Thesis for the degree of Doctor of Philosophy

From Geography to Genes:

Evolutionary Perspectives on Salinity Tolerance in the Brackish Water Barnacle *Balanus improvisus*

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Cover illustration by Anna-Lisa Wrange

To my family and friends
who have supported me
through these years

ABSTRACT

How species respond to changes in their environment is a fundamental question in biology. This has become an increasingly important issue as anthropogenic effects of climate change and biological invasions have major impacts on marine ecosystems worldwide. In this thesis I investigated the role of salinity tolerance from an evolutionary perspective, using a wide range of techniques, spanning from population genetics and common-garden experiments to characterizing potential genes involved in osmoregulation in barnacles. I used the acorn barnacle species *Balanus* (*Amphibalanus*) *improvisus*, which displays a remarkably broad salinity tolerance, to investigate how this trait has influenced the species' potential to establish in new environments, and respond to projected near-future salinity reductions in coastal seas. I also examined physiological and molecular mechanisms that may be involved in osmoregulation in *B. improvisus*. I further analysed population genetic structure using microsatellites and mitochondrial DNA, and related the results to anthropogenic and natural dispersal dynamics on both global and regional (Baltic Sea) scales. I found high genetic diversity in most populations, with many shared haplotypes between distant populations. This supports the hypothesis that maritime shipping is an important vector for the dispersal of the cosmopolitan species *B. improvisus*. Nonetheless, natural larval dispersal is also important on smaller geographical scales, such as within the Baltic Sea. Marked genetic differentiation between northern and southern Baltic Sea populations raises the question whether there is restricted gene flow within the Baltic Sea, creating potential for local adaptations to evolve. To investigate the extent to which the broad distribution of *B. improvisus* along the Baltic Sea salinity gradient is explained by local adaptation *versus* physiological plasticity, I performed a common-garden experiment in which multiple populations were exposed to different salinities and multiple fitness-related phenotypic traits were recorded. The experiment confirmed that phenotypic plasticity, rather than local adaptation, explained the broad distribution of the species along the salinity gradient. Interestingly, all populations of *B. improvisus* performed best at low and intermediate salinities in many fitness-related traits (survival, growth and reproduction), although other traits (e.g. shell strength an juvenile growth) indicated higher costs associated with low salinity. A candidate gene approach was used to investigate the molecular basis of broad salinity tolerance in *B. improvisus* by characterizing the Na^+/K^+ ATPase (NAK) of *B. improvisus* – an ion transporter commonly involved in active osmoregulation in many species. We identified two main gene variants in *B. improvisus* (NAK1 and NAK2), and found that NAK1 mRNA existed in two isoforms that were differentially expressed in different life stages and adult tissues, suggesting an active role in osmoregulation. Lastly, I summarise current knowledge about salinity tolerance in barnacles and outline new research directions to further our understanding of the physiological and molecular mechanisms involved in salinity tolerance in barnacles.

Key words: *environmental change, salinity, evolution, phenotypic plasticity, marine invertebrates, population genetics, functional genomics, barnacles, osmoregulation*

POPULÄRVETENSKAPLIG SAMMANFATTNING

Hur arter kan anpassa sig till förändringar i sin livsmiljö är en grundläggande fråga inom biologi. Det har också blivit en allt viktigare fråga i samband med de stora miljöförändringar som vi står inför idag, där bl.a. klimatförändringar och främmande arter hotar att förändra den marina miljön dramatiskt. Att förstå vilka ekologiska och evolutionära processer som är viktiga för arters framtida överlevnad kan hjälpa oss att begripa hur ekosystemen kommer att förändras och vilka konsekvenser det kan ha för vårt nyttjande av havets resurser. I denna avhandling har jag använt den släta havstulpanen *Balanus (Amphibalanus) improvisus* som uppvisar en anmärkningsvärt bred salthaltstolerans, för att undersöka hur denna egenskap har påverkat artens möjligheter att etablera sig i nya miljöer och tåla framtida miljöförändringar, inklusive potentiellt lägre salthalter i kustnära hav pga. ökad nederbörd. Den släta havstulpanen förekommer framförallt i utsötade miljöer, men växer även gärna på båtar i saltvatten. Artens förmåga att fästa sig på båtskrov har gjort att den spriditts världen över, och det är en av de få arter som klarar av att leva längs hela salthaltsgradienten från Nordsjön till Bottenhavet i Östersjön. Jag använde genetiska markörer för att studera populationsstruktur och spridningsmönster hos den släta havstulpanen och fann hög populationsgenetisk diversitet, men också likheter över stora geografiska avstånd, vilket bekräftar fartygstrafikens betydelse för artens nuvarande utbredning. Min forskning visar även att larvspridning har betydelse för artens spridning, fast på mindre geografiska skalor, som till exempel i Östersjön. Genetisk differentiering mellan norra och södra Östersjön tyder på ett begränsat genflöde inom Östersjön, vilket är en förutsättning för lokal anpassning att utvecklas. Jag använde laboratoriebaserade transplantationsexperiment (s.k. "common garden") för att testa om utbredning i Östersjön kan förklaras av lokal genetisk anpassning eller av fenotypisk plasticitet (dvs. förmågan att ändra sin fenotyp beroende på miljön) och fann att fenotypisk plasticitet, snarare än lokal anpassning, förklarade utbredningen längs salhaltsgradienten. Havstulpanerna presterade överlag bättre i låg salthalt (≤15 ‰), vilket bekräftar att arten är speciellt anpassade till att leva i brackvattenmiljöer. Vidare undersökte jag vilka fysiologiska och molekylära mekanismer som kan förklara bred salthaltstolerans hos *B. improvisus*. Mer specifikt studerade jag en gen som kodar för natriumkalium-pumpen (Na^+/K^+ ATPase) och som är inblandad i osmoreglering hos många arter. Vi fann flera varianter av genen, varav vissa former utrycktes mer i låg salthalt, vilket tyder på inblandning i osmoreglering även hos havstulpaner. Skillnader i genuttryck observerades både mellan vuxna havstulpaner och larver, samt mellan olika vävnader hos vuxna. Slutligen sammanfattar jag aktuell kunskap om salthalttolerans hos olika havstulpanarter och diskuterar andra potentiella fysiologiska och molekylära mekanismer som kan vara inblandade, men som kräver vidare studier. Resultaten från denna avhandling bidrar med ny kunskap om betydelsen av bred tolerans för arters förmåga att hantera förändringar i miljön, utifrån ett evolutionärt perspektiv som sträcker sig från global nivå till gennivå.

LIST OF PAPERS

This thesis is a summary of the following papers, which will be referred to in the text by the roman numbers below:

Paper I:
Wrange, A-L., Charrier, G., Thonig, A., Alm-Rosenblad, M., Blomberg, A., Havenhand, J., Jonsson, P. R., André, C. (*manuscript*). The story of a hitchhiker: population genetic patterns in the invasive barnacle *Balanus* (*Amphibalanus*) *improvisus* Darwin 1854

Paper II:
Wrange, A-L., André, C., Lundh, T., Lind, U., Blomberg, A., Jonsson, P.R., Havenhand, J.N. (*manuscript, submitted*) Importance of plasticity and local adaptation for coping with changing salinity in coastal areas: a test case with barnacles in the Baltic Sea.

Paper III:
Lind U, Rosenblad MA, Wrange AL, Sundell KS, Jonsson PR, Andre C, Havenhand J, Blomberg A (2013). Molecular characterization of the alpha-subunit of Na^+/K^+ ATPase from the euryhaline barnacle *Balanus improvisus* reveals multiple genes and differential expression of alternative splice variants. PLoS ONE 8(10): e77069. doi:10.1371/journal.pone.0077069

Paper IV:
Sundell, K., Wrange, A-L., Jonsson, P.R., Blomberg, A. (*manuscript*) Osmoregulation in barnacles: an evolutionary perspective of potential mechanisms and future research directions

TABLE OF CONTENTS

PAPER I.

PAPER II.

PAPER III.

PAPER IV.

INTRODUCTION

Coping with environmental change

A fundamental question in evolutionary biology is how organisms respond to changes in their environment. This question has become even more critical in light of recent anthropogenic changes to the marine environment, such as global warming, ocean acidification, reduced salinity in coastal areas (due to increased precipitation), pollution and biological invasions (Grosholz, 2002; Byrne et al. 2011; Poloczanska et al. 2013). These changes introduce new challenges for marine organisms, and can potentially result in drastic changes at both local and ecosystem levels (Philippart et al. 2001; Widdicombe and Spicer 2008).

Marine organisms living in shallow coastal areas regularly experience substantial fluctuations in salinity, temperature and pH (Mann 2000). To cope with this environmental heterogeneity on short time scales (hours, days, weeks), organisms have evolved different strategies (including behavioural and/or physiological responses) that provide fitness benefits in their specific environment. In more stable environments, specialists are often favoured over generalists whereas in variable environments, generalists that have the ability to cope with frequent changes will play a more important role (Tienderen 1991; Pigliucci 2001).

Phenotypic plasticity and local adaptation

When facing large-scale, long-term environmental change (months, years), organisms can respond in different ways. Mobile species may respond by migrating to new areas, resulting in distribution range shifts (Parmesan, 2006). Other organisms (especially sessile species), however, adopt alternate strategies to cope with environmental change, which may include phenotypic plasticity and/or local adaptation. Phenotypic plasticity is the ability of a single genotype to alter its phenotype in response to temporal or spatial differences in the environment (Pigliucci 2001; DeWitt and Scheiner 2004; West-Eberhard 2005). Plastic responses can include both morphological and physiological changes as well as shifts in life history and behaviour (Pigliucci 2001). Local adaptation, on the other hand, is the result of divergent natural selection over several generations leading to genetic differentiation between populations (Kawecki and Ebert 2004). Although phenotypic plasticity in itself is not a direct genetic trait, the mechanisms that cause a plastic response may have evolved as a genetic adaptation to existing selection pressures (e.g. fluctuating environments) that favour plasticity over a fixed response (Pigliucci 2001; Ortells et al. 2005; Nussey et al. 2007; McCairns and Bernatchez 2010). Both phenotypic plasticity and local genetic adaptation may contribute to a species' ability to inhabit different environmental conditions. Phenotypic plasticity is usually considered costly (DeWitt et al., 1998; Releya, 2002), whereas local adaptation is considered to be slow, taking generations to respond to environmental change, and being constrained by high gene flow between populations (Waples & Gaggiotti, 2006; Kawecki 2008; Sotka 2012). Consequently, it has been suggested that in more stable environments, where high plasticity is not required, adaptations to a narrower range of

environmental conditions may be selected for, resulting in a loss of phenotypic plasticity (referred to as "genetic assimilation") (Via, 1993, Pigliucci et al., 2006, Lande, 2009).

Plasticity has also been proposed to accelerate evolution by allowing individuals to exploit new environments if they have the capacity to develop a new phenotype suitable for that new environment (Pigliucci 2001; Reed et al. 2011). However, although high levels of plasticity may increase a population's persistence, it can potentially also hinder long-term local adaptation to specific conditions, since changes in phenotype will compensate for environmental change to a certain extent (Price et al. 2003). Phenotypic plasticity has been suggested to play an important role as a response to future environmental change and has also been shown to influence the success in establishment of invasive species (Smith et al. 2009). The relative importance of plasticity and adaptation for coping with environmental change will depend on many factors including the rate and extent at which the environment changes, life history strategies, level of plasticity, dispersal ability and population size (Gienapp et al. 2008; Sotka 2012). Although there is mounting evidence that local adaptation may be common in the marine environment there are few studies on the relative role of evolutionary responses versus plasticity to changing salinity in marine invertebrates (Sotka 2012; Reusch 2014; but see Renborg et al. 2013).

Rapid evolution under environmental change
Traditionally, evolution has been considered a relatively slow process, where random mutations and natural selection drives adaptation (Futuyma 2009). However, there is increasing evidence suggesting that evolution in fact can occur over very short time scales (Hoffmann and Sgro 2011). Although a large number of species throughout geological history have failed to adapt to new environmental conditions, there are examples of organisms that have managed to adapt rapidly to environmental changes, including anthropogenic change, e.g. altered melanism in moths (Lees and Creed 1975) and evolution of PCB tolerance in fish (Wirgin et al. 2011). Most of what we know today about how adaptations evolve is based on laboratory studies and breeding experiments. It is often difficult to extrapolate results from such studies to natural environments since evolution in nature involves greater complexity and operates over multiple time scales. Several field-oriented studies have attempted to infer evolutionary adaptation from correlations between environmental and genetic data (e.g. Bekkevold et al. 2005; McCairns and Bernatchez 2008; DeFaveri et al. 2013). However, confirming that the observed patterns are indeed caused by adaptation requires experimental evaluation. For example, Barrett et al. (2011) studied cold tolerance in sticklebacks experimentally and found that after three generations freshwater sticklebacks were able to tolerate lower minimum temperatures than marine sticklebacks, and that this difference was heritable.

In marginal environments, where species often live at their tolerance limits and encounter extreme selection regimes, new adaptations may arise relatively quickly (Kawecki 2008). Ecologically marginal environments also often show low genetic diversity compared to more central populations, leaving less genetic variation

for selection to act upon (Eckert et al. 2008; Hardie and Hutchings 2010). The Baltic Sea is an interesting example of such a marginal environment where populations of several species show dramatic genetic differentiation from other nearby regions, as well as reduced overall genetic diversity (Johannesson and André 2006; Johannesson et al. 2011).

The Baltic Sea – a strong environmental gradient
One of the main characteristics of the Baltic Sea is the strong salinity gradient (Fig. 1) that determines the composition and distribution of species (Bonsdorff, 2006, Ojaveer et al., 2010). The Baltic Sea is one of the world's largest semi-enclosed brackish seas and reached its present brackish state about 8000 years ago (Björck, 1995, Zillén et al., 2008). Since then, the Baltic Sea has been colonised by organisms from both freshwater and marine environments (Leppäkoski and Olenin, 2000, Zillén et al., 2008). The sea surface salinity along this gradient ranges from < 3 PSU (practical salinity units) in the northern Bothnian Bay (Fig. 1) to approximately 30 PSU at the border to the North Sea (Bonsdorff, 2006). Limited water exchange with surrounding seas and almost no tidal flow make salinity conditions in the Baltic Sea relatively stable compared to many other coastal areas (Omstedt and Axell 2003, Leppäranta and Myrberg, 2009). Species diversity and within-species genetic diversity are markedly lower inside the brackish Baltic Sea compared to adjacent areas – characters that have been attributed to these marginal environmental conditions creating specific selection pressures and promoting the evolution of local adaptations (Johannesson and André, 2006, Johannesson et al., 2011). Less than 3% of the marine macro-invertebrates in the Skagerrak are able to survive the conditions in the Bothnian Bay, indicating how extreme this gradient is for organisms. In addition to natural salinity stress, the large catchment area of the Baltic Sea has resulted in major anthropogenic impact, including pollution and eutrophication, causing additional stress to the organisms living there (Ducrotoy and Elliott 2008). These unique environmental conditions and recent geological history provide a unique framework to study adaptation and other evolutionary processes such as speciation.

Previous work comparing populations within and outside the Baltic Sea has shown significant genetic differences as well as reduced overall genetic variation inside the Baltic for a wide range of genetic markers (Johannesson and André 2006). For example, several fish species including cod (*Gadus morhua*) and herring (*Clupea harengus*) show high levels of genetic differentiation between populations inside and outside the Baltic (Nielsen et al. 2003; Gaggiotti et al. 2009). Despite the clarity of these differences, the evolutionary mechanisms that produce these patterns are often not well understood. Many species in the Baltic Sea also display phenotypic differences compared to populations outside the Baltic, e.g. some fish species have different spawning periods or increased buoyancy of eggs in low salinity environments (Nissling and Vallin 1996), which further promotes reproductive isolation and genetic differentiation.
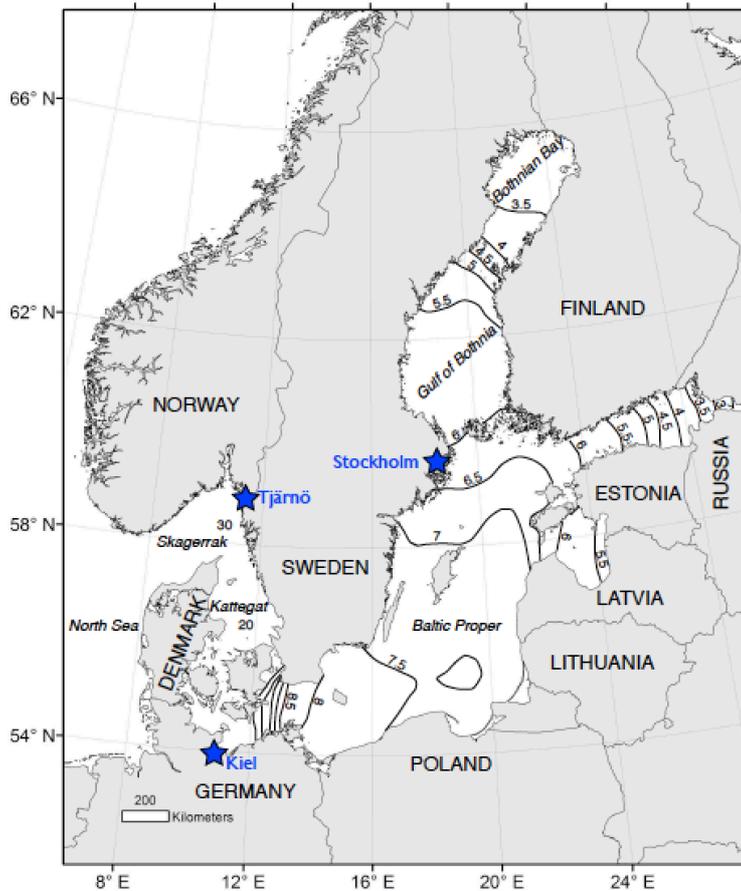
Figure. 1. A map of the Baltic Sea salinity gradient, ranging from <3 PSU in the northern Bothnian Bay, to full marine conditions (>30 PSU) in the Skagerrak and North Sea. The three stars represent sampling sites for **Paper II** in this thesis.

Coping with varying salinity

Salinity plays an important role in shaping coastal marine communities. Intertidal organisms in particular experience fluctuations in salinity e.g. during heavy rainfall and terrestrial run-off. Changes in salinity can have direct or indirect effects on metabolism, growth, reproduction, immune responses and osmotic balance in aquatic organisms (Kinne 1964; Qiu and Qian 1999; Normant and Lamprecht 2006; Lapucki and Nonnant 2008; Birrer et al. 2012). Marine invertebrates with well-defined and closable exoskeletons, such as gastropods, bivalves, and barnacles can display a behavioural escape response to rapid salinity change by closing their shells, thus maintaining a hyperosmotic inner environment (the mantle cavity) relative to a diluted external environment (Davenport 1976; Solokova et al. 2000). Long-term exposure to reduced salinities requires more sophisticated adaptations, however (Henry et al. 2012).

In response to changing salinity, marine organisms either osmoconform, i.e. allow the osmolality of the extracellular fluids to equal that of the surrounding medium, or they can osmoregulate and maintain their extracellular fluids at a constant osmolality relative to the external environment (Pequeux 1995). Osmoregulation can

be performed in several different ways, including active ion transport via ion pumps such as the Na$^+$/K$^+$ ATPase system, or by the production of osmolytes (amino acids and other small molecules that affect the osmotic balance in the cell). Extensive physiological and molecular research in both fish and invertebrates (in particular decapod crustaceans) has revealed a large diversity of physiological and cellular mechanisms involved in osmoregulation (Hendry et al. 2012; McNamara and Faria 2012; Edwards and Marshall 2013), but little is known about osmoregulatory mechanisms in barnacles (Fyhn, 1976; Sundell et al. in prep.).

Ecological and evolutionary implications of broad salinity tolerance
Colonisation of novel habitats: Broad environmental tolerance has been suggested as a key trait in invasive species (Richards et al. 2006; Muth and Pigliucci 2007; Smith et al. 2009; Capps et al. 2011). For example, Zerebecki and Sorte (2011) showed that the invasive ascidian *Diplosoma listerianum* had a broader thermal tolerance range than the native ascidian species *Distaplia occidentalis*, and suggested this reflected greater plasticity in stress-response in the former species.

Estuaries and shallow coastal bays are frequent sites of marine invasions, partly as a result of relatively high frequency of anthropogenic activities. Estuaries are also characterized by fluctuating environmental conditions, including salinity, which requires specific adaptations and therefore limits the diversity of species that can live there. For euryhaline species however, this means that there are potentially more ecological niches available, which facilitates colonisation (e.g. Paavola et al. 2005). Successful invasions in brackish environments are furthermore often associated with a lack of natural predators, competitors and pathogens, which are not able to cope with the harsh estuarine conditions (also known as the "enemy release hypothesis", Keane and Carwley 2002; Grey 2010).

Life history evolution: Osmotic stress can strongly affect larval survival and fitness (Anger 2003), and this selection pressure has resulted in evolution of particular life-history strategies and physiological adaptations in both adults and early developmental stages. In many species, early larval stages are more sensitive to environmental changes than adults (Charmantier 1998). For example, larvae of the green crab *Carcinus maenas* are more sensitive to low salinity (>20 PSU) compared to adults (>4 PSU) (Anger et al. 1998). This limited larval tolerance to low salinities has been suggested to be a limiting factor in the ability of *C. maenas* to become established in brackish habitats (Bravo et al. 2007). Several strategies have evolved to overcome problems that arise as a result of differential tolerance between life stages (Torres et al. 2011), e.g. by adult migration towards higher salinities before reproduction and later re-colonization of freshwater areas by juveniles (Anger 1991).

Coping with climate change: Future climate-driven changes in the marine environment include projection of decreased salinity in many coastal areas due to increased precipitation and enhanced freshwater run-off (Antonov et al., 2002; Boyer et al., 2005; Meier et al., 2012). Despite this, most marine climate change research to

date has focused on the effects of increasing temperatures and ocean acidification, rather than salinity (Doney et al., 2009; Byrne, 2011; Kroeker et al., 2013). However, future alterations in salinity may also pose major ecological challenges to organisms inhabiting intertidal and coastal areas (Wikner and Andersson, 2012). The ability to tolerate a broad range of salinities (i.e. being euryhaline) may play an important role in defining which species will dominate coastal communities in the future.

Role in divergent evolution: Euryhalinity has also been suggested to play an important role in population divergence (Schultz and McCormick 2013). Since euryhaline species can invade a broad range of habitats, e.g. along environmental gradients where different selection pressures operate, this may lead to the evolution of local adaptations and even speciation (Johannesson et al. 2011). In addition, brackish water bodies are often partially isolated from each other, resulting in restricted gene flow among estuaries and brackish seas, which further promotes population differentiation (Bilton et al. 2002). Lastly, selective tidal stream transport in estuaries provides potential for high retention of larvae within estuaries, which also restricts gene flow and promotes differentiation between populations that reside in such areas. Several examples of intraspecific divergence in morphology, physiology, behaviour and life history traits between populations in freshwater, brackish and marine environments have been documented (Bilton et al. 2002).

Why study barnacles?
There are many advantages of using barnacles to study ecological and evolutionary questions. Barnacles form a significant component of intertidal communities (Southward 1987). In this harsh environment they experience a wide range of environmental conditions that include varying temperature, salinity and wave action, all of which expose them to a wide range of selection pressures (Southward 1987). Barnacles are also small and easy to collect and culture in the laboratory, and have a relatively short life cycle with several pelagic larval stages. This makes it possible to study responses in several life stages and follow evolutionary processes in different populations over multiple generations. In addition, because they are major fouling organisms on ships worldwide, a substantial quantity of basic research has already been performed on barnacles, especially with regard to larval behaviour, physiological tolerance and settlement processes (e.g. Turpayeva and Simkina 1961; Berntsson and Jonsson 2003; Holm 2012). Collectively this provides useful background information for ecological and evolutionary studies.

The bay barnacle *Balanus (Amphibalanus) improvisus* (Darwin, 1854) (Fig. 2a) is a prominent filter feeder in many fouling communities (Berntsson and Jonsson 2003) occurring in shallow, tidal areas in both salty and brackish waters (Kennedy and DiCosimo, 1983; Leppäkoski et al., 2002; deRivera et al., 2011). The bay barnacle is the only barnacle species recorded from the Baltic Sea, but it is also found in other coastal areas and estuaries worldwide (Leppäkoski and Olenin 2000). It has been suggested that *B. improvisus* originates from the eastern North America, and it was first recorded in the Baltic Sea in the mid 1800's (Gislén 1950; Blom, 1965). *B.*

improvisus is a facultative, cross-fertilizing, hermaphrodite, in which self-fertilization has been suggested to occur (Furman and Yule 1990), although there are no confirmed records of selfing in this species. Like most barnacles, the species releases free-swimming nauplii larvae into the water, which go through six different moults (six nauplius stages and one non-feeding cypris stage) before settling (attaching and metamorphosing) onto a hard surface after 2-4 weeks (Fig. 2b) (Jones and Crisp, 1954; Thiyagarajan et al., 2003). Compared to most other barnacle species, *B. improvisus* can tolerate an extremely wide range of salinities, from <1 to 35 PSU; however, salinities below 3 PSU have been reported to have long-term negative impacts on fitness (Turpayeva and Simkina, 1961, Fyhn, 1976, Nasrolahi *et al.*, 2012). The molecular and physiological mechanisms behind this broad salinity tolerance are poorly known, although it has been suggested that *B. improvisus* can alternate between being a passive osmoconformer in high salinities (>15 PSU) and actively osmoregulate in salinities below 15 PSU (Fyhn, 1976).
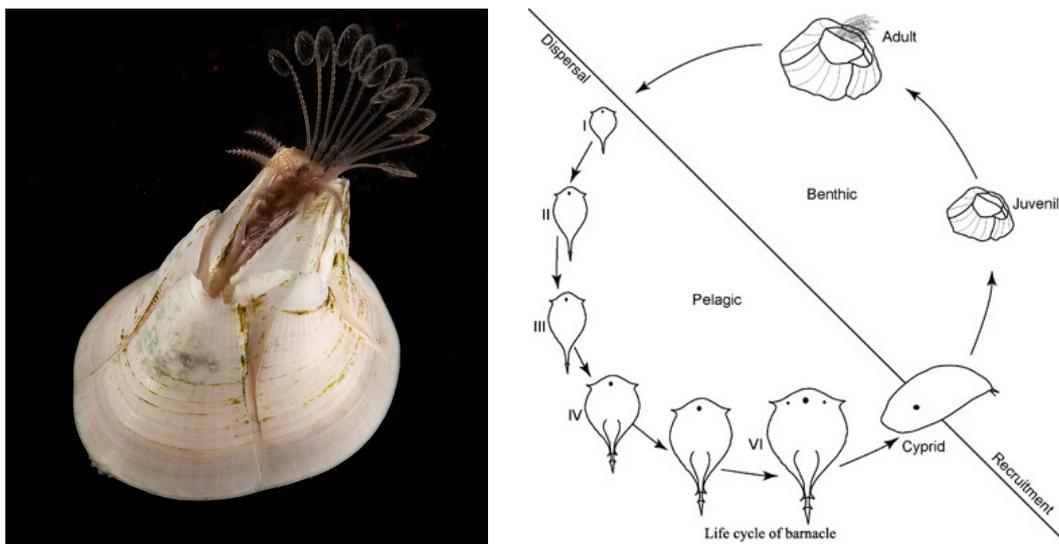

Figure 2. Left: The bay barnacle *Balanus improvisus* (photo: A-L Wrange). Right: The life cycle of *B. improvisus*, with seven different larval stages, including seven nauplii stages and one non-feeding cyprid stages (http://taxo4254.wikispaces.com/Balanus+Amphitrite).

AIM AND APPROACH

Understanding the ecological and evolutionary processes that determine how species cope in their natural environment, as well as how new adaptations arise when facing environmental change, is important for predicting how marine ecosystems will respond in the future. Climate change research to date has focused on the effects of increasing temperatures and ocean acidification (Doney et al. 2009; Byrne 2011; Kroeker et al. 2013). Future salinity shifts in coastal areas (Antonov et al. 2002; Boyer et al. 2005; Meier et al. 2012) may impose strong selection on species inhabiting these areas, which may lead to changes in species composition and the evolution of new adaptations. Yet this area of research has received relatively little attention to date.

Species with a broad geographical distribution are interesting models for studying evolution for several reasons. They inhabit a wide range of environments where they may be exposed to different environmental conditions/selection pressures, which may lead to local adaptations evolving. Furthermore, they often display broad tolerance to environmental factors – providing opportunities to investigate the role of plasticity versus local genetic adaptations, in relation to gene flow. The bay barnacle *B. improvisus* is one of barnacle species with broadest salinity tolerance in the world; however little is known about what physiological and molecular mechanisms that lie behind this broad salinity tolerance, or what consequences euryhalinity may have for its capacity to tolerate future environmental change, including the potential to evolve new adaptations. The species is also a common fouling organism on ships, resulting in high dispersal potential on larger scales than most other sessile species, which combined with free-swimming larvae provides additional opportunities for dispersal.

The overall aim of my PhD project was to address evolutionary questions such as: How do species cope with environmental change and what mechanisms are required? What is the role of phenotypic plasticity in the evolution of local adaptation? What role does broad tolerance play in successful establishment in new areas? More specifically, I focussed on questions related to broad salinity tolerance in barnacles using a combination of population genetic analyses, common-garden experiments and functional genomics.

Specific aims for each paper in the thesis:

Paper I: To characterise the population genetic structure (using microsatellites and mitochondrial DNA) and describe potential dispersal dynamics of the euryhaline barnacle *B. improvisus* on a global and regional scale (the Baltic Sea).

Paper II: To investigate whether the broad distribution of *B. improvisus* along the salinity gradient of the Baltic Sea can be explained by local adaptation or physiological plasticity, by measuring fitness-related phenotypic traits in multiple populations in a common-garden experiment.

Paper III: To use a candidate gene approach to investigate the molecular basis of broad salinity tolerance in *B. improvisus* by characterizing the Na$^+$K$^+$ ATPase of *B. improvisus* – an enzyme involved in active osmoregulation in many species.

Paper IV: To summarize current knowledge about salinity tolerance in barnacles and, based on known physiological and molecular mechanisms involved in osmoregulation in other invertebrates, propose possible osmoregulatory mechanisms in barnacles and outline new research directions to further our understanding of physiological and molecular mechanisms, as well as exploring ecological implications of osmoregulation in barnacles.
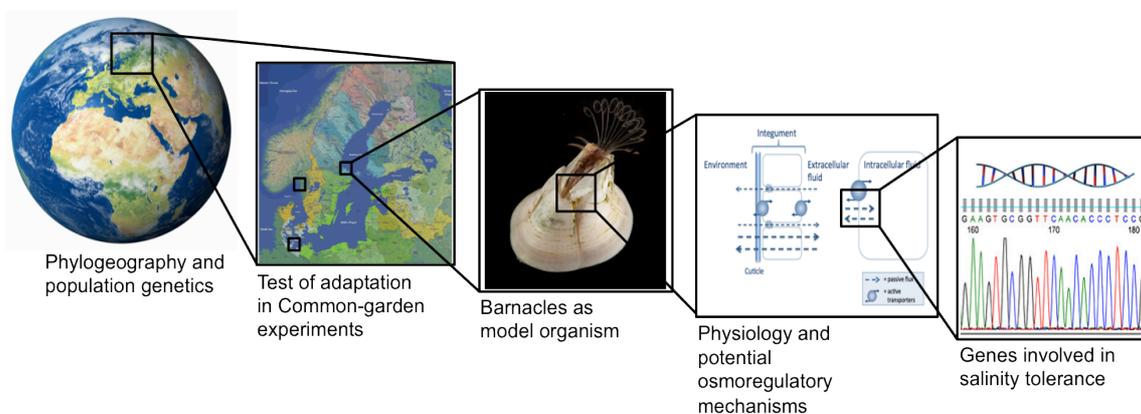

Figure 3. From geography to genes: an overview of the different approaches used in this thesis to study evolutionary questions related to salinity tolerance in barnacles.

METHODS

In this section I briefly present an overview of the methods used throughout the thesis. Detailed descriptions of the methods can be found in each specific paper.

Sampling and culturing barnacles

Barnacles were collected from the field by placing settling panels in the water during a few weeks in summer, onto which barnacle larvae settled (Fig. 4a). Panels were then brought back to the laboratory and the barnacles were maintained in flow-through or re-circulating systems (depending on origin) and fed with *Artemia* and microalgae. Barnacle cultures containing populations from both inside and outside the Baltic Sea (hence from different salinity regimes) were maintained during the course of my PhD. From these cultures, barnacle larvae were obtained (methods modified from Berntsson et al. 2000) (Fig. 4b) and used in the different studies (**Paper II**, **Paper III**).

16

Figure 4. Barnacles were collected in the field on Plexiglass panels and cultured in the laboratory (left), Cyprid larvae were obtained from the barnacle culture, and used in the experiments (right). Photo: A-L Wrange and J. Havenhand.

Genetic markers to study population structure

One of the main goals of the field of molecular ecology is to understand the genetic basis of ecologically important phenotypic variation. In recent years various molecular techniques have been developed, which provide new opportunities to investigate evolutionary processes that underlie population structure, local adaptation and evolutionary change (Feder and Mitchell-Olds 2003; Allendorf et al. 2010). In general, there is no single genetic marker type that is appropriate for all kinds of studies. In many cases, combining different markers increases the possibility to obtain more comprehensive and informative data (Narum et al. 2008; Dawson et al. 2010). Therefore, I combined two types of genetic markers to study the global population genetic structure of *B. improvisus* (**Paper I**).

Mitochondrial DNA

I used a 694 bp sequence of the mitochondrial gene COI (cytochrome oxidase I) to study global population genetic structure of *B. improvisus* (**Paper I**). Mitochondrial DNA (mtDNA) is commonly used in phylogeographic studies, in which sequence information from mitochondrial genes is combined with biogeography to explain historical events as well as present-day population structure and distribution (Avise 2009; Zakas et al. 2009). Mitochondrial DNA is typically maternally inherited and occurs in multiple copies in each cell separate from the nuclear DNA. The lack of recombination coupled with maternal inheritance leads to a four-fold increase in genetic drift, and subsequent population divergence, compared to nuclear DNA. However, mtDNA is a single molecule, which provides a limited view on evolutionary history and it has consequently been suggested that mtDNA markers should be combined with other genetic markers to avoid bias (Zhang and Hewitt 2003). Mitochondrial DNA has also been used to study mechanisms involved in biological invasions (Astanei et al. 2005; Darling et al. 2008).

Microsatellites

In line with the suggestions of Zhang & Hewitt (2003) I developed microsatellite markers to combine with the mtDNA to further clarify the population structure of *B. improvisus* (**Paper I**). Microsatellites are commonly used in population genetic studies due to their abundance in the genome, their high level of polymorphism, and their relatively simple scoring (Selkoe and Toonen 2006). Microsatellites consist of repeats of short sequence motifs (often 2-5 nucleotides, e g. CACACACACA), which are typically repeated 5 to 40 times. Different numbers of repeats results in different allele sizes, which are detected after PCR amplification by gel electrophoresis and sequencing (Selkoe and Toonen 2006). Microsatellites have been criticised because of problems with null alleles and variable mutational patterns introducing ambiguity to the analysis (Morin et al. 2004), but the high level of polymorphism in microsatellite loci also provides higher statistical power compared to many other markers (Larsson et al. 2007). Microsatellites have successfully been used to address a wide range of questions including identifying origins of biological invasions (Darling et al. 2008), parentage analysis (Dakin and Avise 2004), and identifying the evolutionary history of different populations (Mäkinen et al., 2006). However, microsatellites are less useful for inferring older divergence between populations or species, since the risk of homoplasy is higher than with mtDNA (Estoup et al. 2002).

Issues related to microsatellite development

I tested multiple approaches to obtain microsatellite markers for *B. improvisus* including selecting DNA sequences containing microsatellite repeats from EST (expressed sequence tag) libraries, 454- and Illumina-sequencing as well as contracting a commercial company to develop microsatellites and primers. Despite extensive efforts using rigorous primer design criteria (including re-designing primers multiple times) and testing a wide range of PCR conditions, only very few (4 of 108) easily scored polymorphic microsatellites were obtained (**Paper I**). Problems with selected microsatellites (that were later discarded) included: non-amplifying loci (due to possible mutations in primer sites), monomorphic loci (no variation between individuals), alleles of unexpected length (no clear repeat units), and loci with high homozygosity (possibly caused by null alleles or large allele dropout). Heterozygote deficiency is commonly observed in invertebrates (Dupont et al., 2003; Astanei et al., 2005) and the causes and consequences of this deficiency for interpretations of genetic structure have been widely discussed (DeWoody et al., 2006; Chapuis and Estoup, 2007; Brownlow et al., 2008). Some of the difficulties I encountered may be related to the extremely high genetic variation, both within and between individuals, which is currently being revealed in the preliminary genome sequencing data (unpublished; www.cemeb.science.gu.se/research/imago-marine-genome-projects).

Functional genomics – studying "genes in action"

Using "neutral" genetic markers such as microsatellites makes it possible to estimate historical events including migration/gene flow, bottlenecks and genetic drift, leading to increased differentiation between populations over time. However, they provide

little information about adaptations and selective forces acting on specific genes at present. Another approach to more directly study adaptation is to assess the adaptive genes in action ("candidate genes") that are directly involved in responding to e.g. environmental change (Hoffmann and Willi 2008). Candidate genes are selected based on *a priori* knowledge of the gene's biological and functional impact on a trait. Suitable candidate genes are generally selected based on known biological, physiological, or functional relevance to the trait/response in question. This approach is limited by its reliance on existing knowledge about the possible importance of the gene in a specific phenotypic response. In **Paper III** we chose to investigate the potential functional role of the Na^+/K^+ ATPase (NAK) in osmoregulation in *B. improvisus*, since it is known that this ion pump is involved in osmoregulation in both vertebrates and invertebrates (e.g. Lucu et al. 2003; McCormick et al. 2009; Henry et al. 2012). We identified the NAK gene in *B. improvisus* using NAK sequences from other species, characterising the sequence variation, and testing the expression of the gene using qPCR (quantitative PCR). The details of the methodology can be found in **Paper III**. The potential role and function of NAK in barnacles as well as other species, is further discussed in **Paper IV**.

Common-garden experiments
A common-garden experiment is a transplant experiment designed to test whether there is a genetic component to phenotypic differences observed between populations in contrast to phenotypic plasticity (i.e. testing if populations are locally adapted to their environment). Typically, populations from different environments or different geographical locations are grown together in a "common garden" (a common environment, which may include both native and reciprocal environments) and phenotypic traits (growth, survival, reproduction etc.) are measured (e.g. DeFaveri and Merilä 2013). In **Paper II**, we used common-garden experiments to test whether barnacle populations from the Baltic Sea were locally adapted to the low salinity in the Baltic compared to populations from outside the Baltic Sea (Fig. 5a).

Modelling dispersal patterns in barnacle populations
Most benthic marine invertebrates (including barnacles) disperse by pelagic larvae, which are transported for days or weeks over large distances before settling (Thorson 1950, Gilg and Hilbish 2003). For these species, the stability and persistence of populations depends strongly on the success of the larvae reaching a suitable environment in which they can settle and establish. Many factors can affect larval dispersal, including hydrodynamic processes (tides, currents etc.), oceanic boundaries, reproductive success in the source population, duration of the larval stage, planktonic mortality, as well as behaviour and tolerance of the larvae themselves (Sponaugle et al. 2002). Several different approaches have been developed to study larval dispersal and connectivity (Cowen et al. 2007). Oceanographic models (Fig. 5b) have provided insight in recognizing patterns of larval movement and dispersal (Hinrichsen et al. 2001). Recent studies have shown that by combining such models with population genetics it is possible to get new insights about larval dispersal and its influence on

population structure (Gilg and Hilbish 2003; Sotka et al. 2004; Jolly et al. 2009). In **Paper I**, we used an oceanographic model to study potential connectivity between barnacle populations within the Baltic Sea, and compared the results with our population genetic data on *B. improvisus*.
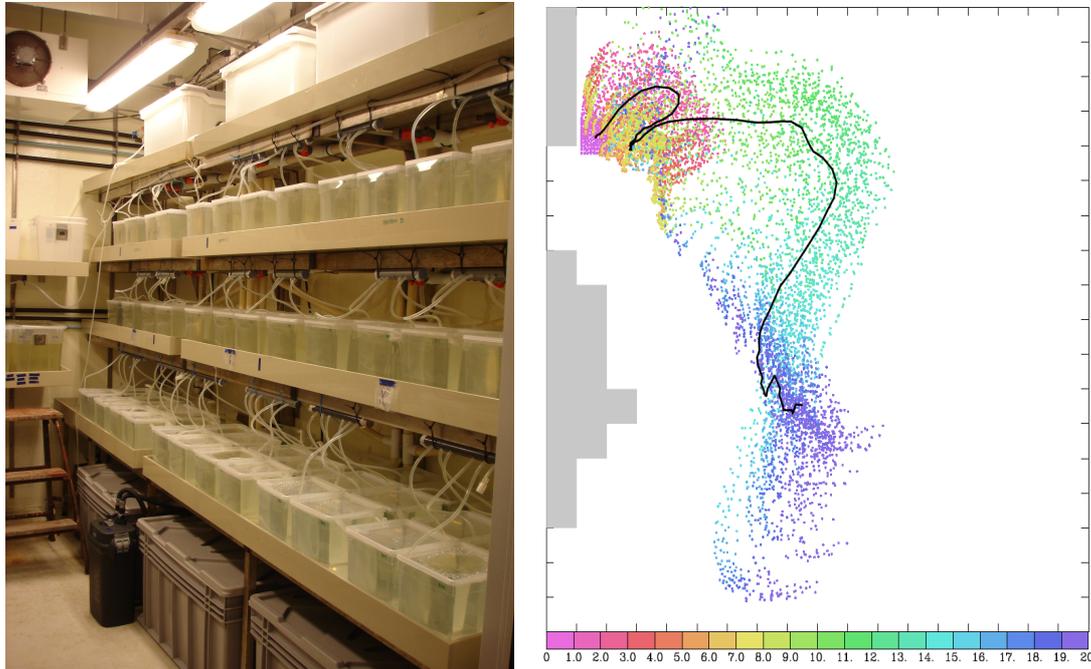

Figure 5. Illustrations of two methods used in this thesis. Left: setup of a common garden experiment in the laboratory, Right: example of larval trajectory clusters obtained from oceanographic modelling (Döös and Jonsson, unpublished) where colours indicate different dispersal time (days) when released from the coast (grey grids). Photo: Anna-Lisa Wrange.

MAIN RESULTS AND DISCUSSION

In the first paper (**Paper I**), I investigated how broad salinity tolerance of *B. improvisus* has influenced the invasion success and population genetic structure of the species on both a global and regional scale. I was further interested in determining how anthropogenic dispersal (mainly through fouling on ships) and natural larval dispersal have influenced the population genetic structure and discuss implications of broad tolerance and multiple modes of dispersal for the potential of adaptations to evolve. On a global scale, I found a high genetic diversity in most populations with many shared haplotypes between populations (Fig. 6), suggesting that human-mediated dispersal (probably fouling on ship hulls) has played an important role in the relatively recent spread of the species (during the last few centuries). Only a few populations – the northern Baltic, the Caspian Sea and the Black Sea – showed signs of reduced genetic variation following introduction (bottlenecks). This suggests that multiple introductions have occurred in many regions, which have helped to maintain

high diversity within introduced populations. The broad salinity tolerance of *B. improvisus* has most likely been an important trait that has contributed to the successful global spread of *B. improvisus*.
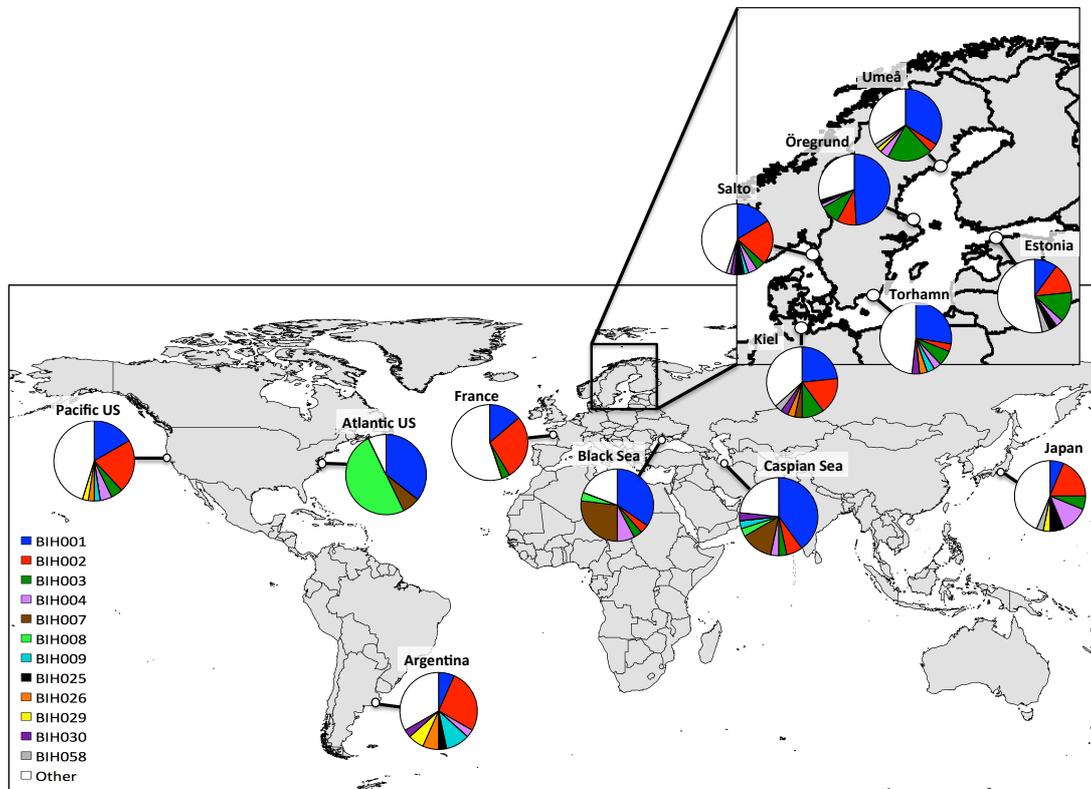

Figure 6. Distribution of haplotypes (based on a sequence from the mitochondrial COI gene) in *B. improvisus* populations worldwide. The twelve most common haplotypes (represented by five or more individuals) are colour-coded and the white proportion contains all the haplotypes represented by five or less individuals in total (**Paper I**).

On a regional scale, oceanographic connectivity through larval dispersal explained more of the genetic structure than simple geographical distance between sampling locations. This indicates that larval dispersal has an important role in the spread of the species on smaller geographical scales (e.g. within the Baltic Sea), in addition to dispersal by anthropogenic vectors. Clear genetic differentiation was found between populations from the northern and southern Baltic, which could partly be explained by restricted gene flow through larval dispersal. If barnacle populations in the northern Baltic are isolated from the southern Baltic, this could lead to the evolution of local adaptations: salinity in the northern Baltic Sea is very low (<4 PSU) and stable, which could impose different selection pressures on these populations to populations in the southern Baltic and Skagerrak. It should be cautioned that describing population genetic structure and connectivity of a species based on "neutral" markers does not provide direct evidence for local adaptation. On the one hand it provides a baseline understanding of the potential for local adaptation because restricted gene flow can

facilitate the evolution of local adaptations (Palumbi 1994; Hellberg 2009). On the other hand, local adaptation can also evolve despite high connectivity, e.g. when non-random dispersal or strong post-settlement mortality occurs (Johannesson et al. 1995; Schmidt and Rand 2001).

In **Paper II**, I set out to investigate whether barnacle populations in the Baltic Sea are locally adapted to live in very low and stable salinity conditions. I was also interested in evaluating if the predicted salinity decrease within the Baltic Sea region will have a negative impact of fitness-related traits in *B. improvisus*. The study confirmed that *B. improvisus* can tolerate a broad range of salinities (6-30 PSU), and revealed almost no population-specific differences in phenotypic responses. These results suggest little local adaptation to Baltic salinity conditions. The only population-specific response observed was in shell strength, in which barnacles from outside the Baltic Sea (Tjärnö) produced significantly stronger shells in high salinity treatments compared to barnacle populations from the inner Baltic Sea (Stockholm) (Fig. 7a). This may suggest local adaptation to differences in predation pressures rather than a direct effect of salinity (since the barnacle shell provides an important protection against predation and most barnacle predators are absent from the inner Baltic Sea). However, other factors such as trans-generational plasticity ("maternal effects"; Marshall et al. 2008) cannot be excluded, since the study did not include multiple generations. All barnacle populations performed slightly better (in terms of survival, growth and reproduction) at low and intermediate salinities, although some presumably negative effects of low salinity were also observed on fitness-related traits such as early growth rate and shell strength. My results therefore suggest the presence of trade-offs between different traits that are contingent on the environmental conditions. This could indicate that there is an energetic cost involved to tolerate low salinities, e.g. in the ability to osmoregulate. To clarify this, physiological and molecular studies testing potential osmoregulatory mechanisms in *B. improvisus* are required.
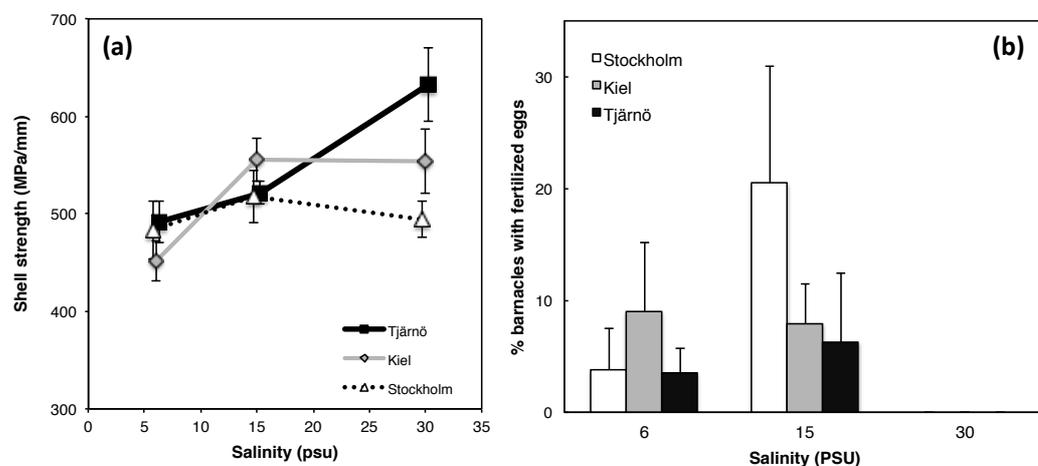

Figure 7. (a) Effects of salinity on shell strength and (b) Effects of salinity on reproduction in barnacles from three different populations (Stockholm, Kiel and Tjärnö) that were grown in different salinity treatments (mean± SE; n = 2–4). (**Paper II**)

In **Paper III**, we investigated the molecular mechanisms involved in osmoregulation in *B. improvisus* by characterizing the Na$^+$/K$^+$ ATPase (NAK) gene in *B. improvisus*. The NAK is an important transporter of ions across cell membranes in many animals, and since it has been suggested that *B. improvisus* also has the ability to osmoregulate in low salinity (Fyhn 1976), it was a promising candidate gene to investigate further. We identified two main gene variants of the NAK in *B. improvisus* (NAK1 and NAK2), which were approximately 70% identical at the protein level. The NAK1 mRNA existed in a long and short variant with the encoded proteins differing only by 27 N-terminal amino acids. Interestingly, the two NAK1 isoforms were differentially expressed in different life stages and in various tissues of adult barnacle: e.g. the long isoform was predominant in cyprids and in adult cirri (Fig. 8a). When barnacle cyprid larvae were subjected to low salinity the expression of the long NAK1 mRNA increased relative to the short isoform (Fig. 8b), indicating that the long Nak1 protein might have a more prominent role in low salinity osmoregulation. These findings provide an interesting baseline for further investigation of the functional roles of the different *B. improvisus* NAKs in barnacle osmoregulation.
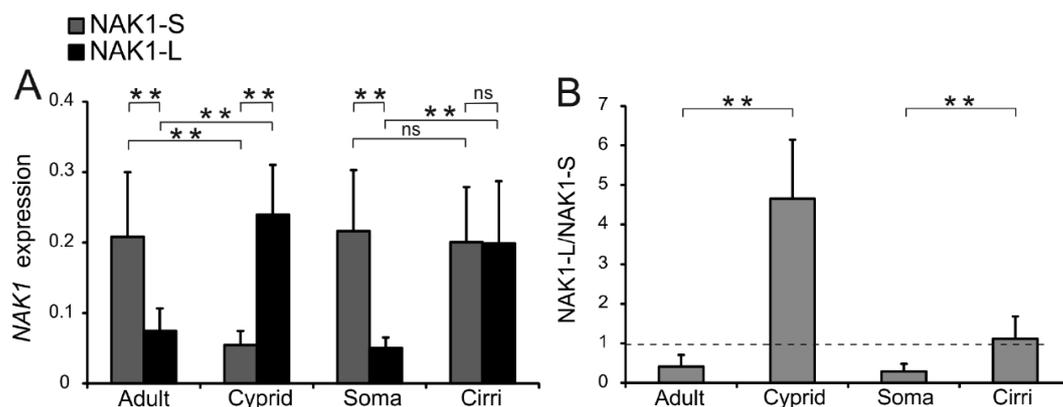

Figure 8. Differential expression of the two splice variants of NAK1 in different life stages and tissues: (A) Cyprids express more of the long than of the short isoform, whereas the short isoform is dominating in adult individuals, (B) The relative expression of the long variant compared to short variant is significantly higher in cyprids than in adults and in cirri compared to soma (body). (**Paper III**)

Although we identified the molecular structure of several NAK gene variants in *B. improvisus*, there are many other physiological and molecular mechanisms that could be involved in the broad salinity tolerance of *B. improvisus*. Therefore, we reviewed the literature on salinity tolerance in barnacles, and compiled information about known osmoregulatory strategies and mechanisms in other invertebrates, focusing mainly on crustaceans (**Paper IV**). Using this information, we outlined a conceptual model of barnacle osmoregulation (Fig. 9) with the aim to clarify future research to identify the exact osmoregulatory tissues and molecular mechanisms that are involved in euryhalinity in barnacles. In brief, we suggest a working hypothesis that involves

euryhaline barnacles hyper-regulating the hemolymph at low salinities, not only by active uptake of monovalent ions, but also using proline or other osmolytes that are produced and excreted from the surrounding cells (Fig. 9). This could be an "ion-saving" mechanism that ensures that most ions are used intracellularly for proper metabolic cell functions but could also merely reflect a low ion-absorbing capacity of *B. improvisus* that instead adopts the strategy to maintain a hyperosmotic hemolymph by the aid of osmolytes.

Figure 9. Hypothetical model (partly based on Fyhn, 1976) for the osmoregulatory strategy of the euryhaline barnacle, *Balanus improvisus*, showing (A) In marine environments: osmoconformation and intracellular volume regulation, using mainly the amino acid proline (Pro), at salinities above 100 mOsm kg^{-1} and (B) In highly diluted seawater: hyper-osmoregulation using active ion transport in combination with Pro as extracellular osmolyte, at salinities below 100 mOsm kg^{-1}. (**Paper IV**)

Table. Summary of the main questions and results from this thesis:

Main question	Main Result	Implications
I. How have broad salinity tolerance and multiple dispersal modes influenced the spread and population genetic structure of *B. improvisus* on a global and regional (Baltic Sea) scale? **What is the role of shipping and larval dispersal for the potential gene flow between barnacle populations?**	High genetic diversity and many shared haplotypes between populations on a global scale indicate importance of dispersal *via* shipping, facilitated by broad salinity tolerance. Larval dispersal influences genetic structure on a more regional scale (Baltic Sea). High global genetic diversity makes it difficult to determine the geographical origin of *B. improvisus*.	Broad salinity tolerance is likely a key trait in barnacles for successful colonisation of coastal areas. Both shipping and larval dispersal are important for the spread of *B. improvisus*. Genetic markers provide useful tools to study the spread of non-indigenous species.
II. Are *B. improvisus* populations in the Baltic Sea locally adapted to different salinity regimes, compared to North Sea populations? **Will predicted future salinity reductions in the Baltic Sea negatively impact *B. improvisus*?**	Phenotypic plasticity rather than local adaptation explains the broad distribution along the Baltic salinity gradient. However, population-specific responses in shell strength indicate possible adaptation to different predation pressures, rather than to salinity.	Future predicted salinity changes in the Baltic Sea will most likely not have major negative effects on *B. improvisus*. Phenotypic plasticity will most likely play an important role in coping with future environmental change.
III. What is the role of the osmoregulatory ion pump Na$^+$/K$^+$ATPase (NAK) in coping with changing salinities in the barnacle *B. improvisus*?	We found two variants of the NAK gene, with multiple splice forms, that were differentially expressed in different life stages and tissues when exposed to various salinities, indicating an osmoregulatory function.	Knowledge of the structure of NAK provides an important baseline for further studies on the functional role of NAK in barnacles.
IV. Based on knowledge from other invertebrates, what are the probable mechanisms involved in osmoregulation in barnacles? **What are the ecological and evolutionary implications of broad salinity tolerance?**	A combination of multiple mechanisms including: ion transport (Na$^+$/K$^+$ ATPase and other ion transporters), osmolyte regulation (mainly proline), epithelial permeability regulation and behavioural responses (valve closure) are likely involved in osmoregulation in barnacles. Exact tissues and mechanisms involved in osmoregulation in *B. improvisus* remains to be elucidated.	Using a combination of methods e.g. immunohistochemistry, functional and comparative genomics based on newly available genome resources will further our understanding of osmoregulation in barnacles. Broad salinity tolerance has facilitated invasion success in many species and likely plays an important role in life history evolution and under future climate change.

CONCLUSIONS AND FUTURE PERSPECTIVES

- The bay barnacle *Balanus improvisus* is a highly euryhaline species with capacity to tolerate a broad range of salinities, primarily through plastic responses
- Broad salinity tolerance (high plasticity) is one of the key factors explaining the successful introduction of *B. improvisus* to non-native regions
- The barnacle *B. improvisus* shows high levels of genetic diversity both within and between populations on a global scale
- A large number of shared haplotypes between distant populations as well as evidence of bottlenecks and demographic expansion in several populations confirms a recent invasion history of *B. improvisus*, in which shipping has probably played an important role
- The geographic origin of *B. improvisus* could not be clearly confirmed, but the results suggest that Argentina is part of the native region
- Oceanographic connectivity, rather than geographic distance, explains the regional genetic structure within the Baltic Sea, highlighting the importance of larval dispersal for further spread after a first introduction has occurred
- Correlation between genetic structure and oceanographic connectivity in the Baltic Sea indicates a possible restriction in gene flow between northern and southern Baltic populations, which could potentially lead to local adaptations
- Recent colonisation to the Baltic Sea (<200 yrs), high dispersal and broad salinity tolerance can potentially explain why few signs of local adaptation were detected
- Population-specific responses in shell strength may be an example of adaptation to different selection pressures between the Baltic and North Sea, although maternal effects cannot be excluded
- Despite broad salinity tolerance, potential trade-offs between fitness-related traits were found depending on environmental conditions, which may be linked to energetic costs of osmoregulation
- *B. improvisus* has the capacity to osmoregulate at low salinities, partly through ion regulation (using the Na^+/K^+ ATPase) but other mechanisms including regulation *via* osmolytes and epithelial permeability may also be of importance
- Future predictions of reduced salinity in coastal areas (including the Baltic Sea) will most likely not impact *B. improvisus* negatively, but rather favour the species
- Barnacles are valuable model species for the study of evolution in changing marine environments

Future perspectives…

In this thesis I have shown that broad salinity tolerance, as well as multiple dispersal modes, plays an important role in successful colonisation of new environments by *B. improvisus*. High genetic diversity on a global scale, with many shared haplotypes, made it difficult to clearly identify the native region of the species, and further sampling of multiple populations from putative native areas is required to clarify this. Plasticity rather than local adaptations seemed to mostly explain the broad distribution of *B. improvisus* in the Baltic Sea, however population-specific responses in shell strength suggest the possibility of local adaptation. Selection experiments using multiple generations of *B. improvisus* would be useful to elucidate the respective roles of phenotypic plasticity, trans-generational effects and local adaptations in response to locally strong selection pressures. Despite being a successful invader in shallow estuarine environments, *B. improvisus* is seldom a dominant species in natural fouling communities in full marine environments (personal observations), indicating that their ability to compete with other species may be limited in this environment. It would be interesting to investigate this further, comparing life history traits between competitor species and evaluate how broad salinity tolerance may influence successful competition with other species. The results from the common-garden experiment also indicate that future climate-driven reductions in salinity in coastal areas will most likely not have major impacts on *B. improvisus* populations. However, further work is needed to clarify the interactions between salinity tolerance and other stressors such as temperature, ocean acidification and food limitation, which also are projected to change in the future. Little is known about how osmoregulatory functions are influenced by other stressors, e.g. temperature and pH. There could e.g. be synergistic effects acting to reduce salinity tolerance at high temperatures, as has been observed between salinity and temperature in marine isopods (Vlasblom et al. 1977). A crucial question is thus whether evolutionary changes in osmoregulatory functions can match the rate of climate change. Despite having characterised the structure and expression of the Na^+/K^+ ATPase in *B. improvisus*, there is still a lot to be learned about the functional role of this ion transporter and other potential genes and regulatory pathways involved in osmoregulation in barnacles. Recent advances in molecular biology, including genome sequencing of non-model organisms such as *B. improvisus*, provide new promising tools to study evolutionary responses in organisms experiencing changing environmental conditions. With these molecular tools it is possible to document genetic variation within and between individuals, study gene expression patterns and molecular pathways underlying the expression of different traits. Barnacles provide an excellent model organism for this future work.

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