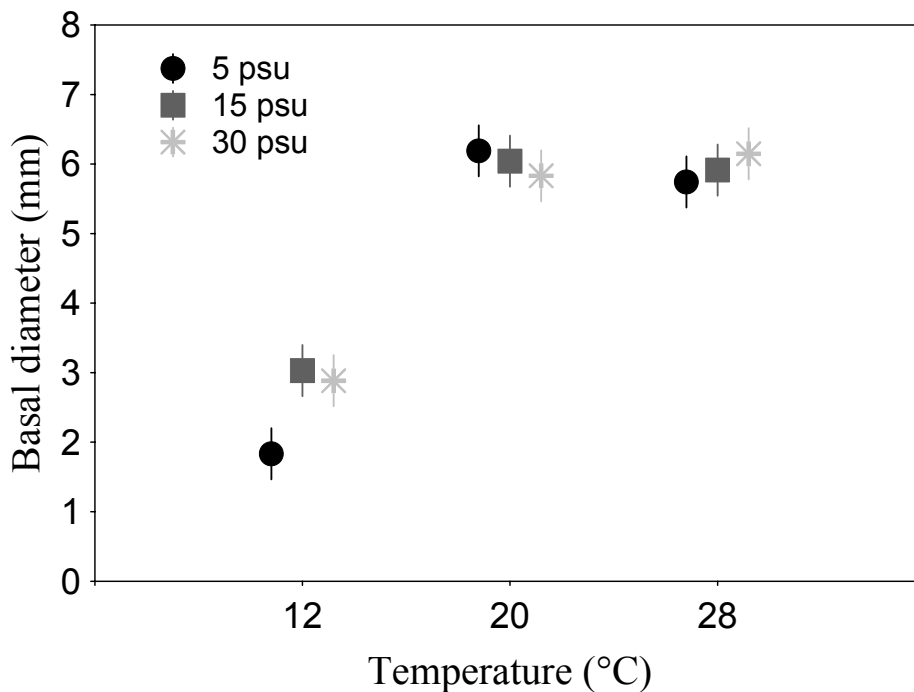


Fig. 3. *Amphibalanus improvisus* - effect of temperature and salinity on the basal diameter of post-larvae at the end of experiment (day 40). Each bar represents the mean (\pm CI) of eight replicates.

Chapter III

There was a significant interaction between temperature, salinity and the duration of assay on mortality (Table 1). Mortality was highest at 5 psu at all applied temperatures but most conspicuously so at 12°C. The lowest mortality occurred at medium temperature and salinity (20°C and 15 psu) (Fig. 1). Two-week old juveniles showed a reduced mortality compared to post-larvae (Fig. 2). At 12°C, about 15% of the early juveniles died between day 14 and day 28 regardless of salinity. Juvenile mortality was even lower (< 10%) at 20 and 28°C (Fig. 2).

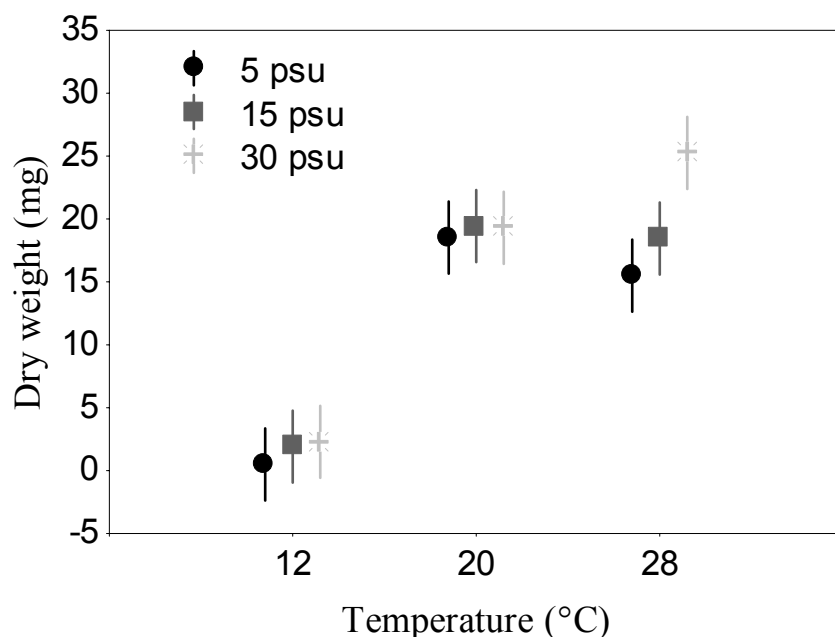


Fig. 4. *Amphibalanus improvisus* - effect of temperature and salinity on dry weight (DW) of juveniles at the end of experiment (day 40). Each bar represents the mean (\pm CI) of eight replicates.

Growth as assessed by basal diameter increment of juveniles at day 40 was significantly affected by interactive effects of temperature and salinity (Table 2). Generally, juveniles grew more in warmer temperature. At the lowest temperature (12°C) growth was lower at low (5 psu) than at medium (15) or high (30) salinity. At medium and high temperature growth rates were highest and unaffected by salinity (Fig. 3). Mean basal diameter was about 50% smaller at 12°C compared to 20 and 28°C (Fig. 3).

There was a significant interaction between temperature and salinity on DW (Table 2). DW of the juveniles followed a similar pattern as seen for the basal diameter increment. However, DW increased with temperature and was positively affected by highest salinity only under the warmest conditions (Fig. 4).

Chapter III

Table 2. Growth of *Amphibalanus improvisus* - two-way ANOVA of the effects of temperature (T) and salinity (S) on basal diameter (BD), dry weight (DW), ash-free dry weight (AFDW) and the organic to inorganic material ratio (OIR) of barnacle post-larvae at day 40.

	Source	SS	df	MS	F
BD	T	184.562	2	92.281	340.605**
	S	2.397	2	1.198	4.423*
	T × S	5.600	4	1.400	5.167*
	Error	17.069	63	0.271	
DW	T	5098.82	2	2549.41	154.894**
	S	204.50	2	102.25	6.212*
	T × S	214.51	4	53.63	3.258*
	Error	1036.92	63	16.46	
AFDW	T	136.574	2	68.287	88.473**
	S	1.171	2	0.585	0.758ns
	T × S	4.396	4	1.099	1.423ns
	Error	48.626	63	0.771	
OIR	T	185.359	2	92.679	89.045**
	S	13.121	2	6.560	6.303*
	T × S	3.888	4	0.972	0.934ns
	Error	65.571	63	1.040	

*p < 0.05.

**p < 0.001.

ns: not significant

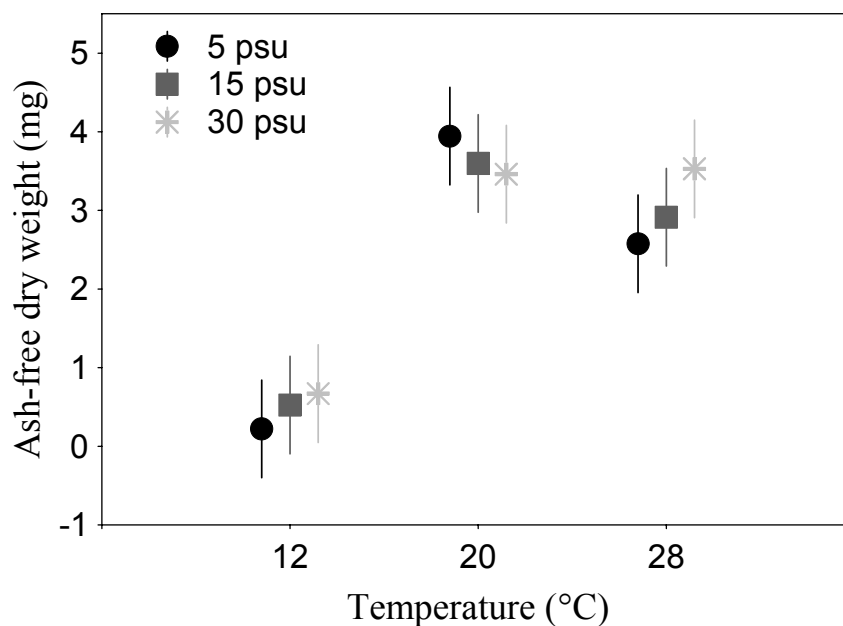


Fig. 5. *Amphibalanus improvisus* - effect of temperature and salinity on ash-free dry weight (AFDW) of juveniles at the end of experiment (day 40). Each bar represents the mean (\pm CI) of eight replicates.

The AFDW was significantly influenced by temperature but not by salinity (Fig. 5, Table 2).

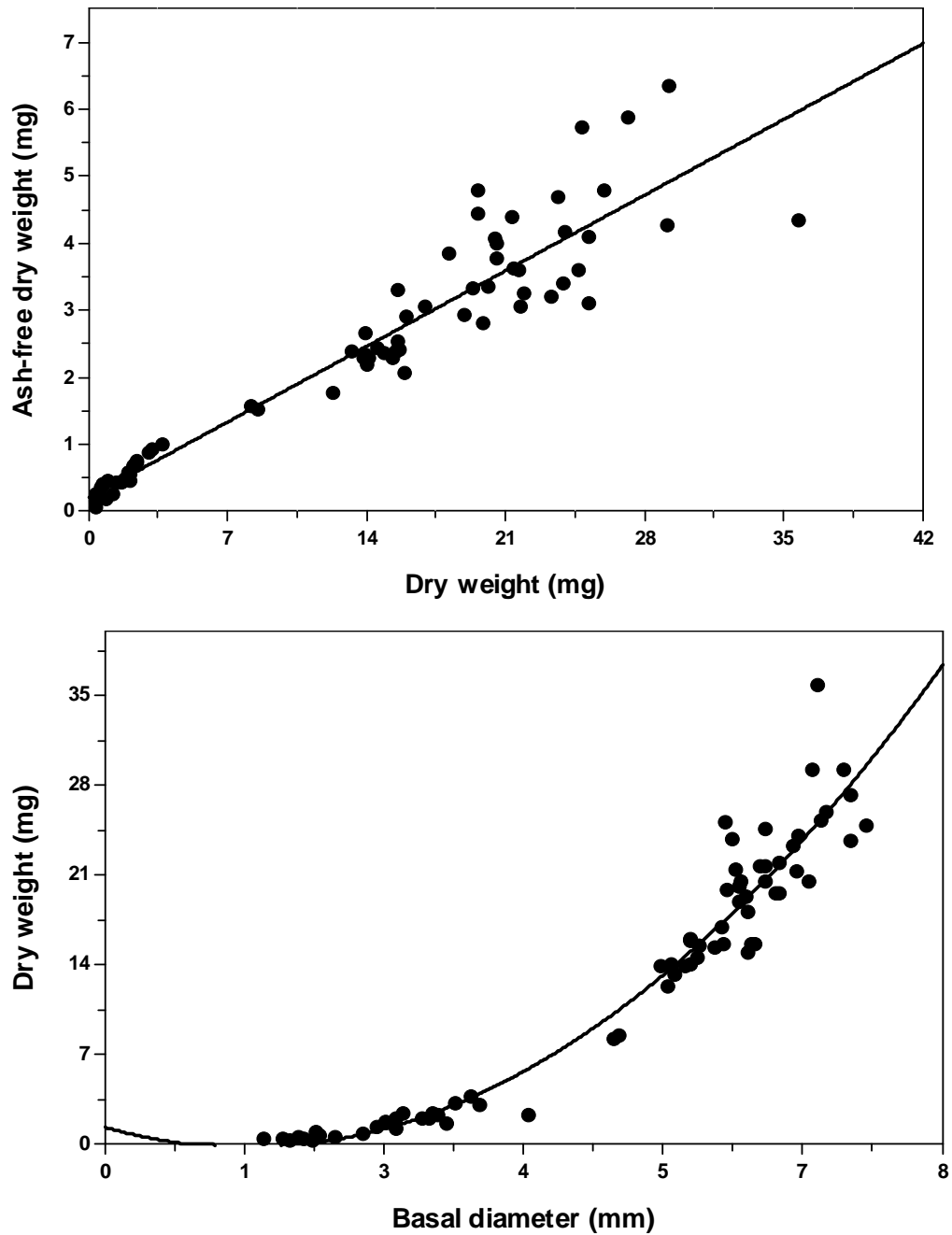


Fig. 6. *Amphibalanus improvisus* - relationship between biomass (mg) and basal diameter (mm) in juvenile barnacles: **A** dry weight as a function of basal diameter and **B** ash-free dry weight as a function of dry weight (over all applied treatment combinations).

Chapter III

The relationship between dry and ash-free dry weight of juveniles was curve-linear (Fig. 6A), whereas a linear relationship between basal diameter and biomass (DW) was obtained (Fig. 6B).

Temperature as well as salinity significantly affected the condition index (organic to inorganic material ratio), without an interaction between the two factors (Table 3). The condition index of juveniles decreased with increasing temperature (Fig. 7). The highest condition index was observed at 12°C and 5 psu, while the lowest ratio was seen at 28°C and 30 psu (Fig. 7). There was a steady tendency towards increased shell materials with enhancing the salinity from 5 to 30 psu (Fig. 7).

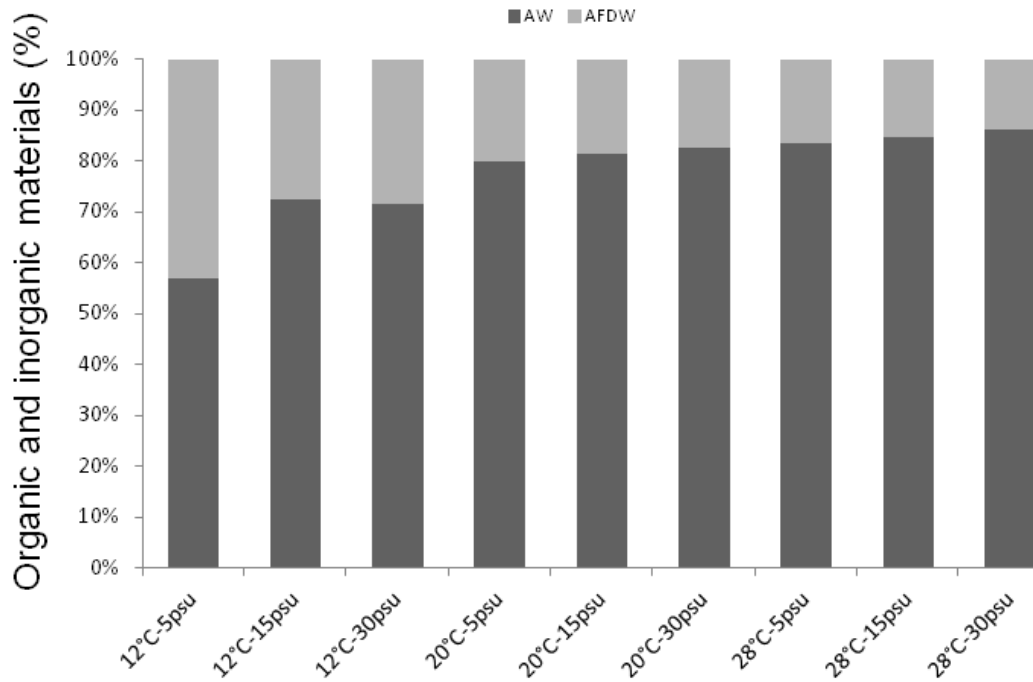


Fig. 7. *Amphibalanus improvisus* - effect of temperature and salinity on the proportion of organic (AFDW, light grey) and inorganic (AW, dark grey) of barnacle post-larvae at the end of experiment (day 40).

Discussion

Post-larval mortality showed to be high during the first few days after settlement, i.e. when the post-larvae were first exposed to the treatments. Approximately 90% of the total mortality occurred during the first week after metamorphosis, i.e. most of the remaining individuals from

Chapter III

the first week survived throughout the following 33 d of the experiment. Apparently, the more sensitive genotypes had succumbed to the treatment(s) while the surviving genotypes were either pre-adapted to the conditions or able to acclimatize in time. The findings of very high post-larvae mortality in our study are consistent with previous reports on barnacles (e.g., Gosselin and Qian 1996; Jarrett 2000; Pineda et al. 2006; Gosselin and Jones 2010). Beside the natural high mortality at early ontogeny, low temperature and low salinity interactively increased the mortality of post-larvae in this study. Mortality was highest at 5 psu being especially so when combined to low temperature (12°C). Barnacle post-larvae utilize energy reserves from the nauplii phase to metamorphose and grow until their digestive system is completely developed after some days (Anderson 1994). A high proportion (38-58%) of these energy reserves is used for metamorphosis (Thiyagarajan et al. 2003). Exposing the post-larvae to stresses such as low temperature or sub-optimal salinity will require energy expenditure for stress compensation (Anger 2003), which is no longer available for metamorphosis. Stress driven post-larval mortality can therefore, constitute a bottleneck for recruitment (Gosselin and Qian 1996) which can potentially have a substantial effect on population dynamics (Jenkins et al. 2008), population size and distribution as well as community structure (Gosselin and Qian 1997; Moran 1999; Osman and Whitlatch 2004). Pineda et al. (2006) found that only a few percent of barnacles survived to reproductive adults and those barnacles settled during a narrow recruitment window. Thus, deviations of environmental factors from species-specific optima during recruitment may lead to, high post-settlement mortality and prevent many settlers from joining the local reproductive pool. If our earlier speculation, i.e. that stress driven mortality eliminates a certain set of genotypes from the group of recruits, holds true, environmental stress during the period of peak settlement may affect the genetic diversity and composition of a population and, thus, its sensitivity to further stress.

Growth rates of early juvenile barnacle may depend on energy reserves carried over from the larval stage, as well as on food availability in the benthic habitat (Jarrett 2003; Thiyagarajan et al. 2003). Delayed metamorphosis is another important factor influencing post-metamorphic performance of the barnacles (Thiyagarajan et al. 2007). In this study however, we used one cohort of cyprids assuming that the difference in their quality is minimized and randomly distributed among the panels used for the experiments. We also fed the post-larvae ad libitum in

Chapter III

the laboratory study. Therefore, the effects observed should be mainly caused by the temperature and salinity treatments applied. Combined effects of temperature and salinity on juvenile growth were measured as basal diameter increment (shell growth), dry weight and ash-free dry weight (tissue growth) in this study. Mean growth in basal diameter increment ranged from 0.05 mm/day at 12°C and 5 psu to 0.15 mm/day at 20 and 28°C regardless of salinity. Thus, an increase of temperature by 8°C (from 12 to 20°C) at 5 psu caused an increase in growth (basal diameter) by 300%. At 15 and 30 psu this increase in growth was 200%. This was, however, not the case between 20 and 28°C. In fact, when temperature drops to 12°C the tolerance of barnacles to salinity changes becomes impaired whereas in moderate and high temperatures they tolerate large ranges in salinity. The effect of low temperature and low salinity on growth was enhanced by each other's effect. Tissue growth (measured as DW and AFDW) was also affected by temperature and salinity in this study. A strong positive relationship between shell and tissue growth of juveniles indicates that basal diameter is a suitable predictor for the effects of temperature and salinity on juvenile growth. These results are consistent with previous studies on the basal diameter and tissue weight of *Balanus amphitrite* (Pechenik et al. 1993; Thiyagarajan et al. 2002, 2003) but are in contrast with the findings of Thiyagarajan et al. (2007) on the effects of delayed metamorphosis on *B. amphitrite* juvenile growth.

In barnacles, traits such as cirral activity, food intake and assimilation are strongly correlated with temperature (Anderson 1994). A rise in seawater temperature causes an increase in metabolic rates and cirral activity (i.e. feeding efficiency) of barnacles (Crisp and Bourget 1985; Skinner et al. 2007), resulting in high growth rates as long as stored energy reserves and/or food availability meet the required additional energy expenditure (Crisp and Bourget 1985; Anderson 1994). Our results of increased growth with increasing temperature corroborate findings of previous studies (Qiu and Qian 1999; Thiyagarajan et al. 2003). We also observed higher feeding rates at higher temperatures than at lower temperature (personal observation on the clearance rates).

Berger et al. (2006) observed a cessation of cirral activity in the juvenile barnacle *Balanus glandula* at low salinities (below 10 psu), leading to reduced growth rates. We observed a similar low growth of *A. improvisus* at low salinity (5 psu) most pronounced at low temperature. Decreased feeding time as a consequence of retracting the feeding apparatus and

Chapter III

potential starvation, in concert with elevated levels of osmotic stress, can probably explain the observed low growth rate at low salinity in this study.

In the present study, low temperature and low salinity stresses caused a reduction in juvenile growth. These detrimental effects on juveniles are ecologically important because slow juvenile growth results in inferior competitive abilities and could also contribute to mortality by prolonging the susceptibility of individuals to predation before attaining a size refuge (Miller and Carefoot 1989; Pechenik 1990). Predators with feeding structures that are effective at handling small post-larvae are less efficient at handling large juveniles (Gosselin and Qian 1997; Osman and Whitlatch 2004; Griffiths and Gosselin 2008). Thus, ontogenetic shifts in susceptibility to predators occur in part because post-larvae progressively grow larger and develop more resistant protective structures such as shells. Refuge from predators may be reached sooner at higher temperatures (as we observed in this study) and therefore makes it more difficult for their predators to handle or crush their prey (Gosselin 1997). In our study, at low temperature and low salinity (12°C/5 psu) shell materials contributed about 56% of total weight of barnacles while this portion at 28°C and 30 psu was about 86%. Shifting the body/shell ratio by environmental factors may therefore alter the barnacle's quality as prey and expose them to different set of predators. The process of calcification and shell deposition in *Mytilus edulis* was observed to be depressed at low salinities (below 12.8 psu) and therefore resulted in reduced shell thickness (Almeda-Villela 1984). In Baltic Sea mussels more biomass was allocated to body mass relative to shell than in North Sea mussels (Kautsky et al. 1990). Low temperature, as another important factor influencing the growth, can reduce filtration rate via increasing the water viscosity (Riisgård and Larsen 2007; Melzner et al. 2011). Herein, low temperature, probably via direct reduction of cirral activity and/or increasing the water viscosity, reduced food consumption resulting in less energy intake. Since more energy costs are associated with calcification in cold water (Melzner et al. 2011), it is likely that under a low energy budget, energy is allocated to more vital processes such as somatic mass maintenance instead of shell conservation.

In conclusion, this study demonstrates that environmental stress (e.g., temperature and salinity) can alter survivorship at critical stage of life such as post-larval stage. Reduced survival rates in combination with altered body/shell ratios and growth rates of the survivors are likely to play an essential role in the population dynamics of barnacles and the composition and

Chapter III

performance of benthic hard bottom communities in general. The predictions for climate change in the Baltic Sea regarding temperature and salinity (higher temperature and lower salinity, BACC 2008), however seem not to drastically affect this species since detrimental effects of low salinity may at least in part be compensated by beneficial effects of high temperature. Other stresses associated with climate change such as ocean acidification and increased hypoxia may, however, add additional pressure on calcifying sessile species such as barnacles. High mortality during first week after settlement in this study brings the idea that the more sensitive genotypes had succumbed to the treatment(s) while the surviving genotypes were either pre-adapted to the conditions or able to acclimatize in time. This would be an interesting topic to be tested.

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A protective coat of microbes on macroalgae: inhibitory effects of bacterial biofilms and epibiotic microbial assemblages on barnacle attachment

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Abstract

Effects of epibiotic bacteria associated with macroalgae on barnacle larval attachment were investigated. Eight bacterial isolates from three macroalga species were cultured as monospecies bacterial films and tested for their activity against barnacle (*Amphibalanus improvisus*) attachment in field experiments (Western Baltic Sea). Furthermore, natural biofilm communities associated with the surface of the brown alga *Fucus vesiculosus* which had been treated at different temperatures (5 °C, 15 °C, and 20 °C) were harvested and subsequently tested. Generally, monospecies bacterial biofilms as well as natural microbial assemblages inhibited barnacle attachment by 20% to 67%. DGGE (Denaturing Gradient Gel Electrophoresis) fingerprints showed that temperature treatment shifted the bacterial community composition and weakened the repellent effects at 20 °C. Repellent effects were absent when settlement pressure of cyprids was high. Nonviable bacteria tended to repel cyprids when compared to the unfilmed surfaces. We conclude that biofilms can have a repellent effect benefiting the host by preventing heavy fouling on its surface. However, severe settlement pressure as well as stressful temperature may reduce the protective effects of the alga's biofilm. Our results add to the notion that the performance of *F. vesiculosus* may be reduced by multiple stressors in the course of global warming.

Key words: Bacterial biofilms, *Fucus*, larval attachment, *Amphibalanus improvisus*, DGGE, temperature effects.

Introduction

In marine habitats nearly all submerged natural and artificial surfaces become covered by biofilm. Microbes, such as bacteria and diatoms, are among the first colonizers (Wahl, 1989) that

Chapter IV

can substantially change the physical and chemical properties of the substratum (Characklis & Cooksey, 1983) making it suitable or unsuitable for colonization by invertebrate larvae (reviewed by Qian *et al.*, 2007). Inhibitory effects of biofilms on larval settlement have been documented in many studies (e.g. Olivier *et al.*, 2000; Lau *et al.*, 2003; Dobretsov & Qian, 2006; Rao *et al.*, 2007; Ganesan *et al.*, 2010). However, inductive or neutral effects have also been reported (e.g. Wieczorek *et al.*, 1995; Harder *et al.*, 2002; Ganesan *et al.*, 2010). The inhibitory effect of biofilms has been mainly attributed to their bacterial components (Maki *et al.*, 1988; Holmström *et al.*, 1992; Avelin Mary *et al.*, 1993; Anil & Khandeparker, 1998; Lau & Qian, 2000; Khandeparker *et al.*, 2002, 2003; Dobretsov & Qian, 2006). Previous studies have shown that bacterial surface chemistry, micro-topography, and a range of bacterial products from small-molecule metabolites to high-molecular weight extracellular polymers mediate cyprid settlement (reviewed by Qian *et al.*, 2007). It is further known that biofilm community composition, which can be a determinant for larval attachment, can be affected by environmental factors such as temperature and that these altered biofilm communities can change the settlement behavior of benthic larvae (Lau *et al.*, 2005). Structural and chemical cues (e.g., 6, 9-heptadecadiene and 12-octadecenoic acid, Hung *et al.*, 2008) from the natural biofilm community play an important ecological role in the natural environment of barnacle larvae by aiding in search of suitable habitat. For instance, barnacle larvae chose to attach to substrate with biofilms from environments that are favorable to barnacle recruitment over biofilms from less favorable environments (Hung *et al.*, 2007). *Amphibalanus amphitrite* cyprids from the mid intertidal can also distinguish between biofilms from different tidal ranges and settle preferentially on intertidal biofilms compared to subtidal biofilms and unfilmed surfaces (Qian *et al.*, 2003). Furthermore, the attachment behavior of the barnacle species *A. amphitrite* and *Balanus trigonus* was shown to depend on the temperature at which biofilms were grown, with more cyprids attaching to biofilms formed at higher temperatures (> 23 °C) than to ones formed at 16 °C (Lau *et al.*, 2005).

Most studies on interactions between biofilms and invertebrate colonizers have focused on biofilms grown on nonliving substrata (e.g. Qian *et al.*, 2003; Hung *et al.*, 2007) in their experiments. In the marine system, however, also living surfaces such as macroalgae thalli are potential suitable substratum for invertebrate larvae in competition for space. Uncontrolled

Chapter IV

colonization of epibiotic microorganisms on the macroalga usually has detrimental effects, e.g. by shading its surface and thus reducing its growth rate (Rohde *et al.*, 2008). To counteract this, the brown macroalga, *Fucus vesiculosus*, can produce secondary metabolites that directly repel invertebrate larvae (Brock *et al.*, 2007; Lachnit *et al.*, 2010). Lachnit *et al.* (2010) suggested that *F. vesiculosus* can also indirectly protect its surface from unwanted colonizers via regulation of a beneficial epibiotic bacterial biofilm community that produces repellent substances. If a potentially beneficial epibacterial community undergoes a shift in its composition (via direct stress and/or via indirect stress on the alga) the attachment of macrofouler larvae could in turn be affected. The immensely important function of biofilms in attachment processes of barnacles (and other invertebrate larvae) and the potential interaction with their macroalgal host led us to investigate the effect of biofilms composed of bacteria epibiotic on macroalgae on the number of attached *Amphibalanus improvisus* cyprids in field assays. Specifically our objectives were 1) to test the effect of monospecies bacterial biofilms originally isolated from macroalgal hosts on attachment of barnacle larvae 2) to investigate how effective these monospecies biofilms are under natural settlement pressures 3) to test the effect of water soluble chemical compounds from a natural assemblage of microorganisms detached from their brown macroalgal host, *F. vesiculosus*, on attachment of barnacle larvae and 4) to test the indirect effects of environmental abiotic stress (i.e. temperature) via a shift in the epibiotic bacterial community composition on attachment of cyprids.

Materials and methods

Monospecies bacterial films

Monospecies bacterial films were developed from eight bacterial strains, which had been isolated from three locally (Kiel Bight, Western Baltic Sea) common macroalgal species, and were tested for their effects on the attachment of cyprids in field assays. Strains were identified as *Pseudoalteromonas mariniglutinosa* (NR_028992), *Pseudoalteromonas tunicata* (Z25522), *Shewanella baltica* (AJ000214) and *Bacillus foraminis* (NR_042274.1) (isolated from the brown macroalga *Fucus vesiculosus* (L., 1753); *Ulvibacter litoralis* (AY243096) and *Photobacterium*

Chapter IV

halotolerans (NR_042975) (isolated from *Fucus serratus* (L., 1753); *Pseudoalteromonas arctica* (NR_043959.1) and *Shewanella basaltis* (EU143361) (isolated from the red alga *Polysiphonia stricta* (Dillwyn) Greville 1824).

Bacterial cultures were grown in sterile nutrient broth (5 g peptone + 3 g yeast in 1 l of filtered seawater). Each strain was inoculated to individual culture flasks containing 50 ml of sterile nutrient broth prepared with 0.22 µm filtered seawater (FSW) and incubated for 45-48 h at 20°C. Prior to the assays, the optical density (OD) of the bacterial cultures was determined with a Beckman Du 650 spectrophotometer at a wavelength of 600 nm, using pure medium as blank. All working cultures were adjusted to an OD of 1.5. Cultures were diluted to the mentioned OD with sterile medium when necessary.

Formation of monospecies bacterial films

Bacteria were harvested from cultures and adjusted to around 10^8 cells ml⁻¹ by turbidimetry. 0.2 µm polycarbonate filters (Sartorius, Germany) with a diameter of 47 mm served as settlement substratum for the bacteria. The sterile filters were placed onto a specially constructed sterile apparatus and fixed tightly with a screw-on lid which partially covered the filter (Fig. 1). This reduced the filter diameter to 35 mm and resulted in an effective attachment area of 9.62 cm² onto which 2 ml of bacterial suspension was pipetted. After 4 h of incubation under a sterile hood at room temperature (ca. 20 ° C) under continuous light the filters were gently rinsed with autoclaved FSW to remove unattached bacteria. Cells attached to the filter surface will be referred to as monospecies bacterial films hereafter and were developed as described above for all experiments.

Preparation of nonviable bacterial films

Monospecies bacterial films, prepared as described above, were pretreated with formaldehyde in order to generate nonviable films. Bacterial films were covered with 2 ml of formaldehyde (3.7% in FSW) for 30 min and were subsequently rinsed five times with autoclaved FSW (Lau *et al.*, 2003). Control dishes did not contain any bacterial film but were treated with formaldehyde following the same procedure as for the bacterial films.

Enumeration of bacterial cells

Chapter IV

Before initiating the attachment bioassay, the density of bacteria on reference filters was enumerated using epifluorescence microscopy (Zeiss Axio Scope A1 fluorescence microscope). After 4 h of incubation, the bacterial films on the filters were fixed with 3.7 % formaldehyde in FSW for 15 min and subsequently stained with 0.2% (v/v) 4,6-diamidino-2-phenylindole (DAPI, Invitrogen) for 15 min at room temperature. For bacterial enumeration, cell numbers in five randomly chosen fields of view were counted at 1000 × magnification. In the fifth experiment we did not enumerate bacteria. Microscopic examination of the filters, however, showed a similar coverage of the filters by bacterial cells as in the other experiments indicating that settlement of bacteria was successful. The relationship between bacterial density and attached cyprids was analyzed. Data from experiment II was excluded from analysis since there was no effect of bacterial films on cyprid attachment due to high settlement pressure.

Barnacle attachment assays

The barnacle life cycle progresses through six naupliar stages followed by a non-feeding cyprid larva, which is specialized for settlement. Peak settlement of *Amphibalanus improvisus* in the Kiel Fjord usually occurs in July and August (Thomsen *et al.*, 2010; Nasrolahi *et al.*, 2012). During this period, barnacles commonly settle in high numbers within a short period of time. However, settlement pressure may fluctuate drastically between days. Daily observation of settlement panels allowed us to visually estimate settlement pressure during the experiments. We ran a series of field experiments during July-August 2011 as follows:

At the beginning of the cyprid settlement period, panels with monospecies bacterial films were deployed for 7-24 h, depending on settlement pressure, subsequently retrieved, and the number of attached cyprids (irreversible attachment) was counted using a binocular microscope. Five individual experiments were conducted (I-V). Experiment I was carried out before the high settlement pressure period (for 24 h). Experiment II was conducted at the very peak settlement of cyprids (for 24 h). Experiment III was done during high settlement pressure but exposure time was reduced to 7 h in order to make it comparable to experiment I in terms of cumulative attachment and in order to examine the role of settlement pressure (comparing experiments II and III) in masking anti-settlement effects. Experiment IV was carried out to test the effect of nonviable bacterial films on cyprid attachment (for 7 h). Since several studies have shown the

Chapter IV

repellent effects of *Pseudoalteromonas* species on marine invertebrate larvae (e.g., Holmström *et al.*, 2002 and references therein) experiment V was run to test the effect of *Pseudoalteromonas* species isolated from *F. vesiculosus* and *P. stricta* on cyprid attachment. 5-7 replicates per bacterial film were used for each experiment depending on the number of bacterial strains used in the respective experiment. The single apparatuses were fixed on a PVC frame which was deployed at a water depth of 1.5 m. The filmed surfaces served as attachment substratum for cyprids and faced downward in order to avoid sinking particles from the water column to accumulate on the filters

Natural assemblage of microorganisms associated with *F. vesiculosus*

Sampling, experimental design and setup

30 *Fucus vesiculosus* thalli, each attached to one small rock (defined as one *Fucus* individual), were collected in the Baltic Sea, Kiel Bight (54°27'N, 10°11'50) in appr. 0.5 m water depth in July 2011. Within two hours of collection, they were brought to the laboratory in individual plastic bags and were transferred to aquaria of the experimental setup in a constant temperature chamber at the IFM-GEOMAR in Kiel. The experimental units (EU) were 20 l plastic aquaria - one for each algal thallus resulting in 30 EUs. Aquaria were filled with filtered seawater from the Kiel Fjord which was maintained at three different temperature levels: 5 °C, 15 °C and 20 °C using a water cooler (Titan 2000, Aqua medic) or 300 and 600 W heating rods (SCHEGO, Germany), respectively. All three temperature levels are experienced by *F. vesiculosus* in its natural habitat during the course of the season. Temperatures above 15 °C enhance biotic stress in Baltic Sea *Fucus* (Wahl *et al.*, 2011). Thus, the 20 °C treatment can be considered stressful for the brown alga and its biofilm. Light was supplied by neon tubes with an intensity of $100 \mu\text{mol m}^{-2}\text{s}^{-1} \pm 5 \mu\text{mol m}^{-2}\text{s}^{-1}$ and a light:dark regime of 16:8. Bubbling stones supplied the tanks with air, and water in the aquaria was exchanged once a week.

Biofilms associated with the surface of *F. vesiculosus* were harvested after 14 days of incubation at the different temperatures for use in the cyprid settlement assays. One large thallus branch of each individual alga was rinsed with sterile seawater for 5 sec in order to reduce loosely attached particles and was swabbed with three sterile cotton swabs. We know from

Chapter IV

microscopic analysis that a biofilm on *Fucus vesiculosus* is, on average, 11 μm thick (M. Fischer, E. Rickert, unpublished data). In order to harvest enough biofilm material to obtain a natural concentration of cells in an experimental volume of 1ml we swabbed a thallus surface of 900 cm^2 ($=1\text{ml}/11\mu\text{m}=1\text{cm}^3/0.0011\text{cm}$) and re-suspended the microbial cells (after washing, see below) in 1ml of sterile SW. By removing a natural biofilm from the alga, the original biofilm state is no longer intact. We therefore will refer to the suspension of microorganisms as “microbial assemblage” and not as biofilm. Each swab was placed in a 2 ml Eppendorf tube containing 1 ml of sterile seawater which was vortexed for 15 sec to remove the biofilms from the swab. After discarding the cotton swab, the microbial assemblage was centrifuged for 5 min at 5000 rpm. The resulting pellet was washed in two steps in order to remove residual metabolites originating from the host alga. First the pellet was washed with 1 ml of sterile seawater, the supernatant was discarded and the centrifugation step was repeated. Again 1 ml of seawater was added. Then 666 μl of the supernatant was pipetted off. The remaining suspensions of microorganisms from three swabs (each 334 μl) originating from one algal individual were combined (resulting volume = 1002 μl) in the cavity of the attachment apparatus (Fig. 1). The filter was placed on top of the cavity containing the microbial assemblage and sealed tightly with a screw-on lid. This construction allowed water soluble metabolites produced by the natural assemblage of microorganisms to diffuse through the filter, but retained all organisms $< 0.2 \mu\text{m}$. The top of the filter served as settlement substratum for the cyprid larvae. The settlement panels were suspended for 24 hours in the Kiel Fjord at a water depth of 1.5 m facing downward, after which the number of attached cyprids were counted at 10 x magnification. The assays were repeated in two separate runs due to limited space on the settlement panels. Both repeats were conducted within five days during which experimental conditions were similar. Therefore, we pooled the data of both repeats (resulting in 10 replicates for statistical analysis).

Enumeration of microbial cells

In order to determine the microbial cell densities in the wells of the attachment apparatus at the beginning of the assays microbial cells (bacteria and cells that were not bacteria i.e. mostly diatoms) in a subsample (100 μl) of the suspensions of 5 replicates per temperature level were enumerated. The samples were prepared and counted using an epifluorescence microscope

Chapter IV

following the procedure mentioned above with the exception that cells in 20 visual fields per sample were counted.

Epibacterial community composition

Sampling

In order to analyze the epibacterial community composition at the different temperatures via DGGE each algal individual was sampled for its associated bacteria. Prior to sampling, approx. 20 cm² (visual estimation) of algal thallus was rinsed with sterile seawater for 5 sec in order to reduce loosely attached particles. Subsequently, the bacteria were harvested with one sterile cotton swab per sample and stored in 2 ml Eppendorf tubes at -80 °C until DNA extraction.

DNA extraction

DNA was extracted with the QIAamp DNA Mini Kit (Qiagen GmbH) following the manufacturers protocol for buccal cotton swabs with an additional incubation step at 95 °C for 8 min after the protocol's second lysis step with buffer AL. DNA was eluted with nuclease-free water (Qiagen GmbH) and stored at -20 °C until PCR-DGGE.

PCR-DGGE (Denaturing Gradient Gel Electrophoresis)

The V3 region of 16 S rRNA gene sequences of bacteria was amplified using the primer pair 341F- GC (5'-[CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G] CCT ACG GGA GGC AGC AG-3') and 534R (5'-ATT ACC GCG GCT GCT GG-3') (Muyzer et al. 1993) (Eurofins MWG Synthesis GmbH). PCR amplifications were prepared with puReTaq™Ready-To-Go™ Beads (GE Healthcare Europe GmbH), 1 µl of each primer pair [10 pmol/µl], and 1 µl of DNA template in a reaction volume of 25 µl per sample). They were performed on a thermocycler (Flex Cycler, Analytik Jena) with following settings: Initial denaturation at 94 °C for 2 min, 10 touchdown cycles with initial annealing at 65 °C for 40 s and a reduction of 1° C per cycle, elongation at 72 °C for 40 s and denaturation at 95 °C for 30 s. This was followed by 25 cycles of annealing at 55° C for 40 s, elongation at 72 °C for 40 s and denaturation at 95 °C for 30 s. Final annealing was at 42 °C for 60 s, and final elongation at 72

Chapter IV

°C for 5 min. Correct sequence length (233 bp, including the 40 nucleotide GC clamp) of 2.5 µl of PCR amplicons per sample was controlled via electrophoresis on a 1.5% agarose gel (1 x TBE). PCR amplicons were then separated according to GC content on a DGGE gel. A double gradient gel was cast with a structural gradient of 6% - 8% acrylamid-bisacrylamid (37.5:1) and with a denaturing gradient of 40% - 80% (100% denaturant is defined as 7 M urea and 40% formamide (v/v)) (Petri & Imhoff 2001). This gradient was shown to be suitable for the analysis of bacteria associated with *F. vesiculosus* (Lachnit *et al.*, 2009). Electrophoresis was run in 0.5 x TAE buffer at 60 °C and 80 V for 14 hours in a C.B.S. Scientific DGGE system. The gel was stained with SYBR Gold (Life Technologies GmbH, Darmstadt, Germany) for 45 min and rinsed with 1 x TAE buffer for 30 min prior to gel documentation via UV transilluminator (Vilber).

Statistical analysis

The assumptions of normality and homogeneity of variances were tested with Shapiro-Wilks W test and Levene's test, respectively. Non-normally distributed data were square root transformed. The settlement assays were analyzed by one-way ANOVA using the Statistica 8 software package (StatSoft). Post-hoc comparisons were done with Tukey's HSD. Bands of the DGGE fingerprint were called with the bare eye and a Bray-Curtis similarity matrix was constructed based on the presence or absence of bands using the PRIMER 6 software package. The matrix served as basis for ANOSIM (Analysis of similarity) and data were visualized by cluster analysis and non-metric multidimensional scaling. R values generated by ANOSIM indicate the degree of separation between groups and usually lie between 0 and 1 (Large R values close to 1 indicate complete separation of groups, small values close to 0 imply that they are not or barely separated. Mid-range values of R (0.5) indicate that groups are distinguishable but overlap to a certain degree (Clarke & Warwick, 2001).

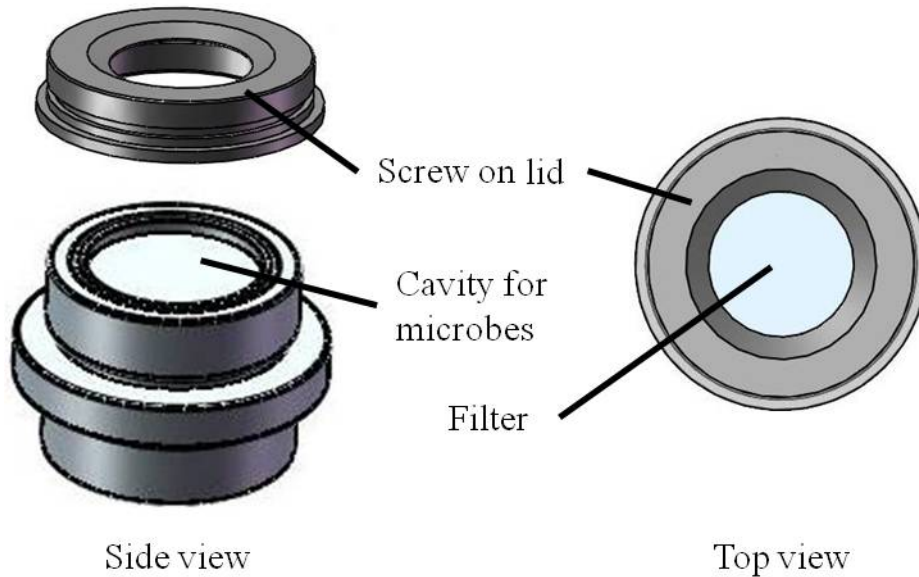


Fig.1. Apparatus used in the cyprid attachment field assays. Natural biofilms were placed in the cavity underneath the filter during assays, monospecies bacteria formed a film on top of the filter, which in all cases was used as attachment substratum for cyprids. The area available for attachment was 9.62 cm². The screw-on lid tightly sealed the filter cavity and held the filter in place. One apparatus was used for one replicate. Individual apparatuses were fixed to a PVC frame and deployed in the Kiel Fjord. Courtesy of Ralf Schwarz.

Results

Larval attachment assays with monospecies bacterial films

In general, a higher proportion of *Amphibalanus improvisus* cyprids settled on unfilmed surfaces than on the surfaces with a monospecies bacterial film.

Cyprid attachment in films of *Shewanella basaltis* significantly differed from attachment on the unfilmed surfaces (Fig. 2). Although the effects of *Photobacterium halotolerans* and *Ulvibacter litoralis* films on cyprid attachment were not significantly different from attachment on the unfilmed surfaces, cyprids tended to attach less to the filmed surfaces than to non-filmed surfaces (Fig. 2).

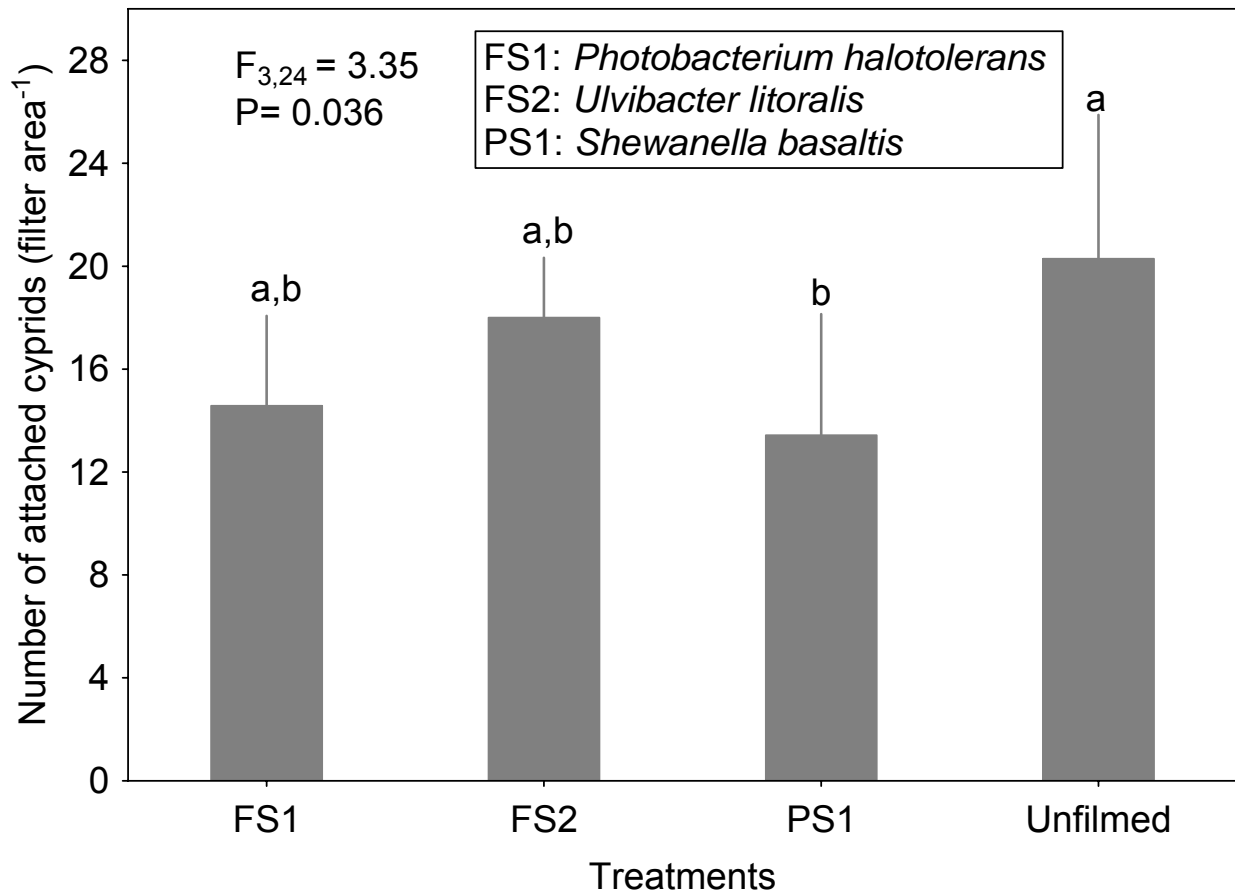


Fig.2. Experiment I. Effect of monospecies bacterial films isolated from *F. serratus* (FS) and *P. stricta* (PS) on cyprid attachment of *Amphibalanus improvisus*. Mean numbers of cyprids attached after 24 h of exposure in the Kiel Fjord. The unfilmed surface was a filter substrate without bacterial film. Each bar represents the mean of seven replicates. Error bars represent 95% CI. Significant differences at $\alpha = 0.05$ in Tukey's test are indicated by different letters above the bars

Under high settlement pressure the initially observed inhibitive effects of bacterial films on cyprid attachment of all bacterial films disappeared (Fig. 3). When exposure time was reduced (at high settlement pressure) cyprid attachment on all filmed surfaces differed significantly from the unfilmed surfaces (Fig. 4). The repellent effect of *S. basaltis* tended to be strongest (Fig. 4). No significant difference of cyprid attachment between filmed and unfilmed surfaces was seen after bacterial films were killed (Fig. 5).

Chapter IV

Table 1. Density of attached bacteria and the number of attached cyprids on the filter area of the apparatus

Bacteria	Mean density of bacteria × 10 ⁶ cells filter area ⁻¹	Mean number of cyprid attachment
PS1	7.699154	13.42
FS1	3.508008	12.5
FS2	7.963794	18
PS1	6.499046	5.8
FS1	12.40112	11.6
FS2	16.30916	12.4
FV1	2.289437	11.2
FV2	13.2935	11
PS1	19.29404	10
FS1	1.261652	8.6
FS2	12.5919	8
FV1	8.413065	7.5

Data on attached bacterial density and number of attached cyprids from experiment I, III and IV in the monospecies bioassay. PS1: *Shewanella basaltis*, FS1= *Photobacterium halotolerans*, FS2= *Ulvibacter litoralis*, FV1= *Bacillus foraminis*, FV2= *Shewanella baltica*. The mean density of bacteria was based on counts of 5 fields at 1000 x magnification under an epifluorescence microscope. There is no correlation between bacterial density and the amount of larval attachment ($r^2 = 0.007$, $p = 0.417$).

During the experiments with *Pseudoalteromonas*, cyprid attachment in the field was low and on average only 4 cyprids attached after 24h (Fig. 6). Cyprid attachment on *P. mariniglutinosa* and *P. tunicata* films did not differ in comparison to the unfilmed surfaces. These two films even showed a slight but non-significant inductive effect on cyprid attachment (Fig. 6). In contrast, *Pseudoalteromonas arctica* repelled cyprids (Fig. 6).

Table 2. Analysis of Similarity (ANOSIM) pairwise tests results of epibacterial community samples (n = 6 per temperature level).

Groups	R Statistic	Significance Level	Possible Permutations	Actual Permutations	# Observed permuted statistics > or = to R
5°C, 15°C	0.501	0.002	462	462	1
5°C, 20°C	0.756	0.002	462	462	1
15°C, 20°C	0.256	0.024	462	462	11

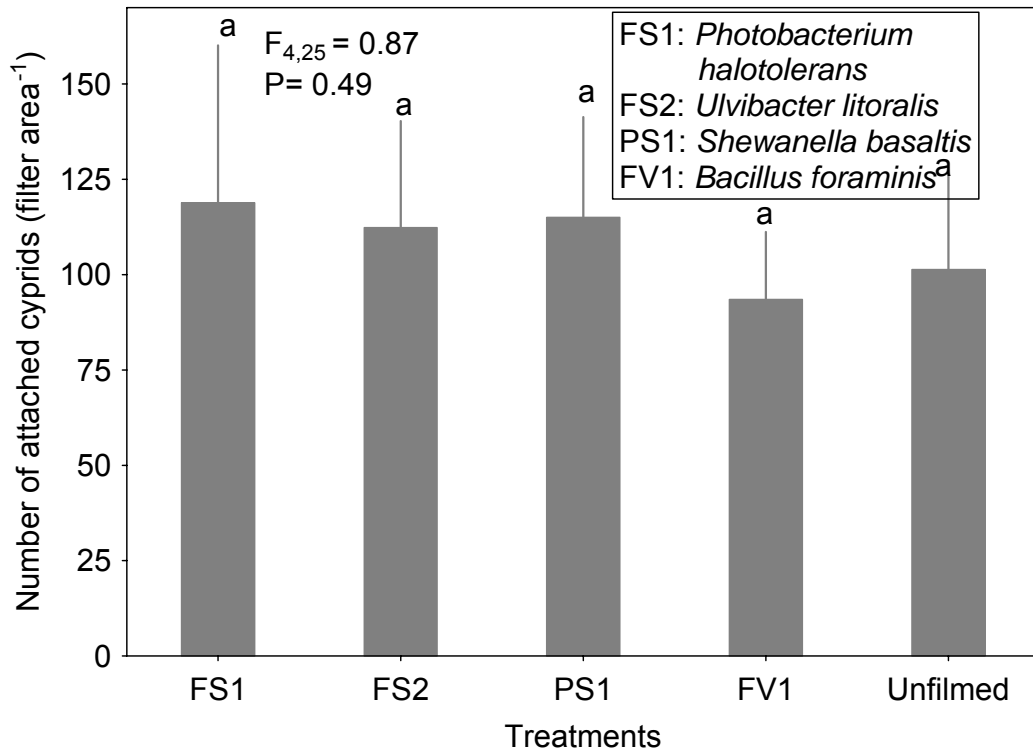


Fig.3. Experiment II. Effect of monospecies bacterial films isolated from *F. vesiculosus* (FV), *F. serratus* (FS) and *P. stricta* (PS) on cyprid attachment of *Amphibalanus improvisus* at the very peak settlement: Mean numbers of cyprids attached after 24 h of exposure in the Kiel Fjord. The unfilmed surface was a filter substrate without bacterial film. Each bar represents the mean of six replicates. Error bars represent 95% CI. Significant differences $\alpha = 0.05$ in Tukey's test are indicated by different letters above the bars.

Larval attachment assays with microbial assemblages

The cyprid attachment assays revealed that on average 24% - 54% fewer cyprids attached to substrata with differently treated microbial assemblages than to the filters without microbes underneath (controls). Lowest numbers of attached cyprids were found on substrata with microbial assemblages developed at 5 °C and 15 °C, an effect which was significant relative to

Chapter IV

control substrata but not relative to the substrata with microbial assemblages developed at 20 °C (Fig. 7). The 20 °C microbial assemblages showed a repellent trend but this result was not significantly different from the control.

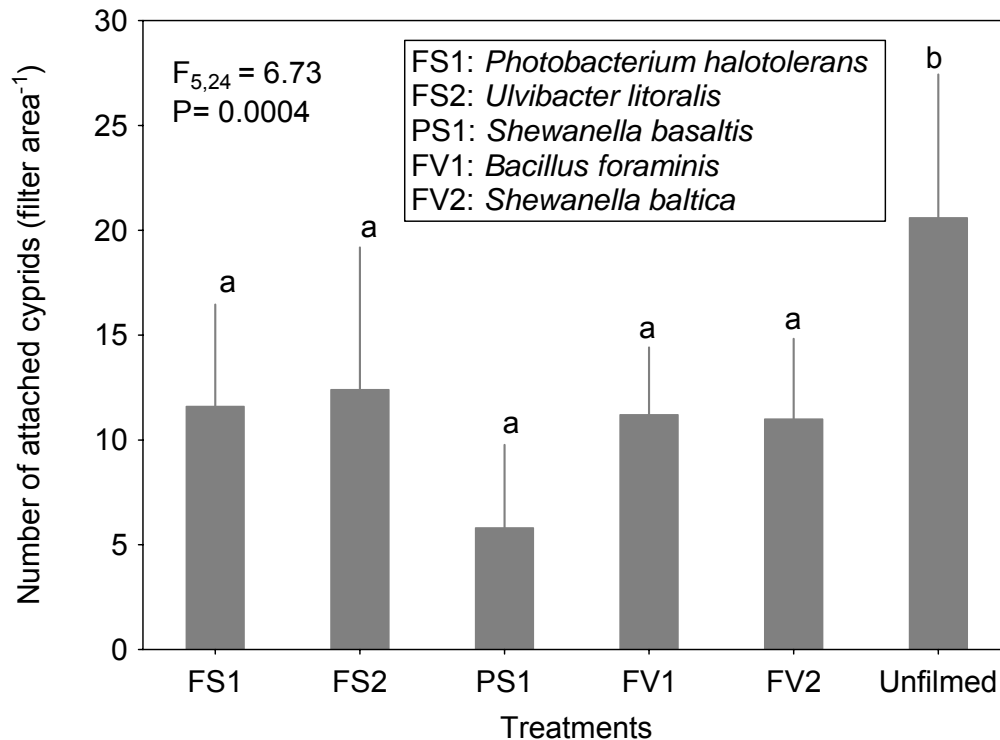


Fig.4. Experiment III. Effect of monospecies bacterial films isolated from *F. vesiculosus* (FV), *F. serratus* (FS) and *P. stricta* (PS) on cyprid attachment of *Amphibalanus improvisus*: Mean numbers of cyprids attached after 7 h of exposure in the Kiel Fjord. The unfilmed surface was a filter substrate without bacterial film. Each bar represents the mean of five replicates. Error bars represent 95% CI. Significant differences at $\alpha = 0.05$ in Tukey's test are indicated by different letters above the bars.

Epibacterial community composition

Analysis of the DGGE banding pattern (Fig. 8) of 16S rRNA gene sequences of bacteria sampled from their host, *F. vesiculosus*, showed that the different temperature treatments led to significant shifts in the epibacterial community composition (ANOSIM global $R = 0.524$, $p = 0.001$). The bacterial communities from the different temperature treatments formed clusters with different degrees of similarity (Fig. 9), as shown by the results of the pairwise permutation tests (Table 2).

Chapter IV

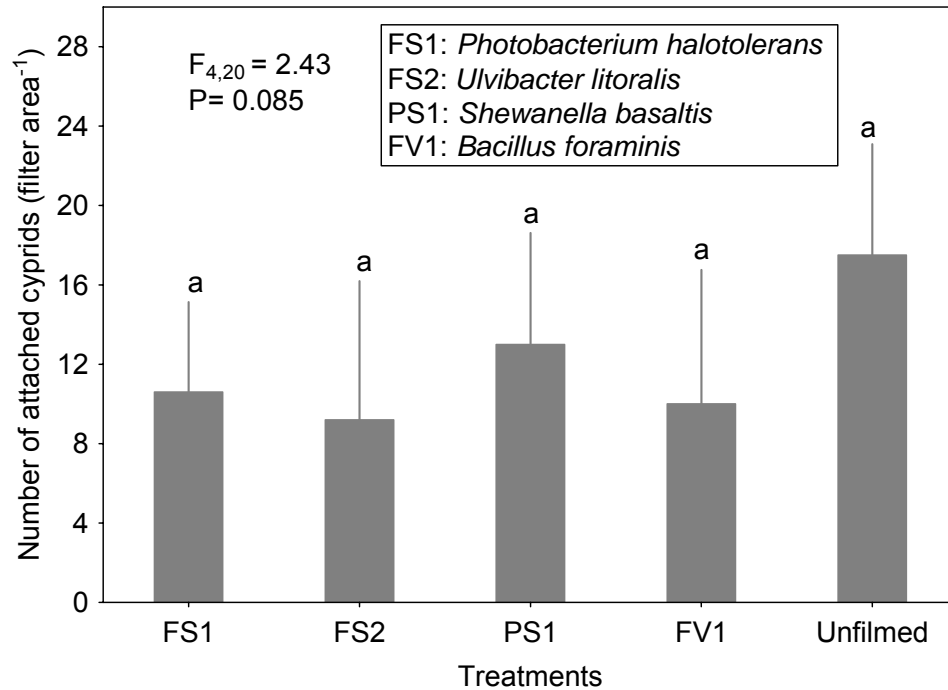


Fig.5. Experiment IV. Effect of nonviable monospecies bacterial films isolated from *F. vesiculosus* (FV), *F. serratus* (FS) and *P. stricta* (PS) on cyprid attachment of *Amphibalanus improvisus*: Mean numbers of cyprids attached after 7 h of exposure in the Kiel Fjord. The unfilmed surface was a filter substrate without bacterial film. Each bar represents the mean of six replicates. Error bars represent 95% CI. Significant differences at $\alpha = 0.05$ in Tukey's test are indicated by different letters above the bars.

The bacterial communities of 5 °C and 20 °C differed as indicated by a high value of R (0.756) (Table 2); The bacterial communities of 5 °C and 15 °C differed slightly less (R = 0.501), whereas communities from the 15 °C and 20 °C treatments largely overlapped (R = 0.256). The number of bacterial cells in the cavities of the filter apparatuses during the field attachment assays did not significantly differ between the temperature treatments and were on average 270- 360 *10⁶ cells /cavity. The number of other microbes (mostly diatoms) was on average approx. 100 fold smaller and also did not significantly differ between treatments (Fig. 10 a and b).

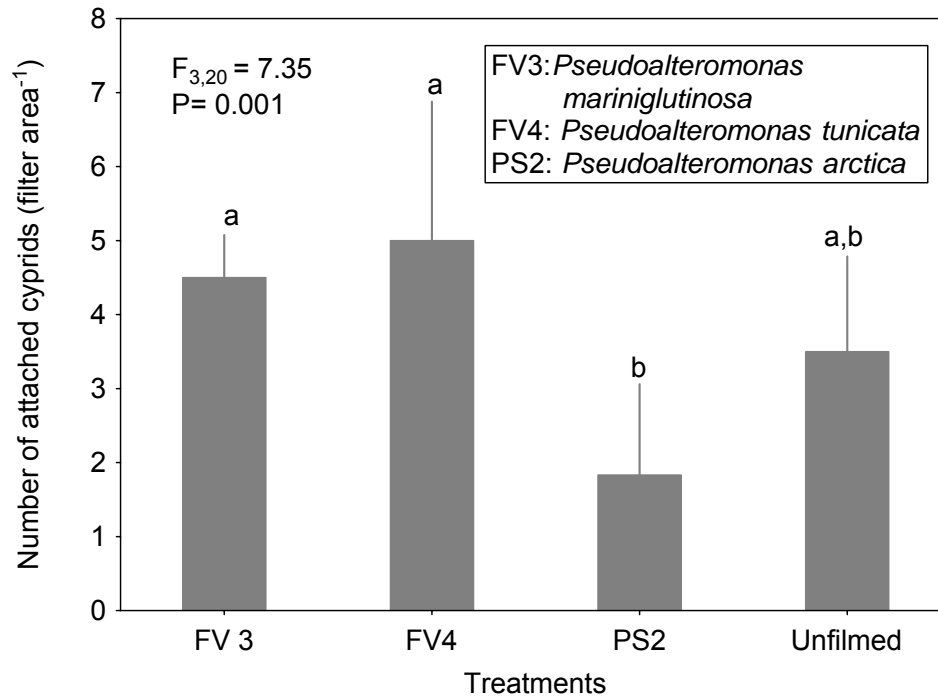


Fig.6. Experiment V. Effect of *Pseudoalteromonas* species isolated from *Fucus vesiculosus* (FV) and *Polysiphonia stricta* (PS) on cyprid attachment of *Amphibalanus improvisus*: Mean numbers of cyprids attached after 24 h of exposure in the Kiel Fjord. The unfilmed surface was a filter substrate without bacterial film. Each bar represents the mean of five replicates. Error bars represent 95% CI. Significant differences at $\alpha = 0.05$ in Tukey's test are indicated by different letters above the bars.

Discussion

Knowing that bacteria are key components of the biofilms especially with regard to their inhibitory effects on macrofoulers (e.g. Khandeparker *et al.*, 2002, 2003; Dobretsov & Qian, 2006), we used bacterial strains isolated from macroalgal surfaces and tested their effects on larval attachment of *Amphibalanus improvisus* in field experiments. Since only about 5% of bacteria from the natural environment are culturable (Eilers *et al.*, 2000) and therefore the results of experiments with bacterial isolates may not reflect interactions in the field we also used natural microbial assemblages which were harvested from their host *Fucus vesiculosus* grown under different temperature conditions.

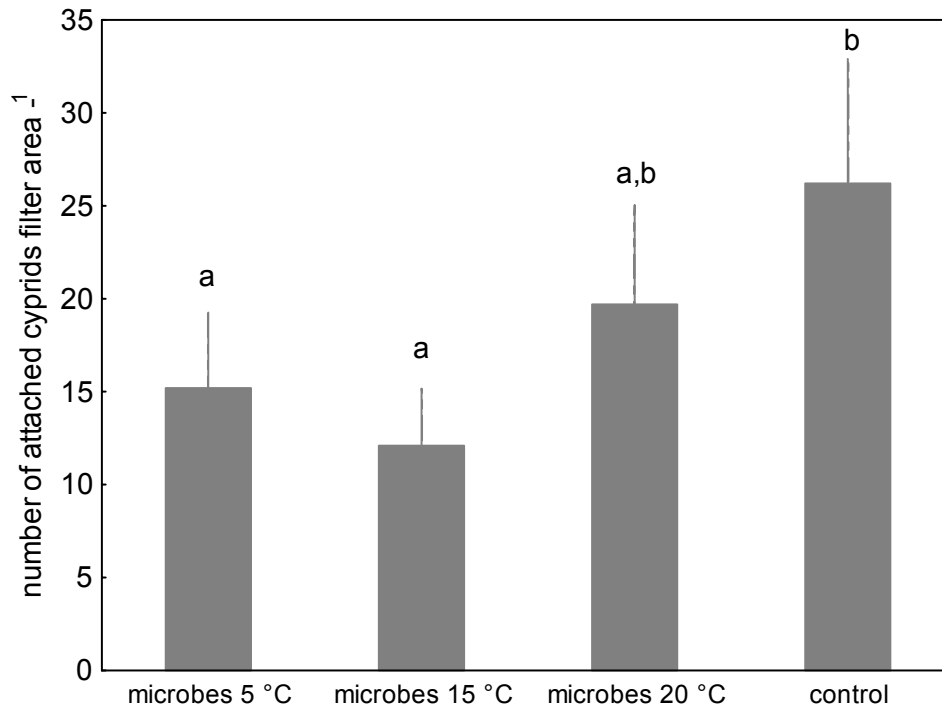


Fig.7: Mean number of attached *Amphibalanus improvisus* cyprids on apparatus with polycarbonate filters during exposure in the Kiel Fjord for a maximum of 24 h. Biofilms were detached from their macroalgal host, *Fucus vesiculosus*, which had been cultured at different temperatures (5 °C, 15 °C, and 25 °C). Filters without the harvested microbial assemblage served as a control substrate. Each bar represents the mean of 10 replicates. Error bars represent 95% CI. Significant differences at $\alpha = 0.05$ in Tukey's test are indicated by different letters above the bars.

Marine bacteria associated with basibionts have the potential to protect hosts from micro- and macrofouling (Holmström *et al.*, 1992; Holmström & Kjelleberg, 1999; Steinberg *et al.*, 2002; Dobretsov & Qian, 2004). Our results showed that there were inhibitory effects of monospecies bacterial films on cyprid attachment of *Amphibalanus improvisus*. Six isolates from the surfaces of macroalgae tested in this study were inhibitive and two were non-inhibitive. O'Connor & Richardson (1996) developed laboratory and field assays with the same barnacle species used in our study but with different monospecies bacterial films (*Deleya marina*, *Alteromonas macleodii*, and *Pseudomonas fluorescens*). The authors also found repellent effects on barnacle attachment for all tested strains, although this was substratum-dependent. It seems that several different strains can repel *A. improvisus* as shown by the study mentioned above and supported by our results.

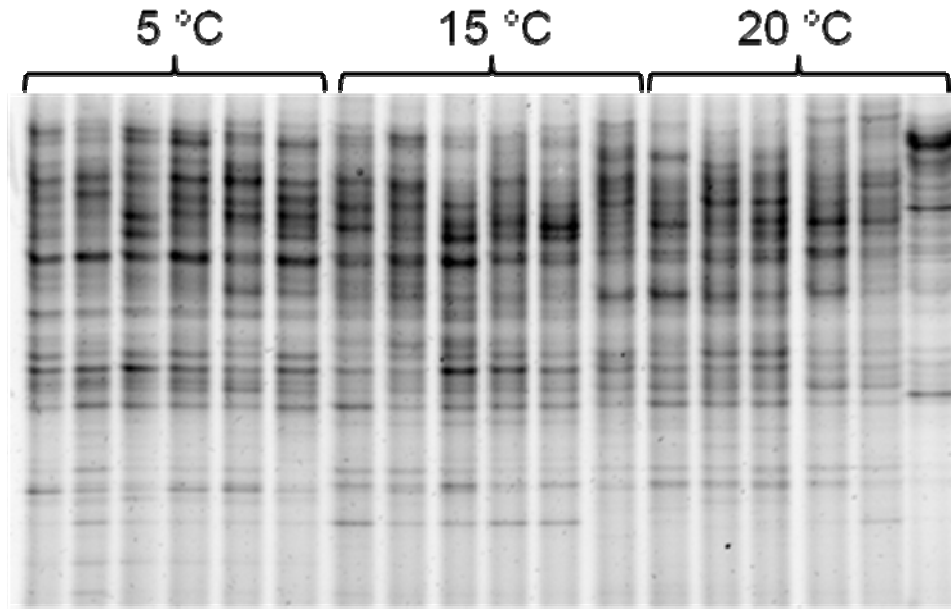


Fig.8: DGGE fingerprint of 16S rRNA gene sequences of epibacteria from *Fucus vesiculosus* cultured at different temperatures (5, 15, and 20 °C; n = 6 per temperature level).

In the present study, *Shewanella basaltis* and *Pseudoalteromonas arctica*, isolated from a biofilm associated with the red alga *Polysiphonia stricta*, were the most potent inhibitors among all tested isolates. They reduced the number of attached cyprids by 35%-67%. *Shewanella baltica* and *Bacillus foraminis* isolated from *Fucus vesiculosus* had inhibitory effects (~ 48%) similar to the effects of bacterial strains isolated from *F. serratus* (*Vibrio* sp. and *Ulvibacter littoralis*). *Pseudoalteromonas tunicata* and *Pseudoalteromonas mariniglutinosa* isolated from *F. vesiculosus* did not have any repellent effects on cyprid attachment. *P. tunicata*'s neutral effect on attachment of cyprids is surprising because many studies have reported inhibitive effects of this species (e.g. Holmström *et al.*, 2002 and references therein). However, inductive effects of some other *Pseudoalteromonas* species on marine larval invertebrates have been shown. For instance, *Pseudoalteromonas luteoviolacea* was identified to be one of the strongest settlement inducers for an Australian echinoid, *Heliocidaris erythrogramma* (Hugett *et al.*, 2006). Some bacteria express larval specific activity, i.e. inhibit settlement of one species and induce settlement of another species (Maki *et al.*, 1989, 1992; Lau *et al.*, 2003). Different species of barnacle cyprids may also respond differently to similar or even identical cues (e.g. Johnson & Strathmann, 1989; Keough & Raimondi, 1996). In our study *P. tunicata* did not have any

Chapter IV

repellent effect on *A. improvisus* larvae whereas this isolate strongly repelled *A. amphitrite* larvae in the study done by Holmström *et al.* (2002). This target organism specificity can be one explanation for the discrepancy of results from different studies.

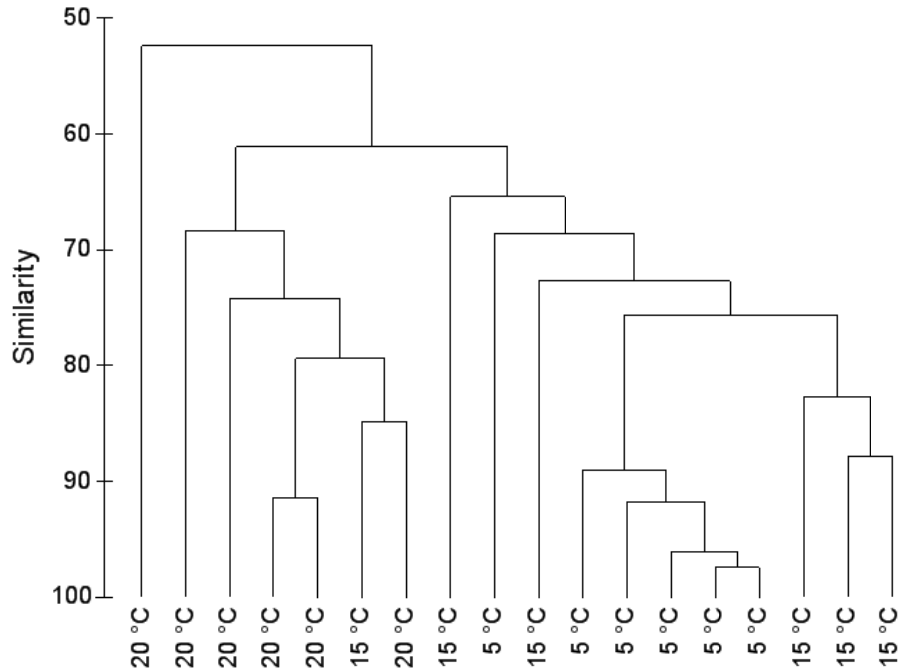


Fig.9: Dendrogram based on DGGE epibacterial community fingerprint showing similarities in % (group-average linking of Bray-Curtis similarities).

Our results show that reduction of barnacle attachment by bacterial films was not density-dependent. These observations coincide with findings of Kavouras & Maki (2004) on the inhibitive effect of monospecies biofilms on zebra mussel larvae. Results of our study are, however, in disagreement with that of Lau *et al.* (2003) who found a negative correlation between bacterial density and the percent settlement of cyprids. It seems that a certain cell density evokes a repellent effect and above this density there is no additional activity. There may have been slight changes of the microbial cell densities and also of the bacterial community composition during the course of the assays that we could not measure. We experienced difficulties in sampling the microbial assemblages at the end of the assays without cell loss and microscopic enumeration of cells densities was not possible due to particles and barnacles

Chapter IV

attached to the filters. However, the assays were of relatively short duration, so we assume that the changes are negligible.

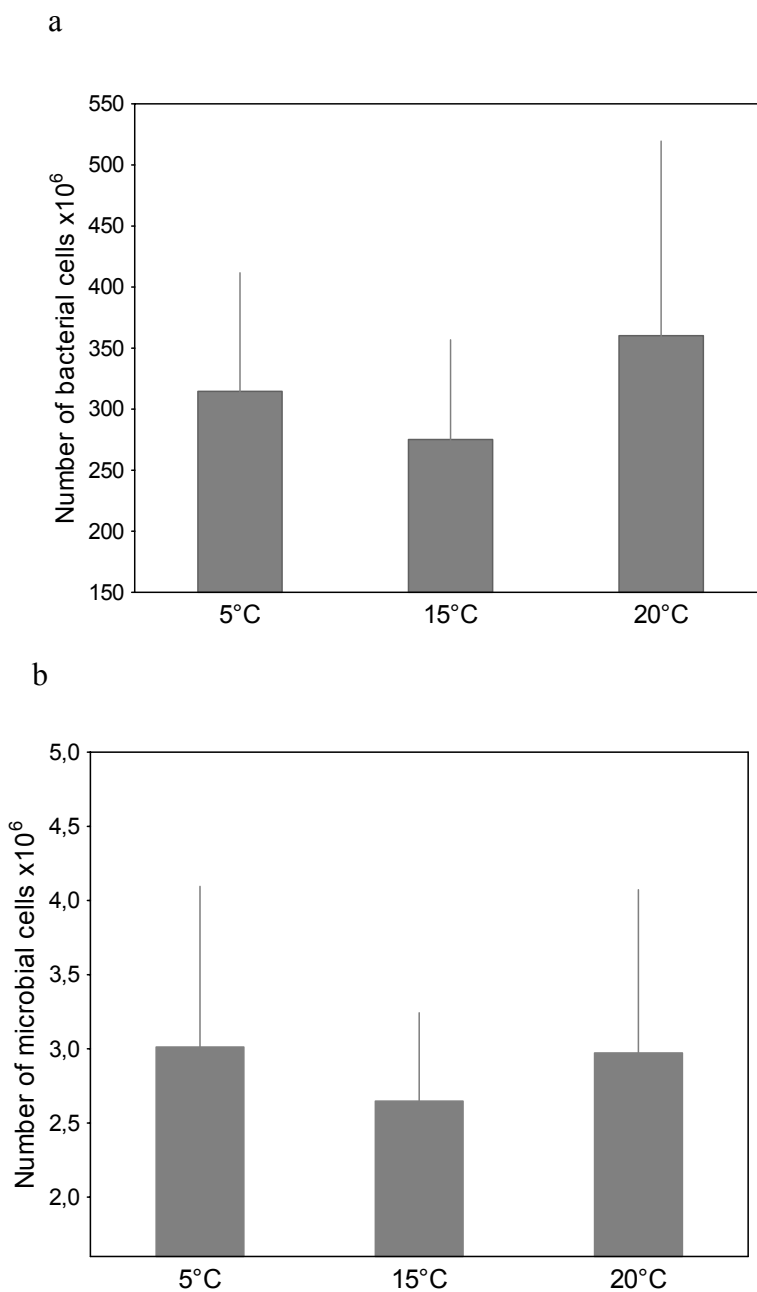


Fig.10: a) Mean number of bacterial cells and b) mean number of other microbial cells (mostly diatoms) used in the field attachment assays per temperature level. Cells were enumerated by epifluorescence microscopy. Each bar represents the mean of five replicates per temperature level 95% CI; N.S.

Chapter IV

In this study, we used the procedure of Lau *et al.* (2003) and tested cellular viability as a parameter for the settlement inhibition activity of bacterial films. Lau *et al.* (2003) found that cyprid settlement of *A. amphitrite* showed no difference on live and nonviable bacterial films. Another study by Lau & Qian (2000) showed that inductive effects of *Rhodovulum* sp. (α -Proteobacteria) on larval settlement of the marine polychaete, *Hydroides elegans*, were strictly dependent on bacterial viability. In our study, inhibitive effects of nonviable (i.e. no metabolic activity) bacterial films on cyprid attachment were weaker than that of live bacterial films. However, all isolates still exhibited inhibitive trends on larval attachment compared to the unfilmed surfaces, suggesting that the inhibitive effect was mediated by both bacterial metabolic activity and direct larval contact with the bacterial film surface (physical existence of bacterial cells).

In our study, inhibitive effects of bacterial films were not observed when cyprid settlement pressure in the field was high. In contrast to our results, O'Connor & Richardson (1998) reported that settlement of *A. amphitrite* on bacterial films was independent of the number of cyprids they used in their assays in the laboratory. During surface exploration, barnacle cyprids use a proteinaceous secretion to attach temporarily and walk across surfaces, leaving behind a so-called "footprint". This secretion also functions as settlement cue for subsequently exploring larvae (Dreanno *et al.*, 2006 a, b). Barnacles often settle gregariously (Crisp, 1974), which could explain the high settlement rates of cyprids when large numbers of cyprids are present in the field. In laboratory experiments however, usually cyprid density is lower than that in natural habitats during peak settlement. Consequently, the magnitude of settlement observed in lab experiments may not be ecologically representative (Gotelli, 1990; Clare *et al.*, 1994)

During peak settlement of barnacles in the Kiel Bight we have observed that *F. vesiculosus* thalli can become heavily fouled by *A. improvisus* (Rhode *et al.*, 2008). This observation indicates that results from the monospecies bacterial films, i.e. no repelling effect on cyprids when settlement pressure was severe, reflect realistic scenarios in the field. The thallus surface is essential for light and nutrient uptake (Wahl, 2008). Severe overgrowth by macrofoulers can therefore be disadvantageous for the host alga because photosynthesis is

Chapter IV

reduced and thus growth (Rohde *et al.*, 2008). Obviously, *F. vesiculosus* is not covered by a monospecies biofilm in the field but by a complex bacterial community (Lachnit *et al.*, 2011). Therefore, in addition to the monospecies films we tested the effect of natural epibiotic microbial assemblages on cyprid attachment, which closer resemble the natural biofilm state than monospecies films do. We could not conduct the attachment assays with biofilms that are 100% identical with those on the alga, because we removed the biofilm from its host, possibly losing biofilm members in the cotton swabs. We followed this procedure in order to exclude or at least minimize the potential direct effects of the host alga on barnacle attachment. Despite this limitation the microbial assemblages had repellent effects on barnacle larvae, most pronounced with the microbes from algae treated at 5 °C and 15 °C. The repellent effect of bacterial biofilms on invertebrate larval attachment can be explained by bacterial abundance (e.g. Dobretsov & Qian, 2006) and by the bacterial community composition (e.g. Qian *et al.*, 2003), both of which were analyzed in our study. Neither the number of bacterial cells nor the number of other microbes (mostly diatoms) differed between the biofilms from different temperatures. Apparently, the number of microbial cells was not responsible for the strongest repellent effects of the 5 °C and 15 °C microbial assemblages. These results coincide with previous studies on the attachment of two barnacle species which were not affected by the abundance of microbes (Qian *et al.*, 2003, Thiagarajan *et al.*, 2006). Our results can rather be explained by the bacterial community composition, which differed most between 5 °C and 20 °C and least between 15 °C and the 20 °C, respectively. The 15 °C microbial assemblage repelled on average about 37% more and the 5 °C microbes on average 22% more barnacle larvae than the 20 °C microbial assemblage did. We would therefore have expected the microbial communities from the lower (5 °C and 15 °C) and high (20 °C) temperature treatments to form distinct communities responsible for the observed effects on cyprid attachment. However, the picture was not as clear as expected: the 15 °C and 20 °C microbial communities did not form distinct clusters but largely overlapped in their community composition. In a previous study temperature had a direct effect on the bacterial community composition on artificial substrate which in turn affected the attractiveness of the biofilm to barnacle cyprids (Lau *et al.*, 2005). In their study the bacterial communities differed more amongst each other than the ones in our study did. Thus, our results indicate that not large but small shifts in the community composition (which in our study resulted from a

Chapter IV

temperature shift of 5 °C) or in the relative abundance of single community members may account for a stronger repellent effect of the microbial assemblage. It remains to be tested which members of the bacterial community are responsible for the repellent effects. Candidates could be the single strains that showed repellent effects in the study at hand. However, it remains unclear whether they would play the same role in a complex community. We cannot rule out that other members of the microbial assemblage (e.g. diatoms) or metabolites from the host algae may have caused or at least contributed to the repulsion of barnacles. *F. vesiculosus* has been shown to produce secondary metabolites (phlorotannins) with the potential to directly repel cyprids (Brock *et al.*, 2007). However, the microbial assemblages used in our study were thoroughly washed in order to remove residual host algal water soluble phlorotannins and other water soluble compounds. Microalgal (mostly diatoms) cells were present in the tested microbial community. They were not identified further and thus we do not know whether bioactive species were present. In any case diatom cells were outnumbered by bacteria by far (100 fold). Considering the repellent effect of several monospecies biofilms in this study, and the undisputed key role of bacteria in larval settlement (reviewed in Hadfield, 2011) it is likely that the bacteria in the natural microbial assemblages were at least in part responsible for the repellent effects against barnacle larvae. There is evidence that bacteria produce water soluble compounds play an inhibitive role in the attachment of the polychaete *H. elegans* (Dobretsov & Qian, 2002). To our knowledge, most studies (e.g. Qian *et al.*, 2003; Lau *et al.*, 2005) on the inhibitive effects of complex biofilms on barnacle attachment conducted assays in which the larva were in direct contact with the biofilm thus testing surface bound and water soluble compounds simultaneously. In our study the attachment apparatus was constructed in a way that only allowed water soluble compounds from the natural microbial assemblage to diffuse through the filters. The observed repellent effect thus must have been caused by one or several water soluble compounds emitted by the bacteria (and possibly diatoms) and perceived by the cyprids rather than by surface bound compounds of the EPS (extracellular polymeric substances). The nature of the compounds was not analyzed and their identification will need to be addressed in future studies.

Many antibacterial compounds have been extracted from algae (reviewed in Goecke *et al.*, 2010) indicating that many algae species actively defend their surface from epibiosis (for

Chapter IV

examples see Harder, 2008). Some algal species have been shown to regulate their surface associated bacteria to their own benefit (*Delisea pulchra*: Maximilien *et al.*, 1998; *Bonnemaisonia asparagoides*: Nylund *et al.*, 2010; *Dictyosphaeria ocellata*: Sneed & Pohnert, 2011). This regulatory capacity has also been suggested for *F. vesiculosus* (Lachnit *et al.*, 2010). In our study the microbial assemblages from algae treated at the lower temperatures (5 and 15°C) were the most repellent. Therefore, it seems possible that the alga is in a healthy state at lower temperatures, allowing it to maintain a beneficial epibacterial community and that the alga's chemical control of the biofilm could be impaired at higher temperatures causing stress. Although this idea is speculative we find it deserves attention in future studies investigating effects of climate change. In the course of climate change, water surface temperatures in the Baltic Sea could increase by up to 4 °C by 2100 (BACC, 2008). This rise in temperature could indirectly reduce the general performance of *F. vesiculosus* via weakening of the protective biofilm leading to higher fouling of its surface and consequently reduced available solar energy. Besides this, higher water temperatures during the summer months coincide with high settlement pressure of barnacles as well as increased grazing pressure and therefore can generate synergistic stressful interactions (Wahl *et al.*, 2011).

We conclude that the complex biofilm associated with macroalgae can protect its host from unwanted colonizers. This indirect defense mechanism may strengthen the direct antifouling defense of the macroalga against macrofoulers. As indicated by the results of the monospecies films, this protective effect may not withstand severe fouling pressure but may minimize biotic stress for the algae caused by heavy fouling of its surface at times when fouling pressure is moderate. Temperature stress may also reduce the protective effect of the alga's biofilm. Thus an increase in water temperature due to global warming may result in multiple stressors reducing the performance of the macroalgae.

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Chapter IV

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Chapter IV

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Chapter IV

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Chapter IV

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General discussion

General discussion

In this study, the performance of early stages of two different populations of *Amphibalanus improvisus* in the Baltic Sea (one at Kiel Fjord, Germany and another one at Tjärnö, Sweden) was evaluated. The transitional period between the cyprid and the adult stage of barnacles (juvenile stage) is critical and relatively neglected. Thus, in this study, sensitivity to temperature and salinity of early benthic phase was also observed. Concerning the extremely important function of biofilms in attachment processes of barnacles and the potential interaction with their macroalgal host, the effect of biofilms composed of bacteria epibiotic on macroalgae on the number of attached cyprids in field assays was furthermore, investigated.

Responses of larvae of *A. improvisus* from Kiel population were relatively different from that of Tjärnö population (Table 1). In Kiel population, naupliar duration was shortest at 28°C (regardless of salinity), metamorphosis success and overall survival (from start to end of experiment) was best at 5 psu (regardless of temperature) and the proportion of settled cyprids, i.e., nauplii that completed their entire larval development and successfully transformed into settled, juvenile barnacles, was highest at the “home” conditions of 20°C/15 psu. Settlement success of “naive” cyprids, i.e., when nauplii were raised in the absence of stress (20°C/15 psu), was less impacted by stressful temperature/ salinity combinations than that of cyprids with a stress history. Here, settlement success was highest at 30 psu particularly at low and high temperatures.

Tjärnö population larvae of *A. improvisus* showed more persistent responses pattern to temperature and salinity combination in comparison with Kiel population (Table 1). Nauplii to cyprid metamorphosis success, survival and settlement were always lowest at low temperature and high salinity (12°C/30 psu). Nauplii performed following a consistent pattern of temperature and salinity (i.e., temperature: 28> 20> 12°C; salinity: 15> 5> 30 psu). Naupliar duration was shortest at 28°C (regardless of salinity). In a separate experiment, when cyprids reared at 26°C/30 psu were exposed to the same temperature and salinity combinations, almost all cyprids survived (>95%). Cyprid settlement, in contrast, was impacted by salinity and was lowest at 30 psu regardless of temperature.

General discussion

Table 1. Response variables of *A. improvisus* from Kiel and Tjärnö populations to temperature and salinity. Cyprids are considered as „naive“ when nauplii were raised at 20°C/15 psu and at 26°C/30psu for Kiel and Tjärnö populations, respectively.

Response variables	Kiel	Tjärnö
Naupliar duration	Tem (°C): 12>20>28 Sal (psu): 5~15~30	Tem (°C): 12>20>28 Sal (psu): 5~15~30
Nauplii to cyprid metamorphosis success	Tem(°C): 12<20~28 Sal 5~15~30	Tem(°C): 12<20<28 Sal (psu): 5<15>30
Survival	Tem(°C): 12~20~28 Sal(psu): 5>15~30	Tem(°C): 12<20<28 Sal (psu): 5<15>30
Settlement	Tem(°C): 12<20<28 Sal (psu) 5<15>30	Tem(°C): 12<20<28 Sal (psu): 5<15>30
Naive cyprid survival	Tem (°C): 12~20~28 Sal (psu): 5~15~30	Tem (°C): 12~20~28 Sal (psu): 5~15~30
Naive cyprid settlement	Tem (°C): 12~20~28 Sal (psu): 5~15<30	Tem (°C): 12~20~28 Sal (psu): 5~15>30

Juvenile mortality was high (42 - 63%) during the first week following attachment, regardless of the temperature and salinity treatments. Subsequently, mortality and growth were interactively influenced by temperature and salinity. Highest mortality and lowest growth of juveniles occurred at lowest temperature (12°C) and salinity (5 psu). Juvenile barnacles constructed more shell material compared to body mass at low temperature and low salinity while a reverse situation was observed at high temperature and high salinity.

Barnacle attachment was reduced by monospecies bacterial biofilms and natural microbial assemblages by 20% to 67%. However, the repellent effects of bacterial biofilms were no longer observed when settlement pressure was high. The composition and abundance of biofilms on macroalga (*Fucus vesiculosus*), a potential host for barnacles, were affected by temperature which led to a reduction of their barnacle repellent effects by 60% at high temperature.

General discussion

Biological and ecological roles of the various ontogenetic phases

Barnacle larvae begin life in the first of several nauplius stages, which feed in the plankton before metamorphosing to the, non-feeding cyprid stage. The cyprid stage is specialized for benthic habitat selection and in the presence of suitable substratum and environmental stimuli, cyprids attach and metamorphose into sessile juveniles and grow and become adult (Thiyagarajan and Qian, 2008). Early development stages of marine invertebrates (e.g., barnacles) are often the most sensitive life phases to environmental stresses (Pörtner and Farrell, 2008; Melzner et al., 2009; Dupont et al., 2010). Recruitment success depends firstly on the survival of the embryos and larvae (López et al., 1998) and, consequently, any decrease in embryos and larval survival or delay in development can reduce population long-term viability (Morgan, 1995). Many benthic marine organisms rely on the dispersal of propagules to colonize new surfaces and recruit to new populations. Supply and settlement of planktonic larvae are therefore determinants of benthic invertebrate population dynamics (e.g., Menge 2000).

Because adult barnacles are sessile, finding a good spot to settle (the location it will reside in for the rest of its life) is very critical. Barnacle species, using chemo- and neuro-receptors, often exhibit gregariousness when larval settlement occurs (Dreano et al. 2006a). Gregariousness means that newly-arriving larvae settle closer to existing individual conspecifics than would be expected by chance. This has an important influence in space-limited systems, where the spatial pattern of sessile organisms can change from randomly to uniformly distributed populations as new colonisers occupy available space (Wetthey, 1984).

Barnacle post-settlement mortality prevents many settlers from joining the local reproductive pool (reviewed by Gosselin and Qian 1997). Once a barnacle settles on the shore, it must deal with a suite of physical (e.g., food availability, temperature, salinity, etc) and biological factors (e.g., competition and predation) in order to survive, grow, and reproduce. Regardless of which factors or combination of factors play a role in determining recruitments, barnacles must reach the adult and reproductive stage to maintain the population. Variations in recruitment may influence the population dynamics and ultimately, community structure (Carroll 1996).

General discussion

Temperature and salinity effects on barnacle performance

Temperature and salinity are known to regulate survival, duration of larval development and recruitment in many marine invertebrates (reviewed by Pechenik, 1987). Temperature can prolong or shorten the naupliar duration of barnacle larvae. This may be a direct consequence of the increase in metabolic rate at higher temperatures (to a certain amount of increased temperature, Neuheimer et al. 2011) and low assimilation of energy at lower temperatures (Anil et al. 2001). Low temperature is known to reduce the feeding efficiency (Scheltema and Williams 1982) and the filtration rate via increasing the water viscosity (Riisgård and Larsen 2007; Melzner et al., 2011) in barnacles and other marine suspension feeders. Therefore, low temperature, via one or either ways, can reduce food consumption resulting in less energy intake. Consequently, a low energy budget may result in the low rate of transformation from nauplius to cyprid and high mortality under cold conditions. In barnacles, traits such as cirral activity, food intake and assimilation are strongly correlated with temperature (Anderson 1994). A rise in seawater temperature causes an increase in metabolic rates and cirral activity (i.e. feeding efficiency) of barnacles (Crisp and Bourget 1985; Skinner et al. 2007), resulting in high growth rates as long as stored energy reserves and/or food availability meet the required additional energy expenditure (Crisp and Bourget 1985; Anderson 1994). On the other hand, low temperature via reduced food consumption and less energy intake may result in reduced shell/body mass ratio. Since more energy costs are associated with calcification in cold water (Melzner et al. 2011), it is likely that under a low energy budget, energy is allocated to more vital processes such as somatic mass maintenance instead of shell conservation. Salinity stress has been also shown to affect the growth and survival of a wide range of marine invertebrate larvae and, in turn, subsequent metamorphic competence and juvenile survival (see Pechenik, 1987; Pechenik 2006 for reviews). However, the ability to exist at varying salinity depends on different adaptations (e.g., osmoregulation). Various Balanomorph species including; *Amphibalanus improvisus*, *A. amphitrite*, *Balanus balanus*, *B. balanoides*, *B. crenatus*, and *B. glandula* all regulate hyperosmotically (Newman, 1964; Foster, 1970; Fyhn, 1976). These species will osmoregulate when in seawater less than 500mOsm and osmoconform in seawater greater than 500mOsm. Blood osmolality is always kept hyperosmotic to that of seawater, even when osmoconforming, which is necessary for urine production and excretion of nitrogenous wastes.

General discussion

Barnacle larval stages are however generally more sensitive than adults to changes in salinity (Qiu and Qian, 1999). Therefore, suboptimal salinity conditions may cause osmotic stress and reduce energy budget resulting in lower larval performance. Barnacle cyprids may be more resistant to osmoregulatory stress than nauplii and thus may be unable to successfully metamorphose if exposed to lower salinity than the salinity they are adapted to (Tindle et al. 2004).

Findings and interpretation of different early life stages

In the field, temperature and salinity change simultaneously and, thus, may jointly affect the development of barnacle larvae (Harms, 1986; Anil et al., 1995). The interactive effect of putative stresses such as temperature and salinity on larval performance and survival of barnacles has been subject of only a few studies (Harms 1986; Anil et al. 1995; Qiu and Qian 1999; Thiagarajan et al. 2003) and this study is the first one investigating multiple stresses (temperature and salinity) relevant for global change impact on early life stages of *A. improvisus* in the Baltic Sea specially. In this study, naupliar duration of both populations of barnacle was impacted by temperature only and was shortened by increasing temperature. This was similar with previous findings on barnacle species well (e.g., *Amphibalanus amphitrite*: Anil et al. 1995, 2001; Qiu and Qian 1999, *B. trigonus*: Thiagarajan et al. 2003, *A. eburneus*: Scheltema and Williams 1982, *Elminius modestus*: Harms 1984, 1986, and *Semibalanus balanoides*: Harms 1984). Independency of naupliar duration to salinity may reflect the euryhalinity of this species (Fyhn 1976; Gohad et al. 2009).

Naupliar duration as a response to these stress factors is ecologically crucial. The length of larval life in organisms with a planktonic dispersal phase determines their dispersal abilities, e.g. the potential to be widely distributed by water currents. On the other hand, planktonic mortality is high and increasing larval phase enhances the risk of predation. Moreover, the chances of transport to unsuitable locations could be enhanced as well (Miner, 2005; Elkin and Marshall, 2007). Furthermore, many ecological processes are synchronized. For example, barnacle larvae are released in the plankton to coincide with the spring bloom. If the development of nauplii larvae is delayed as suggested by Findlay et al. (2009), synchrony with

General discussion

the algal bloom will not be achieved. The timing of recruitment is also crucial and late settlers often experience the lowest survival (Kendall et al., 1985).

Nauplii to cyprids metamorphosis success in this study was very low at low temperature and increased with warming. Here, low success is because of enhanced naupliar duration, high mortality and low cyprid quality. In this treatment, despite the fact that mortality was high, there was still 5-20% survivors but almost no settled. Anil et al. (1995) reported that during the winter *A. amphitrite* larvae were present in the water but failed to develop successfully because of lower water temperature and food supply. Salinity was another important factor that influenced metamorphosis success in our study. High salinity (30 psu) resulted in lowest rate of metamorphosis at all temperature levels in both populations. Low salinity (5 psu) and intermediate salinity (15 psu) caused highest successful metamorphosis for Kiel and Tjärnö population, respectively. This was surprising because larvae of both populations metamorphosed greater at a lower salinity than their local salinity condition at their peak settlement season (15 and 30 psu, respectively). Larval survival of both populations followed the same pattern as metamorphosis. Settlement of cyprid larvae of Kiel population however, was best at home condition (20°C/15 psu). In contrast, cyprids from Tjärnö population settled least at home salinity (30 psu) and most at intermediate salinity (15 psu). The response of marine invertebrate developmental stages to perturbations often reflects the environmental history of the maternal parent (Hamdoun and Epel 2007). This is however, not always the case as we observed deviation from this hypothesis. If the barnacle populations we investigated were optimally adapted to the local conditions, we would have expected best performance (e.g., survival and settlement) among those individuals that experienced conditions prevailing during peak settlement. Low performance of barnacle larvae at their local conditions implies that these populations are apparently maladapted to their habitat.

The effects of stress experienced in one life-stage can influence the performance of the subsequent stages of barnacles (Qiu and Qian 1999). This in fact, has been however, relatively neglected and understudied so far. In this study, these carry-over effects were evaluated by comparing the settlement of pre-stressed and non-stressed (naive) larvae from the Kiel population of *A. improvisus*. On average, 20% more of naive cyprids settled than of pretreated

General discussion

cyprids. Osmotic stress experienced as found by Qiu and Qian (1999), in one life stage of a barnacle can be passed over to the next life stage. Corresponding to this, we saw that settlement of cyprids with a stress history (at the nauplius stage) was less than 15% of the initial number of nauplii, while in naive larvae settlement and survival were much higher (relative to the initial number of cyprids, not nauplii). Such carryover effects could potentially work in two directions: either stress-induced selective mortality at the naupliar stages results in a preselection for stress resistance in the (surviving) cyprids population (composed of the more resistance genotypes) or stress conditions throughout the naupliar stages lead to unfit cyprids, which are low in energy, have a retarded development and exhibit higher mortality. Since we did not observe strong carryover effect, we assume that the two opposing effects compensated each other.

Post-larval mortality showed to be high during the first few days after settlement, i.e. when the post-larvae were first exposed to the treatments (approximately 90% of the total mortality). Apparently, the more sensitive genotypes had succumbed to the treatment(s) while the surviving genotypes were either pre-adapted to the conditions or able to acclimatize in time. The findings of very high post-larval mortality in this study are consistent with previous reports on barnacles (e.g., Gosselin and Qian 1996; Jarrett 2000; Pineda et al. 2006; Gosselin and Jones 2010). Mortality was highest at 5 psu being especially so when combined to low temperature (12°C). Barnacle post-larvae utilize energy reserves from the nauplii phase to metamorphose and grow until their digestive system is completely developed after some days (Anderson 1994). A high proportion (38-58%) of these energy reserves is used for metamorphosis (Thiyagarajan et al. 2003). Exposing the post-larvae to stresses such as low temperature or sub-optimal salinity will require energy expenditure for stress compensation (Anger 2003), which is no longer available for metamorphosis. Stress driven post-larval mortality can therefore, constitute a bottleneck for recruitment (Gosselin and Qian 1996) which can potentially have a substantial effect on population dynamics (Jenkins et al. 2008), population size and distribution as well as community structure (Gosselin and Qian 1997; Moran 1999; Osman and Whitlatch 2004). Pineda et al. (2006) found that only a few percent of barnacles survived to reproductive adults and those barnacles settled during a narrow recruitment window. Thus, deviations of environmental factors from species-specific optima during recruitment may lead to, high post-settlement mortality and prevent many settlers from joining the local reproductive pool. If our

General discussion

earlier speculation, i.e. that stress driven mortality eliminates a certain set of genotypes from the group of recruits, holds true, environmental stress during the period of peak settlement may affect the genetic diversity and composition of a population and, thus, its sensitivity to further stress.

Mean growth in basal diameter increment of juveniles ranged from 0.05 mm/day at 12°C and 5 psu to 0.15 mm/day at 20 and 28°C regardless of salinity. Thus, an increase of temperature by 8°C (from 12 to 20°C) at 5 psu caused an increase in growth (basal diameter) by 300%. This corroborates findings of previous studies (Qiu and Qian 1999; Thiyagarajan et al. 2003). Growth was least at low salinity (5 psu) in this study. This was similar to the results of Berger et al. (2006) who observed a cessation of cirral activity in the juvenile barnacle *Balanus glandula* at low salinities (below 10 psu). Decreased feeding time as a consequence of retracting the feeding apparatus and potential starvation, in concert with elevated levels of osmotic stress, can probably explain the observed low growth rate at low salinity in this study.

Growth rates play a critical ecological role in determining vulnerability to predation as well as in the outcome of competitive community interactions (Miller and Carefoot 1989; Gosselin and Qian 1997; Osman and Whitlatch 2004; Urban 2007; Pechenik et al. 2010). In the present study, low temperature and low salinity stresses caused a reduction in juvenile growth. These detrimental effects on juveniles are ecologically important because slow juvenile growth results in inferior competitive abilities and could also contribute to mortality by prolonging the susceptibility of individuals to predation before attaining a size refuge (Miller and Carefoot 1989; Pechenik 1990). Predators with feeding structures that are effective at handling small post-larvae are less efficient at handling large juveniles (Gosselin and Qian 1997; Osman and Whitlatch 2004; Griffiths and Gosselin 2008). Thus, ontogenetic shifts in susceptibility to predators occur in part because post-larvae progressively grow larger and develop more resistant protective structures such as shells. In the Western Baltic, early juveniles are substantially decimated by the “bulldozing” activity of the snail *Littorina littorea*, larger individuals are preyed upon by the seastar *Asterias rubens* and the crab *Carcinus maenas* (Wahl and Hoppe 2002; Enderlein and Wahl 2004). For the snails and seastars prey size matters, for the crabs shell stability is more relevant (Enderlein and Wahl 2004, Appelhans et al. accepted). Refuge from

General discussion

these predators may be reached sooner at higher temperatures (as we observed in this study) and therefore makes it more difficult for their predators to handle or crush their prey (Gosselin 1997). In this study, at low temperature and low salinity (12°C/5 psu) shell materials contributed about 56% of total weight of barnacles while this portion at 28°C and 30 psu was about 86%. Shifting the body/shell ratio by environmental factors may therefore alter the barnacle's quality as prey and expose them to different set of predators. Reduced shell thickness at low salinities has been previously observed for the mussels (Almeda-Villela 1984; Kautsky et al. 1990).

Barnacle cyprids are specialized for settlement (attachment and metamorphosis; Thiyagarajan and Qian 2008) by exploring the substrates before settlement. Cyprids use both live (e.g. macroalgal thalli) and non-viable (rocks, pebbles, etc) substrates to settle. Bacterial biofilms associated with these substrates have an undisputed key role in this larval settlement (reviewed in Hadfield, 2011). Inhibitory effects of biofilms on larval settlement have been previously documented in many studies (e.g. Olivier *et al.*, 2000; Lau *et al.*, 2003; Dobretsov & Qian, 2006; Rao *et al.*, 2007; Ganesan *et al.*, 2010). In this study, Barnacle attachment was reduced by monospecies bacterial biofilms and natural microbial assemblages (originally isolated from macroalgal hosts) by 20% to 67%. Warming by 5 °C in this study resulted in a shift of community composition of microbial assemblage associated with *F. vesiculosus* surface and consequently caused an increase of barnacle settlement by 37%. Therefore, in the course of climate change, global warming may indirectly ease barnacle settlement by stress-driven shifts in epibiotic biofilms.

Significance of cumulative impacts of ontogenetic stages

The persistence and success of populations requires all ontogenetic stages be completed successfully and, due to their sensitivity to environmental stressors, developmental stages may be a population bottleneck in a changing ocean (Byrne 2011). Recruitment in marine species with pelagic larvae is sensitive to variation in larval supply, settlement rates, and early settler mortality thus, the relative proportion of these processes and the dynamic of larval stages may regulate the dynamics of adults (Bertness et al. 1992). Developmental failure, regardless of stage will cause recruitment failure with negative flow on effects for marine populations and ecosystems (Przeslawski et al., 2008; Brierley and Kingsford, 2009; Hofmann et al., 2010).

General discussion

Relatively small perturbations in recruitment can translate to large alterations of adult populations (Uthicke et al., 2009). The impacts of environmental stressors and climate change on adults means little if development is compromised (Byrne 2011). In barnacles, recruitment success substantially affects the composition and diversity of benthic communities (Dürr and Wahl 2004) and, thus, ultimately their ecosystem services.

Overall impact of stress combinations

In this study, *A. improvisus* larval mortality (nauplii + cyprids) from Kiel population was about 90%. Of the post-larvae an additional 55% died. Thus, cumulative mortality of early life stages (larvae + early juvenile) reached approximately 95%. Larval mortality of the Tjärnö population was about 71%. The post-larval mortality of Tjärnö barnacles was not investigated. Assuming similar values as found in Kiel, cumulative mortality could reach 85% in the Tjärnö population. Each stage of a species' life cycle is exposed to a variety of physical, chemical and biological conditions and processes which affect the duration of each stage and the probability that a given individual will make the transition to the next stage. Therefore, intra- and interspecific competition for space or food, predation, other physiological stress (e.g., acidification or oxygen concentrations), and disease or parasites (Eckman 1996) may contribute to the above mentioned mortality of barnacles. Different life stages have different optima and if the environmental factors shift to suboptimal conditions, larval survival and final recruitment will be probably even less and consequently can reduce population long-term viability and influence the whole community (Morgan, 1995).

Possible responses of the barnacles to climate change

A temperature increase of more than 0.7°C during the last century has been documented for the Baltic Sea and a further warming by up to 6°C is expected until 2100 (BACC 2008). Simultaneously, a decrease in salinity (up to -45%) due to higher precipitation is predicted (BACC 2008). For Kiel Bight in the Western Baltic and during the season of peak barnacle settlement, this would correspond to a warming from 20 to 26°C and a desalination from 15 to a still unknown value between 14 and 8 psu. For the Swedish west coast temperature would shift from 20- 25°C to 23-29°C and a desalination from 22-30 psu to a still unknown value between 13

General discussion

and 18 psu (“up to -45%”, BACC 2008; Meier et al. 2011). If the Kiel population of *A. improvisus* does not adapt to these changes, both factors would slightly accelerate larval development (if food is not limiting).

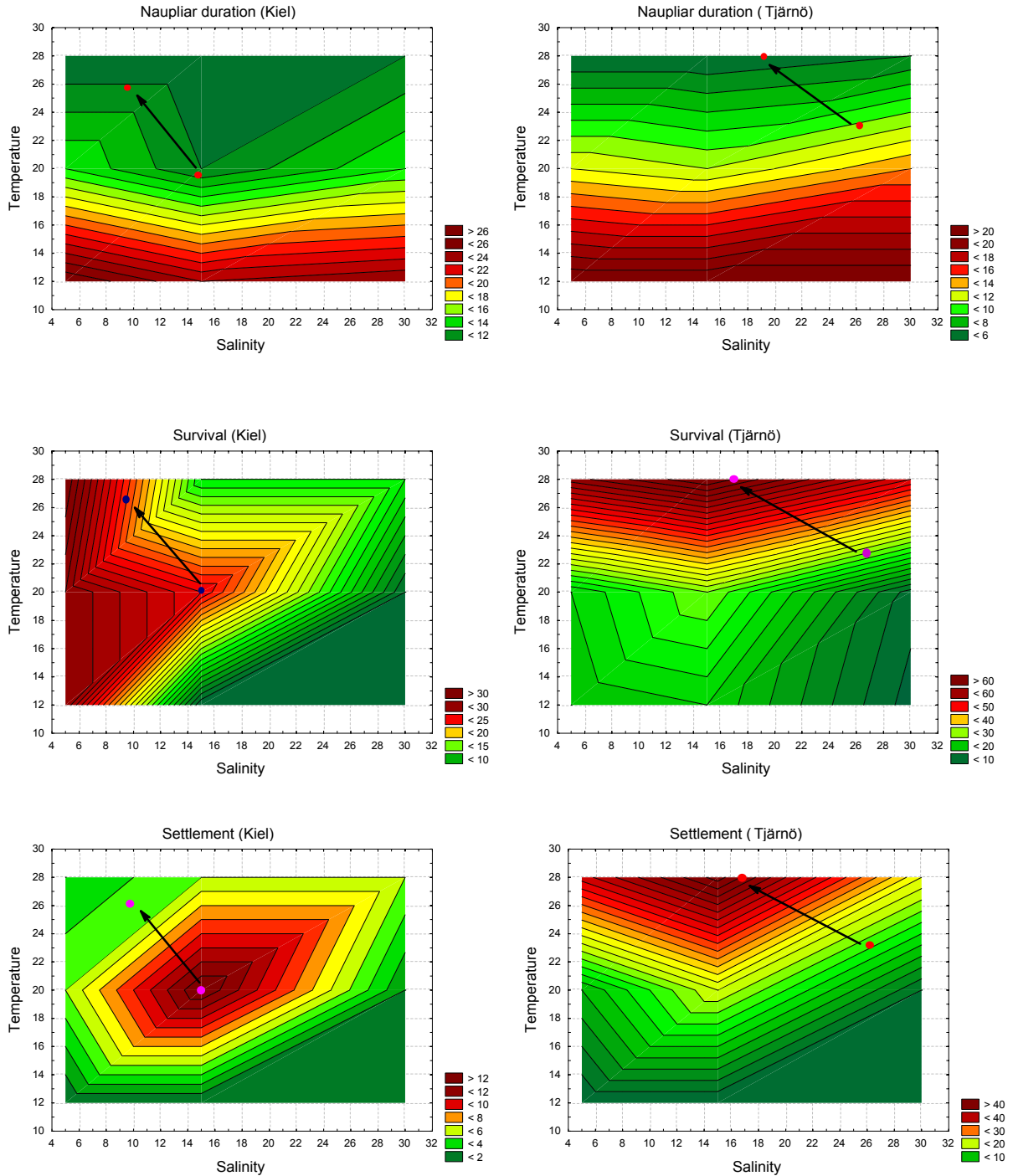


Fig. 1. Response of *A. improvisus* to climate change prediction in the Baltic Sea (warming and desalination).

General discussion

Survival might not change as suggested by the rates found for 20°C/15 psu (25%) and 28°C/5 psu (37%), respectively. Furthermore, our results suggest that settlement success of cyprids might decrease by ~75% (Fig. 1). Thus, the beneficial effects of climate change on larval development may be overruled by the detrimental effect on settlement success. Both warming and desalination may however, favor *A. improvisus* from Tjärnö population. This thesis shows that predicted climate change would lead to an acceleration of larval development by more than two folds which could reduce larval mortality due to external factors such as food limitation or predation. Likewise, survival and settlement might increase by two folds (Fig. 1). Therefore, warming and desalination would additively or synergistically increase the performance of this population dramatically. Warming of 5°C indirectly via shift in epibiotic biofilms may also facilitate settlement of barnacles on macroalgae such as *Fucus vesiculosus*. Other stresses associated with climate change such as ocean acidification and increased hypoxia may, however, add additional pressure on calcifying sessile species such as barnacles.

Consequences for the ecosystem

Climate change can profoundly affect the abundance and distribution of species through both the direct effects of environmental factors on survival, and also by altering important negative interactions through shifting competitive balances and essentially weakening (or strengthening) competitors or predators (Poloczanska et al. 2008). In Kiel Fjord during summer, substrata often become monopolized by mussels or barnacles (Reusch and Chapman, 1997, Dürr and Wahl 2004). These species are strong competitors for space. Shore crab *Carcinus maenas* and the starfish *Asterias rubens* are the main predators in this system (Dürr and Wahl 2004). If *A. improvisus* become less abundant during the course of climate change, it is expected that mussels will be more dominant still because of reduced competition. As a consequence of this competitive asymmetry, diversity will decrease if monopolization of mussels is not controlled by predation. Therefore, the dominance of mussels will depend on how its predators are impacted by climate change. Unlike the Kiel population of barnacle, Tjärnö population of *A. improvisus* will perform better and make larger population as a consequence of climate change. Increased abundance of barnacles can, in turn, outcompete its competitors (mussels) in this habitat. Here

General discussion

again, by exclusion or weakening of one of the competitors, species richness and community diversity will reduce unless the predation damps barnacle monoculture.

Conclusion & looking ahead

Conclusion

After this study, we have now a better understanding of how early developmental stages of *A. improvisus* are sensitive to selected environmental conditions. This study showed that mortality of this barnacle species is high during early life stages. Mortality cumulates during ontogenetic stages and can constitute a bottleneck for recruitment which can potentially have substantial effects on population dynamics, population size and distribution as well as community structure. This study further indicates that sensitivity of this species to temperature and salinity differs between life stages. When comparing the two populations, this sensitivity is also different between the two sites. The interaction between stresses, life stage sensitivity and population may therefore enhance or buffer climate change effects. The interactions of warming and desalination with other environmental variables expected to shift (pCO₂, pH, UV, nutrients etc) should be considered before any prediction about climate change effects on species performance can be made.

Looking ahead

The prediction of climate change effects on early life stages of barnacle in this study, disregard the fact that *A. improvisus* has the potential to adapt to climate change to some, yet unexplored, extent, and it ignores further potential interactions of future salinity and temperature regimes with other changing environmental variables. Therefore, any prediction about climate change effects on species performance remains uncertain as long as we do not know about the compound effect of the whole suite of shifting environmental factors (temperature, salinity, nutrients, pCO₂, pH, UV, stratification, predation, parasitism, etc.) and the eco-evolutive potential, at all of their ontogenetic stages, of the affected species to adapt to these shifts (e.g., Reusch and Wood 2007; Wahl et al. 2011). Future studies on marine invertebrates under climate change scenarios should therefore focus more on the interactive effects of different environmental stressors considering all ontogenetic stages (including adults).

Climate change can also indirectly alter negative interactions through shifting competitive balances and essentially removing dominant competitors or predators (Poloczanska et al. 2008). Hence, the complex, indirect effects of climate change need to be taken into account

Conclusion & looking ahead

if we are to accurately forecast the long-term effects of global warming. To do so, key species (e.g., dominant competitors) should be also included in the experimental designs.

Given the global distribution of *A. improvisus* (Weidema 2000), different response of populations of the same species to climate change (Chapter II) and the likelihood of local adaptation (Sanford and Kelly 2011) – especially in different salinity regimes – there remains a need for repeated assessment of global change impacts on multiple separate populations of the same species. Thus, along with studies on single population, multiple populations should be more often examined. Only then can local results be extrapolated to regional or global scales.

Last but not least, the transition between the cyprid and the adult stage (settlement, metamorphosis and early juvenile growth) is recognised as a critical period in the life cycle of barnacles and can constitute a bottleneck for recruitment because of high mortality (Gosselin and Qian 1996). Here, there may be a preselection for the more resistance genotypes. However, genetic approaches were not used in this study. Therefore, it would be interesting to see whether selective mortality results in preselection of genotypes and consequently more stress resistance.

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel und Quellen verwendet habe. Ich habe bisher keinen anderen Promotionversuch unternommen, und diese Arbeit hat weder ganz noch teilweise im Rahmen eines anderen Prüfungsverfahrens vorgelegen. Bei der Erstellung dieser Abhandlung habe ich mich an die Regeln guter wissenschaftlicher Praxis gehalten.

Kiel, den
