

Bioenergetics of calcification in mytilid bivalves along the Baltic Sea salinity gradient

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zur Erlangung des Doktorgrades der Mathematisch-Naturwissenschaftlichen
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Vorgelegt von
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Bioenergetics of calcification in mytilid bivalves
along the Baltic Sea salinity gradient

Bioenergetik der Kalzifizierung in Mytiliden
Muscheln entlang des Salinitätsgradienten der
Ostsee

Dissertation zur Erlangung des akademischen Grades

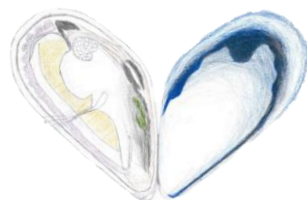
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Summary

The Baltic Sea is a semi-enclosed, brackish water body with a decreasing salinity gradient from almost fully marine in the west, to below 3 practical salinity units (psu) in the northern and eastern basins. Marine calcifying mussels in the genus *Mytilus* have extensively colonised the coastal Baltic benthos down to salinities of 4.5 psu and play a key role as ecosystem engineers and nutrient recyclers. Despite this, growth rates are severely reduced below salinities of ~ 11 psu with maximum shell lengths reaching only half that of fully marine populations. This has been attributed to increased metabolic costs associated with osmotic stress, however, along this salinity gradient, $[Ca^{2+}]$ and dissolved inorganic carbon (C_T) availability also decrease, theoretically hindering calcification. It is not fully understood how low salinity impacts calcification and whether reduced ability to biomineralize $CaCO_3$ structures ultimately limits growth of mytilid mussels at low salinity.

In the first and second chapters of this thesis, the environmental gradients of the south-western Baltic Sea were utilised to investigate how environmental salinity impacts calcification in Baltic *Mytilus*. A series of laboratory experiments were conducted in chapter one to investigate which factor (salinity, $[Ca^{2+}]$ or C_T) is primarily responsible for impeding calcification rates of Baltic *Mytilus* at low salinities. Juvenile mussels were exposed to a range of salinities (6 – 16 psu), Ca^{2+} concentrations (0.5 – 4 mmol kg⁻¹) and HCO_3^- (a proxy for C_T availability) concentrations (300 – 2100 μmol kg⁻¹) to identify at which level these factors begin to limit calcification. Results reveal that $[Ca^{2+}]$ is the chief factor limiting calcification in juvenile Baltic mytilids with calcification rates reduced below 4 mmol kg⁻¹ corresponding to ~ 11 psu. Laboratory experiments and field monitoring of juvenile growth rates, salinity and carbonate chemistry at three sites along the Baltic salinity gradient reveal that C_T availability probably does not limit calcification in Baltic *Mytilus* and high coastal total alkalinity (A_T) may buffer potential impacts of ocean acidification. Field monitoring data also revealed that slow growth at an 11 psu site is accompanied by low food availability, suggesting $[Ca^{2+}]$ alone cannot sufficiently explain field growth rate patterns between sites and other factors (e.g. environmental variability, food availability, biotic interactions) are important in influencing Baltic *Mytilus* calcification rates.

Chapter two further investigated how low calcification rates potentially limit growth in Baltic mussels by estimating the energetic cost of calcification at three salinities (6, 11 and 16 psu) and two temperatures (8 and 18 °C) in juvenile Baltic *Mytilus*. Whole animal energy budgets

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were calculated for mussels over three feeding regimes, used to induce a wide range of calcification rates. Energy available for calcification was plotted against calcification rate to estimate the costs of calcification for the first time in non-larval bivalves. Results revealed that a considerable proportion of available growth energy (29 – 58 %) is allocated to calcification, signifying it is a major energy consuming process in Baltic *Mytilus*. Findings also indicate that costs of calcification are ~ 2-3-fold higher at low salinity and temperatures suggesting growth of Baltic *Mytilus* below ~ 11 psu is severely impeded by high costs of biomineralisation.

Baltic *Mytilus* are all hybrids of *M. edulis*-like genotypes and *M. trossulus*-like genotypes with higher frequencies of *M. edulis*-like alleles at high salinities and higher *M. trossulus*-like allele frequencies at low salinities. The steepest cline in allele frequencies coincides with the steepest section of the salinity gradient, implying salinity plays a role in shaping the genetic structure of Baltic *Mytilus*. This suggests physiological differences between Baltic *Mytilus* populations along the Baltic salinity gradient may result from either acclimation or local adaptation to habitat salinity regimes. However, it has not yet been empirically tested whether genetic differences between hybrid populations result from salinity driven selection and whether local adaptation to low salinity is facilitated by higher frequencies of *M. trossulus* alleles. To test this, two larval experiments were conducted on three Baltic *Mytilus* populations. The first experiment compared the salinity tolerance of a *M. edulis*-like (16 psu) and *M. trossulus*-like (7 psu) population, by quantifying growth, mortality and settlement success at high (16 psu) and low (7 psu) salinity. The second experiment investigated natural selection by low salinity for *M. trossulus* alleles by utilising a highly genetically admixed population of Baltic *Mytilus* (similar frequencies of both *M. edulis*-like and *M. trossulus*-like alleles) from the centre of the genetic transition zone (11 psu). Results from these experiments indicate that low salinity selects against *M. edulis*-like alleles, increasing frequencies of *M. trossulus*-like alleles compared to simulated selection predictions. This natural selection was complimented by evidence of local adaptation to low salinity in *M. trossulus*-like genotypes, due to better larval survival and settlement success at 7 psu compared to *M. edulis*-like genotypes. These findings highlight salinity as a strong selective force shaping the genetic structure of Baltic *Mytilus* populations and driving local adaptation in Baltic *Mytilus* populations.

In marine osmoconforming invertebrates, salinity tolerance is determined by an organisms' ability to adjust the concentrations of its intracellular organic osmolyte and inorganic ion pools. It therefore seems likely, that better salinity tolerance and local adaptation of low salinity *M. trossulus* populations results from adaptive changes in osmolyte pools. However, this has not

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yet been demonstrated experimentally. Consequently, the salinity tolerance and impacts of salinity on intracellular osmolyte pools were investigated in two salinity adapted populations of Baltic *Mytilus* to investigate if local adaptation is facilitated by qualitative and/or quantitative adjustments of intracellular osmolyte pools. A 16 psu population (Kiel) and a 7 psu population (Usedom) were exposed to salinities between 16 and 4.5 psu and their organic and inorganic osmolyte pools were investigated using metabolomic profiling (¹H-NMR spectroscopy) and flame photometry. Metabolomic profiles revealed that the low salinity population exhibits lower concentrations of total organic osmolytes mostly due to significantly lower taurine content. Compared to the high salinity population, the low salinity population also exhibited higher intracellular cation concentrations across all salinities. These findings were complemented by lower mortality rates in low salinity *M. trossulus* populations, suggesting that increased intracellular cation concentrations may be an adaptive response in facilitating local adaptation to extremely low salinities. This is potentially a means to reduce the detrimental impacts of low intracellular ion concentrations on cellular biochemical processes and these findings provide first insights into the mechanisms of adaptation to low salinities in marine bivalves.

In conclusion, it appears that slow growth rates in Baltic mytilid mussels results from limited availability of Ca²⁺ coupled with high costs of calcification. Findings from laboratory experiments reveal these constraints on calcification only become significant below 11 psu, however field monitoring suggests other environmental factors such as food availability and salinity/carbonate chemistry variability are likely important factors governing calcification in Baltic *Mytilus*. It has also emerged that low salinity populations of *M. trossulus*-like genotypes exhibit local adaptation to extremely low salinities, likely facilitated in part by adaptive changes in intracellular osmolyte pools. These findings provide a vital contribution to our understanding of how environmental salinity can drive physiological adaptation as well as genetic segregation in an ecologically and economically important species complex. Climate and hydrological models predict rapid warming and desalination of the Baltic Sea over the next 80 years potentially leading to severe shifts in Baltic mytilid distributions. Therefore, understanding the mechanisms behind potential ecological changes enable us to make better predictions on how climate change might influence coastal ecosystems. Future work should aim to dig deeper into understanding the cellular mechanisms behind changes in growth rates and osmolyte pools with salinity, to broaden our knowledge regarding the processes of adaptation to a rapidly changing ocean.

Zusammenfassung

Die Ostsee ist ein Binnenmeer mit einem starken Salinitätsgradienten, von nahezu marinen Bedingungen im Westen, bis zu Salinitäten unter 3 psu im Nordosten. Marine, kalzifizierende Muscheln der Gattung *Mytilus* sind ein großer Bestandteil des Benthos und tolerieren Salzgehalte bis hinunter zu 4,5 psu. Sie sind eine Schlüsselart in küstennahen Ökosystemen und maßgeblich an der Nährstoffumsetzung beteiligt. Allerdings sind die Wachstumsraten der Muschelschalen bei Salinitäten unter 11 psu halb so schnell wie die von vollständig marinen Populationen. Dies wurde seit jeher auf höhere Stoffwechselkosten bei geringerem Salzgehalt zurückgeführt, die mit osmotischem Stress assoziiert sind. Jedoch sinkt mit dem Salzgehalt auch die Verfügbarkeit von $[Ca^{2+}]$ und gelöstem inorganischen Kohlenstoff (C_T), was ebenso die Kalzifizierung beeinflussen kann. Bisher ist ungeklärt, wie genau niedrige Salinitäten Kalzifizierung beeinflussen, und ob eine verringerte Fähigkeit $CaCO_3$ zu biomineralisieren, das Wachstum von mytiloiden Muscheln bei niedrigen Salzgehalten letztlich limitiert.

Im ersten und zweiten Kapitel dieser Dissertation wird beschrieben, wie der natürliche Umweltgradient der südwestlichen Ostsee genutzt wurde, um zu untersuchen, wie der Salzgehalt die Kalzifizierung von Baltischen *Mytilus* beeinflusst. In Kapitel Eins wird anhand von Laborexperimenten erläutert, welcher Faktor (Salinität, $[Ca^{2+}]$ oder C_T) primär für die geringeren Kalzifizierungsraten von Baltischen *Mytilus* bei niedrigen Salinitäten verantwortlich ist. Dazu wurden juvenile Muscheln einer Reihe von Salzgehalten (6 – 16 psu), $[Ca^{2+}]$ -Konzentrationen (0.5 – 4 mmol kg⁻¹) und $[HCO_3^-]$ -Konzentrationen (als Indikator für C_T Verfügbarkeit; 300 – 2100 μmol kg⁻¹) ausgesetzt, um einen Schwellenwert für Kalzifizierung zu ermitteln. Die Ergebnisse weisen auf $[Ca^{2+}]$ als entscheidenden Faktor bei der Kalzifizierung juveniler Miesmuscheln in der Ostsee hin. Dies äußert sich durch verringerte Kalzifizierungsraten ab 4 mmol kg⁻¹, welches einer Salinität von ca. 11 psu entspricht. Laborversuche und regelmäßige Feldmessungen von Wachstumsraten juveniler Muscheln, Salinität und Karbonatchemie an drei Standorten entlang des Salinitätsgradienten der Ostsee ergaben, dass die natürliche C_T Verfügbarkeit wahrscheinlich keine Auswirkung auf den Kalzifizierungsprozess von Miesmuscheln in der Ostsee hat. Zudem könnte eine hohe Gesamtalkalinität (A_T) im Küstenbereich potentielle Effekte der Ozeanversauerung dämpfen. Feldmessungen zeigen, dass langsames Wachstum bei 11 psu auch mit niedriger Nahrungsverfügbarkeit einhergehen. Dies deutet daraufhin, dass $[Ca^{2+}]$ alleine *in situ* Wachstumsraten und Unterschiede zwischen Standorten mit unterschiedlichen Salzgehalten

nicht ausreichend erklärt, und auch andere Parameter (wie z.B. Variabilität der Umweltbedingungen, Nahrungsverfügbarkeit, biotische Interaktionen) einen wichtigen Einfluss auf die Kalzifizierungsraten von Baltischen *Mytilus* haben.

In Kapitel Zwei wird untersucht, wie niedrige Kalzifizierungsraten das Wachstum von Baltischen *Mytilus* limitieren können. Dazu wurden die energetischen Kosten des Kalzifizierungsprozesses bei verschiedenen Salzgehalten (6, 11 und 16 psu) und zwei Temperaturen (8 und 18°C) in juvenilen Miesmuscheln ermittelt. Drei verschiedene Fütterungsprotokolle wurden eingesetzt, um die Bandbreite der Kalzifizierungsraten weiter zu erhöhen, und die Energiebilanz der Muscheln als Ganzes zu berechnen. Ein beträchtlicher Anteil der verfügbaren Energie (29 – 58 %) ist für Kalzifizierung verfügbar. Dies unterstreicht, dass Kalzifizierung einen maßgeblichen Teil der Energie in Baltischen *Mytilus* verbraucht. Die Ergebnisse weisen darauf hin, dass die Kosten der Kalzifizierung bei niedrigen Salinitäten und Temperaturen ca. 2-3-fach höher sind. Das Wachstum bei Muscheln unter einer Salinität von 11 psu ist damit durch die hohen Kosten der Biomineralisation ernsthaft eingeschränkt.

Die Baltischen *Mytilus* sind Hybriden aus *M. edulis*-ähnlichen und *M. trossulus*-ähnlichen Genotypen. Hierbei sind die *M. edulis*-ähnlichen Allele häufiger bei hohen Salinitäten zu finden, wohingegen *M. trossulus*-ähnliche Allele vermehrt bei niedrigen Salinitäten vorkommen. Dabei fällt die extremste Änderung in den Allelfrequenzen mit dem steilsten Abschnitt des Salzgehaltsgradienten zusammen, was darauf hindeutet, dass der Salzgehalt eine Rolle in der genetischen Struktur von *Mytilus* in der Ostsee spielt. Physiologische Unterschiede zwischen den verschiedenen *Mytilus* Populationen entlang des Salzgradienten kommen daher entweder durch Akklimatisierung oder durch lokale Anpassung an die Salinität zustande. Es ist jedoch noch nicht empirisch getestet worden, ob genetische Unterschiede zwischen Hybridpopulationen aus der salinitätsgetriebenen Selektion resultieren und ob die lokale Anpassung an einen niedrigen Salzgehalt durch Häufung von *M. trossulus*-ähnlichen Allelen erleichtert wird. Um dies zu überprüfen, wurden zwei Larvenversuche an drei Baltischen *Mytilus* Populationen durchgeführt. Im ersten Experiment wurde die Salinitätstoleranz einer *M. edulis*-ähnlichen (16 psu) und einer *M. trossulus*-ähnlichen (7 psu) Population durch Quantifizierung von Wachstum, Mortalität und Fortpflanzungserfolg bei hohem (16 psu) und niedrigem (7 psu) Salzgehalt verglichen. Im zweiten Experiment wurde die natürliche Selektion durch einen geringen Salzgehalt auf *M. trossulus*-ähnliche Allele untersucht. Dazu wurde eine genetisch gemischte Baltische *Mytilus* Population (gleiche Häufigkeiten von *M. edulis*-ähnlichen und *M. trossulus*-ähnlichen Allelen) aus der Mitte der genetischen

Übergangszonen (11 psu) gewählt. Die Ergebnisse beider Experimente zeigen, dass ein geringer Salzgehalt gegen *M. edulis*-ähnliche Allele selektiert, wodurch die Häufigkeiten von *M. trossulus*-ähnlichen Allelen im Vergleich zu simulierten Selektionsprognosen erhöht werden. Diese natürliche Selektion zeigt sich auch in einer lokalen Anpassung von *M. trossulus*-ähnlichen Genotypen an einen niedrigen Salzgehalt, wie z.B. höhere Überlebensraten der Larven und erfolgreiches Ansiedeln bei 7 psu im Vergleich zu *M. edulis*-ähnlichen Genotypen. Die Ergebnisse betonen den Salzgehalt als eine starke selektive Kraft, die die genetische Struktur der Baltischen *Mytilus* Populationen beeinflusst und zu einer lokalen Anpassung führt.

Bei marinen Osmokonformern wird die Salinitätstoleranz durch die Fähigkeit eines Organismus definiert, die Konzentrationen der intrazellulären organischen Osmolyte und der anorganischen Ionen anpassen zu können. Möglicherweise resultiert eine bessere Toleranz gegenüber niedrigem Salzgehalt von *M. trossulus*-ähnlichen Populationen aus Anpassungen der Osmolyte. Dies konnte jedoch bisher noch nicht nachgewiesen werden. In diesem Experiment wurden die Auswirkungen des Salzgehaltes auf die lokale, qualitative und/oder quantitative Anpassung des intrazellulären Osmolytpools in zwei salinitätsadaptierten Baltischen *Mytilus* Populationen untersucht. Eine Kieler Population (16 psu) und eine Population aus Usedom (7 psu) wurden Salinitäten zwischen 16 und 4,5 psu ausgesetzt und ihre organischen und anorganischen Osmolyte mittels Metabolom-Profilings (¹H-NMR-Spektroskopie) und Flammenphotometrie untersucht. Metabolomische Profile zeigen, dass die niedrig saline Population insgesamt niedrigere Konzentrationen von organischen Osmolyten aufweist, hauptsächlich aufgrund eines signifikant niedrigeren Tauringehaltes. Verglichen mit der Population aus 16 psu, weist die niedrig saline Population zudem höhere intrazelluläre Kationenkonzentrationen in allen Salzgehalten auf, möglicherweise um die schädlichen Auswirkungen niedriger intrazellulärer Ionenkonzentrationen auf biochemische Prozesse zu reduzieren. Zusammen mit niedrigeren Mortalitätsraten in *M. trossulus* Populationen aus Usedom deutet dies darauf hin, dass erhöhte intrazelluläre Kationenkonzentrationen eine adaptive Antwort zur lokalen Anpassung an extrem niedrige Salzgehalte sein können. Diese Ergebnisse liefern erste Einblicke in die Mechanismen der Anpassung an niedrige Salzgehalte in marinen Bivalvia.

Zusammenfassend lässt sich sagen, dass langsame Wachstumsraten in Baltischen *Mytilus* auf eine begrenzte Verfügbarkeit von [Ca²⁺] bei gleichzeitig hohen Kalzifizierungskosten zurückzuführen sind. Ergebnisse von Laborexperimenten zeigen, dass diese Einschränkungen während des Kalzifizierungsprozesses erst unterhalb einer Salinität von 11 psu signifikant

werden. Jedoch deutet das Feldmonitoring darauf hin, dass andere Umweltfaktoren, wie die Verfügbarkeit von Nahrung und Salinitäts- bzw. Karbonatchemieschwankungen, wahrscheinlich wichtige Faktoren für die Kalzifizierung bei Baltischen *Mytilus* sind. Außerdem hat sich gezeigt, dass Populationen von *M. trossulus*-ähnlichen Genotypen eine lokale Anpassung an extrem niedrige Salinitäten zeigen, was wahrscheinlich teilweise durch adaptive Veränderungen in intrazellulären Osmolytpools erreicht wird. Diese Ergebnisse liefern einen wichtigen Beitrag zu unserem Verständnis, wie der Salzgehalt die physiologische Anpassung sowie die genetische Segregation in einem ökologisch und ökonomisch wichtigen Artenkomplex vorantreiben kann. Klimatische und hydrologische Modelle sagen eine rasche Erwärmung und Entsalzung der Ostsee in den nächsten 80 Jahren voraus, was möglicherweise zu einer starken Verschiebung der Verbreitung von Baltischen *Mytilus* führen könnte. Wenn wir die Mechanismen hinter den potenziellen ökologischen Veränderungen verstehen, können wir bessere Vorhersagen treffen, wie der Klimawandel die Küstenökosysteme beeinflussen könnte. Zukünftige Arbeiten sollten darauf abzielen, die zellulären Mechanismen hinter den Veränderungen der Wachstumsraten und Osmolyte mit dem Salzgehalt genauer zu verstehen, um unser Wissen über die Anpassungsprozesse an einen sich schnell verändernden Ozean zu erweitern.

List of abbreviations

$^1\text{H-NMR}$	Proton nuclear magnetic resonance
ACC	Amorphous calcium carbonate
A_T	Total alkalinity
Ca^{2+}	Calcium ion
$\text{Ca}^{2+}\text{-ATPase}$	Calcium ATPase
<i>chl-a</i>	Chlorophyll- <i>a</i>
CO_2	Carbon dioxide
CO_3^{2-}	Carbonate ion
C_T	Total dissolved inorganic carbon
DOC	Dissolved organic carbon
dpf	Days post fertilisation
DW	Dry weight
EPF	Extra pallial fluid
FAA	Free amino acid
F_{ST}	Fixation index
H^+	Proton
HCO_3^-	Bicarbonate ion
K^+	Potassium ion
K_{sp}	Calcium carbonate dissociation constant
Na^+	Sodium ion
NKA	Sodium potassium ATPase
O_2	Oxygen
OO	Organic osmolyte
pCO_2	Carbon dioxide partial pressure
PCR	Polymerase chain reaction
PMCA	Plasma membrane Calcium ATPase
psu	Practical salinity unit
SfG	Scope for Growth
SfSG	Scope for Shell Growth
SIR	Substrate inhibitor ratio
SMP	Shell matrix protein
SW	Seawater
Ω	Saturation state
$\Omega_{\text{aragonite}}$	Aragonite saturation state
Ω_{calcite}	Calcite saturation state

1. Introduction

1.1 Climate change and the Baltic Sea

The Baltic Sea is a semi-enclosed brackish water body in Northern Europe with a surface area of $\sim 377,000 \text{ km}^2$ and a drainage basin covering ~ 2 million km^2 (Mörth et al. 2007). At least 85 million people inhabit the catchment area of the Baltic Sea with tourism alone providing almost € 15 billion in income to Baltic Sea countries (HELCOM 2017). The Baltic Sea is connected to the Kattegat, Skagerrak and North Sea via the Danish Belt Seas which provides periodic inflows of oxygen rich, high saline water. Freshwater input from rivers supply water to the central and Northern Baltic Sea basins which subsequently results in a lower salinity compared to the South-West Baltic Sea near the Danish straits. The resulting spatial asymmetry of salt and freshwater input has led to a salinity gradient ranging from ~ 25 practical salinity units (psu) in the Danish Belt Seas to < 6 psu in the far eastern Basins and the Gulf of Bothnia

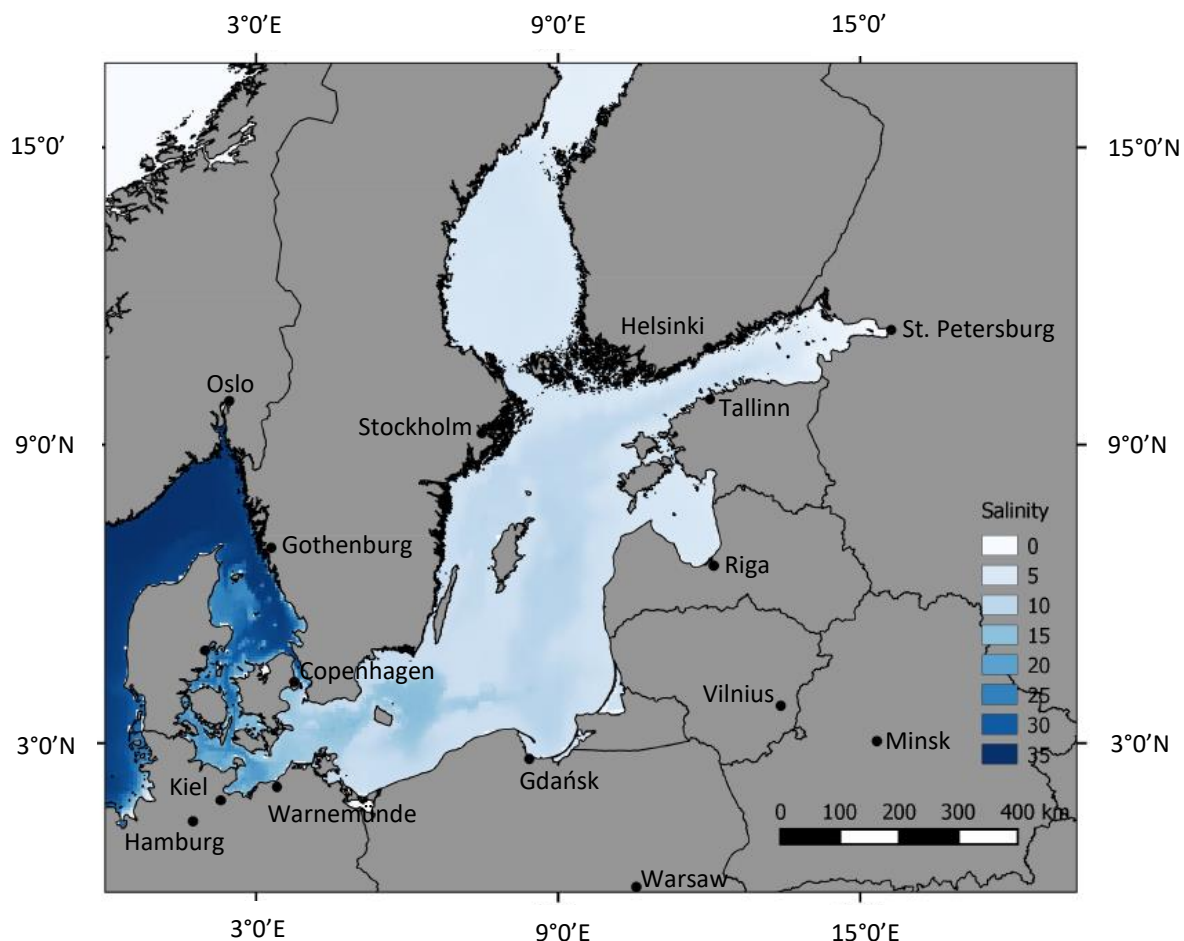


Fig. 1.1 Map of the Baltic Sea depicting the surface salinity gradient (in psu) from high salinities in the Skagerrak and Kattegat near the North Sea to low salinities in the Central and Eastern Baltic (figure adapted from Podbielski et al. 2016).

in the far north (Janssen et al. 1999, Fig. 1.1). Intense agriculture in the drainage basin means the riverine input is rich in nitrogen and phosphorous leading to high levels of nutrient enrichment (eutrophication) in the majority of the Baltic Sea. The resulting eutrophication coupled with water stratification and limited oxygenated saltwater input results in both severe, seasonal hypoxia in coastal zones and persistent anoxia in deep water layers (Conley et al. 2011; Lehmann et al. 2014). Long term monitoring data has, however, revealed that nitrogen and phosphorous input has declines by about 50 % since 1980 (Bange et al. 2011; HELCOM 2017). The Baltic Sea is also one of the fastest warming aquatic ecosystems on the planet with the sea surface temperature (SST) showing an increase of 1.35 °C between 1982-2006 (Belkin 2009). Climatic models predict increased precipitation in the Baltic Sea drainage basin and therefore increased riverine input over the next century. How the frequency and intensity of Baltic saltwater inflows will continue in the future is less clear. However, it is generally accepted that saltwater inflow will decrease by ~ 50 % over the next century. Due to these two factors, the mean surface salinity in the Baltic Sea is predicted to decrease by 0.5 – 3 psu over the next 50 – 80 years (Gustafsson 2000; Meier 2006; Gräwe et al. 2013).

Increased anthropogenic release of CO₂ into the atmosphere and subsequent absorption by oceans leads to a phenomenon termed ocean acidification (Caldeira & Wickett 2003). Increased seawater [CO₂] leads to a shift in carbonate system speciation and results in lower [CO₃²⁻] and therefore a lower saturation state of calcium carbonate (Ω)

$$\text{(equation 1)} \quad \Omega = [\text{Ca}^{2+}][\text{CO}_3^{2-}] / K_{\text{sp}}^*$$

which can be calculated from the salinity and temperature dependant stoichiometric solubility product (K_{sp}^*) (Feely et al. 2004). Values of $\Omega < 1$ lead to thermodynamically favourable dissolution of CaCO₃ structures and will theoretically have a negative impact on organisms with CaCO₃ exoskeletons (Orr et al. 2005; Ries et al. 2016). The increase in [H⁺] and subsequent reduction in pH and Ω can be buffered to an extent by the total amount of dissolved inorganic proton acceptors (termed total alkalinity – A_T). A_T decreases linearly with salinity (S) and is therefore lower in the Baltic Sea compared to fully marine conditions (Lee et al. 2006; Fig. 1.2). Coupled with salinity, [Ca²⁺] are also much lower in the Baltic Sea, thus also lowering Ω according to equation 1 (Kremling & Wilhelm 1997). These factors, in combination with very low winter temperatures lead to extended periods of undersaturation of the CaCO₃ polymorph aragonite ($\Omega < 1$, Tyrrell et al. 2008) which is the mineral many biogenic carbonates consist of (Orr et al. 2005). Low A_T and thus low buffering capacity of the Baltic Sea suggests

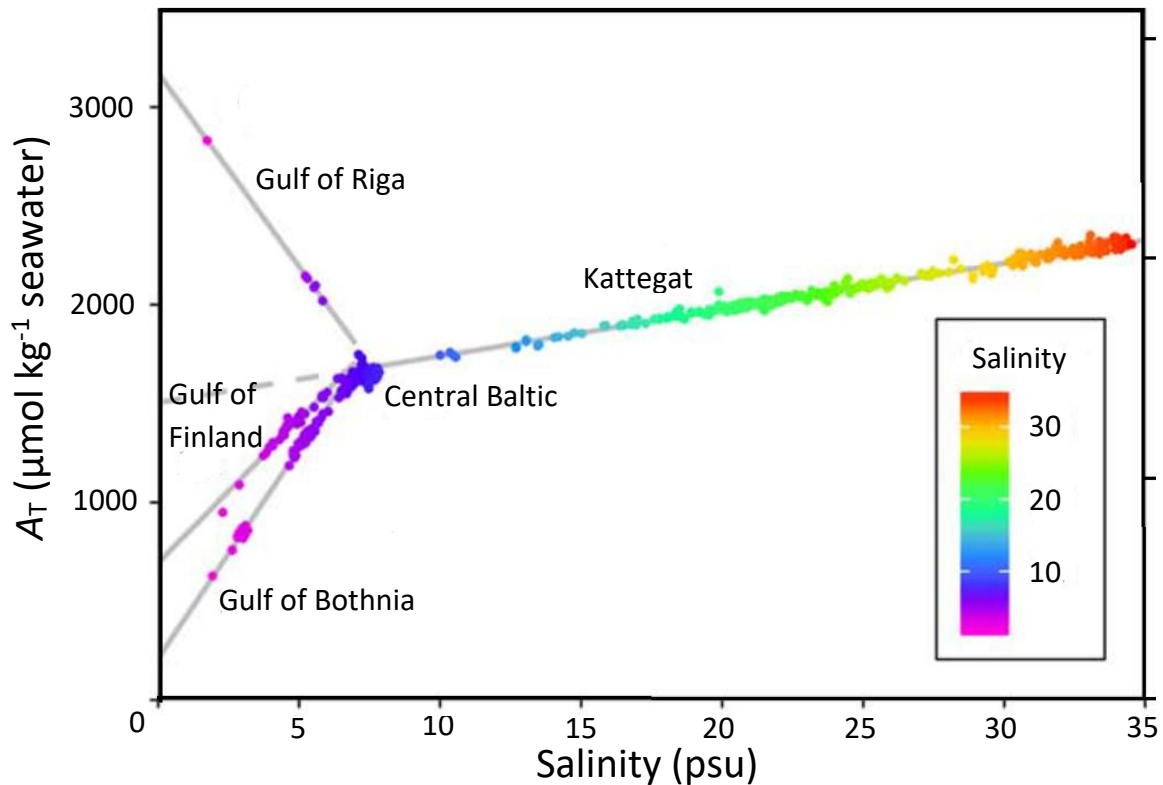


Fig. 1.2 The relationship between salinity and A_T in the Baltic Sea and the main gulfs and basins from data collected in 2008-2009. Different relationships for the different Baltic basins reflect different riverine inputs of dissolved inorganic carbon (figure adapted from Müller et al. 2016).

the Baltic is more vulnerable to ocean acidification compared to oceanic systems. However, the A_T of the Central Baltic Sea is $\sim 70\%$ that of mean oceanic A_T despite salinity being only $\sim 18\%$ that of oceanic salinity (Beldowski et al. 2010). Additionally, historical analysis of Baltic Sea A_T trends has revealed an increase over the last 100 years between $3 - 7 \mu\text{mol year}^{-1}$ and subsequent buffering of acidification by $50 - 100\%$ (Müller et al. 2016). Biogeochemical models predict that Baltic Sea pH will decrease in all the major basins and seasonal variability will increase, despite possible predicted increases in A_T (Omstedt et al. 2009; Omstedt et al. 2012). However, accurately predicting future acidification trends in the Baltic Sea remains difficult due to unclear future A_T trends and a substantial contribution of organic alkalinity to Baltic Sea buffering capacity (Kuliński et al. 2014). Understanding future trends in carbonate chemistry is vital to accurately determine and assess the impacts of climate change on marine organisms, especially calcifying organisms. Coastal zones, particularly the Baltic Sea, experience a multitude of carbonate chemistry variations due to terrestrial input of dissolved inorganic ions, upwelling and photosynthesis activity (Melzner et al. 2013; Saderne et al. 2013). This makes open ocean models relatively obsolete when applied to coastal systems and impedes the ability of scientists to make accurate predictions of future environmental changes. More *in situ* monitoring of coastal marine ecosystems is required to obtain an accurate

understanding of the local environmental conditions experienced by coastal organisms (Vuroinen et al. 2015) and allow more relevant experiments to be designed to improve our predictive ability and functional knowledge of coastal ecosystems.

1.2 Bivalves and calcification

The phylum Mollusca is the second most diverse invertebrate phylum on Earth with the class Bivalvia being the most speciose class of molluscs (Adamkewicz et al. 1997). Bivalves are characterised by a calcified exoskeleton forming two halves or “valves” which provide protection for the soft tissue from predation as well as isolation from the environment during extreme environmental conditions (mostly in epifaunal bivalves). It is thought that the rapid expansion of the Mollusca after the Cambrian period (ca. 545 million years ago) is partly due to the presence of a calcified exoskeleton (Jackson et al. 2010). Bivalve shells typically consist of an outer prismatic layer comprised of either calcite or aragonite and an inner nacreous layer comprised of polygonal aragonite tablets with the external shell surface covered by an organic, protein layer called the periostracum which is involved in shell secretion, mechanical and

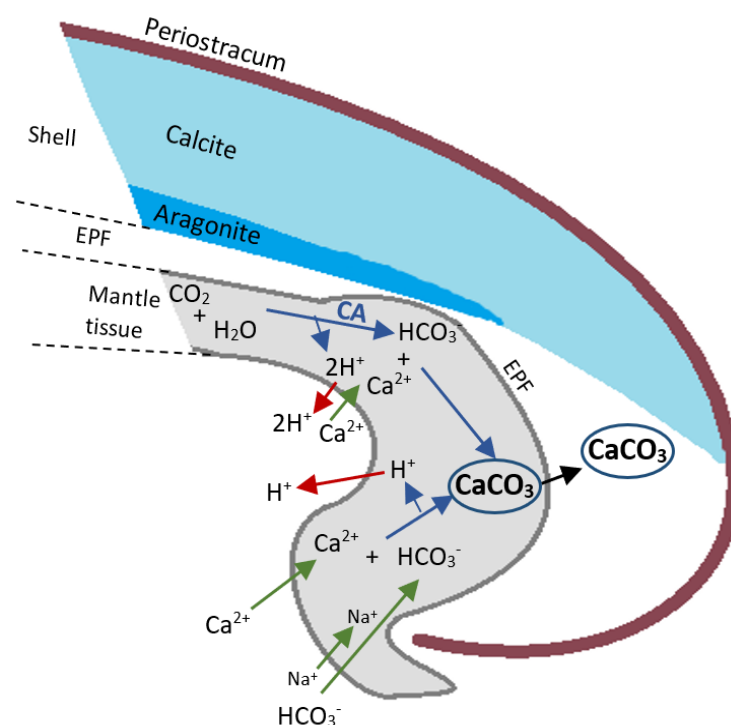
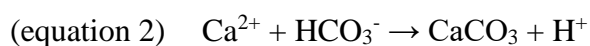


Fig. 1.2 A cross section of a mollusc body and shell with proposed ion transport pathways for calcification assuming calcification proceeds intracellularly. EPF is the extrapallial fluid which is the extracellular space between the mantle tissue and the site of calcification and CA is the enzyme carbonic anhydrase which is involved in the conversion of metabolic CO₂ to HCO₃⁻. Green arrows represent uptake of ions through ion transport proteins, red arrows represent excretion of protons through ion transport proteins and blue arrows show chemical reactions. What is not shown is the Na⁺/K⁺-ATPase which is responsible for maintaining the Na⁺ gradient for HCO₃⁻ uptake. The figure is adapted from McConnaughey & Whelan 1997, with pathways proposed from work by Cohen & McConnaughey 2003, Wang et al. 2008 and Zoccola et al. 2015.

chemical protection (Waite et al. 1979; Harper 1997; Kobayashi & Samata 2006; Maria de Paula & Silveira 2009). Shell matrix proteins (SMPs) and chitin are embedded within the calcium carbonate layers and play an important role in determining calcium carbonate polymorphs and crystal arrangement, as well as the mechanical properties of shells (Weiner & Traub 1984; Belcher et al. 1996; Suzuki et al. 2009). Recent work has also identified their potential role in immunity (Arivalagan et al. 2016). Calcification is the formation of calcium carbonate minerals which requires the substrates, calcium and bicarbonate to form CaCO_3 , subsequently producing protons as end products (McConnaughey & Whelan 1997) via the reaction:



Experiments using isotopically labelled calcium suggest that seawater is the primary source of calcium in marine calcifying bivalves (Bevelander 1952; Sillanpää et al. 2016). Isotope analysis has also revealed that seawater accounts for > 90 % of inorganic carbon substrate for calcification with the other < 10 % deriving from metabolically excreted CO_2 (Lorrain et al. 2004; Gillikin et al. 2006; McConnaughey & Gillikin 2008). Biomineralisation of CaCO_3 structures has been revealed to be a highly complex, regulated process proceeding either intracellularly or extracellularly. Very little is known about these mechanisms in bivalves and there is evidence suggesting both intracellular and extracellular pathways may be utilised in molluscan calcification. The mechanisms of CaCO_3 deposition in molluscs are most likely controlled by an organ called the mantle (analogous to the shell field organ in bivalve larvae) which is responsible for secreting SMP's as well as transforming and transporting calcification substrates to the site of calcification (Addadi et al. 2006). Intracellular calcification models suggest CaCO_3 is initially formed as amorphous calcium carbonate (ACC), which is transported in vesicles across the mantle epithelium and exocytosed to the site of calcification where nucleation and crystal formation occurs at the nucleation site on the organic matrix (Weiner & Hood 1975; Weiss et al. 2002, Addadi et al. 2006; Weiner & Addadi 2011, Fig. 1.3). Recent work however, suggests mineral formation may occur extracellularly with substrates for CaCO_3 mineralisation being transported to the calcifying space (CS) (Ramesh et al. 2017). This is supported by work on trace element concentrations (Sr/Ca) in adult bivalve shells where transepithelial transport of calcium to the CS has also been proposed (Carré et al. 2006). These findings are supported by further evidence suggesting no major role of an ACC precursor molecule in biomineralisation (Saruwatari et al. 2009; Ramesh et al. 2018). However,

it cannot be ruled out that ACC containing vesicles are present on nanometre scales and rapidly transform into crystalline structures (DeVol et al. 2015).

Understanding the mechanisms of molluscan calcification have major implications for both our understanding of the evolution and costs of calcification, as well as the field of mineral science. Supporting evidence for both intracellular and extracellular processes of calcification as well as the presence of ACC precursors makes it difficult to understand the physiological costs and evolutionary mechanisms of molluscan calcification. The use of shell damage/repair experiments coupled with ‘omics approaches (proteomics, genomics and transcriptomics) have provided valuable insights into the proteins and genes utilised during molluscan biomineralisation (Zhang et al. 2012; Sleight et al. 2016; Arivalagan et al. 2017). However, these approaches do not enable us to understand the physiological cellular processes involved in biomineralisation and how these processes can be impacted by environmental conditions. Only by coupling these powerful ‘omics approaches with both intracellular and whole animal physiological measurements can true conclusions be obtained regarding the costs of calcification and subsequent fitness implications of environmental change.

1.3 Costs of Calcification

Costs of calcification can be defined in two ways as either 1) direct energetic costs of CaCO_3 production and transport or 2) indirect ecological costs of growth limitation (Palmer 1981). Much focus has been given to estimating the energetic costs of calcification in molluscs due to the increasing threat of climate change and the perceived impacts of ocean acidification on calcifying shellfish (Orr et al. 2005; Gazeau et al. 2007; Talmage & Gobler 2009). In order to understand how much energy is required to create a shell, it is first vital to understand the energy demanding physiological mechanisms involved in molluscan calcification.

Calcifying marine molluscs need to take up and accumulate Ca^{2+} and HCO_3^- from seawater through transepithelial or vesicular pathways (Fig. 1.3). *In situ* hybridisation has shown that plasma membrane Ca^{2+} -ATPase's (PMCA's) are expressed in epithelial cells of pearl oysters and corals near the site of calcification (Zoccola et al. 2004; Wang et al. 2008). This may suggest these proteins are involved in Ca^{2+} uptake during calcification, however no direct link between their occurrences and their role in calcification has been established in molluscs. In corals, HCO_3^- uptake is believed to proceed via membrane bound bicarbonate transporters of the solute carrier 4 (SLC4) family (via the $\text{Na}^+/\text{HCO}_3^-$ cotransporter and/or the Na^+ driven $\text{Cl}^-/\text{HCO}_3^-$ exchanger) or, membrane bound transporters in the solute carrier 26 (SLC26) family

(Parker & Boron 2013; Zoccola et al. 2015). Although co-transporters and exchangers do not require ATP, the Na^+ and Cl^- gradients required for their function are provided by the membrane bound Na^+/K^+ -ATPase, which requires a considerable fraction (19 – 28 %) of the cellular energy budget (Rolfe & Brown 1997; Pan et al. 2015; Ip et al. 2015). Calcification also produces H^+ (equation 2) which need to be eliminated to prevent pH reductions and inhibition of calcification. This can be achieved via pathways such as the V-type H^+ -ATPase or the $\text{Ca}^{2+}/\text{H}^+$ antiporter which also involves the uptake of Ca^{2+} (Niggli et al. 1982; Waldbusser et al. 2013; Toyofuku et al. 2017). Ion transport and regulation is likely an integral process in molluscan biomineralisation and subsequently, constitutes a considerable energetic cost due to the activity of membrane bound ATPases. Calcification may also entail other potential energetic costs such as carbonic anhydrase activity (the enzyme catalysing the reaction of CO_2 to HCO_3^-) or, potentially, transformation and vesicular transport of ACC transforming precursor phases to crystals (ACC) (Radha et al. 2010). These aforementioned cellular processes are still not completely understood in several calcifying invertebrate phyla and without more concrete knowledge on the energy consuming pathways involved in biomineralisation, estimating the costs of calcification remains difficult.

At this point, it is important to state that the costs of calcification (CaCO_3 deposition) is not the same as costs of shell production (CaCO_3 deposition + shell associated organic material production). As mentioned previously, bivalve shells contain 1-5 % organic material (mostly protein) of which synthesis and excretion of these shell matrix proteins (SMPs) to the calcification site is energetically costly and is thought to constitute a significant proportion of shell production costs (Palmer 1983). Estimates of the costs of calcification have resulted in values of 1-2 J mg^{-1} CaCO_3 for gastropods (Palmer 1992), 1.6 J mg^{-1} CaCO_3 for oyster larvae (Waldbusser et al. 2013; Frieder et al. 2016) and ~ 0.15 J mg^{-1} CaCO_3 for corals (Anthony et al. 2002). A common finding amongst all estimates of calcification costs is that inorganic CaCO_3 precipitation is relatively energetically cheap when compared to the costs of organic matrix production or soft body tissue growth (Palmer 1983; Hawkins 1985; Bayne & Hawkins 1997; Spalding et al. 2017). Costs of inorganic CaCO_3 deposition combined with the cost of shell organic matrix production (~ 29 J mg^{-1} : Palmer 1983) are estimated to constitute 4 – 5 % of the total energy budget in gastropods and bivalves (Watson et al. 2017).

Bottom-up methods of estimating the costs of calcification based on ion transport models rely on assumptions of the cellular processes of calcification (Anthony et al. 2002; Waldbusser et al. 2013; Frieder et al. 2016). These ion transport models use conversions of ion fluxes to ATP

and subsequently to energy in Joules. Although the biochemical conversions of 1 mole of ATP to energy (Joules) are conserved across most phyla (Gnaiger & Bitterlich 1984), calcification mechanisms may not be. Additionally, we do not fully understand all energy consuming processes directly involved in calcification as well as indirect costs of ion transport and regulation such as H⁺ extrusion and maintenance of an alkaline environment at the calcification site (Ramesh et al. 2017). Top-down methods of estimating calcification costs using whole animal energetic fluxes can provide complimentary evidence to bottom-up methods and give a clearer picture of energy consumption in calcification (Palmer 1992). Additionally, very few experiments have investigated how variation in abiotic factors and climate change may impact the costs of calcification marine organisms. Calcification is intrinsically linked to growth which in turn is linked to fitness, therefore our ability to understand the costs of calcification have potentially major evolutionary implications.

1.4 Bivalve growth in the Baltic Sea

Despite the extreme aquatic environment in the Baltic Sea (low salinity, high seasonal temperature and pH variation), marine organisms have still colonised many parts of the Baltic Sea benthos. Bivalves, particularly species within the genus *Mytilus* form extensive benthic reefs in the Central and Southern Baltic Sea facilitating biodiversity and playing an important role in biological nutrient recycling (Kautsky & Wallentinus 1980; Norling & Kautsky 2008). Baltic mytilids occur throughout the Baltic Sea down to a minimum salinity of ~ 4.5 psu in the Gulf of Finland and Gulf of Bothnia. Despite the extensive colonisation of mytilids in the Baltic Sea, growth rates are severely reduced compared to fully marine populations with growth (measured as the increase in shell length per unit time) below 11 psu being < 20 % of that of North Sea populations (Kossak 2006; Riisgård et al. 2014). Additionally, Baltic mytilids reach only half the maximum size of North Sea populations and Baltic mytilid shells are also significantly thinner than those of oceanic populations (Kautsky et al. 1990; Westerbom et al. 2002). Transplant experiments, laboratory experiments and modelling studies have shown that these slow growth rates are due to the extremely low salinity in the Baltic Sea (Tedengren et al. 1990; Riisgard et al. 2013; Telesca et al. 2018), however the physiological mechanisms behind these low growth rates have still not been fully resolved.

There is no evidence that low salinity increases basal metabolic rates in Baltic mytilids as oxygen consumption has been found to be stable across a range of salinities (Landes et al. 2015; Maar et al. 2015). This suggests growth rates are not depressed due to increased metabolic

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costs associated with low salinity. Significantly higher nitrogen excretion rates and lower O:N ratios have been documented in Baltic mytilids when compared to North Sea populations (Tedengren & Kautsky 1986). This suggests less efficient protein turnover which may contribute to higher costs of growth and therefore reduced growth rates (Bayne & Hawkins 1997). However, growth rates of soft tissue have been shown to be similar at both high and low salinities suggesting limited tissue growth rates are not responsible for overall stunted growth (Kautsky et al. 1990; Riisgård et al. 2014).

Marine mytilids are osmoconformers meaning the osmolality of their body tissues remains iso-osmotic with that of the external media. Iso-osmolality is maintained by adjusting intracellular concentrations of organic osmolytes usually in the form of free amino acids (FAA's) or fatty acid derivatives (Yancey 2005). In bivalves these compounds are typically taurine, glycine, alanine and betaine compounds and are termed compatible organic solutes as changes in their concentrations do not detrimentally impact cellular processes (Pierce & Greenberg 1972; Hochachka & Somero 2002; Kube et al. 2006). Intracellular concentrations of these organic osmolytes are higher at high salinities and lower at low salinities to mediate changes in seawater osmolality and prevent major changes in cell volume during salinity fluctuations (Silva & Wright 1994). Bivalves breakdown endogenous protein to accumulate intracellular FAA's or excrete intracellular FAA's in response to salinity changes to maintain cell volume (Hawkins & Hilbish 1992). In fluctuating salinities, the energetic cost of this cell volume regulation in bivalves is significant with estimates suggesting it constitutes up to 30 % of the metabolic rate during salinity acclimation (Hawkins & Hilbish 1992; Neufeld & Wright 1996). These costs are only prominent however, in habitats with constantly fluctuating salinity. The central Baltic has a very stable salinity of ~ 6 psu therefore these cell volume regulation costs probably do not play a role in limiting growth in Baltic mussels.

Inorganic ions are also utilised by marine bivalves as osmolytes, however, intracellular concentrations of these ions tend to stay more stable due to their impacts on cellular enzyme function and activity (Hochachka & Somero 2002). Intracellular concentrations of inorganic ions are modulated during short term exposure to salinity stress, however they return to control levels after a period of acclimation (days) (Silva & Wright 1994). Despite these findings, intracellular concentration of cations (such as Na⁺ and K⁺) have been shown to change with long term (weeks) acclimation to low salinity (Willmer 1978; Berger & Kharazova 1997). Changes in intracellular concentrations of K⁺ and Na⁺ have been found to negatively impact activities of enzymes involved in amino acid metabolism and glycolysis as well as

mitochondrial metabolism (Ballantyne & Moyes 1987; Ballantyne & Berges 1991). All experiments investigating changes in intracellular cations and osmolytes have utilized short-term acclimation over hours or weeks with no long-term acclimation or multigenerational studies available on osmolyte concentration modulation. It is therefore not known how long-term adaptation of Baltic *Mytilus* to extremely low salinities may have impacted the organic osmolyte pool or intracellular cation concentrations. Understanding these potential physiological adaptations may unveil previously unknown mechanisms of salinity tolerance and shed light on the mechanisms of adaptation to low salinity estuarine habitats.

As introduced previously (in section 1.1), the Baltic Sea is a truly challenging environment for calcifiers to inhabit. Low seawater $[Ca^{2+}]$ and A_T coupled with low temperatures in winter result in $\Omega < 1$ and therefore increased dissolution risk to $CaCO_3$ structures. Limiting or more costly shell production therefore seems a potential explanation for the slow growth rates of Baltic mytilids. This has been suggested by Riisgård et al. 2014 where laboratory and field experiments revealed that low salinity did not impact the growth of body tissue but only the growth of shells. It has also been shown that $[Ca^{2+}]$ becomes limiting at $< 3 \text{ mmol kg}^{-1}$ (full strength seawater has a $[Ca^{2+}]$ of $\sim 10 \text{ mmol kg}^{-1}$) for shell growth in larval Baltic mytilids which corresponds to a salinity of $\sim 8 \text{ psu}$ (Thomsen et al. 2018). Due to the limited buffering capacity of the Baltic Sea, severe variations in carbonate chemistry parameters (such as those experienced during upwelling of deep hypercapnic water in Kiel Fjord: See Thomsen et al. 2013) would also be expected to impede calcification and growth. However, high food availability appears to be able to mitigate the negative impacts of low pH and Ω , on calcification and growth in Kiel mussels (Thomsen et al. 2013). Due to differential energy investment between shell and soft body tissue, limited ability to produce $CaCO_3$ can impede overall growth and thus present a considerable fitness cost, for example, reduced growth and reproductive output (Palmer 1981). Other non-energetic ecological costs may also entail reduced calcification and growth rates in Baltic Sea mytilids such as risk of predation due to decreased shell strength and competition for space with other benthic invertebrates (Reimer & Ringdahl 2001). These non-energetic costs associated with reduced calcification rates are difficult to quantify which subsequently makes it difficult to predict the how reduced calcification rates will impact an organism's fitness.

Desalination and warming in the Baltic Sea is predicted to lead to species distribution shifts and subsequent ecological consequences (Vuorinen et al. 2015). Due to the economic and ecological importance of *Mytilus* spp. in the Baltic Sea, it is vital to understand the mechanisms

of growth and calcification at low salinity to better predict potential ecological consequences of climate change. This will also contribute to the fundamental knowledge behind the mechanisms and costs/limits of adaptation and evolution.

1.5 Bivalve hybridization in the Baltic Sea

The unique environmental conditions of the Baltic Sea and its geographic isolation have resulted in populations of Baltic Sea organisms experiencing limited gene flow, low genetic diversity and high genetic differentiation from neighbouring North Sea populations (Johannesson & André 2006). In the bivalves, *Mytilus* spp. and *Macoma balthica*, further unique genetic characteristics are present in the form of extensive hybrid zones or hybrid swarms (Riginos & Cunningham 2005; Nikula et al. 2008). In mytilids, this hybrid zone is between *Mytilus edulis* and *Mytilus trossulus* with *Mytilus edulis*-like alleles being dominant in the higher salinities in the west and *M. trossulus*-like alleles being dominant in the low saline Central and Eastern Baltic (Väinölä & Hvilson 1991). This is in stark contrast to the Canadian Atlantic coast where pure *M. edulis* and *M. trossulus* live in sympatry and show a mosaic-like genetic structure with limited hybridisation (reviewed by Riginos & Cunningham 2005). In the Baltic Sea, the genetic transition zone (where the steepest cline in allele frequency occurs and the highest admixture of both species) is between Rügen on the German Baltic Sea coast and the Swedish coast of Öresund (Väinölä & Strelkov 2011; Stuckas et al. 2017, Fig. 2.4). The geographic extent of the hybrid zone and spatial width of the genetic transition zone for both *M. balthica* and *Mytilus* spp. is very broad suggesting both limited genome incompatibilities and limited reproductive isolation. Additionally, asymmetry in clinal trends between different genetic markers along the transition zone of the German Baltic coast suggests neutral genetic processes such as genetic drift, are not responsible for the observed patterns of gene introgression between *M. edulis* and *M. trossulus* (Stuckas et al. 2009). Modelling of larval dispersal patterns has also indicated that hydrology and geographic barriers limiting larval dispersal do not explain the structure of the genetic transition zone in Baltic *Mytilus* (Stuckas et al. 2017). Although spatial dynamics in the hybrid zone cannot be ruled out as mechanisms driving clinal gene patterns (Stuckas et al. 2009; Strelkov et al. 2017), it seems likely that natural selection is the driving force in maintenance of the Baltic *Mytilus* population structure (Johannesson et al. 1990). Non-neutral genetic forces (natural selection) have also been identified as a driver for the genetic structure of the hybrid swarm between Atlantic *M. balthica* and Pacific *M. balthica* in the Baltic Sea (Luttikhuisen et al. 2012). It has been suggested that hybridisation enhances genetic diversity by forming new allele combinations and can therefore

facilitate adaptive radiation and colonisation of new habitats (Seehausen 2004). When viewed from this perspective, the new allele combinations resulting from hybrid genotypes may have allowed colonisation of the Baltic Sea and better performance than pure genotypes.

Colonisation of the Baltic Sea by marine organisms began when an ancient Baltic lake opened to the North Sea ~ 8000 years ago (Donner 1995). The existence of an ancient *Mytilus* hybrid zone resulting from secondary contact between *Mytilus edulis* and *Mytilus trossulus* might have enabled rapid colonisation and genetic divergence of Baltic and Skagerrak/Kattegat populations from analogous European marine populations (Riginos & Cunningham 2005; Väinölä & Strelkov 2011; Stuckas et al. 2017). *Mytilus* spp. are also unique in their mitochondrial inheritance whereby females receive a mitochondrial genome from the maternal lineage (F) and males receive both a maternal and paternal (M) mitochondrial genome with both M and F mitochondrial genomes exhibiting ~ 20 % genetic divergence (Skibinksi et al. 1994; Zbawicka et al. 2007). Genetic analysis has revealed that all mtDNA of *M. trossulus* origin has been lost in the Baltic Sea and replaced exclusively by mtDNA of *M. edulis* origin

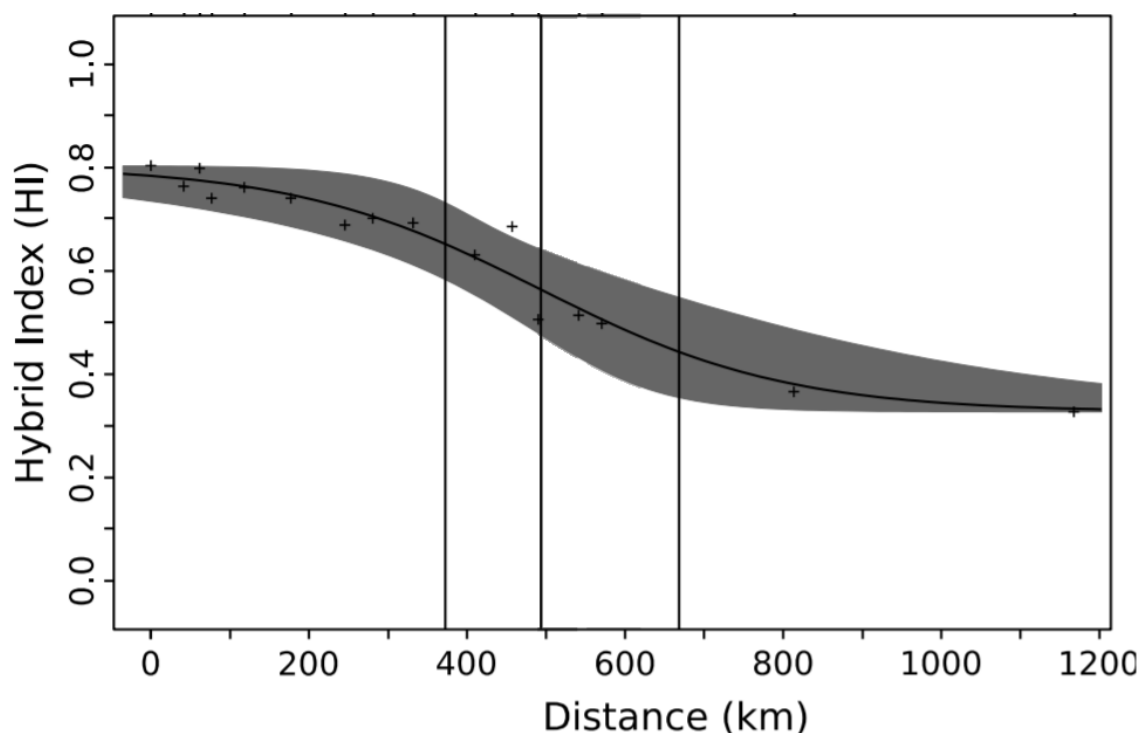


Fig. 1.3 Mean hybrid indices from 16 sampled populations along the Baltic Sea salinity gradient from Flensburg (~ 0 km) on the German Baltic Sea coast to Hel (~ 1200 km) on the Polish Baltic Sea coast. A hybrid score of 1.0 indicates pure *Mytilus edulis* whilst a hybrid score of 0.0 indicates pure *Mytilus trossulus*. Hybrid scores were calculated based on number of *M. edulis* specific alleles at the loci Glu-5', EFbis, MAL-1 and M7 lysin. The left and right most vertical lines represent the confidence intervals of the cline centre (region where the steepest change in hybrid indices occur) with the central most vertical line depicting the cline centre. The shaded area represents the 2 log likelihood confidence interval of the fitted curve. The figure is adapted from Stuckas et al. 2017 with further details of genetic analyses therein.

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(Kijewski et al. 2006). Additionally, the M mitochondrial genome has been replaced by a paternally transmitted female mitochondrial genome, thus resetting the divergence between these two mitochondrial genomes in heteroplasmic males (Quesada et al. 2003). These unique characteristics of Baltic mtDNA inheritance may provide support to the idea that hybridisation is linked to adaptive radiation and increased fitness. However, the physiological and evolutionary mechanisms behind these nuclear and mitochondrial genome anomalies in Baltic *Mytilus* still remain elusive. Currently, no studies have empirically tested how salinity impacts natural selection of certain allele combinations or how hybridisation influences fitness at different salinities in Baltic *Mytilus*. This is vital for our understanding of how hybridisation impacts physiology and for understanding how evolution of physiological processes ensues in a rapidly changing environment.

1.6 Research questions

The aim of this thesis is to investigate how salinity impacts calcification and drives local adaptation of Baltic *Mytilus* in the Baltic Sea.

The south-western Baltic Sea provides an excellent system to investigate these topics due to the salinity gradient and genetic gradient in *Mytilus* allele frequencies. Three Baltic *Mytilus* populations at three sites along the salinity gradient were utilised throughout this thesis to investigate how salinity shapes physiology, growth, local adaptation and genetic structure of Baltic *Mytilus* in laboratory experiments.

Chapter 1 – Salinity and Calcification

Decreasing salinity is coupled with decreasing $[Ca^{2+}]$ and C_T , all of which impact calcification in marine mussels. It is not fully clear which of these 3 factors is chiefly responsible for the low calcification rates of Baltic mussels. Additionally, temporal and spatially environmental variability is high in coastal Baltic habitats and it remains elusive how these and other abiotic conditions which govern growth, ensue in south-western Baltic reefs and how they may impact field growth rates of Baltic *Mytilus*. Consequently, laboratory experiments were conducted looking at calcification rates under different salinities, $[Ca^{2+}]$ and C_T to answer the question: **What factor (salinity or calcification substrate availability) is chiefly responsible for decreased calcification in Baltic *Mytilus*.** Additionally, environmental monitoring of salinity, carbonate chemistry and food availability was conducted at the three Baltic sites to answer the question: **Is salinity primarily responsible for different calcification rates in south-western Baltic reefs?**

Chapter 2 – Energetic costs of calcification

Low salinity is thought to reduce growth rates in bivalves due to energetic costs associated with higher osmotic stress. However, body tissue growth rates appear to be unimpacted by salinity whereas conditions for calcification at low salinities are poor due to low substrate availability and seasonal $CaCO_3$ undersaturation. These sub-optimal conditions for calcification may result in costlier shell production and subsequently slower growth rates as seen in Baltic Sea mussels. To investigate how calcification costs may be impacted by salinity, laboratory experiments were utilised to answer the question: **What are the costs of calcification in Baltic mussels and are these costs higher at low salinities?** Energy budgets of three locally adapted populations were calculated under a multitude of controlled feeding levels to estimate the costs

of calcification at different temperatures and salinities and estimate the relative cost of shell production as a proportion of available growth energy.

Chapter 3 – Salinity, selection and local adaptation

Physiological differences between different salinity adapted populations of Baltic *Mytilus* may result from either physiological acclimation or adaptive evolution to prevailing environmental conditions. The presence of the Baltic *Mytilus* hybrid swarm and the observed genetic transition zone along the salinity gradient may suggest that local adaptation of low salinity populations results from salinity driven selection for *M. trossulus* genotypes at low salinities. However, this has never been systematically tested under controlled laboratory conditions. Subsequently, the final chapter in this thesis answers the question: **Are *M. trossulus* genotypes locally adapted to low salinity and does low salinity select for *M. trossulus* alleles?** To investigate this, two laboratory experiments were performed on Baltic *Mytilus* larvae, the first of which compared growth, mortality and settlement of a high and low salinity adapted population of Baltic *Mytilus* to investigate potential local adaptation of *M. trossulus* genotypes to low salinities. Secondly an artificial selection experiment was conducted on Baltic *Mytilus* larvae from a hybrid population to investigate if natural selection by low salinity favours *M. trossulus* alleles.

Chapter 4 – Mechanisms of salinity adaptation

As well as sub-optimal conditions for calcification, low salinities also invoke cellular changes in organic osmolyte and cation concentrations which have been linked to reduced enzyme activities and mitochondrial metabolic efficiency. Although these short-term mechanisms of cellular osmotic adjustment have been extensively studied, little is known regarding adaptive changes in intracellular cation and osmolyte pools during long term adaptation to low salinity habitats. Potential adaptive changes in osmolyte pools may be related to different salinity tolerances and underlie the mechanisms of adaptation to low salinities. Consequently, a population comparison experiment was conducted to answer the question: **Is local adaptation to low salinity facilitated by adaptive changes in intracellular osmolyte pools?** This was investigated by comparing a high salinity and a low salinity population of Baltic *Mytilus*. Qualitative and quantitative changes in intracellular organic and inorganic osmolyte pools were investigated as well as salinity tolerance at extremely low salinities.

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2. Chapters

Declaration of contribution

List of thesis chapters and the declaration of my contribution to the work conducted in each chapter:

Chapter 1 – Salinity and calcification

Trystan Sanders, Jörn Thomsen, Jens Müller, Gregor Rehder, Frank Melzner. Calcium, rather than dissolved inorganic carbon and salinity, limits calcification in Baltic *Mytilus* in low salinity habitats. To be submitted to Biogeosciences.

I designed and conducted the laboratory experiments together with Jörn Thomsen and Frank Melzner. I analysed the experimental data and field monitoring data while Jens Müller analysed the field carbonate chemistry data. I wrote the manuscript and all authors contributed to revisions.

Chapter 2 – Energetic costs of calcification

Trystan Sanders, Lara Schmittmann, Jennifer Nascimento-Schulze, Frank Melzner. High calcification costs limit mussel growth at low salinity. Submitted to *Frontiers in Marine Science*

I conceived the experimental design together with Frank Melzner and conducted the experiments. Lara Schmittmann and Jennifer Nascimento-Schulze helped with experimental procedures. I analysed the data and wrote the manuscript and all co-authors contributed to manuscript revisions.

Chapter 3 – Salinity, selection and local adaptation

Loreen Knöbel*, Jennifer Catherine Nascimento-Schulze*, **Trystan Sanders***, Dominique Zeus, Jörn Thomsen, Claas Hiebenthal, Heiko Stuckas+, Frank Melzner+. Allele shift under laboratory selection and local adaptation to low salinity in Baltic *Mytilus* hybrids (*M. edulis*, *M. trossulus*). To be submitted to *Proceedings of the Royal Society B Biological Sciences*.

*co-first authors, + co - senior authors

Loreen Knöbel (LK), Jennifer Nascimento-Schulze (JNS) and I share first authorship of this manuscript. Frank Melzner (FM) and Heiko Stuckas (HS) developed the hypothetical framework for the study. I designed and conducted one of the two larval experiments together

with Claas Hiebenthal. JNS, Jörn Thomsen (JT), and LK conducted the spawning and fertilization part of experiment 2 which was run by JNS and co-supervised by me and JT. JNS and I analysed the larval data and genotyping, modeling and genetic analysis was carried out by LK, Dominique Zeus and HS at Senckenberg Natural History Collection Dresden. Writing was coordinated by LK and all first authors, FM and HS wrote the first manuscript draft. All co-authors contributed significantly to manuscript revisions.

Chapter 4 - Mechanisms of salinity adaptation

Trystan Sanders, Christian Bock, Lara Schmittmann, Frank Melzner. Local adaptation of estuarine mussels facilitated by organic and inorganic osmolyte modulation

I designed the study together with my advisor, Frank Melzner. I carried out experimental incubations and performed tissue extractions and extracellular fluid sampling at GEOMAR. Tissue metabolomes were analyzed together with Christian Bock and Lara Schmittmann at the AWI Bremerhaven NMR laboratory while ions and osmolarity were analysed in Markus Bleich's lab at the Institute of Physiology at CAU, Kiel. I analysed the data, wrote the manuscript and all authors contributed to manuscript revision

Chapter 1 – Salinity and calcification

Calcium, rather than dissolved inorganic carbon and salinity, limits calcification in Baltic *Mytilus* in low salinity habitats.

Calcium, rather than dissolved inorganic carbon and salinity, limits calcification in Baltic *Mytilus* in low salinity habitats.

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Keywords: *Baltic Sea, calcification, calcium, dissolved inorganic carbon, Mytilus, total alkalinity.*

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Abstract

The Baltic Sea is characterised by a decreasing salinity gradient from the Danish straits to the northern and eastern basins. Along this salinity gradient, C_T and $[Ca^{2+}]$ also decrease, leading to sub-optimal conditions for calcifying mussels that utilise these substrates for $CaCO_3$ deposition. Climate models predict a rapid desalination of the central Baltic over the next 80 years with the potential to seriously alter Baltic ecosystems via distribution shifts of key Benthic organisms. Baltic mytilid mussels are dominant ecosystem engineers in the Baltic benthos and exhibit very slow calcification rates at extremely low salinities in the Central and Eastern Baltic. It is not clear to what extent these abiotic factors (salinity, $[Ca^{2+}]$ and C_T) contribute to reduced calcification rates in non-larval bivalves. Additionally, our knowledge of the ubiquity of these carbonate chemistry parameters in near-shore Baltic mussel reefs is scarce which is vital for our understanding of how climate change in the Baltic will impact the distribution of Baltic mytilids. In this study a series of laboratory experiments were conducted where calcification rates of juvenile Baltic mytilids were analysed across a series of salinities (16 – 6 psu) and seawater $[HCO_3^-]$ (proxy for C_T ; 300-2100 $\mu\text{mol kg}^{-1}$) and $[Ca^{2+}]$ (0.5-4 mmol kg^{-1}). Additionally, salinity, carbonate chemistry and food availability were monitored in three Baltic mussel reefs along the south-western Baltic Sea salinity gradient. Findings reveal that calcification rate decreases linearly with $[Ca^{2+}]$ below 4 mmol kg^{-1} but is stable down to salinities of 11 psu and $[HCO_3^-]$ of $\sim 900 \mu\text{mol kg}^{-1}$. Coupled with monitoring data from three Baltic mussel reefs, this suggests Ca^{2+} availability rather than inorganic carbon availability is likely limiting growth rates of Baltic mytilids. Monitoring of field mytilid calcification rates also suggests that Ca^{2+} availability can not exclusively explain reduced calcification in Baltic mussels with food availability and environmental variability likely being important factors to consider when predicted the impacts of large scale environmental changes on Baltic ecosystems.

Introduction

The Baltic Sea is a semi-enclosed brackish water body with a decreasing salinity gradient from > 25 practical salinity units (psu) in the Kattegat to < 3 psu in the Gulf of Bothnia and the Gulf of Finland (Meier 2006; Neumann 2010). Total alkalinity (A_T : seawater pH buffering capacity) decreases linearly with salinity in the Baltic Sea from $\sim 2300 \mu\text{mol kg}^{-1}$ to $\sim 1600 \mu\text{mol kg}^{-1}$ at 6 psu in the Baltic Proper (Dickson 1992; Müller et al. 2016). Dissolved inorganic carbon (C_T) species (HCO_3^- and CO_3^{2-}) constitute $\sim 90 \%$ of A_T meaning A_T is intrinsically linked to

carbonate chemistry and thus, CaCO_3 saturation state (Ω) which defines the stability of CaCO_3 structures in seawater (Ries et al. 2016). Values of $\Omega < 1$ theoretically led to thermodynamically favourable dissolution of CaCO_3 structures and have been observed to have negative impacts on calcifying organisms (Orr et al. 2005).

Climate models predict severe physiochemical changes in the Baltic Sea over the next 50-100 years with warming at 2–4 times the global average (Belkin 2009) and rapid desalination by up to 5 psu in the Baltic Proper due to increased extreme weather events, precipitation and freshwater run-off (Gräwe et al. 2013). Although A_T has shown a steady increase over the past 100 years, predicting future trends in A_T remains difficult due to significant impacts from dissolved organic carbon (DOC) and high variability in CO_2 system constituents (Kuliński et al. 2014; Müller et al. 2016). Projected patterns of warming and desalination may severely impact marine species distributions and Baltic ecosystems (Vuorinen et al. 2015).

The dominant benthic macrofauna in the Baltic Sea are the calcifying marine mytilid mussels (from here on: Baltic *Mytilus*) which are an integral reef-forming foundation species responsible for high biodiversity and nutrient recycling (Kautsky & Wallentinus 1980; Norling & Kautsky 2008). Baltic *Mytilus* exhibit extremely slow growth and calcification rates < 10 psu (Kautsky et al. 1990; Vuorinen et al. 2002) which has been attributed to both osmotic stress and limited ability to biomineralize shells (Tedengren & Kautsky 1986; Riisgård et al. 2014). Calcification relies on the seawater availability of the two main calcification substrates Ca^{2+} and HCO_3^- (McConnaughey & Gillikin 2008) which decrease in concentration with salinity and A_T along the Baltic Sea salinity gradient (Kremling & Wilhelm 1997; Beldowski et al. 2010). Reduced concentrations of these ions (Ca^{2+} and HCO_3^-) have been shown to limit calcification in bivalve larvae (Thomsen et al. 2015; Thomsen et al. 2018) however, little is known if the effect of substrate limitation is the same for non-larval bivalves. Low salinities also induce a degree of cellular osmotic stress in osmo-conforming invertebrates and it is not currently known which primary mechanism (Ca^{2+} availability, HCO_3^- availability or osmotic stress) low salinity impacts growth of calcifying organisms.

The coastal Baltic Sea is characterized by extreme temporal variations in microalgae abundance, temperature and pCO_2 (Viitasalo et al. 1995; Thomas et al. 1999; Melzner et al. 2013). Salinity is also variable due to sporadic North Sea inflow events of highly saline water and seasonal variation in riverine freshwater input due to snow melt (Lass et al. 1996; Graham 2004). Spatial variability is high due to the salinity gradient as well as varying degrees of

eutrophication resulting from differences in riverine nutrient-load (Stålnacke et al. 1998; HELCOM 2014). These high levels of eutrophication lead to seasonal microalgae blooms which vary in intensity between Baltic basins providing food for secondary consumers (Pitarch et al. 2016). High food availability has been shown to correlate with improved reproductive condition in mussels (Wołowicz et al. 2006) and counteract the effects of low pH on calcification in Baltic *Mytilus* in Kiel Fjord (Melzner et al. 2011; Thomsen et al. 2013). However, it is not fully understood how food availability may be able to mediate negative impacts of low or fluctuating salinity. How these factors (variability, salinity stress and food) interplay and impact calcification is even less understood and subsequently, bivalve growth models have often omitted calcification. This is because models struggle to accurately predict calcification in response to food and salinity variations (Bergström et al. 2015; Maar et al. 2015). Due to the significant ecological importance of *Mytilus* populations in Baltic ecosystems and climate predictions, it is vital to identify how these key environmental factors impact *Mytilus* calcification and thus growth and fitness.

The aim of this study is to identify and highlight the key environmental variables impacting calcification rates in Baltic *Mytilus* juveniles along the south western Baltic Sea salinity gradient. This was achieved through: 1) Laboratory experiments where Ca^{2+} and HCO_3^- availability was decoupled from salinity to identify the mechanisms by which low salinities reduce calcification rates in Baltic *Mytilus*. 2) Monitoring annual environmental conditions and field mussel growth rates from three mussel reefs along the German Baltic salinity gradient to understand how significant salinity is for predicting calcification rates compared to other environmental factors.

Material and Methods

Animal collection and maintenance conditions

Freshly settled 0.6-1.5 mm shell length (SL) Baltic *Mytilus* were collected from wooden piers in Ahrenshoop, Germany (54° 23' 7.3032" N, 12° 25' 24.0564" E. salinity = 10.8 psu, temperature = 19.3 °C, Depth = < 1 meter) in July 2014 and July 2016. Within 6 hours, specimens were transported in chilled aerated cool boxes to GEOMAR, Kiel. Animals were stored in four, 20 L plastic aquaria (16 °C) for three weeks prior to experiments and fed twice daily with *Rhodomonas salina* at a concentration of >10 000 cells ml⁻¹. Water changes were done every three days and the correct salinity was obtained by mixing 0.22µm filtered seawater (FSW) from Kiel fjord (~16 psu) with distilled water with A_T adjusted to natural values by the

addition of 1 M NaHCO₃ solution. pH_{NBS} and salinity/temperature were monitored daily with a WTW pH 3110 and a WTW Cond 315i probe, respectively. Juvenile mussels were kept in square, 2 L plastic aquaria or 50 ml plastic cylindrical flasks held in 16 °C water baths (details in following section). Live microalgal food (*Rhodomonas salina*) was added twice or thrice daily depending on clearance rates which were monitored weekly using a Multisizer 3 Coulter Counter (Beckman, Germany). Water changes were conducted thrice weekly to prevent significant deviations in seawater carbonate chemistry parameters due to calcification, shell dissolution or the addition of microalgae cultures.

Laboratory experiments

Two separate laboratory experiments were conducted, a C_T limitation experiment in 2014 and a Ca²⁺ limitation experiment in 2016. The C_T limitation experiment involved exposing juveniles (~ 1600 animals per tank, n = 4) to three salinities (16, 11 and 6 psu) and 5 bicarbonate ion concentrations (300, 600, 900, 1500, and 2100 μmol kg⁻¹) in a fully crossed design lasting 70 days. [HCO₃⁻] was used as a proxy for C_T availability as HCO₃⁻ constitutes ~ 90 % of C_T and is proportional to [CO₃²⁻] (Zeebe & Wolf-Gladrow 2001; Table S1 and S2). Therefore, C_T availability is expressed in terms of [HCO₃⁻] throughout the manuscript. Manipulation of C_T also changes pH (Table S1) between treatments. pH as a single factor has been shown to have no impact on mollusc calcification (Waldbusser et al. 2014; Thomsen et al. 2015), therefore we can attribute differences in calcification rates between treatments primarily to C_T availability. The second experiment in 2016 involved the same experimental design but with fewer animals per replicate tank (2 animals per tank, N = 4). 50 ml plastic aquaria were used in this experiment due to limited ability to produce large amounts of artificial seawater. Fewer animals were therefore used to limit impacts of calcification and food addition on water carbonate chemistry. This experiment consisted of the same three salinities but 5 different [Ca²⁺] (0.5, 1, 2, 3, 4 mmol kg⁻¹) lasting for 37 days. At the end of both experiments growth rates were calculated (see: body and shell mass) and mortality was calculated for the 2014 experiment only as the 2016 experiment incurred no mortality. The salinities were chosen based on the salinity range along the German Baltic coast. [Ca²⁺] and [HCO₃⁻] were selected to cover the range from saturated concentrations to below the natural concentrations found at the environmental salinity limit of Baltic *Mytilus*.

Seawater chemistry

For the 2014 experiment, FSW from Kiel fjord was adjusted to 16, 11 and 6 psu with distilled water and aerated for 24 hours. Total inorganic carbon (C_T) was weekly determined in stock FSW and experimental FSW using an AIRICA CO₂ analyser (Kiel, Germany) and verified by measuring certified reference material (Dickson et al. 2003). Seawater carbonate system parameters (A_T , $[H^+]$, $[HCO_3^-]$, $[CO_3^{2-}]$, Ω_{calcite} and $\Omega_{\text{aragonite}}$) were calculated (with C_T and pH_{NBS} as inputs) using the CO2SYS program with KHSO₄, K1 and K2 dissociation constants (Dickson et al. 1990; Roy et al. 1993). To achieve experimental HCO_3^- concentrations, 1M NaHCO₃ or 1M HCl was added to increase or decrease $[HCO_3^-]$, respectively. Water was prepared 24 hours before water changes and aerated with normal air to maintain a pCO_2 of 400 μatm . For the 2016 experiment, Ca^{2+} free artificial seawater (ASW- Ca^{2+}) was prepared (Kester 1967) for salinities of 16, 11 and 6 psu by adding NaCl, NaSO₄, KCl, NaHCO₃, KBr, H₃BO₃, MgCl₂ and SrCl₂ to deionised water. In addition, all treatments contained 5 % FSW from the collection site adjusted to experimental salinities to ensure presence of trace elements. At each salinity level, $[Ca^{2+}]$ was adjusted through the addition of a 1M CaCl₂ stock solution. Water samples were taken for C_T and $[Ca^{2+}]$ measurements from the experimental units and from freshly prepared seawater. $[Ca^{2+}]$ was measured using a flame photometer (EFOX 5053, Eppendorf) calibrated with urine standards (Biorapid).

In addition to saturation state (Ω), the ratio of calcification substrate ($[HCO_3^-]$ and $[Ca^{2+}]$) to inhibitor ($[H^+]$) termed the extended substrate inhibitor ratio (SIR) has also been shown to correlate strongly with calcification. Extended SIR was calculated as described in Thomsen et al.s 2018 with the following equation:

$$\text{(equation 1) SIR} = [HCO_3^-][Ca^{2+}] / [H^+]$$

Body and shell mass

50 mussels were taken and frozen at the beginning of each experiment for a time 0 reference. After each experiment all mussels were gently removed from experimental tanks using a plastic scraper and frozen at $-20\text{ }^\circ\text{C}$ for later mass analysis. For this, two mussels of different sizes from each replicate tank were placed individually into pre-weighed and pre-dried aluminium foil cups and dried at $60\text{ }^\circ\text{C}$ for 12 hours. Samples were then weighed and placed in a muffle furnace and ashed at $450\text{ }^\circ\text{C}$ for 4 hours to remove organic content. The remaining inorganic mass was re-weighed to determine the ash mass which was used as a proxy for CaCO₃ mass. Calcification rates were expressed as the total mass of CaCO₃ deposited per individual

throughout the duration of the experiment divided by the total number of days expressed as $\mu\text{g CaCO}_3 \text{ day}^{-1}$.

Field monitoring

Three monitoring sites were selected along the German Baltic Sea coast spanning the steepest geographic region of the salinity and Baltic *Mytilus* genetic gradient (Fig. 1). These sites were: Kiel (54° 19' 48.846" N, 10° 8' 59.6436" E), Ahrenshoop (54° 23' 7.3032" N, 12° 25' 24.0564" E) and Usedom (54° 3' 20.5668" N, 14° 0' 40.0572" E).

Salinity, temperature and seawater ions

CTD (conductivity, temperature, depth) miniature loggers (Star-Oddi, Iceland) were placed on wooden pillars at each site between July and September 2015 at a depth of 1 meter. These were replaced every 2-3 months over two years. $[\text{Ca}^{2+}]$ was calculated from salinity values using $[\text{Ca}^{2+}]$ -chlorinity relationships from Kremling & Wilhelm 1997 and a chlorinity-salinity relationship from Millero 1984. Additionally, every 2-3 months, 2 x 500 ml water samples were taken from Ahrenshoop and Usedom at 1 meter and immediately poisoned with saturated MgCl_2 for pH , A_T and C_T measurements at the Leibniz Institute for Baltic Sea Research, Warnemünde. Kiel carbonate chemistry data was taken from published monitoring data for 2015 (Hiebenthal et al. 2017). Sample pH_{Total} was measured spectro-photometrically at 25 °C with non-purified m-cresol purple as indicator dye (dye pH -perturbation corrected after Hammer et al. 2014). C_T was determined with a SOMMA system (Single Operator Multi-Parameter Metabolic Analyzer) operated at 15°C and A_T was determined by open-cell titration at 20°C (Dickson et al. 2007). Seawater carbonate system parameters for each site ($[\text{HCO}_3^-]$, $[\text{CO}_3^{2-}]$, Ω_{calcite} and $\Omega_{\text{aragonite}}$) were calculated (with C_T and A_T as inputs) using the CO2SYS program with KHSO_4 , K_1 and K_2 dissociation constants (Dickson et al. 1990; Roy et al. 1993).

Field mussel growth

In March 2016, settlement panel structures ($n = 3$ per site) were placed at each site to monitor the growth of the spring cohort of settled juveniles over the course of 15 months. The structures were made from a 35 cm long cylinder of grey PVC pipe with a diameter of 10 cm. 20 x 2cm diameter holes were drilled into the sides and a 0.2 mm gridded nylon net (standard seed sock for mussel aquaculture) was placed diagonally across the inside to increase surface area allowing for maximum settlement. These settlement structures were cable tied to wooden

pillars at each site at ca. 1 m depth and samples of 50 juvenile mussels were taken for shell length measurements from each site every 2–3 months (see Fig. S1).

Food availability

Chlorophyll-*a* (chl-*a*) concentrations ($\mu\text{g L}^{-1}$) are used as a proxy for phytoplankton food availability in surface waters (Wasmund et al. 2011). These values were generated for each site from model predictions taken from CMEMS datasets (EU, Copernicus Marine Service). Daily means for surface waters were calculated with a 2 km x 2 km resolution between Oct 2015 and Oct 2017. Additionally, raw chl-*a* monitoring data at the closest location to each site were compared to model predictions. Field monitoring data for chl-*a* concentrations in Kiel Fjord was supplied by the Landesamt für Landwirtschaft, Umwelt und ländliche Räume (LLUR) sampled from Mönkeberg (54° 21' 7.105" N, 10° 10' 35.836" E, 2.5 km from the field monitoring site in Kiel Fjord). Chl-*a* monitoring data for Ahrenshoop and Usedom were supplied by the Landesamt für Umwelt, Naturschutz und Geologie (LUNG) sampled from near Fischland (54° 20' 42.983" N, 12° 26' 54.542" E, 1 km from the sampling site in Ahrenshoop) and Zinnowitz (54° 4' 56.531" N, 13° 54' 57.165" E, 4 km from the monitoring site in Usedom).

Data analysis

All data analysis was conducted using R (version 3.4.1.). For growth and calcification rates linear or log regression models were used based on the best statistical fit and analysed with ANCOVA with salinity as a factor and $[\text{HCO}_3^-]$ or $[\text{Ca}^{2+}]$ as co-variates. Annual cycles of salinity, temperature, A_T , C_T , chl-*a* were plotted (2015-2018) for each site and total mean values were compared between sites using a one-way ANOVA with locations as factors.

Results

Experimental results

$[\text{HCO}_3^-]$ had a significant effect on calcification rates (ANCOVA, $F_{(1,53)} = 26.3$, $p < 0.001$) with calcification steeply declining below 900 $\mu\text{mol kg}^{-1}$ (Fig. 1D). Salinity also had a significant effect on calcification rate (ANOVA, $F_{(2,53)} = 131.8$, $p < 0.001$) with calcification rates being severely reduced at 6 psu compared to 11 and 16 psu even at high $[\text{HCO}_3^-]$ (Fig. 1B). There was also a significant interaction effect (ANOVA, $F_{(2,53)} = 6.3$, $p = 0.003$) between $[\text{HCO}_3^-]$ and salinity. Shell length growth was maintained down to lower $[\text{HCO}_3^-]$ than calcification but was substantially reduced at 300 $\mu\text{mol kg}^{-1}$. Mortality was significantly higher at lower $[\text{HCO}_3^-]$ compared to higher concentrations (ANOVA, $F_{(1,54)} = 54.1$, $p < 0.001$) with

the 16 psu treatment having significantly lower mortality rates compared to the 11 and 6 psu treatments (ANOVA, $F_{(2,54)} = 13.1$, $p < 0.001$, Fig. S6).

Calcification rate decreased linear with $[Ca^{2+}]$ (ANOVA, $F_{(1,54)} = 106.9$, $p < 0.001$) at all salinities (Fig. 1C) however, salinity had no significant effect on calcification rates (ANOVA, $F_{(2,54)} = 0.8$, $p = 0.44$). There was also a significant interaction between salinity and $[Ca^{2+}]$ (ANOVA, $F_{(2,54)} = 3.38$, $p = 0.041$) with the 6 psu treatment exhibiting ~ 40 % lower calcification rates at high $[Ca^{2+}]$ (Fig. 1C). Shell length growth showed a logarithmic decline with $[Ca^{2+}]$ (ANCOVA, $F_{(1,45)} = 24.2$, $p < 0.001$, Fig. 1A) compared to the linear decline observed with $[Ca^{2+}]$ and calcification rate. Calcification rates were comparable between both experiments, however at 6 psu, calcification was significantly reduced at all $[HCO_3^-]$ in the bicarbonate experiment compared to the 6 psu treatment in the calcium experiment. Additionally, in the bicarbonate experiment, shell length growth was ~ 50 % of that in the calcium experiment, likely a result of smaller initial shell lengths.

Shell length growth rates from field monitoring over 15 months showed different patterns of growth with salinity (Fig. 2) with Kiel Fjord mussels (mean salinity: 15.2 psu) having much higher shell length growth rates than Ahrenshoop and Usedom populations at lower salinities (ANOVA, $F_{(2,832)} = 1771$, $p < 0.001$). Shell length growth rates were very low at both Ahrenshoop and Usedom with no significant difference between them (Tukey post-hoc, $p = 0.463$), despite laboratory experiments revealing large differences in calcification rates between 11 and 6 psu. These differences in shell length growth correspond to enormous differences in calcification rates ($mg\ CaCO_3\ month^{-1}$) with Kiel exhibiting calcification rates ~ 40-fold higher than Ahrenshoop and Usedom (Fig. S4)

Field data

Salinity data from the three monitoring sites showed significantly different mean salinities between Kiel (15.15 psu), Ahrenshoop (10.89 psu) and Usedom (7.23 psu) (Krusal-Wallis, $Chi^2 = 7797$, $p < 0.001$, Fig. 3). Kiel had the largest salinity range being 17.1 psu followed by Ahrenshoop at 16.1 psu and Usedom the lowest at 5.8 psu with winter exhibiting higher salinities on average than summer (Fig. 3). Annual temperature cycles and seasonal means were similar between the three sites with Usedom showing the highest annual temperature range (23.0 °C) and Kiel the lowest (20.9 °C) (Fig. 4).

Modelled chl-*a* concentrations were significantly different between all sites (Kruskal-Wallis, $Chi^2 = 449$, $p < 0.001$) with Kiel having the highest annual mean of 5.08 $mg\ L^{-1}$ followed by

Usedom with 2.44 mg L^{-1} Ahrenshoop with 1.34 mg L^{-1} (Fig. 5). The high chl-*a* concentrations in Kiel were mostly due to extremely high values in summer which is also shown in the high annual variability in chl-*a* concentrations (Table S3). Raw chl-*a* monitoring data produced values within the range of modelled data apart from Usedom where raw chl-*a* values were over 2 x higher than modelled data (Fig. 6). Subsequently, chl-*a* concentration from monitored data showed similar annual means between Usedom and Kiel and only significantly lower values in Ahrenshoop (ANOVA, $F_{(2,77)} = 13.8$, $p < 0.001$). Annual A_T was higher in Kiel (mean $A_T = 2071 \text{ } \mu\text{mol kg}^{-1}$) compared to the other two sites (ANOVA, $F_{(2,43)} = 42.7$, $p < 0.001$, Fig. 7). However, the magnitude of annual variation in A_T was much smaller in comparison to salinity at all sites suggesting a decoupling of A_T and salinity during salinity fluctuations.

Discussion

Carbonate chemistry and growth

The effects of altered carbonate chemistry on calcifying organisms has been well documented over the last two decades but mainly from the point of increased CO_2 concentrations (Gazeau et al. 2013). Until now, only a few studies have investigated the effects of how substrate (C_T and Ca^{2+}) limitation impacts calcification rates in molluscs (Thomsen et al. 2015; Thomsen et al. 2018). Our experiment with C_T limitation revealed impeded calcification $< 900 \text{ } \mu\text{mol kg}^{-1} \text{HCO}_3^-$ in line with findings from *Mytilus edulis* larvae (Thomsen et al. 2015). This may result from either: (1) lack of available HCO_3^- substrate limiting calcification rates or (2) physiological constraints on calcification mechanisms through changes in ion transport in the calcifying epithelia. Although CO_3^{2-} ions ultimately react with Ca^{2+} ions to form CaCO_3 , it is thought that in marine animals the main carbon source for calcification is HCO_3^- . This is because firstly: seawater $[\text{HCO}_3^-]$ is 10-fold higher than $[\text{CO}_3^{2-}]$ and secondly: HCO_3^- transporters (via the $\text{Na}^+/\text{HCO}_3^-$ -cotransporter or the Na^+ driven $\text{Cl}^-/\text{HCO}_3^-$ -exchanger) have been localized in the calcifying epithelia of marine invertebrates (Jury et al. 2010; Thomsen et al. 2015; Bach 2015; Zoccola et al. 2015). It cannot be ruled out however, that low $[\text{CO}_3^{2-}]$ and high $[\text{H}^+]$ (Table S2) in our experiment may also be negatively impacting calcification by impeding optimum physiological conditions within the calcifying space as has been documented in bivalve larvae and corals (Allison et al. 2014; Ramesh et al. 2017). High physiological stress and increased costs of homeostasis may also explain the high mortality rates ($> 50 \%$, Fig. S6) observed $< 900 \text{ } \mu\text{mol HCO}_3^- \text{ kg}^{-1}$ compared to no mortality in the calcium limitation experiment at very low $[\text{Ca}^{2+}]$. Although it may also be possible that calcium

limitation only becomes lethal after longer exposure times or concentrations lower than 0.5 mmol kg⁻¹

Genetic factors may also explain calcification and mortality differences in our experiments. The sampled population (Ahrenshoop) lies directly in the middle of the steepest cline of the genetic transition zone between Baltic *M. edulis* and Baltic *M. trossulus* (Stuckas et al. 2017). Therefore, genetic diversity is high at Ahrenshoop due to high admixture between *M. edulis* and *M. trossulus* genotypes. Interannual salinity and temperature variation, particularly during the larval season, may act on this genetic variation and lead to significant genetic differences between settling larval cohorts (Knoebel et al. in prep). This has already been a proposed mechanism for shifts in the Baltic *Mytilus* hybrid zone in Öresund, Sweden (Strelkov et al. 2017). This is further supported by findings from Thomsen et al. 2018 where a low salinity adapted population of Baltic *Mytilus* larvae was found to be more tolerant of low [Ca²⁺]. These genetic differences between sampling years may lead to differing salinity tolerances and could explain the different mortality and shell length growth rates between both experiments.

Inorganic carbon limitation

Calcification limiting concentrations of HCO₃⁻ are unlikely under fully marine conditions due to high and stable A_T in ocean waters (Millero et al. 1998). However, estuarine and brackish water environments at lower salinities are accompanied by lower A_T which lower the seawater buffering capacity leading to higher variations in carbonate chemistry parameters (Miller et al. 2009). Despite the low salinity, the Baltic Sea is characterized by relatively high A_T due to the large riverine input of terrestrially derived C_T, particularly in the southern basins. The impacts of riverine input of C_T may be more severe at locations close to the shore do to the influence of submarine groundwater discharge, such as the sampling locations in this study (Kuliński et al. 2017). This may explain why measured A_T at our monitoring sites was higher than predicted from published Baltic A_T - salinity relationships (Beldowski et al. 2010; Müller et al. 2016). Dissolved organic carbon (DOC) species also constitute ~ 3.5 % of Baltic Sea A_T and may contribute more to A_T in coastal environments where terrestrial influence and biological activity is higher than more offshore environments (Kuliński et al. 2014). This high C_T and A_T in our measured coastal habitats, coupled with predicted trends of increasing A_T (~ 7 μmol kg⁻¹ yr⁻¹) over the last century (Müller et al. 2016) may significantly mitigate the threat of ocean acidification and suggests that low calcification rates in coastal Baltic mussel reefs in the south western Baltic are not a result of seawater carbon limitation.

Calcium limitation

In this study, calcification rates decreased linearly with $[Ca^{2+}]$ with reduced rates even below 4 mmol kg^{-1} . This is similar to previous findings where significantly impaired shell formation in Baltic *Mytilus* larvae was observed $< 3 \text{ mmol kg}^{-1}$ (Thomsen et al. 2018). Limited $[Ca^{2+}]$ below 4 mmol kg^{-1} corresponds to a salinity of ~ 11 psu and concurs with previous findings where calcification was found to be significantly reduced below 11 psu (Kossak 2006). It is also important to state here that although calcification rates showed linear decreases with $[Ca^{2+}]$ below 4 mmol kg^{-1} , shell length growth did not exhibit a linear fit but rather a logarithmic fit with calcification rates decreasing more steeply at lower $[Ca^{2+}]$. This may suggest a tipping point in shell length growth with $[Ca^{2+}]$, although it is possible this level may be higher than $4 \text{ mmol Ca}^{2+} \text{ kg}^{-1}$ in this experiment. This also potentially demonstrates that shell length growth and total CaCO_3 deposition may be differentially impacted by calcium availability. This is discussed in more detail in the following 2 paragraphs.

In molluscs, the cellular mechanisms surrounding calcification are largely unknown and potential calcification pathways are often based on findings from other marine invertebrate phyla. Therefore, suggesting the potential mechanisms by which seawater CaCO_3 substrate limits calcification must be done with caution. Bivalve molluscs are poor at regulating the ion composition of their extracellular fluid and therefore seawater carbonate chemistry is tightly coupled with the carbonate chemistry of the extracellular fluid (Heinemann et al. 2012). Sub-optimal carbonate chemistry in the extracellular fluid will therefore be in direct contact with the mantle epithelia and likely impact ion transport processes involved in calcification. There are two potential mechanisms for reduced calcification at low $[Ca^{2+}]$: 1) reduced rates of calcium uptake by plasma membrane Ca^{2+} ATPases (PMCA) due to limiting seawater $[Ca^{2+}]$ (Niggli 1981; McConnaughey & Whelan 1997). Reduced $[Ca^{2+}]$ leads to a lower calcification substrate-inhibitor ratio which has been shown to be tightly correlated to calcification rates (Bach 2015; Fassbender et al. 2016; Thomsen et al. 2018). 2) low $[Ca^{2+}]$ can lead to reduced aragonite saturation states ($\Omega_{\text{aragonite}}$) which impedes calcification either through kinetic constraints on mineral deposition, or increased dissolution of mineralized structures (Waldbusser et al. 2014).

Shell length growth vs total calcification

Molluscan calcification is a product of increasing shell length and also increasing shell thickness of both the prismatic (calcite) layer and the nacreous (aragonite) layer. Different

relationships between shell length growth and calcification with substrate limitation point towards possible changes in shell condition (shape or thickness). Reduced shell thickness under ocean acidification has been observed in multiple bivalve studies with evidence suggesting this is primarily due to decreased thickness of the inner nacreous layers (Melzner et al. 2011; Fitzer et al. 2015). The inner nacreous layer is particularly susceptible to dissolution because (1) it is comprised primarily of aragonite which dissolves more readily than calcite and (2) it is in contact with EPF which has a higher CO₂ partial pressure than the surrounding water due to the demands of metabolic CO₂ excretion (Melzner et al. 2011; Heinemann et al. 2012).

Shell length growth is more energetically costly than shell thickness growth due to the synthesis and excretion of shell matrix proteins (SMP's) and the periostracum. This organic fraction of bivalve shells (up to 10 % in juveniles) comprises a considerable proportion of shell production costs (Palmer 1983; Thomsen et al. 2013). Higher nitrogen excretion and lower O:N ratios have been recorded in Baltic *Mytilus* suggesting a reduced efficiency of protein turnover and absorption (Tedengren & Kautsky 1986). This could impede shell length growth due to decreased energetic efficiency of SMP and periostracum synthesis reducing growth at 6 psu in the calcium limitation experiment, even at higher calcium ion concentrations. Future work should focus on understanding how salinity impacts protein turnover and how this impacts growth efficiency and individual fitness in variable coastal habitats.

Field monitoring

Salinity is maintained in the Baltic Sea through inflow of high saline North Sea water through the Danish straits. Major inflow events such as the one recorded in 2014 (Mohrholz et al. 2015) are vital for oxygenation of Baltic bottom water and fill the deep Baltic basins with high saline water compared to the less saline surface water layers maintaining a permanent halocline. These inflows occur mostly in winter due to atmospheric conditions and contribute to higher winter salinities in the south-western Baltic (Schimke & Matthäus 1997). In addition, wind driven upwelling of high saline Baltic bottom water contributes to salinity fluctuations and variation in carbonate chemistry (Melzner et al. 2013; Saderne et al. 2013). Salinity variation imposes a significant energetic cost on osmoconformers due to cellular mechanisms of volume regulation (Neufeld et al. 1996). To remain iso-osmotic with external seawater during hypersalinity exposure, marine bivalves increase intracellular concentrations of organic osmolytes (mostly free amino acids - FAA's) by breaking down endogenous protein reserves. During low salinity exposure, cellular excretion of FAA's occurs to decrease intracellular

osmolality (Hawkins & Hilbish 1992). Regular salinity fluctuations (as seen in the South-West Baltic Sea) would invoke constant breakdown and excretion of endogenous body protein, constituting a significant energetic cost. It is possible these energetic costs can be mediated by high food availability as has been found in Kiel fjord during periods of high $p\text{CO}_2$ (Melzner et al. 2011; Thomsen et al. 2013). However, Ahrenshoop exhibits significantly lower annual chl-*a* concentrations compared to Kiel and Usedom, which coupled with fluctuating and periods of very low salinity (< 8 psu) and limiting $[\text{Ca}^{2+}]$, may explain the lower calcification rates compared to Kiel animals. It is therefore likely that the low calcification rates at Ahrenshoop are due to energy limitation, whereas the low calcification rates in Usedom are due to substrate limitation and therefore cannot be counteracted by the high food availability as evidenced by the monitoring data. The 2-fold higher chl-*a* concentrations at Usedom from the monitoring data compared to the modelled chl-*a* data suggests regional scale models may not be accurate at predicting environmental conditions in near-shore coastal habitats.

Conclusions

Our findings clearly show that reduced availability of calcium limits calcification and growth in juvenile Baltic *Mytilus* at salinities ~ 11 psu. Field monitoring of 3 Baltic mussel reefs also reveals high salinity and temperature variability with high food availability potentially being a mitigator of the negative impacts of environmental variability as long as salinity isn't below critical levels (~ 11 psu). Our findings also suggest sub-optimal carbonate chemistry in near-shore coastal Baltic habitats may be buffered by riverine input of dissolved inorganic carbon which may potentially decrease future acidification risk of these habitats. Future work should investigate the extent of this high C_T and A_T in coastal environments and the potential drivers of these patterns. Additionally, the impacts of riverine input and the availability of Ca^{2+} in coastal habitats should be better understood in the Baltic, as this appears to be the limiting factor in Baltic *Mytilus* growth. To accurately understand how multiple environmental factors interact, monitoring of many more coastal sites is needed particularly in estuarine and brackish systems such as the Baltic Sea.

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Author contributions

TS, FM and JT designed the laboratory experiments conducted by TS and JT. TS, FM, GR and JM implemented the environmental monitoring and TS collected and analysed field monitoring data. JM and GR analysed carbonate chemistry samples. TS wrote the manuscript and all authors contributed manuscript comments.

Competing interests

The authors have no competing interests.

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Figures

Fig. 1. The top two panels present mean shell length growth of each replicate tank plotted against $[\text{Ca}^{2+}]$ (**A**) and $[\text{HCO}_3^-]$ (**B**) with logarithmic relationships. The bottom two panels present mean calcification rate of each replicate tank plotted against $[\text{Ca}^{2+}]$ with linear trend lines (**C**), and against $[\text{HCO}_3^-]$ with logarithmic trend lines (**D**). The colours represent the three different experimental salinities (in psu).

Fig. 2 Field shell lengths (mm) from three Baltic Sea sites over 15 months starting in May 2016 (Kiel ~ 16 psu, Ahrenshoop ~ 11 psu and Usedom ~ 7 psu). 20 animals were sampled at each timepoint with all shell lengths plotted and linear trendlines fit.

Fig. 3 Salinity monitoring data from the three Baltic Sites (Kiel: blue, Ahrenshoop: green and Usedom: red) from August 2015 to January 2018 (**A**). The boxplot in panel (**B**) presents mean salinity values for each site with the interquartile ranges.

Fig. 4 Temperature monitoring data from the three Baltic Sites (Kiel: blue, Ahrenshoop: green and Usedom: red) from August 2015 to January 2018 (**A**). The boxplot in panel (**B**) presents mean temperature values for each site with the interquartile ranges.

Fig. 5 Modelled surface chl-*a* data for the three Baltic Sites (Kiel: blue, Ahrenshoop: green and Usedom: red) from October 2015 to January 2018 (**A**). The boxplot in panel (**B**) presents mean chl-*a* concentrations for each site with the interquartile ranges. Modelled data was accessed from the Copernicus Marine Services data base (EU, Copernicus Marine Services).

Fig. 6 Surface chl-*a* concentrations near the three Baltic Sites (Kiel: blue, Ahrenshoop: green and Usedom: red) from January 2015 to January 2018 (**A**). The boxplot in panel (**B**) presents mean chl-*a* concentrations for each site with the interquartile ranges. Monitoring data originated from on-going plankton monitoring conducted by the LLUR and the LUNG.

Fig. 7 Surface A_T values from the three Baltic Sites (Kiel: blue, Ahrenshoop: green and Usedom: red) from January 2015 – January 2018 (**A**). Symbols represent different years: 2015 (●), 2016 (▲), 2017 (■) and 2018 (+). The boxplot in panel (**B**) presents mean A_T for each site across all years with the interquartile ranges.

Figure 1

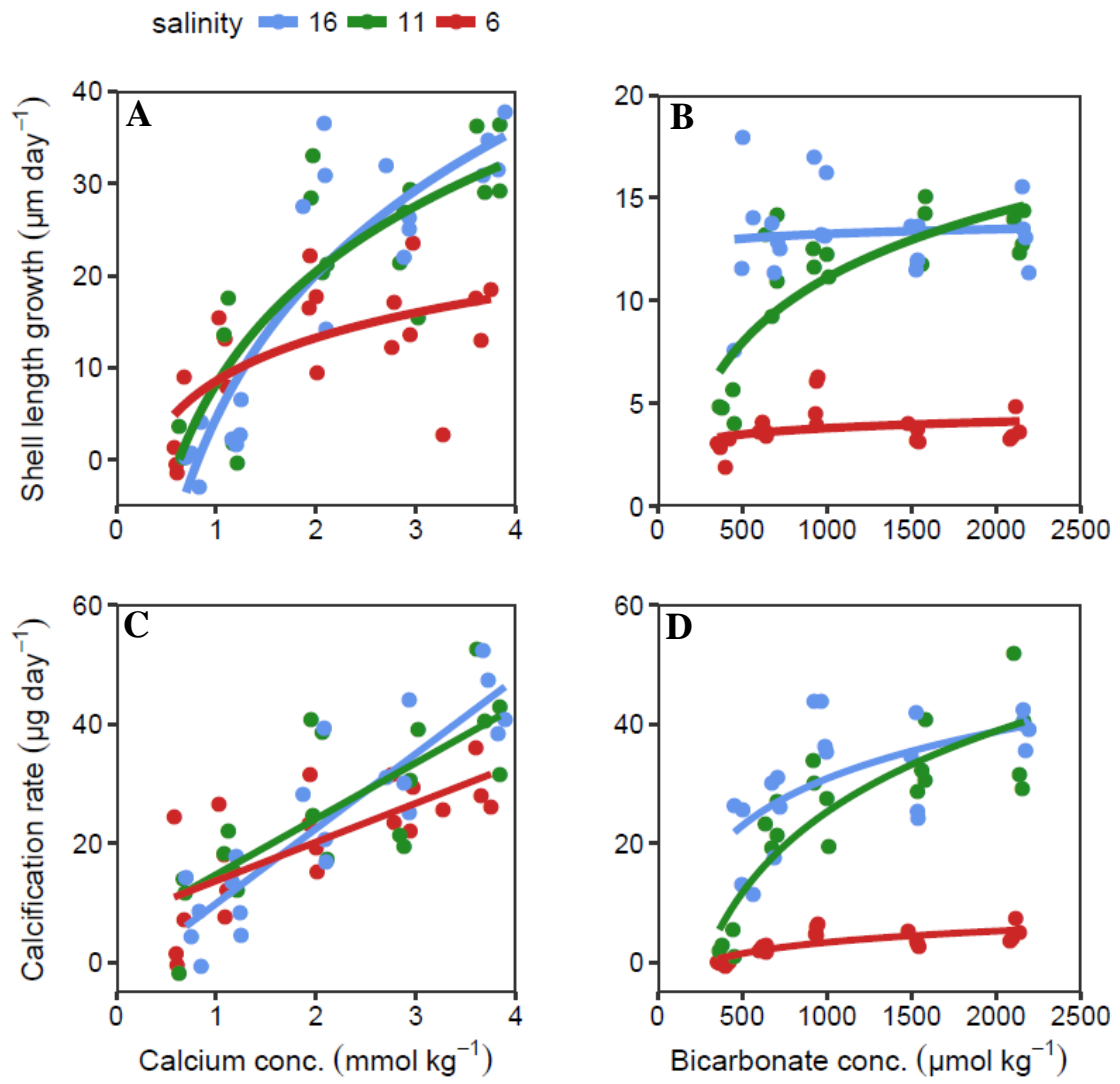


Figure 2.

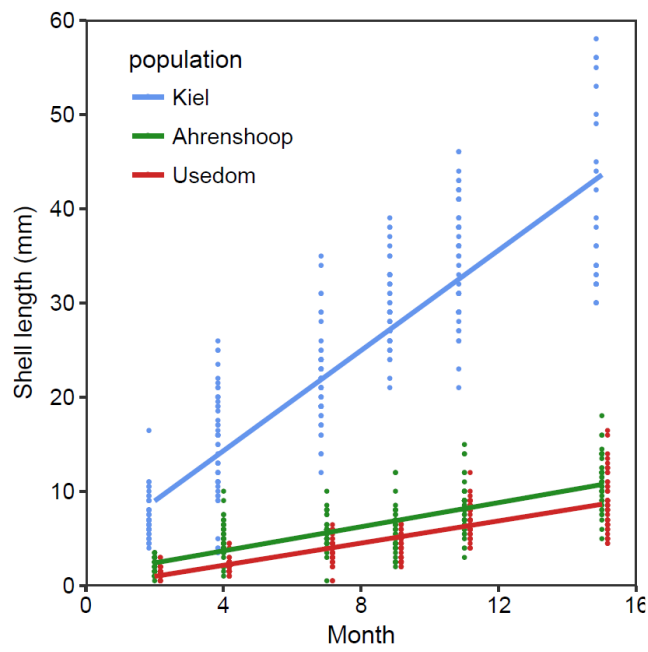


Figure 3

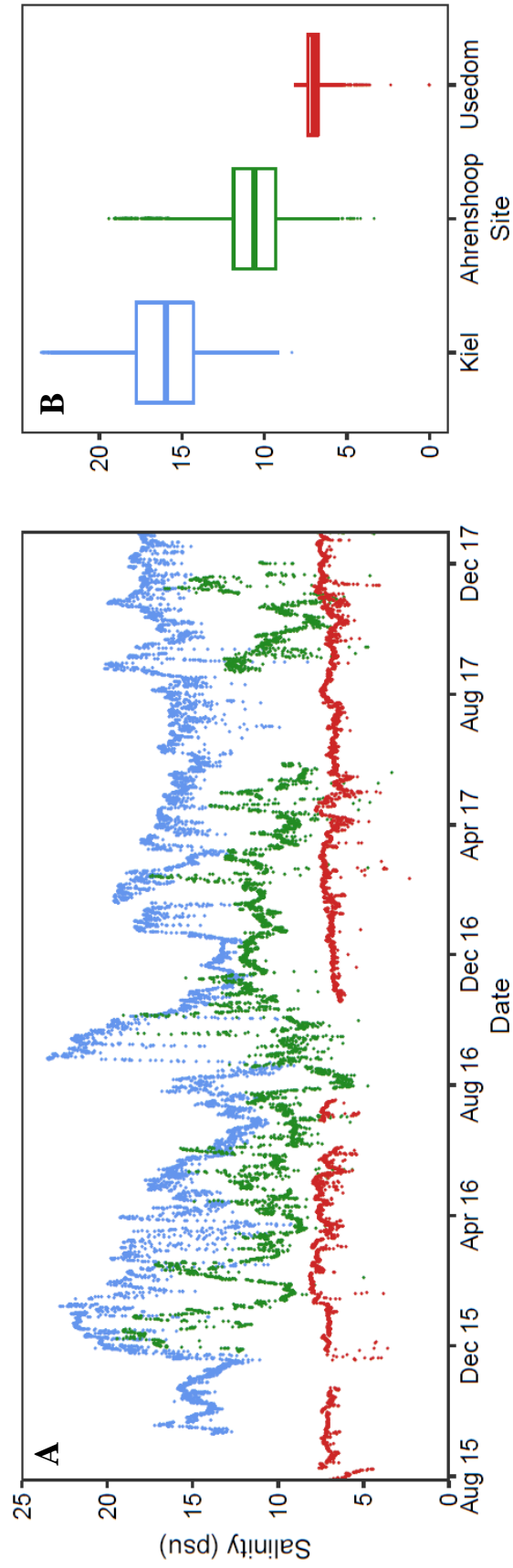


Figure 4.

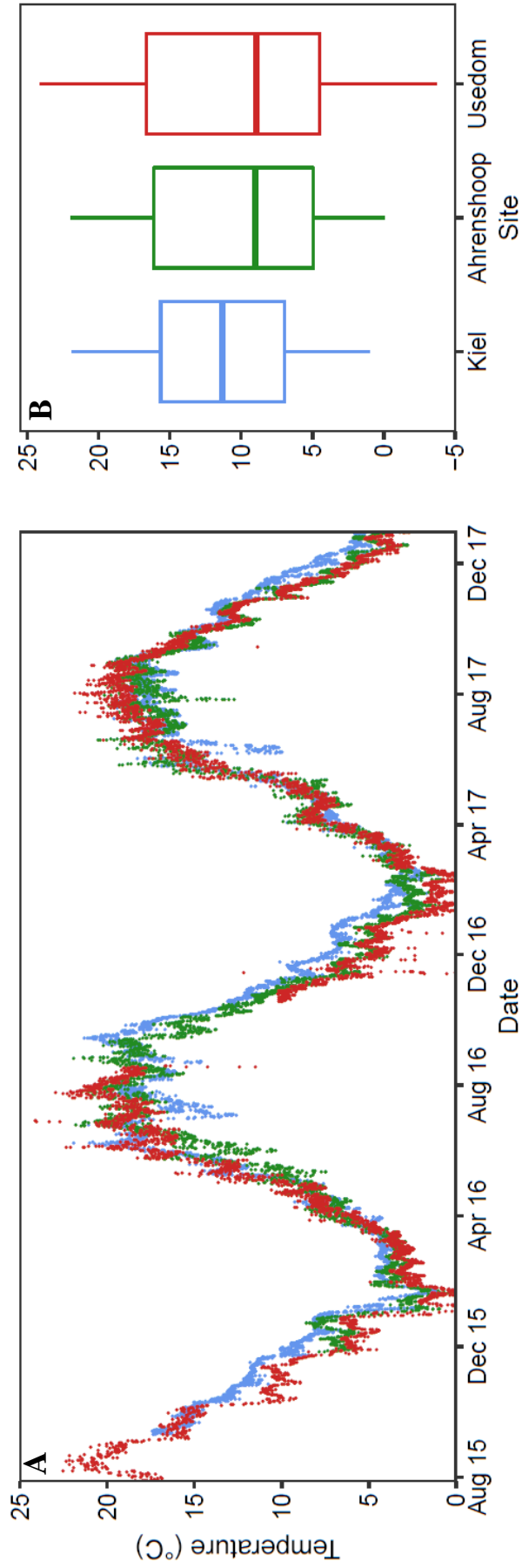


Figure 5

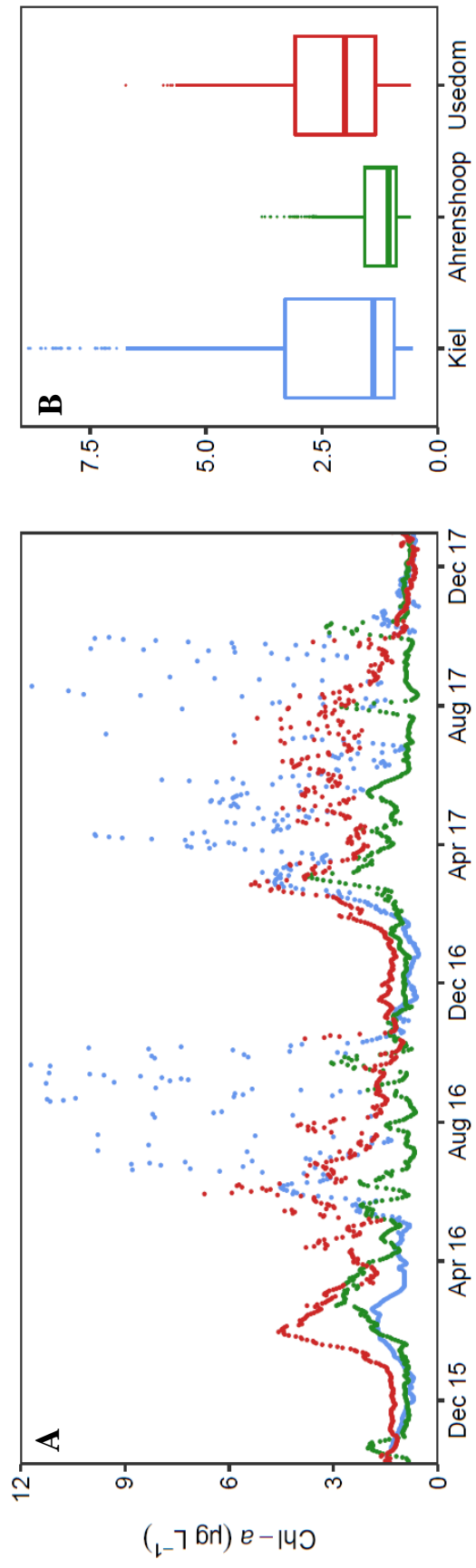


Figure 6

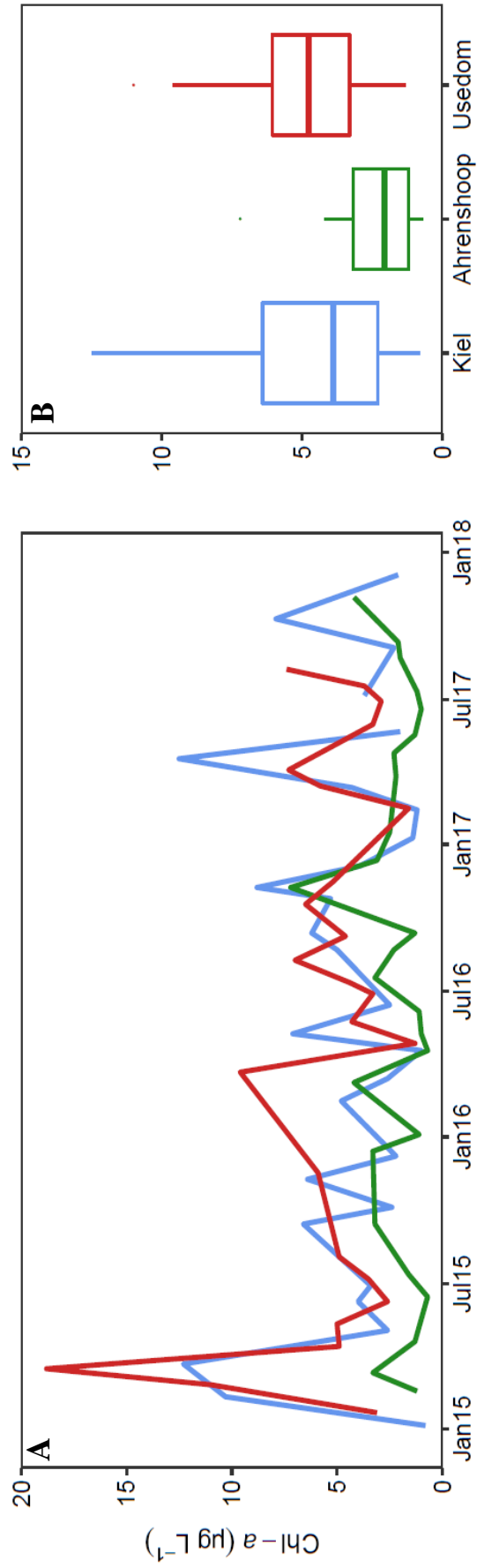
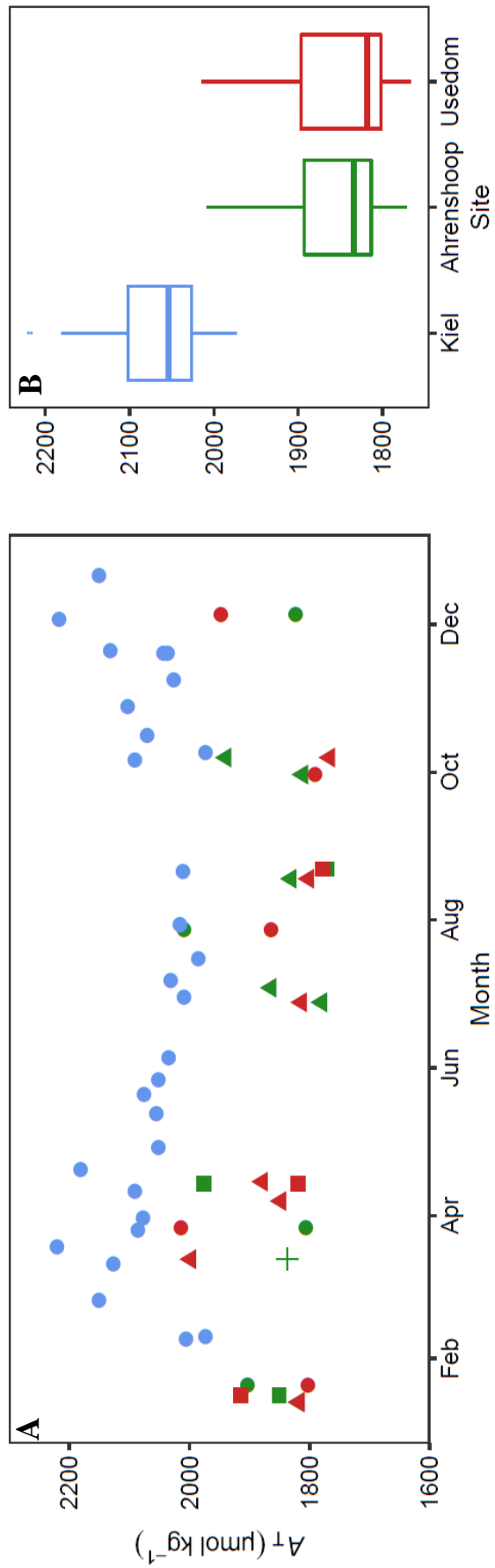


Figure 7



Supplementary Material

Figure S1.

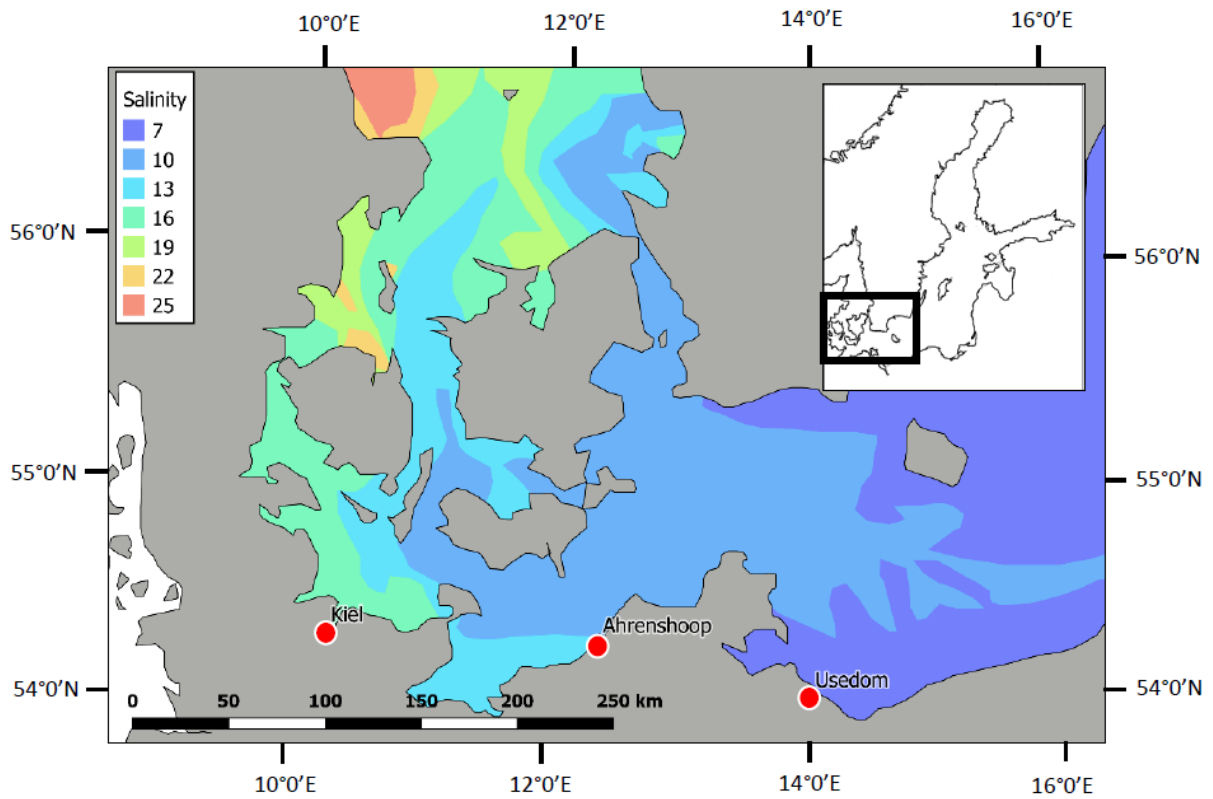


Fig. S1 Map of the South-West Baltic Sea depicting the decreasing salinity gradient from the Kattegat to the Baltic Proper. The red dots represent the three sampling sites along the salinity gradient. Salinity data was taken from open source monitoring data (EU, Copernicus Marine Services).

Figure S2.

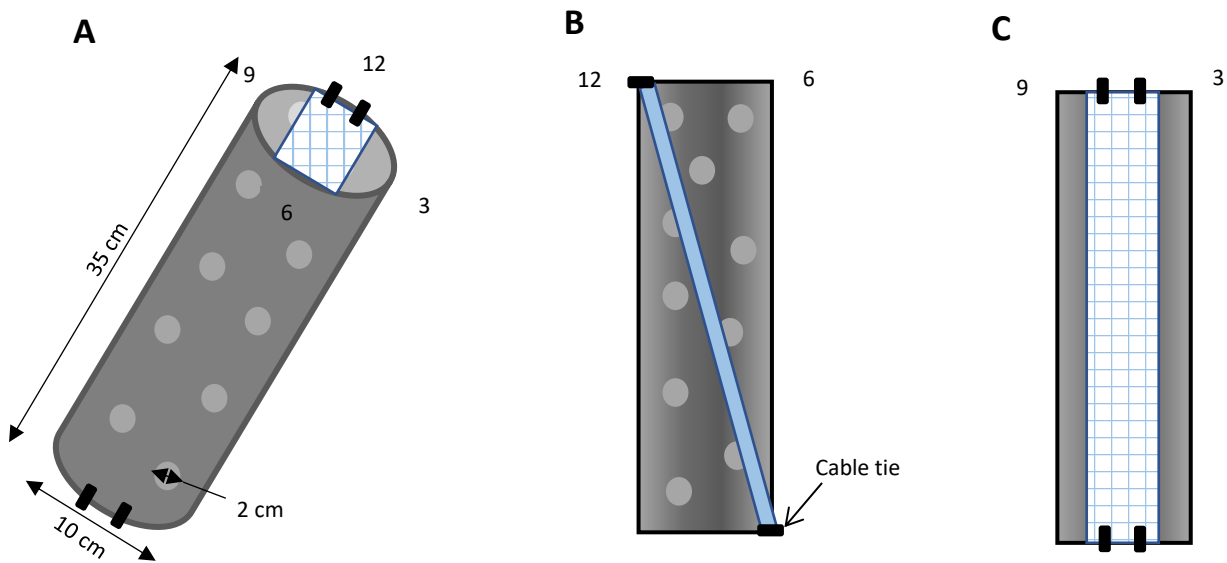


Fig. S2 (A) Settlement structures deployed at all three sites (Kiel, Ahrenshoop and Usedom) in March 2016. Numbers at the top of the diagram represent the position of the cross sections in panels B and C. **(B)** A cross section of the settlement structures from 12 o'clock to 6 o'clock showing the orientation of the mesh net inside the cylinder. **(C)** A cross section of the settlement structures showing the width of the mesh net inside the cylinder. A total of 20 x 2 cm diameter holes were drilled into each cylinder and a 0.2 cm pore size cotton/nylon spat sock was diagonally positioned across the inside of the cylinders using cable ties. Numbers at the top of each figure represent the orientation of the cross sections in panels **B** and **C**.

Figure S3.

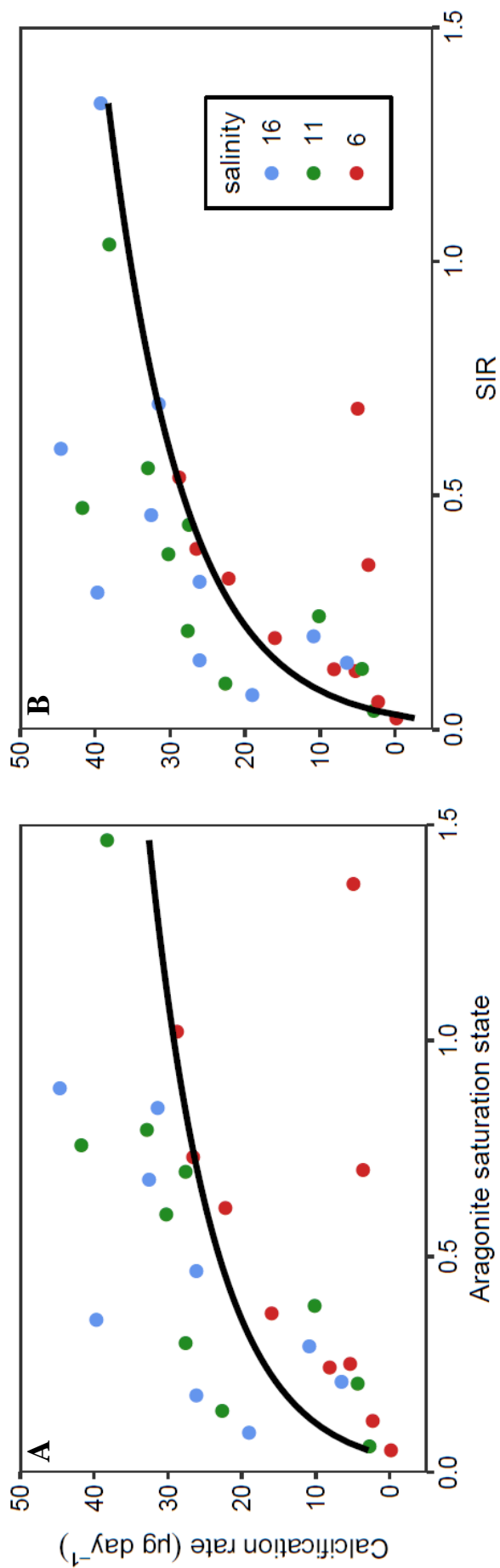


Fig. S3 Calcification rates from both Ca^{2+} and HCO_3^- limiting experiments plotted against $\Omega_{\text{aragonite}}$ (A) and the extended substrate inhibitor ratio (B) with logarithmic trendlines fit ($\Omega R^2 = 0.372$, $\text{SIR } R^2 = 0.487$). Colours represent the different salinities.

Figure S4

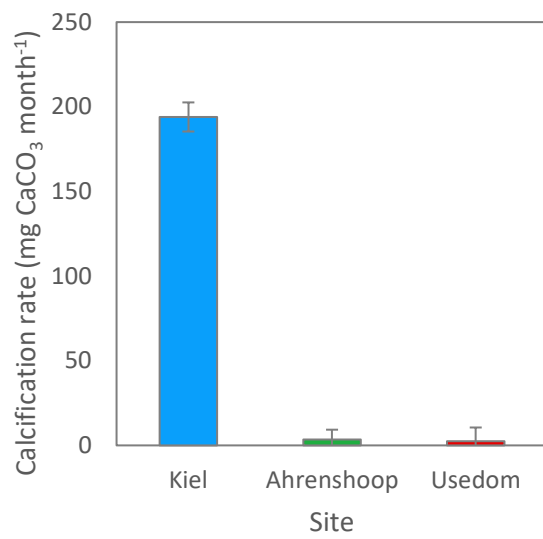


Fig. S4 Field calcification rates (mg CaCO₃ month⁻¹) converted from shell length increase per month. These conversions were done using shell length-shell mass relationships from Kautsky et al. 1990. values are based on the slopes of linear regressions of shell mass against time (months) with error bars representing standard error of the regressions.

Figure S5

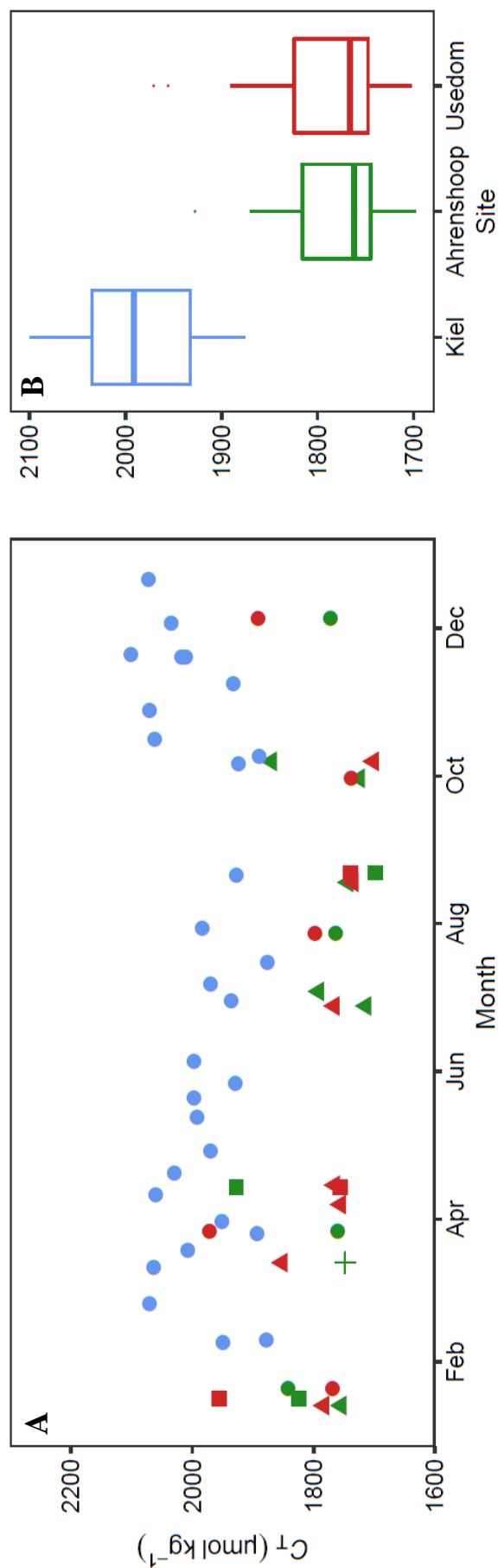


Fig. S4 Surface C_T values from the three Baltic Sites (Kiel: blue, Ahrenshoop: green and Usedom: red) from January 2015 – January 2018 (A). Symbols represent different years: 2015 (●), 2016 (▲), 2017 (■) and 2018 (+). The boxplot in panel (B) presents mean C_T for each site across all years with the interquartile ranges.

Figure S6

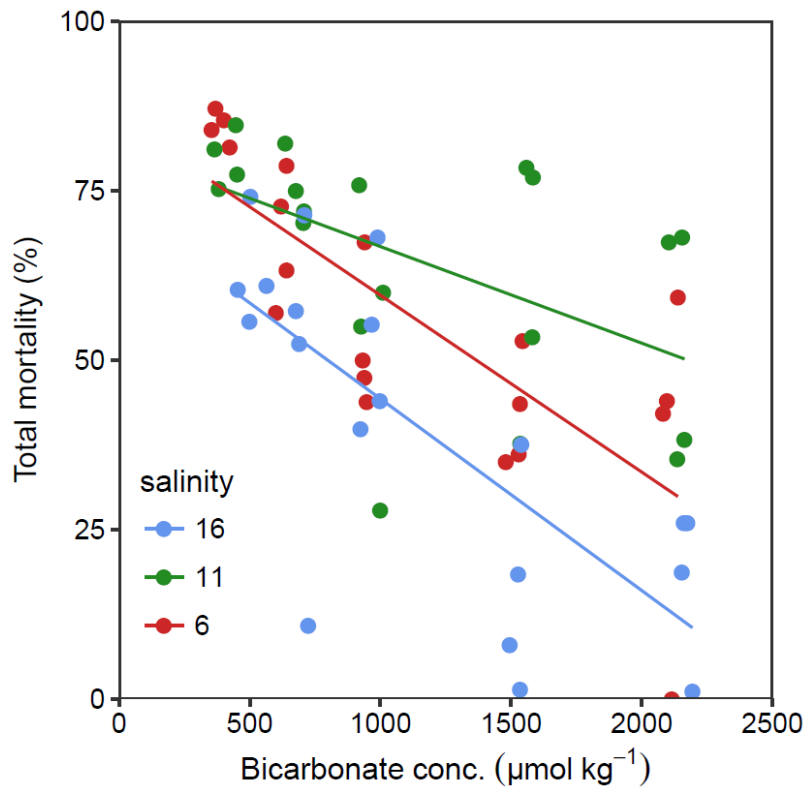


Fig. S6 Total mortality at the end of the bicarbonate limitation experiment per replicate tank as a percentage of the initial number of juveniles. Linear trendlines are shown with colours representing the different salinity treatments.

Table S1 Water chemistry parameters for the bicarbonate ion manipulation experiment. Columns from left to right represent: salinity, bicarbonate ion concentration, carbonate ion concentration, pH, CO₂ partial pressure, total alkalinity, calcium ion concentration, extended substrate inhibitor ratio and aragonite saturation. Carbonate chemistry parameters were calculated using pH and C_T as inputs and values are shown ± standard deviation.

Salinity	[HCO ₃ ⁻] (μmol kg ⁻¹)	[CO ₃ ²⁻] (μmol kg ⁻¹)	pH _{NBS}	pCO ₂ (μatm)	A _T (μmol kg ⁻¹)	[Ca ²⁺] (mmol kg ⁻¹) 1)	SIR	Ω _{aragonite}
6.31 ± 0.18	387 ± 16	3.01 ± 1.3	7.43 ± 0.11	676 ± 77	397 ± 76	2.41 ± 0.01	0.03 ± 0.01	0.05 ± 0.02
	625 ± 10	7.05 ± 1.3	7.61 ± 0.05	713 ± 50	644 ± 55	2.41 ± 0.01	0.06 ± 0.01	0.12 ± 0.02
	940 ± 3	14.84 ± 2.7	7.76 ± 0.07	770 ± 134	973 ± 66	2.41 ± 0.01	0.13 ± 0.02	0.25 ± 0.04
	1522 ± 14	41.37 ± 4.5	8.00 ± 0.04	716 ± 59	1614 ± 66	2.41 ± 0.01	0.35 ± 0.04	0.70 ± 0.07
	2108 ± 12	80.52 ± 6.5	8.15 ± 0.03	701 ± 42	2279 ± 85	2.4 ± 0.01	0.69 ± 0.07	1.36 ± 0.11
11.13 ± 0.12	411 ± 22	3.69 ± 1.6	7.4 ± 0.13	689 ± 69	425 ± 89	3.96 ± 0.00	0.04 ± 0.02	0.06 ± 0.03
	681 ± 17	8.7 ± 1.6	7.58 ± 0.06	785 ± 124	707 ± 61	3.95 ± 0.01	0.1 ± 0.02	0.14 ± 0.03
	964 ± 24	17.99 ± 3.7	7.75 ± 0.06	739 ± 80	1010 ± 104	3.95 ± 0.01	0.21 ± 0.05	0.30 ± 0.06
	1565 ± 11	47.6 ± 9.6	7.96 ± 0.06	731 ± 73	1671 ± 166	3.94 ± 0.00	0.57 ± 0.12	0.79 ± 0.16
	2140 ± 13	87.81 ± 12.2	8.09 ± 0.05	735 ± 46	2330 ± 161	3.94 ± 0.00	1.05 ± 0.16	1.46 ± 0.20
16.33 ± 0.42	505 ± 22	5.6 ± 3.0	7.42 ± 0.10	742 ± 98	523 ± 138	5.61 ± 0.01	0.08 ± 0.04	0.09 ± 0.05
	699 ± 10	10.82 ± 2.8	7.586 ± 0.06	713 ± 42	731 ± 110	5.6 ± 0.01	0.15 ± 0.04	0.18 ± 0.05
	970 ± 17	21.49 ± 5.6	7.74 ± 0.07	694 ± 31	1028 ± 159	5.62 ± 0.01	0.3 ± 0.08	0.35 ± 0.09
	1524 ± 10	51.24 ± 13.9	7.92 ± 0.08	720 ± 43	1647 ± 256	5.62 ± 0.01	0.72 ± 0.20	0.84 ± 0.23
	2171 ± 9	100.88 ± 24.3	8.06 ± 0.07	739 ± 52	2358 ± 346	5.57 ± 0.00	1.41 ± 0.35	1.62 ± 0.42

Table S2 Water chemistry parameters for the calcium ion manipulation experiment. Columns from left to right represent: salinity, calcium ion concentration, pH, CO₂ partial pressure, total alkalinity, bicarbonate ion concentration, carbonate ion concentration, extended substrate inhibitor ratio and aragonite saturation. Carbonate chemistry parameters were calculated using pH and C_T as inputs and values are shown ± standard deviation.

Salinity	[Ca ²⁺] (mmol kg ⁻¹)	pH _{Total}	pCO ₂ (μatm)	A _T (μmol kg ⁻¹)	[HCO ₃ ⁻] (μmol kg ⁻¹)	[CO ₃ ²⁻] (μmol kg ⁻¹)	SIR	Ω _{aragonite}
6.1 ± 0.29	0.651 ± 0.03	7.99 ± 0.12	651 ± 198	1972 ± 18	1836 ± 28	64.33 ± 15.0	0.13 ± 0.06	0.24 ± 0.11
	1.108 ± 0.03	7.94 ± 0.11	716 ± 200	1961 ± 21	1840 ± 25	57.27 ± 12.7	0.2 ± 0.09	0.37 ± 0.16
	1.968 ± 0.04	7.90 ± 0.12	797 ± 213	1954 ± 18	1840 ± 27	53.55 ± 14.7	0.32 ± 0.17	0.61 ± 0.34
	2.818 ± 0.05	7.85 ± 0.08	848 ± 154	1942 ± 26	1847 ± 20	44.66 ± 8.3	0.39 ± 0.15	0.73 ± 0.30
	3.656 ± 0.05	7.88 ± 0.09	799 ± 150	1948 ± 28	1846 ± 20	48.12 ± 9.7	0.54 ± 0.22	1.02 ± 0.41
10.7 ± 0.47	0.727 ± 0.03	7.95 ± 0.10	645 ± 145	1994 ± 40	1839 ± 12	70.26 ± 15.3	0.13 ± 0.06	0.21 ± 0.09
	1.185 ± 0.03	8.01 ± 0.10	565 ± 108	2010 ± 44	1832 ± 18	80.77 ± 22.0	0.24 ± 0.13	0.39 ± 0.21
	2.035 ± 0.03	7.97 ± 0.09	602 ± 107	1999 ± 38	1838 ± 14	72.97 ± 15.6	0.37 ± 0.16	0.60 ± 0.26
	2.967 ± 0.04	7.89 ± 0.05	708 ± 85	1978 ± 29	1849 ± 15	58.37 ± 17.2	0.44 ± 0.11	0.70 ± 0.17
	3.864 ± 0.06	7.81 ± 0.04	840 ± 70	1961 ± 25	1853 ± 17	48.77 ± 5.7	0.47 ± 0.10	0.76 ± 0.18
15.3 ± 0.41	0.836 ± 0.03	7.95 ± 0.07	585 ± 81	2020 ± 51	1837 ± 24	80.69 ± 13.2	0.14 ± 0.05	0.21 ± 0.07
	1.222 ± 0.03	7.94 ± 0.05	599 ± 66	2015 ± 46	1840 ± 25	77.01 ± 9.6	0.2 ± 0.05	0.29 ± 0.07
	2.019 ± 0.03	7.92 ± 0.04	614 ± 46	2011 ± 43	1842 ± 26	74.33 ± 7.7	0.32 ± 0.07	0.47 ± 0.10
	3.04 ± 0.13	7.90 ± 0.05	647 ± 63	2007 ± 45	1843 ± 26	71.82 ± 9.1	0.46 ± 0.12	0.68 ± 0.17
	3.828 ± 0.03	7.91 ± 0.07	647 ± 98	2010 ± 52	1840 ± 24	74.81 ± 13.4	0.6 ± 0.21	0.89 ± 0.32

Table S3. Summary of environmental conditions at the three monitoring sites (Kiel, Ahrenshoop, Usedom). Parameters from left to right: sampling site, salinity, temperature, chlorophyll *a* concentration, calcium ion concentration, total alkalinity, bicarbonate ion concentration and aragonite saturation state. Value are presented \pm standard deviation.

Site	Salinity (psu)	Temperature (°C)				Chlorophyll <i>a</i> mg L ⁻¹		[Ca ²⁺] (mmol kg ⁻¹ SW)	A _T (mmol kg ⁻¹ SW)	[HCO ₃ ⁻] (μmol kg ⁻¹ SW)	Ω _{aragonite}
		spring	summer	autumn	winter	Model data	Monitoring data				
Kiel	15.15 ± 3.07	8.35	17.06	13.3	5.43	5.08 ± 7.98	4.68 ± 3.23	4.99 ± 0.91	2071 ± 67	1871 ± 74	1.30 ± 0.54
Ahrenshoop	10.89 ± 2.34	7.84	17.92	12.08	3.91	1.34 ± 0.58	2.25 ± 1.45	3.87 ± 0.75	1858 ± 68	1661 ± 58	1.50 ± 0.84
Usedom	7.02 ± 0.55	8.34	18.48	10.45	2.83	2.44 ± 1.05	5.52 ± 3.59	2.63 ± 0.17	1884 ± 83	1713 ± 78	1.39 ± 0.50

Table S4. ANCOVA results from the relationships between calcification rates and both $[Ca^{2+}]$ and $[HCO_3^-]$ in the 2 laboratory experiments.

		Calcification rate					
		Sum					
Response	factor	Df	Sq	Mean Sq	F value	P value	
Calcium	Calcium	1	7160	7160	106.93	<0.001	
	Salinity	2	110.9	55.5	0.829	0.442	
	Calcium:Salinity	2	452.4	226.2	3.38	0.041	
	Residuals	54	3615.9	67			
Bicarbonate	log(Bicarbonate)	1	0.206	0.498	26.339	<0.001	
	Salinity	2	2.063	1.031	131.784	<0.001	
	log(Bicarbonate):Salinity	2	0.099	0.049	6.308	0.003	
	Residuals	53	0.415	0.008			
						Tukey post-hoc	
						Factor	P-value
						11 - 6	<0.001
						16 - 6	<0.001
						16 - 11	0.002

Table S5. ANCOVA results from the relationships between shell length growth rate and both $[\text{Ca}^{2+}]$ and $[\text{HCO}_3^-]$ in the 2 laboratory experiments.

		Shell length growth				
Response	factor	Df	Sum Sq	Mean Sq	F value	P value
Calcium	Log(Calcium)	4	44.70	11.17	24.18	<0.001
	Salinity	2	0.56	0.28	0.60	0.551
	Calcium:Salinity	8	6.37	0.80	1.72	0.119
	Residuals	45	20.79	0.46		
		factor	Df	Sum Sq	Mean Sq	F value
Bicarbonate	Bicarbonate	4	109.2	27.3	10.2	<0.001
	Salinity	2	983.5	491.8	183.8	<0.001
	Bicarbonate:Salinity	8	117.7	14.7	5.5	<0.001
	Residuals	45	120.4	2.7		
						Tukey post-hoc
					Salinity	p-value
					11 - 6	<0.001
					16 - 6	<0.001
					16 - 11	<0.001

Table S6. Result of ANCOVA on field shell length growth rates from the three monitoring sites with Tukey HSD post-hoc analysis on significant factors.

Field shell length growth					
	Sum	Mean			
Factor	df	sq	sq	F value	p-value
time	1	232.28	232.28	1709.882	<0.001
population	2	481.1	240.551	1770.773	<0.001
time:population	1	1.63	1.63	12.002	<0.001
Residuals	832	113.02	0.136		
			Factor	population	p-value
				Kie:Ahp	<0.001
				Use:Ahp	0.463
				Use:Kie	<0.001

Table S7. Results of ANCOVA on mortality rates in the bicarbonate limitation experiment with post-hoc analysis run on salinity as a significant factor

		ANCOVA - Mortality				
Response	factor	Df	Sum Sq	Mean Sq	F value	P value
Mortality	Bicarbonate	1	12243.2	12243.2	54.14	<0.001
	Salinity	2	6263	3131.5	13.08	<0.001
	Bicarbonate:Salinity	2	875.6	437.8	1.83	0.17
	Residuals	54	12927.8	239.4		
					Tukey post-hoc	
					Factor	p-value
					11 - 6	0.175
					16 - 6	0.006
					16 - 11	<0.001

Table S8. Results of non-parametric ANOVA (Kruskal-Wallis) on mean annual salinities between each of the three monitoring sites with nemenyi post-hoc analysis.

Annual mean salinity			
	df	Chi²	p-value
Site	2	7797.3	< 0.001
nemenyi post-hoc analysis			
	p-value		
sites			
Kiel-Ahrenshoop			< 0.001
Usedom-Ahrenshoop			< 0.001
Usedom-Kiel			< 0.001

Table S9. (A) Results of non-parametric analysis using the Kruskal-Wallis test for modelled chl-*a* data with nemenyi post-hoc analysis. **(B)** ANOVA on field monitoring data for chl-*a* concentrations with Tukey post-hoc analysis

A

Modelled chl-<i>a</i> concentrations			
factor	df	chi²	p-value
Site	2	448.978	<0.001
nemenyi post-hoc analysis			
Site	p-value		
Kiel-Ahrenshoop	<0.001		
Usedom-Ahrenshoop	<0.001		
Usedom-Kiel	0.001		

B

Raw chl-<i>a</i> concentrations from monitoring					
factor	df	Sum sq	Mean sq	F value	p-value
Site	2	11.41	5.7	13.8	<0.001
Residuals	77	31.82	0.41		
Tukey post-hoc					
Site					p-value
Kiel-Ahrenshoop					<0.001
Usedom-Ahrenshoop					<0.001
Usedom-Kiel					0.35

Table S10. ANOVA on mean annual A_T at each of the three monitoring sites with subsequent Tukey post-hoc tests. Kiel = Kie, Ahrenshoop = Ahp and Usedom = Use.

ANOVA- Total alkalinity					
factor	df	Sum sq	Mean sq	F value	p-value
Site	2	425827	212914	42.678	<0.001
Residuals	43	215422	4989		
	comparison	difference	lower	upper	p-value
	Kie-Ahp	212.234	143.763	280.705	<0.001
	Use-Ahp	25.604	-57.708	108.917	0.738
			-	-	
	Use-Kie	-186.63	252.051	121.208	<0.001

Chapter 2 – Energetic costs of calcification

High calcification costs limit mussel growth at low salinity

High calcification costs limit mussel growth at low salinity

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Abstract

In coastal temperate regions such as the Baltic Sea, calcifying bivalves dominate benthic communities playing a vital ecological role in maintaining biodiversity and nutrient recycling. At low salinities, bivalves exhibit reduced growth and calcification rates which is thought to result from physiological constraints associated with osmotic stress. Calcification demands a considerable amount of energy in calcifying molluscs and estuarine habitats provide sub-optimal conditions for calcification due to low concentrations of calcification substrates and large variations in carbonate chemistry. Therefore, we hypothesise that slow growth rates in estuarine bivalves result from increased costs of calcification, rather than costs associated with osmotic stress. To investigate this, we estimated the cost of calcification (in $\text{J mg}^{-1} \text{CaCO}_3$) for the first time in benthic bivalve life stages and the relative energy allocation to calcification in three *Mytilus* populations along the Baltic salinity gradient. Our results indicate that calcification rates are significantly reduced only in 6 psu populations compared to 11 and 16 psu populations, coinciding with ca. 2 – 3-fold higher calcification costs. This suggests that reduced growth of Baltic *Mytilus* at low salinities results from increased calcification costs rather than osmotic stress related costs. We also reveal that shell growth (both calcification and shell organic production) demands 33 - 65% of available assimilated energy from food, which is significantly higher than previous estimates. Energetically expensive calcification represents a major constraint on growth of mytilids in estuarine and coastal seas where warming, acidification and desalination are predicted over the next century.

Introduction

The Baltic Sea is characterized by a natural salinity gradient exists decreasing from fully marine in the West to almost freshwater (< 3 practical salinity units (psu)) in the East (Meier 2006). This gradual salinity gradient provides an excellent model to investigate the impacts of salinity on physiology of marine invertebrates. Marine mytilid mussels dominate the Baltic benthos and play an important ecological role in benthic nutrient recycling and as a foundation species (Kautsky & Wallentinus 1980; Norling & Kautsky 2008). Baltic mytilid mussels (from here on: Baltic *Mytilus*) are all hybrids of *Mytilus edulis*-like genotypes and *M. trossulus*-like genotypes with *M. edulis*-like genotypes being dominant in the high saline western Baltic and *M. trossulus*-like genotypes dominating the low saline eastern Baltic (Väinölä & Hvilson 1991). The genetic transition zone where dominance shifts from *M. edulis*-like to *M. trossulus*-like genotypes or vice-versa, is in the south-western Baltic Sea located near Ahrenshoop on the

German coast (Fig. S1, Stuckas et al. 2017). At salinities < 10 psu Baltic *Mytilus* exhibit significantly reduced growth rates and smaller maximum sizes of ~ 35 mm in shell length compared to > 70 mm at 33 psu. While comparisons between Baltic *M. edulis*-like and *M. trossulus*-like populations have revealed local adaptation to habitat salinities (Kossak 2006; Kautsky et al. 1990), reciprocal acclimation experiments have demonstrated that environmental salinity explains the majority of salinity induced changes in growth and feeding rates (Tedengren et al. 1990; Riisgård et al. 2013). Reduced growth rates at low salinities have been suggested to be a result of unfavourable protein metabolism and higher physiological costs associated with osmotic stress (Tedengren & Kautsky 1986; Maar et al. 2015). However, oxygen consumption rates have not been found to be impacted by salinity suggesting no increased metabolic maintenance costs at low salinity (Landes et al. 2015). Concentrations of Ca^{2+} and HCO_3^- relevant for calcification also decrease with salinity which, coupled with low winter water temperatures, leads to extended periods of aragonite undersaturation ($\Omega_{\text{arag}} < 1$: Beldowski et al. 2010; Tyrrell et al. 2008). This imposes kinetic constraints on calcification and can cause net dissolution of CaCO_3 structures (Melzner et al. 2011; Thomsen et al. 2015). Low seawater concentrations of calcification substrate coupled with kinetic constraints on calcification may increase the energetic costs of calcification and subsequently limit growth at low salinities.

Calcium carbonate exoskeletons are extensive throughout the animal kingdom and in bivalve molluscs, shells can constitute over 70 % of total animal mass (Eklöf et al. 2017). Despite this, the energetic costs of CaCO_3 biomineralisation are still not fully understood. This is because 1) animals can modulate energy investment between shell and body tissue growth in response to a range of environmental factors (Anthony et al. 2002; Brookes & Rochette 2007) and 2) there is a limited understanding of the mechanisms of calcification at the tissue and cellular level, especially in bivalves. Consequently, estimating calcification costs is problematic and no direct estimates for shell formation costs in benthic bivalve life-stages currently exist. This makes it difficult to assess the impacts of environmental change on bivalve energetics, growth and fitness. This is particularly important in the Baltic Sea where bivalve molluscs play an important ecological role. Climatic and oceanographic models predict desalination of the Baltic Sea over the next 50-100 years due to more extreme precipitation events and increased freshwater run-off from the Baltic Sea basin (Meier 2006; Gräwe et al. 2013). Projected decreases in salinity by ca. 2 psu may lead to extensive distribution shifts of Baltic *Mytilus* westwards by several hundred kilometres, particularly in the central part of the Baltic, where the salinity ranges

between 5 and 8 psu (Gräwe et al. 2013). This could have large scale implications for benthic communities in the future. It is thus of great interest to better understand the physiological and energetic mechanisms that underlie reduced growth in the low salinity parts of the Baltic Sea.

In this study, we tested whether calcification costs, rather than costs associated with osmotic stress are the main factor limiting mussel growth at low salinities (6 psu) characteristic for the central Baltic Sea. To investigate this, energy budgets of three locally adapted populations of Baltic *Mytilus* along the natural salinity gradient were determined in the lab at their respective native salinities (16, 11 and 6 psu). Two temperatures were used (8 and 18°C) to simulate seasonal differences and impose kinetic constraints on calcification by decreasing CaCO₃ saturation state at low temperature. By using a regression approach across three feeding regimes, energy available for calcification (Scope for Shell Growth) was correlated with calcification rates to obtain direct estimates for calcification costs for the first time in juvenile mussels.

Materials and Methods

Experimental design

We factored potential local adaptation of Baltic *Mytilus* populations to low salinity into our experimental design by testing three separate, populations originating from different salinities along the south-western Baltic salinity gradient (fig. S1) at their native salinities (Usedom 6 psu; Ahrenshoop 11 psu; Kiel 16 psu; table S1). Animals were reared at two temperatures (8 °C and 18 °C) and three feeding regimes (high food: 6000 microalgae cells ml⁻¹, medium food: 3000 cells ml⁻¹ and low food: 500 cells ml⁻¹) with 4 replicate, 2 L tanks per treatment (40 animals per tank, total of 72 replicate tanks, 2880 animals in total; experimental design depicted in Fig. S2). Three feeding regimes were used to induce a wide range of growth and calcification rates for regression analyses in order to calculate calcification costs and were not included as a factor in the experimental design except when analysing growth rates (see materials and methods: Statistics). We used a discrete feeding method as it was vital to ensure uniform energy intake between salinities. It was also important to ensure maximum particle retention to accurately quantify energy intake, therefore 6000 cells ml⁻¹ was chosen as the high food treatment as higher cell concentrations result in production of pseudofaeces and < 100% particle intake (Riisgård et al. 2013: Fig. S3). Animals were fed twice daily for the duration of the experiment (total 8 weeks) with a live microalga mix of *Isochrysis galbana*, *Tetraselmis suecica* and *Rhodomonas salina* at a ratio of 1:1:1 (supplementary methods). Water was changed

weekly with water prepared in relation to the specific carbonate chemistry at each site (supplementary methods, Table S2). During water changes, tanks were cleaned, animal numbers per tank were counted and any dead animals removed. Mortality was minimal (< 3.5 %) and factored into calculations.

Growth and calcification rates

Shell length was measured weekly from a random sub-sample of 10 individuals per tank. Five animals from each tank were removed at week 0, 4 and 8 and stored at - 80 °C for tissue and shell mass analyses. For this, individual animals (shell and tissue) were placed in pre-weighed tin foil boats and dried at 60 °C for 12 hours. Samples were then weighed to determine the total dry mass of shell and tissue (DM, mg). A second sub-sample was defrosted and briefly microwaved to enable the removal of all soft tissue from the inside of the shell under a stereomicroscope. Empty shells were then placed in pre-weighed foil boats and dried at 60 °C for 12 hours and weighed to determine the shell mass (SM, mg). These shells were then ashed at 450 °C for 4 hours and weighed again to determine the ash mass (AM, mg) which is the weight of the inorganic material of the shell. This was used as a proxy for CaCO₃ mass. The ash-free dry mass was used as a proxy for the shell organic mass (SOM, mg):

$$(1) \text{ SOM} = \text{SM} - \text{AM}$$

M. edulis soft tissue also contains approximately 13 % inorganic material (Dare & Edwards 1975). However, this only constitutes ~ 2 % of total mass and was therefore excluded from our calculations. For each salinity and temperature, total dry mass (Fig. S4), shell mass (Fig. S5) and ash mass (proxy for CaCO₃ mass) were plotted against shell length with power functions showing the highest R² values (Table S3). Continuous experimental measurements of shell length were converted to mass values using these relationships. Soft tissue dry mass (TM, mg) was calculated using the following equation:

$$(2) \text{ TM} = \text{DM} - \text{SM}$$

Growth rates were calculated as the difference between mass at the start of the experiment and mass at the end of the experiment divided by the experimental period, expressed in µg day⁻¹. Calcification rates are expressed as deposition rate of inorganic shell material in µg CaCO₃ day⁻¹ excluding the mass of shell organic material.

Oxygen consumption

O₂ consumption rates were measured every 2 weeks at progressively larger shell lengths (week 0: 4 mm, week 2: 5 mm, week 4: 6 mm and week 6: 7 mm). O₂ consumption was quantified by measuring O₂ depletion within sealed chambers using a 24 channel SDR SensorDish Reader (PreSens, Germany). Prior to measurements, mussels were starved for 18-24 hours to ensure measurements during basal metabolism and reduce the effects of feeding on metabolism. Respiration water was prepared 2 hours before measurements using freshly filtered (0.22 µm) seawater (FSW) from Kiel Fjord adjusted to the correct salinity, total alkalinity (A_T) and temperature and oxygenated appropriately to ensure 100 % air saturation. Mussels were placed individually in custom made (Eydam, Germany) 700 µl, airtight glass vessels. Lids were sealed without air bubbles and mussels were left for 30 minutes before measurements commenced to ensure opening of valves and active aerobic metabolism. Runs during which mussels closed their valves for more than 10 % of the time were excluded. Depletion of oxygen within the vessels over time was measured in dark temperature-controlled rooms and measurements were terminated when air saturation reached < 70 %. Empty vessels were used as controls for background seawater O₂ flux and subtracted from experimental vessels. Individual O₂ consumption rates per hour were plotted against soft tissue mass with O₂ consumption expressed as nmol O₂ individual⁻¹ hr⁻¹.

Energetics, calcification cost and energy allocation

All following energy fluxes were calculated as mean values per individual with tanks as replicates and expressed as total values over the 8-week experiment. Scope for Growth (SfG, J), which is the energy available for growth, was calculated using the following equation:

$$(3) \text{ SfG} = (A * \text{AE}) - M$$

Where A is the energy intake (J), AE is the absorption efficiency (ratio: 0-1) and M is the metabolic energy loss (J). A was calculated as the total number of cells added to each tank multiplied by an energetic conversion factor of 19 µJ ng⁻¹ microalgae based on the organic content of the 3 cultured microalgae species (Renaud et al. 2002). Clearance was measured to ensure sufficient feeding rates to allow complete clearance of algae cells between feeding events (Fig. S3). Absorption efficiency was not measured due to logistical constraints and an average literature value for Baltic *Mytilus* of 0.58 was used (Tedengren et al. 1990). For metabolic energy loss, oxygen consumption rates were converted to energy using the conversion factor 1 µmol O₂ = 450 mJ (Gnaiger & Bitterlich 1984). Ammonia excretion was excluded from our calculations as energy lost through this process contributes < 3 % to energy

budgets in *M. edulis* (Sanders et al. 2014). Final Scope for Growth (SfG, J) was expressed in Joules per individual over the entire experiment (8 weeks).

The energy available for calcification termed Scope for Shell Growth (SfSG, J) was calculated by subtracting the energy allocated to shell organic mass growth (G_{SOM} , J) and soft tissue mass growth (G_{TM} , J) from the SfG:

$$(4) G_{TM} = TM * 23.96$$

$$(5) G_{SOM} = SOM * 23.64$$

$$(6) SfSG = SfG - (G_{TM} + G_{SOM})$$

G_{SOM} was calculated using an energetic conversion value of 23.64 J mg⁻¹ protein as SOM is > 95 % protein (Gnaiger & Bittlerich 1984; Weiner & Traub 1984). G_{TM} was calculated with an energetic conversion value of 23.96 J mg⁻¹ based on published values of soft tissue biochemical composition in bivalves outside the reproductive season (10.0 % lipid; 53.8% protein; 19.5 % carbohydrate) with energetic conversions of 39.54 J mg⁻¹ (lipid), 23.64 J mg⁻¹ (protein) and 17.15 J mg⁻¹ (carbohydrate) (Gnaiger & Bittlerich 1984; Celik et al. 2014). At the end of the experiment, total values of SfSG, were plotted against total calcification for each tank with all food treatments pooled to allow regressions across a wide range of calcification rates and SfSG values (Fig. 3A). The slopes of linear regressions represent the cost of calcification in J mg⁻¹.

Available assimilated energy ($A * AE$) termed E, was divided into allocation to metabolism, calcification ($G_{calcification}$, J), shell organic mass growth and soft tissue mass growth with excess energy termed unaccounted energy (X, J):

$$(7) E = (M + G_{calcification} + G_{SOM} + G_{TM}) + X$$

These values were calculated as a proportion of total assimilated energy (E) per tank but exclusively for the high food treatments. High food treatments induced high growth rates, thus enabling accurate determination of energy partitioning to growth processes. The low food treatment was designed to support little more than energy needed for maintenance of body mass.

Statistics

All statistical analysis was conducted using R Software (v.3.4.3). All data was tested for normality using Shapiro-Wilk tests and for homogeneity of variances using Levene's test. If data deviated from normality or homogeneity of variances it was transformed using a log transformation. Soft tissue mass growth rates and calcification rates ($\mu\text{g day}^{-1}$) were analysed

parametrically using 3-way ANOVA with temperature, salinity and food as factors (with tanks as replicates). Food was only included as a factor when analysing growth rates to ensure the food levels were sufficiently different to induce a wide range of growth rates. Energy allocation to calcification was analysed using a 2-way ANOVA with temperature and salinity as factors. Energy allocation was analysed for the high food treatments only as medium and low food treatments did not allow maximum energy allocation to growth processes. All feeding treatments were pooled for ANCOVA and not analysed separately. This was because different food levels were only used to induce a wide range of growth rates for more accurate regression analyses. Oxygen consumption rates were analysed using 2-way ANCOVA with soft tissue mass as a co-variable and temperature and salinity as factors. Costs of calcification (SfSG vs calcification) were analysed statistically using 2-way ANCOVA with calcification as a co-variable and temperature and salinity as factors. For ANCOVA analyses, all food treatments were pooled within each salinity and temperature treatment to allow a wide range of masses for more accurate regression analyses. All statistical results are summarized in Table S4 (ANCOVAs) and Table S5 (ANOVAs).

Results

Calcification and soft tissue growth

Calcification rate was lower at 6 psu with an average of $43 \mu\text{g day}^{-1}$ compared to an average of $76 \mu\text{g day}^{-1}$ at 11 and 16 psu (ANOVA, $F_{(2,54)} = 13.3$, $p < 0.001$; Fig. 1A). The absence of differences in calcification rates between 11 psu and 16 psu suggests a non-linear relationship between salinity and calcification rate. Interestingly, temperature had no effect on calcification rate but did significantly reduce soft tissue growth at 8°C (ANOVA, $F_{(1,54)} = 29.2$, $p < 0.001$). Calcification rate ($\mu\text{g CaCO}_3 \text{ day}^{-1}$) was significantly impacted by food (ANOVA, $F_{(2,54)} = 194.8$, $p < 0.001$) being different between all food treatments (Fig. 1A). Food also had the same effect on soft tissue growth (ANOVA, $F_{(2,54)} = 240$, $p < 0.001$) indicating there were sufficient differences in growth rates between feeding regimes for subsequent regression analyses to calculate calcification costs.

Oxygen consumption and energetics

Each of our experimental populations showed high levels of variation in O_2 consumption with R^2 values of linear regressions between 0.49-0.80 (Fig. 2). O_2 consumption correlated significantly with tissue mass and was lower at 8°C compared to 18°C (ANCOVA, $F_{(1,268)} =$

323, $p < 0.001$). Oxygen consumption rates were not different between salinities (ANCOVA, $F_{(1,268)} = 1.7$, $p = 0.177$).

Scope for Shell Growth (SfSG) correlated with calcification rate with R^2 values being between 0.59 – 0.93 with Ahrenshoop 11 psu 18 °C showing a weaker relationship with an R^2 of 0.39 (Fig. 3A). Costs of calcification were not impacted by temperature (ANCOVA, $F_{(1,60)} = 0.05$, $p = 0.831$) however, calcification costs were impacted by salinity (ANCOVA, $F_{(2,60)} = 3.238$, $p = 0.041$), with higher costs observed at 6 psu (17 - 56 J mg⁻¹) compared to 11 and 16 psu (10 - 14 J mg⁻¹, Tukey test, $p < 0.001$: Fig. 3B). Energy allocation to calcification as a percentage of the total energy budget ranged between 30 – 59 % (Fig. 4). Low temperature invoked an increase in allocation of energy to calcification compared to higher temperature (ANOVA, $F_{(1,18)} = 8.27$, $p = 0.010$) however, salinity had no impact on the relative allocation of energy to calcification (ANOVA, $F_{(1,18)} = 2.24$, $p = 0.135$).

Discussion

The findings in this study indicate that reduced growth of Baltic mytilids at 6 psu likely results from reduced calcification rates and increased costs of calcification, rather than costs associated with osmotic stress. We also reveal that calcification (CaCO₃ production) encompasses a significant proportion of the energy budget in Baltic *Mytilus* constituting 29 – 58 % of total energy allocation.

Growth and physiological energetics

The reduced calcification rates in our study are in accordance with previous findings in *M. edulis* where calcification rate starts to rapidly decline below salinities of ~ 10 psu when compared to 25 psu (Kossak 2006; Maar et al. 2015; Riisgård et al. 2014). These vastly reduced growth rates have been suggested to result from lower soft tissue growth rates due to increased protein metabolism at low salinities (Tedengren & Kautsky 1986). However, previous work on Baltic *M. trossulus*-like individuals has recorded stable growth rates of soft tissue between high salinities (24 - 33 psu) and low salinities (6-7 psu) (Kautsky et al. 1990; Riisgård et al. 2014). This suggests low salinity limits calcification rather than body tissue growth in Baltic *Mytilus*. For molluscs with exoskeletons, this ultimately limits total growth and body size as has been found in marine gastropods (Palmer 1981) which would explain the low tissue mass growth at 6 psu compared to higher salinities in our study

Previous studies found O₂ consumption to be relatively insensitive to salinity in Baltic *Mytilus* (Maar et al. 2015; Landes et al. 2015), which contrasts with observed reductions in O₂ consumption at low salinity in more stenohaline bivalve species (Hutchinson & Hawkins 1992). A Dynamic Energy Budget (DEB) model was employed to predict growth of Baltic *Mytilus* at 8 psu and found the best growth estimates were attained when assuming a significant increase in costs of osmoregulation despite no measured changes in respiration rate (Maar et al. 2015). These costs were suggested to result from decreased protein turnover efficiency and lower O:N ratios as suggested by Tedengren & Kautsky 1986. However, energetic costs of calcification were excluded from the DEB model in Maar et al. 2015 and as our findings show, shell production (both calcification and shell organic production) demands up to 65 % of the available assimilated energy in Baltic *Mytilus*. Previous work has estimated calcification constitutes < 10 % of the energy budget in marine gastropods and bivalves, substantially lower than in this study (Watson et al. 2017). However, calcification costs were not calculated in Watson et al. 2017 and values for calcification costs were taken from published literature values on marine gastropods (1-2 J mg⁻¹ CaCO₃; Palmer 1992). These values may not be applicable to other organisms and environmental conditions, for example low salinity adapted mussels.

Osmotic stress at salinities < 8 psu may also invoke intracellular changes in ion concentrations which can impact growth efficiency. Although *Mytilus* spp. are osmoconformers and favour adjusting intracellular organic osmolyte concentrations rather than inorganic ions to remain iso-osmotic to seawater (Silva & Wright 1994), reduced intracellular ion concentrations (particularly K⁺) at salinities < 8 psu have been recorded (Willmer 1978a; Berger & Kharazova 1997). Changes in intracellular ion concentrations have been shown to impact numerous cellular processes, including glutamate oxidation and rates of electron transport in bivalve mitochondria (Ballantyne & Moyes 1987). These intracellular changes at salinities < 8 psu may partially be responsible for reduced enzyme activities and subsequently reduced growth at 6 psu but not at 11 and 16 psu.

Costs of calcification

The cost of calcification calculated in our study was 10 – 14 J mg⁻¹ CaCO₃ in both 11 and 16 psu treatments and 17 – 58 J mg⁻¹ CaCO₃ at 6 psu, significantly higher than other estimates of 1-2 J mg⁻¹ for adult gastropods (Palmer 1992), 1.6 J mg⁻¹ for oyster larvae (Waldbusser et al. 2013; Frieder et al. 2016) and ~ 0.15 J mg⁻¹ in coral species (Anthony et al. 2002). Our method of estimating calcification costs partially resembles that used by Palmer 1992 in that we used a linear regression of available energy correlated with calcification. The method presented in this

study is further optimised by using the SfG model and subtracting the energetic content of soft tissue from available growth energy, thus incorporating variations in tissue growth and metabolism into these estimates. Additionally, large differences in calcification rates between feeding treatments and precise knowledge of food intake resulted in substantially higher R^2 values than previous work by Palmer 1992, enabling us to obtain more robust estimates of calcification costs. Growth efficiency is impacted by changes in protein absorption rates (protein synthesis rate – protein degradation rate) which vary with season and genetic heterozygosity (Hawkins et al. 1986; Bayne et al. 1997). Although this can impact the efficiency of body tissue growth it can also impact shell growth efficiency as mytilid shells contain up to ~ 6 % organic material composed mostly of protein (Thomsen et al. 2013). This is even more important for Baltic *Mytilus* as *M. trossulus*-like populations have been observed to have higher proportions of shell organic than North Sea *M. edulis* (Kautsky et al. 1990). This organic fraction is known to make up a large proportion of the costs of shell growth (Palmer 1983) and thus indicates another source of increased costs of shell production in Baltic *Mytilus*.

Costs of calcification arise from several key processes such as Ca^{2+} and HCO_3^- ion uptake, H^+ extrusion and enzymatic conversion of CO_2 and HCO_3^- to CO_3^{2-} . Previous estimates of calcification costs in non-feeding ontogenetic stages of larval oysters have been based on metabolic energy loss minus the energetic cost of protein synthesis and Na^+/K^+ -ATPase activity, which were attributed to be processes independent of calcification (Frieder et al. 2016). Similarly, other calcification cost estimates in larval oysters were based on Ca^{2+} -ATPase driven costs of H^+ extrusion, a coral calcification model adapted from findings in human erythrocyte membranes (Waldbusser et al. 2013; Niggli et al. 1982). While these ion transport processes contribute considerably to cellular ATP demand, it is not yet clear what proportion of their total activity is involved in calcification as well as how conserved the mechanisms of calcification are between different phyla. In the absence of current understanding of the exact cellular mechanisms and pathways for calcification related ion transport in bivalves, such approaches to estimating calcification costs must remain explorative and associated with a high degree of uncertainty.

At salinities < 8 psu, low Ca^{2+} concentrations (< 3 mmol kg^{-1}) have been found to reduce calcification in Baltic *Mytilus* larvae (Thomsen et al. 2018). This is a likely mechanism by which calcification is limited at 6 psu in this study. Calcium uptake via the Ca^{2+} -ATPase is energetically costly and is more expensive at lower calcium ion concentrations (Niggli et al. 1982; McConnaughey & Whelan 1997) highlighting a potential source of increased

calcification costs at 6 psu. Increased trans-membrane ion transport activity via the Na^+/K^+ -ATPase has also been recorded at 8 psu in mussel neuronal and gill tissue (Rola et al. 2017; Willmer 1978b). This has implications for calcification as the Na^+/K^+ -ATPase is important in the uptake of HCO_3^- (via the $\text{Na}^+/\text{HCO}_3^-$ cotransporter and the Na^+ driven $\text{Cl}^-/\text{HCO}_3^-$ -exchanger of the solute carrier (SLC) 4 family) and in proton extrusion (via Na^+/H^+ exchangers of the SLC9 family) which have been localized in marine invertebrate calcifying epithelia (Laurent et al. 2013; Zoccola et al. 2015). Together with our findings, this suggests that very low salinities invoke changes in ion transport activity which may interfere with the mechanisms of calcification in Baltic *Mytilus* and lead to reduced calcification rates and increased calcification costs.

Calcification rates are also impacted by the degree of shell dissolution, which depends on shell mineralogy (aragonite vs calcite) and the seawater saturation state (Ω) of calcium carbonate (Ries et al. 2016). Low salinity and temperatures reduce CaCO_3 saturation states ($\Omega = [\text{Ca}^{2+}][\text{CO}_3^{2-}]/K_{sp}$; where K_{sp} is the temperature dependent solubility product of CaCO_3) due to decreased $[\text{Ca}^{2+}]$, $[\text{CO}_3^{2-}]$ and K_{sp} with net dissolution of CaCO_3 structures occurring when $\Omega \leq 1$. In molluscs, the pH of the extra-pallial fluid (EPF) under the shell valve is even lower than ambient seawater due to high pCO_2 . This results in low EPF Ω leaving internal shell surfaces more prone to dissolution than external shell surfaces (Melzner et al. 2011, Heinemann et al. 2012). In our study, seawater Ω_{arag} was < 1 in the 11 and 6 psu treatments at all temperatures suggesting that calcification may need to work against dissolution of the internal nacreous layer. Higher costs of epithelial transport of Ca^{2+} in the 6 psu treatment, coupled with kinetic constraints on calcification ($\Omega_{\text{arag}} < 1$), may both act together to induce significantly higher costs of calcification, potentially limiting overall growth rates in low salinity Baltic *Mytilus*.

This is the first study to estimate the cost of calcification in bivalves using a regression method which encompasses all major energy consuming processes (metabolism and body tissue growth). Our findings show that both low salinity (6 psu) and low temperatures (8 °C) invoke higher energetic costs of calcification severely constraining growth and thus fitness in an ecologically and economically important coastal species. We also highlight that calcification demands a considerable amount of energy in Baltic *Mytilus* at all temperatures and salinities, significantly more than previously suggested in bivalves. With climate projections suggesting many coastal systems to experience desalination and acidification in the coming decades, future work should aim to understand the mechanisms behind these elevated costs in the context of a rapidly changing ocean.

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Author contributions

TS, and FM, conceived and designed the study. TS, JNS, and LS, conducted the experiments and TS, analysed data. TS, wrote the manuscript and all authors contributed to revisions.

Conflict of interests

Authors have no conflicts of interest.

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Figures

Fig. 1 Mean calcification rate (A) and growth of soft tissue (B) in μg per day \pm standard error. Salinity treatments are represented with red (6 psu), green (11 psu) and blue (16 psu) colours and “low”, “med”, and “high” labels represent food levels described in the methodology section. 8 °C treatments are shown on the left and 18 °C are shown on the right of each graph. Letters represent statistically significant salinities

Fig. 2 Oxygen consumption plotted against soft tissue dry mass at 18 °C (A) and 8 °C (B) measured across a range of mussel sizes. Linear regressions were calculated across all food levels and salinities are represented by red (6 psu) green (11 psu) and blue (16 psu). There were no significant differences between salinities.

Fig. 3 Linear regressions across all food treatments between total Scope for Shell Growth (SfSG) and total calcification (A) and the corresponding costs of calcification (B). Salinities are represented by red (6 psu) green (11 psu) and blue (16 psu) with open circles and dashed regression lines depicting 8 °C and closed circles with solid regression lines representing 18 °C. Slopes of the linear regressions \pm standard error are shown in panel B which represent the cost of calcification. Letters represent groupings of statistically significant treatments.

Fig. 4 Pie charts representing the relative energy partitioning (%) to different processes. Processes shown are: calcification (blue), shell organic (white), soft tissue (red), metabolism (lilac diagonal lines) and unaccounted-for energy (green checkered) in all temperature and salinity treatments for the high food treatments only. Unaccounted energy refers to leftover available energy not used for the above processes. This can include processes such as feeding, digestion and protein synthesis.

Figure 1

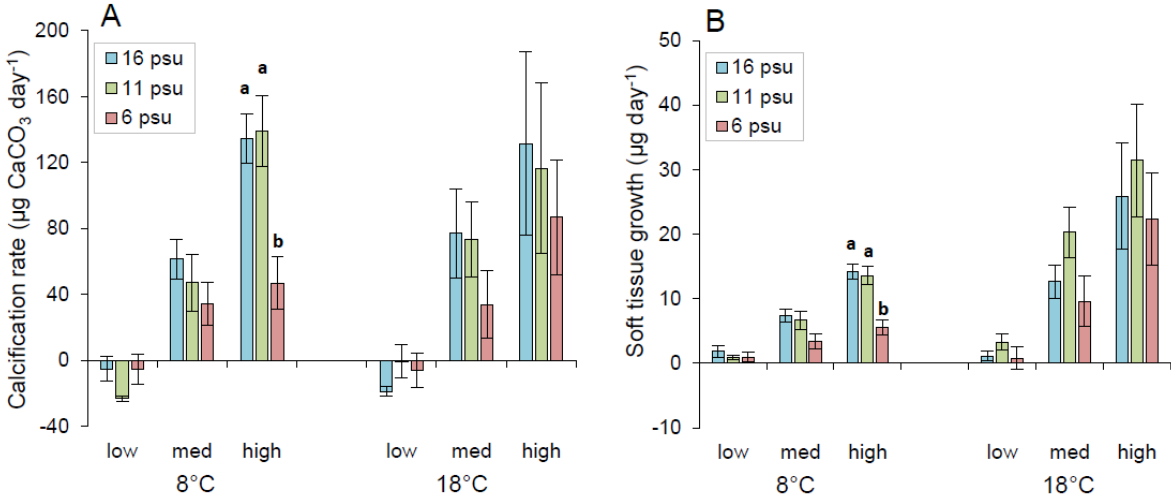


Figure 2

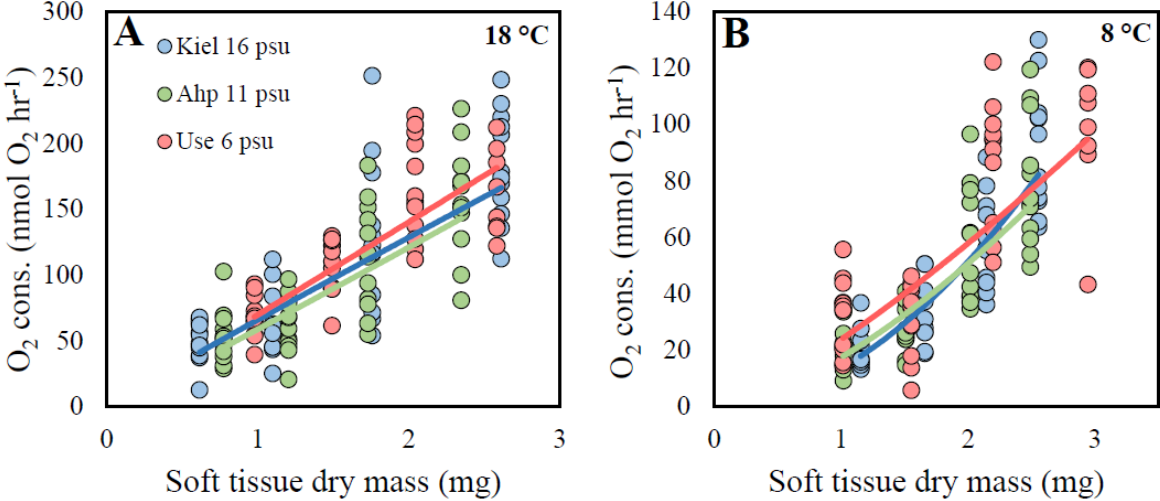


Figure 3

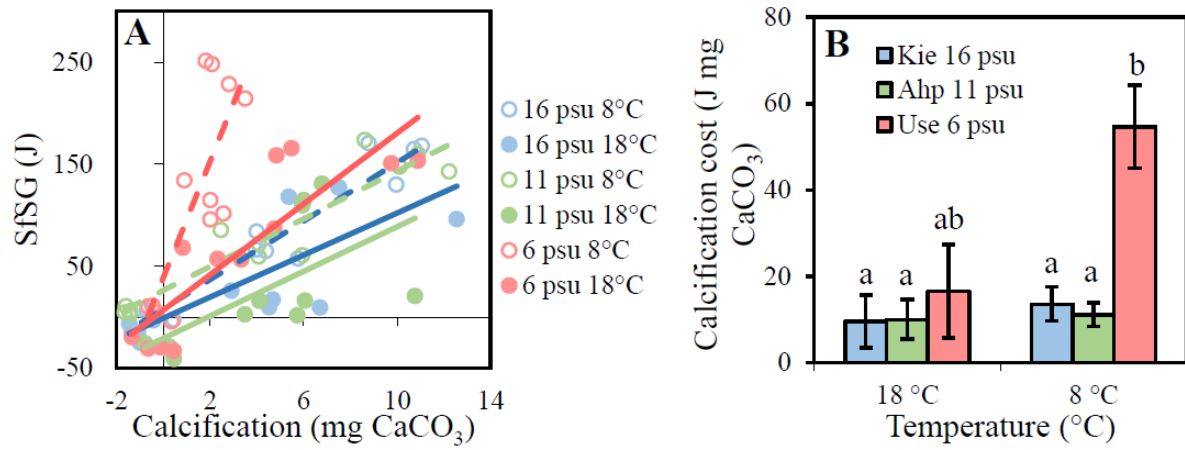
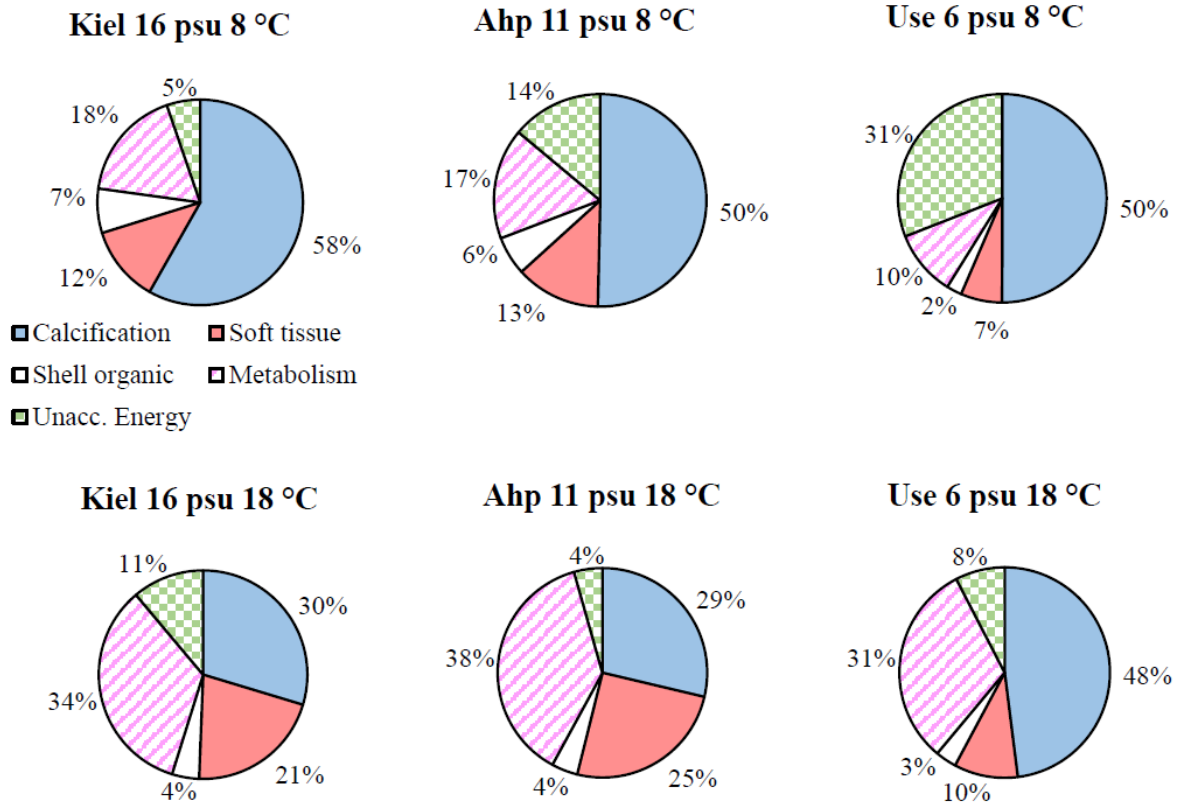


Figure 4



Supplementary material

Supplementary methodologies

Animal collection

Juvenile mussels were chosen for investigation as they have higher relative growth and calcification rates compared to adults. Mussels (shell length (SL) 4.8 ± 0.4 mm) were collected in September 2015 from 3 subtidal populations of Baltic *Mytilus* along the Baltic Sea salinity gradient from 1 m water depth at Kiel (*M. edulis*-like, $54^{\circ} 19' 48.846''$ N, $10^{\circ} 8' 59.6436''$ E), Ahrenshoop (hybrid transition zone, $54^{\circ} 23' 7.3032''$ N, $12^{\circ} 25' 24.0564''$ E) and Usedom (*M. trossulus*-like, $54^{\circ} 3' 20.5668''$ N, $14^{\circ} 0' 40.0572''$ E, Figure S1). Animals were transported in cool boxes to GEOMAR labs within 5 hours after collection (see: supplementary methods).

Animal acclimation

Animals were kept at 15° C in climate chambers in 10 L aerated plastic aquaria ($n = \sim 500$ per aquarium) and fed twice daily with $15,000$ cells ml^{-1} *Rhodomonas salina* for 4 weeks prior to the experiment. Complete water changes were done three times weekly with water prepared 24 hours before and aerated at the correct experimental temperature (for details on water preparation see supplementary section: Water chemistry). Salinity at each of the three collection sites was monitored over 2 years using *in situ* CTD (conductivity, temperature, depth) loggers (StarOddi, Iceland) at 1 meter depth with a measurement frequency of 3 hours.

Water chemistry

Water was prepared by filtering ($0.22 \mu\text{m}$) Kiel fjord water and salinity was adjusted to 16, 11 and 6 psu with deionized water using a Cond 3110 conductivity meter (WTW, Germany). Dilution of salt water with deionized water also dilutes the total alkalinity (A_T) which influences calcification rate. The A_T was therefore increased with 1 M HCO_3^- solution to natural levels found in the Baltic Sea at the respective experimental salinities using a published salinity- A_T relationship (Beldowski et al. 2010). pH was monitored twice weekly using a 340i pH meter (WTW, Germany). Every two weeks, total inorganic carbon (C_T) samples were taken and analysed using an AIRICA C_T analyser (Marianda, Germany) and aragonite saturation state (Ω_{arag}) was calculated using pH_{NBS} and C_T values input into the CO2sys_v2.1 programme using the KHSO_4 constant (Dickson 1990) and carbonate system dissociation constants K_1 and K_2 (Dickson 1987).

Microalgae food cultures

Microalgae food was cultured at 25 °C and three salinities (16, 11 and 6 psu) using F2 medium and vitamins. At the beginning of the experiment, microalgae cell dry mass was calculated by filtering cultures onto pre-ashed GF/F filters and dried at 60 °C and weighed. Filters were then ashed at 450°C for 4 hours to calculate the organic content per algal cell. As organic content was found to differ significantly between culture salinities food rations were mixed between salinities to achieve an average salinity of 11 psu and a cell number ratio of 1:1:1 for all three species prior to feeding to ensure an equal ration of organic content for all treatment tanks. Culture cell densities and cell diameter was measured daily using a Multisizer 3 Coulter Counter (Beckman Coulter, USA). Field chlorophyll *a* concentrations were obtained from model predictions acquired from the publicly available EU database, CMEMS (EU, Copernicus Marine Service).

Filtration

Filtration rate measurements were not used in SfG calculations as food was not applied *ad libitum*, but measured to ensure complete clearance of microalgae food within each treatment tank. Measurements were conducted in experimental plastic aquaria (2 L) with sufficient bubbling to ensure constant mixing of algal cells. *Rhodomonas salina* (concentrations: 6000, 3000 or 1000 cells ml⁻¹ for high medium and low food treatments, respectively) were used exclusively to measure filtration rate to keep cell size uniform and was measured weekly in two 25 ml samples taken 1 hour apart measured using a Coulter Counter. Filtration rate measurements indicated that all tanks were feeding at high enough rates to consume all food before algae cells stagnated and died (Figure S6).

References

1. Dickson, A.G., Millero, F.J. (1987). A comparison of the equilibrium constants for the dissociation of carbonic-acid in seawater media. *Deep Sea Res.* 34, 1733–1743.
2. Dickson, A.G. (1990). Standard potential of the reaction – AgClS+1/2 H₂ = AgS+HClAq and the standard acidity constant of the ion HSO₄ – in synthetic sea-water from 273.15-K to 318.15- K. *J. Chem. Thermodyn.*, 22, 113–127.

Supplementary Figures and Tables

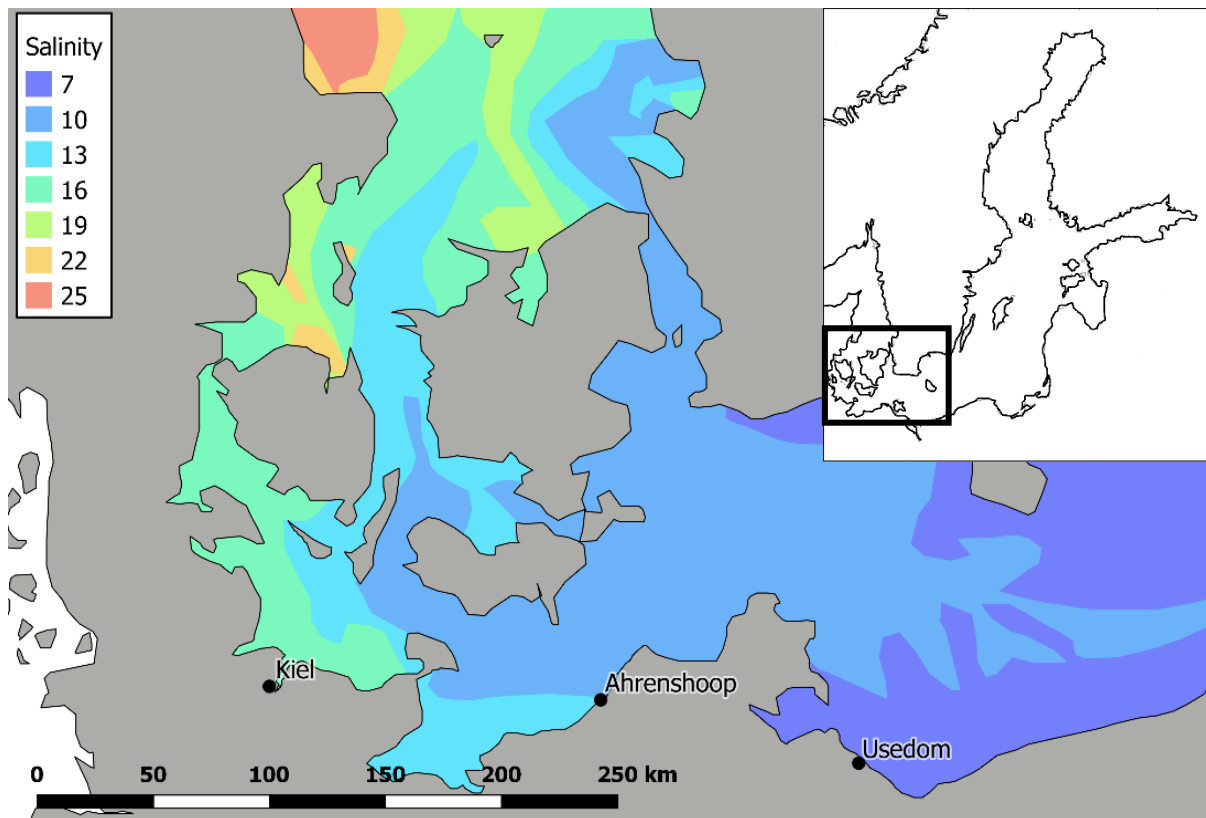


Fig. S1 Map of the south west Baltic Sea showing surface salinity distribution and the three sampling sites on the German Baltic Sea coast. Inlay shows the sampling area in relation to the entire Baltic Sea. Salinity data taken from: EU Copernicus Marine Service. (2017). Copernicus marine environment monitoring service - CMEMS. Available at: <http://marine.copernicus.eu/>. Last accessed 10 October 2017.

Table S1. Environmental conditions at each of the three sampling sites on the day of sampling. A_T represents the total alkalinity which is the sum of all inorganic bases. Standard errors for salinities were calculated from 2 years of monitoring data which will be published in a separate manuscript. Details on calculating saturation states (Ω) are given in the supplementary methods.

Collection site	Salinity (psu)	Temperature (°C)	pH _{NBS}	A_T ($\mu\text{mol kg SW}^{-1}$)	Chl ($\mu\text{g l}^{-1}$)	a	$\Omega_{\text{aragonite}}$
Kiel	15.5 (± 3.07 se)	16.5	7.82	1972.76	5.08		1.29
Ahrenshoop	10.5 (± 2.34 se)	18.5	7.93	1811.72	1.34		1.46
Usedom	7.1 (± 0.56 se)	17.9	7.83	1790.41	2.44		1.13

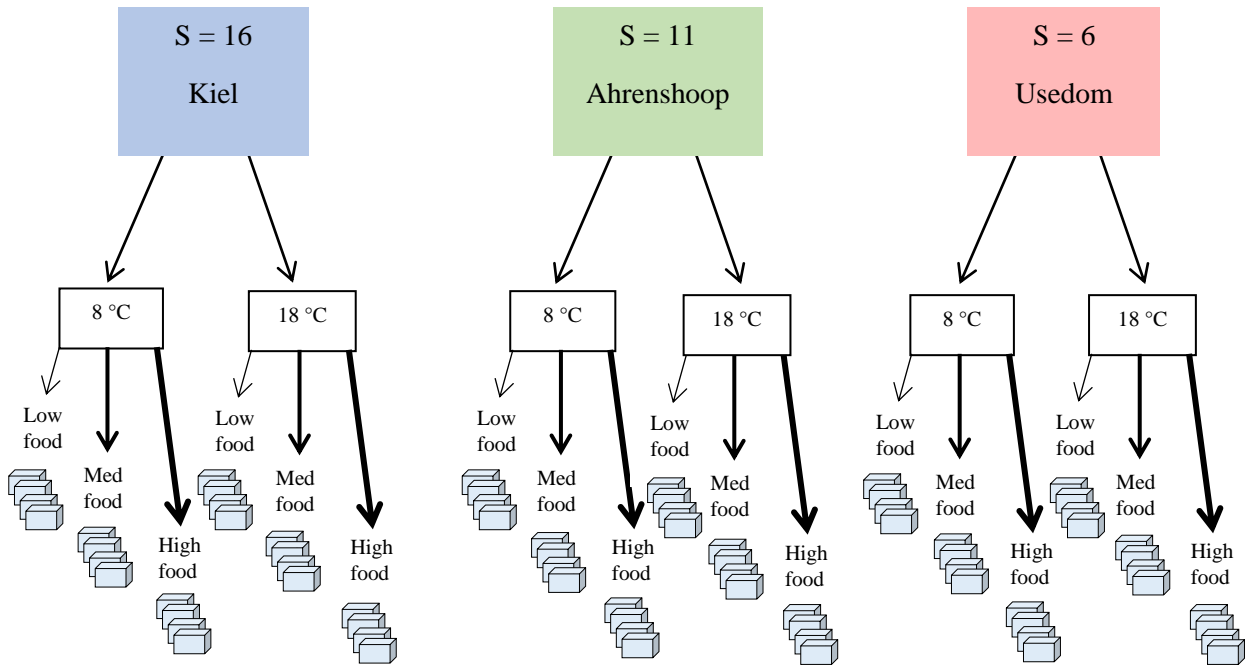


Fig. S2 Schematic of experimental design showing the three salinities/populations, two temperatures and three food treatments with 4 replicate tanks, each containing 40 mussels. The total number of tanks = 72. Measurements were averaged or summed to a total value (depending on measurement) per tank and statistically analysed with tanks as replicates.

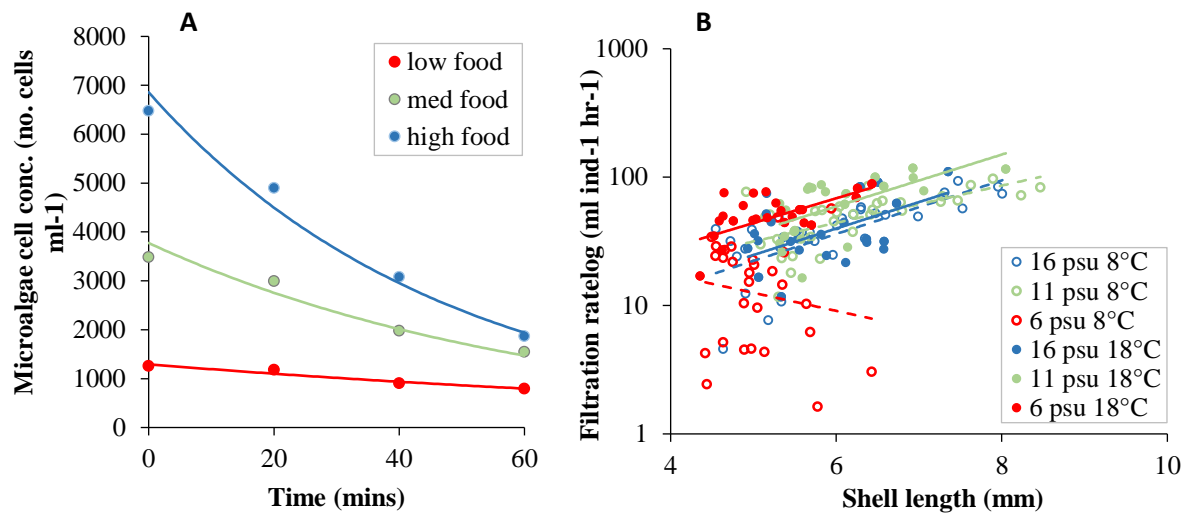


Fig. S3 (A) An example of a single replicate tank clearance measurement for all food levels with samples taken every 20 minutes for 1 hour. Trendlines show an exponential decrease in algae concentrations in all food treatments. (B) Filtration rate measured throughout the course of the experiment on a log scale plotted against shell length with exponential trend lines for all temperature and salinity treatments. Open circles and dotted trend lines represent 8 °C whereas closed circles and solid lines represent 18 °C. Clearance rates show sufficient feeding to clear water of all particles between feeds.

Table S2. Mean water chemistry parameters within each treatment for salinity, temperature, pH, total inorganic carbon (C_T), calcite saturation state (Ω_{calcite}) and aragonite saturation state ($\Omega_{\text{aragonite}}$) over the entire experiment +/- standard error.

Salinity (psu)	Temperature (°C)	pH _{NBS}	C_T ($\mu\text{mol kgSW}^{-1}$)	Ω_{calcite}	$\Omega_{\text{aragonite}}$
6.16 ± 0.01	18.07 ± 0.02	7.98 ± 0.02	1694 ± 68	1.72 ± 0.08	0.96 ± 0.05
6.10 ± 0.02	8.09 ± 0.02	8.13 ± 0.02	1670 ± 71	1.56 ± 0.08	0.85 ± 0.04
11.10 ± 0.02	18.19 ± 0.02	7.91 ± 0.02	1629 ± 70	1.66 ± 0.05	0.95 ± 0.03
11.21 ± 0.02	8.03 ± 0.02	8.06 ± 0.02	1647 ± 72	1.53 ± 0.05	0.86 ± 0.03
16.42 ± 0.08	18.08 ± 0.02	7.90 ± 0.02	1952 ± 13	2.05 ± 0.08	1.22 ± 0.45
16.64 ± 0.05	8.08 ± 0.02	8.05 ± 0.02	2037 ± 6	1.91 ± 0.07	1.12 ± 0.04

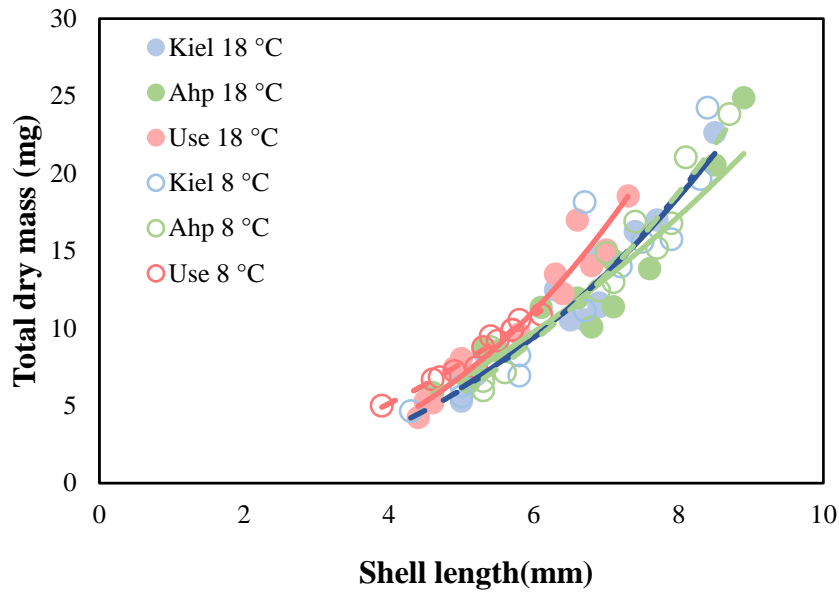


Fig. S4 Total dry mass vs shell length with all food treatments pooled at the end of the experiment. Power relationships showed the highest R^2 values. Dotted lines represent 8 °C treatments and solid lines represent 18 °C treatments

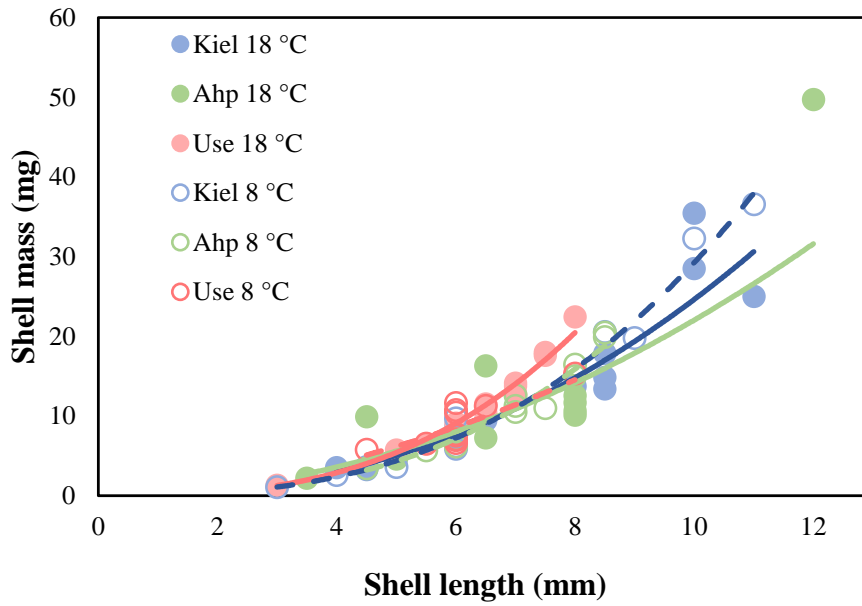


Fig. S5 Total shell mass including organic vs shell length with all food treatments pooled at the end of the experiment. Power relationships showed the highest R^2 values. Dotted lines represent 8 °C treatments and solid lines represent 18 °C treatments. Relationships between CaCO_3 mass and shell length are not shown as shell organic mass did not differ statistically between treatments.

Table S3. R^2 values for all regression analyses using ANCOVA. Costs represent calcification costs from linear regressions. All other regressions were performed on power relationships.

Temperature	Salinity	R^2			
		Costs	O₂	DM	SM
18 °C	16 psu	0.57	0.627	0.948	0.943
18 °C	11 psu	0.37	0.578	0.918	0.745
18 °C	6 psu	0.74	0.702	0.953	0.993
8 °C	16 psu	0.92	0.798	0.917	0.981
8 °C	11 psu	0.88	0.675	0.948	0.97
8 °C	6 psu	0.63	0.491	0.969	0.597

Table S4 Statistical results from ANCOVA's (sum of squares method) with co-variable significance shown. Post-hoc analyses (Tukey HSD) results are also shown for significant factors. Shell length = SL, Significant *p*-values are shown in bold.

	factor	<i>df</i>	Sum sq	Mean sq	F-value	<i>p</i> -value
Total dry mass vs shell length	SL	1	12.21	12.21	702.02	< 0.001
	salinity	2	0.26	0.13	7.57	0.001
	temperature	1	0.00	0.00	0.09	0.767
	salinity*temperature	2	0.02	0.01	0.49	0.614
	residuals	60	1.04	0.02		
	post-hoc analyses					
					16-11	0.992
				salinity	6-11	< 0.001
					6-16	< 0.001
Total shell mass vs shell length	SL	1	28.14	28.14	352.45	< 0.001
	salinity	2	1.11	0.55	6.95	0.002
	temperature	1	0.26	0.26	3.27	0.076
	salinity*temperature	2	0.82	0.41	5.12	0.009
	residuals	60	4.79	0.08		
	post-hoc analyses					
					16-11	0.166
				salinity	6-11	0.059
					6-16	0.001
Oxygen consumption	TM	1	247627	247627	322.69	< 0.001
	temperature	1	27767	27767	36.18	< 0.001
	salinity	2	2627	1336	1.74	0.177
	salinity*temperature	2	484	242	0.32	0.730
	residuals	268	205659.00	767.00		
Calcification cost	calcification	1	132855	132855	79.82	< 0.001
	temperature	1	148	148	0.09	0.766
	salinity	2	11743	5872	3.53	0.036
	salinity*pop	2	21975	10988	6.60	0.003
	residuals	60	99860	1664		

	post-hoc analyses	
	16-11	0.872
salinity	6-11	< 0.001
	6-16	< 0.001

Table S5. Statistical results from ANOVA's (sum of squares method) with post-hoc analyses (Tukey HSD) results are also shown for significant factors. Significant *p*-values are shown in bold.

	factor	<i>df</i>	Sum sq	Mean sq	F-value	<i>p</i> -value	
CaCO ₃ growth	temperature	1	1.51	1.51	0.7	0.408	
	salinity	2	57.39	28.7	13.26	< 0.001	
	food	2	843.39	421.7	194.83	< 0.001	
	temp*sal	2	27.01	13.5	6.24	0.004	
	temp*food	2	0.96	0.48	0.22	0.802	
	sal*food	2	51.47	12.87	5.94	< 0.001	
	temp*sal*food	4	59.29	14.82	6.85	< 0.001	
	residuals	54	116.88	2.16			
		post-hoc analyses					
					16-11	0.967	
	salinity			6-11	< 0.001		
				6-16	< 0.001		
				low-high	< 0.001		
	food			med-			
				high	< 0.001		
				med-low	< 0.001		
Soft tissue growth	temperature	1	0.28	0.28	29.21	< 0.001	
	salinity	2	0.29	0.15	15.17	< 0.001	
	food	2	4.62	2.31	239.90	< 0.001	
	temp*sal	2	0.23	0.12	12.05	< 0.001	
	temp*food	2	0.13	0.06	6.60	< 0.001	
	sal*food	2	0.26	0.06	6.70	< 0.001	
	temp*sal*food	4	0.04	0.01	0.97	0.434	
	residuals	54	0.52	0.01			
		post-hoc analyses					
					16-11	0.084	
	salinity			6-11	< 0.001		

					6-16	0.005
					low-high	< 0.001
				food	med-	
					high	< 0.001
					med-low	< 0.001
Energy allocation to calcification	temperature	1	1916	1916	13.55	0.002
	salinity	2	347	173	1.23	0.317
	temperature*salinity	2	745	372	2.6	0.099
	residuals	18	2545	141		

Chapter 3 – Salinity, selection and local adaptation

Allele shift under laboratory selection and local adaptation to low salinity in
Baltic *Mytilus* hybrids (*M. edulis*, *M. trossulus*)

Allele shift under laboratory selection and local adaptation to low salinity in Baltic *Mytilus* hybrids (*M. edulis*, *M. trossulus*)

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Abstract

The capacity to exhibit adaptive evolution in a changing environment is dictated by the severity of the environmental change and the genetic variation which natural selection can act on. The Baltic Sea provides an excellent system to test these relationships due to a steep salinity gradient in the South-West and highly divergent, and in many cases, hybridising taxa. Mytilid mussels in the Baltic form an extensive hybrid zone with Baltic *Mytilus edulis* genotypes being more common at high salinities (Western Baltic) and Baltic *M. trossulus* more common at low salinities (Eastern Baltic). In this context, we investigated the extent to which salinity as a natural selection agent can shape the genetic structure of the Baltic Sea *Mytilus edulis*, *Mytilus trossulus* hybrid zone as well as the role it plays in driving local adaptation to extremely low salinities. We performed laboratory experiments comparing fitness parameters (growth, survival, settlement success) in larvae from a high salinity Baltic *M. edulis* population and a low salinity Baltic *M. trossulus* population. Additionally, by utilisation the high genetic diversity in the centre of the genetic transition zone, we performed an artificial selection experiment investigating the extent by which simulated desalination naturally selects for Baltic *M. trossulus* alleles. Results demonstrate that Baltic *M. trossulus* larvae are locally adapted to low salinities exhibiting lower mortality and higher settlement rates compared to Baltic *M. edulis*. Simulated desalination of a genetically diverse population (similar proportions of Baltic *M. edulis* and Baltic *M. trossulus* alleles) led to allele shifts towards Baltic *M. trossulus*. We conclude, despite some observable plasticity to salinity in eastern Baltic *M. trossulus*, Baltic *Mytilus* populations are locally adapted to native habitat salinities and salinity driven selection shapes the distribution of species specific alleles in the face of pervasive gene flow. These results suggest strong environmentally driven selection can drive local adaptation to extreme conditions and provides a baseline for future work into the mechanisms behind observed adaptation to environmental change.

Introduction

Mussels of the *Mytilus edulis* species complex (*M. edulis*, *M. galloprovincialis*, *M. trossulus*) are distributed worldwide in the northern hemisphere and function as an important foundation species for marine benthic communities. In geographic areas of sympatry, they normally form stable mosaic-like hybrid zones, i.e. patches of parent species and interspecies hybrids.

However, an exception to this pattern is in the brackish waters of the Baltic Sea. Analyses on shell morphology and allozymes allowed classifying mussels in the Western Baltic (Kattegat) as *M. edulis* and Eastern Baltic (Baltic proper) as *M. trossulus* (Väinölä & Hvilson 1991). Subsequent analyses using nuclear and mitochondrial DNA markers revealed pervasive asymmetric interspecific gene flow to an extent that Eastern Baltic *M. trossulus* are best described as a hybrid swarm (Riginos & Cunningham 2005) (Figure 1A). Similarly, introgression is also substantial in Western Baltic *M. edulis* resulting in substantial genetic divergence to their genetically pure congeners in the North Sea (Stuckas et al. 2017; Steinert et al. 2012) (Figure 1A). Considering these high levels of introgression in Baltic *Mytilus* populations and to distinguish Baltic *Mytilus* from their genetically pure congeners, we refer to them as “Baltic *M. edulis*” and “Baltic *M. trossulus*”. Despite substantial genetic introgression at several nuclear loci, Western Baltic *M. edulis* and Eastern Baltic *M. trossulus* still maintain substantial dissimilarities in shell morphological traits and physiology (Kautsky et al. 1990; Tedengren et al. 1990). However, the mechanisms underlying the maintenance of these phenotypic differences in the face of pervasive introgression, are not fully understood. Apart from physical factors such as barriers to larval drift (Stuckas et al. 2017) or weak reproductive barriers (Riginos & Cunningham 2005; Stuckas et al. 2009; Stuckas et al. 2017), non-neutral processes have been proposed, such as local adaptation through natural selection by salinity (Riginos & Cunningham 2005; Stuckas et al. 2017).

The brackish waters of the Baltic Sea are physiologically challenging for both marine and fresh water organisms and *Mytilus* represent one of the few marine species that extensively colonised this environment (Johannesson et al. 2011). Previous studies have revealed several indirect lines of evidence that Eastern and Western Baltic *Mytilus* mussels are differentially adapted to their differing native salinities (Stuckas et al. 2009; 2017; Riginos & Cunningham 2005). This is also supported by the fact that frequencies of species specific alleles in both hybridizing species strongly correlate with the salinity gradient ranging from 25-12 psu (Western Baltic) to 8-4.5 psu (Eastern Baltic) (Figure 1A, supplementary Figure S1) (Stuckas et al. 2009; 2017). The genetic transition zone between both Baltic *Mytilus* species (i.e., the area of most intense genetic change) lies in the Darss Sill area where salinity drops to below 10 psu (Figure 1A, supplementary Figure S1). This seems to be a critical threshold as physiological energetics of larval and adult *Mytilus* are severely impacted below ~ 11 psu (Kossak 2006; Thomsen et al. 2018), causing reduced growth and calcification rates (Riisgård et al. 2014). Interestingly, pure Atlantic *M. edulis* and *M. trossulus* larvae (fully marine conditions, ~33 psu) are unable to settle and metamorphose at salinities below 10 psu (Qiu et al. 2002). This suggests local adaptation

of Baltic *Mytilus* species to low salinity regimes. Given the high level of introgression in Baltic mussel species, the putative role of introgressive hybridisation for low salinity adaptation (adaptive hybridisation) has been previously discussed (Stuckas et al. 2017).

Disentangling whether adaptation to extremely low salinity drives the maintenance of distinct phenotypes is not only interesting in the field of evolutionary biology, but is also vital in the light of anthropogenic global change. Climate models predict desalination and a westward shift of the salinity gradient in the Baltic with a reduction of 1.5 to 2 psu in sea surface salinity (Meier et al. 2006; Gräwe et al. 2013). In addition, the Baltic Sea has been identified to be the fastest warming coastal marine ecosystem on the planet (Belkin 2009) with a predicted increase in sea surface temperature of 1.9 - 4 °C by the end of this century (HELCOM 2013; Meier et al. 2012; Gräwe et al. 2013). Baltic mytilids are a dominant foundation species forming extensive reefs, particularly in the Eastern Baltic, where predators such as sea stars and crabs are absent. These reefs provide refuge for numerous other species and are important in organic matter transformation, as well as benthic-pelagic coupling (Reusch et al. 1994; Kautsky & Wallentinus 1980; Kautsky & Evans 1987). Therefore, if there is a causal relationship between abiotic factors (salinity, temperature) and the spatial distribution of Baltic *Mytilus*, climate change may have severe consequences for Baltic ecosystems,

One key aspect in understanding the influence of abiotic factors on species integrity and distribution is to disentangle the relative importance of phenotypic plasticity and adaptive evolution. This problem can be approached by utilising selection experiments investigating the effects of changing salinity on free-swimming planktonic larval stages. Mussel early life stages have been observed to be more susceptible to abiotic stressors (e.g. salinity, carbonate chemistry) than adults, thus acting as a bottleneck for species abundance and distribution (Thorson 1950; Underwood et al. 2000; Thomsen et al. 2015). As there is no data available on the effects of low salinity on Baltic *Mytilus* larvae, we performed laboratory experiments simulating desalination and temperature changes that mussel larvae may encounter both presently, and in the future, during their pelagic planktonic developmental stage prior to settlement. Experimental larvae populations were bred from adults originating from three genetically distinct populations of Baltic *Mytilus* species: Western Baltic *M. edulis* (Kiel, 16 psu), Eastern Baltic *M. trossulus* (Usedom, 7 psu), and transition zone mussels (Ahrenshoop, 11 psu) (Figure 1B). If phenotypic plasticity explains local physiological phenotypes, larvae would be expected to exhibit similar growth rates, mortality and settlement under different

salinity and temperature conditions and the genetic composition of larval cohorts would not be expected to shift during mortality in the larval phase. Alternatively, in the case of local adaptation, differential larval performance would be expected and/or allelic frequency shifts in larval cohorts resulting from mortality.

We first tested these assumptions in a **population comparison experiment** using a common garden experimental design. Proxies for fitness (survival, growth and settlement) of Baltic *M. edulis* and Baltic *M. trossulus* larvae were quantified at both native and crossed salinity regimes to gauge how larvae perform under different salinity conditions. A second experiment was performed to simulate desalination at two different temperatures encompassing the temperature range normally experience by Baltic *Mytilus* larvae. This **simulated desalination experiment** utilised a population from the genetic transition zone where species admixture is highest and monitored larval performance and potential allelic shifts to uncover whether selection by low salinity favours Baltic *M. trossulus* or Baltic *M. edulis* alleles.

Material and Methods

Broodstock sampling and maintenance

Adult *Mytilus* (ca. 30 mm shell length) were collected from three locations along the Baltic Sea coastline (Kiel, Ahrenshoop, Usedom; Figure 1B, Table S1) in Spring 2016. Animals were transported to GEOMAR climate chambers in aerated cooling boxes and kept at 10 °C in 10 l plastic aquaria (N=20 per aquarium) with 20 µm-filtered sea water (FSW) at their native salinities for at least 15 days (Table S1). FSW from Kiel fjord (16 psu) was diluted for Ahrenshoop and Usedom mussels using de-ionised water. Total alkalinity (A_T) was adjusted to field levels for each population by adding 1M HCO_3^- solution. Water changes were conducted 3x weekly and animals were fed 3x daily with lab cultured *Rhodomonas salina* (25 °C, 16 psu) to achieve a feeding rate of ca. 5 % dry mass day^{-1} . All microalgae used in this study were cultured at the Kiel Marine Organism Culture Centre (KIMOCC) algae culture facility at GEOMAR.

Population comparison experiment

Spawning of Kiel (16 psu, Western Baltic) and Usedom (7 psu, Eastern Baltic) broodstock, at their native salinities, was induced by increasing water temperature to 18 °C over 30 minutes. Spawning individuals were immediately isolated in 100 ml glass beakers with gametes mixed

periodically and later quality checked (active sperm, circular eggs) before fertilisation. Within each population, female (N = 4) gametes were pooled and mixed with pooled male (N = 6) gametes (1:100 ratio; egg to sperm), producing a mixture of families. Fertilisations were conducted at native salinities and fertilisation success was determined by the presence of a polar body and/or cell cleavage. Embryos from each population were subsequently split into two salinity treatments (16 psu and 7 psu) with six replicates each and adjusted to the experimental salinities at a rate of 3 psu per day by conducting 50 % water changes every day for the first 3 days (details of water changes below). Larvae were cultured in 10 L glass Duran bottles (Schott) at a density of 15 larvae ml⁻¹ at 17 °C for the first 36 hours until shell formation as preliminary trials showed that the conical culture units used for the remainder of the larval stage, induced significant mortality during the early, non-swimming embryonic stages. After 36 h, shelled larvae were added to custom made 14 L plastic conical culturing vessels at a density of 10 larvae ml⁻¹ with aeration optimised to prevent settlement of larvae but prevent mechanical damage to larvae. After 3 days post fertilisation (dpf) water changes were conducted every 3rd day by gently passing larvae through cylindrical 65 µm mesh filters and submerged in freshly prepared FSW before filters were inverted and submerged into fresh FSW and larvae were gently rinsed back into the culturing vessels using FSW. Pilot experiments showed this method did not induce significant larval mortality. Larvae were fed daily from 3 - 16 dpf with live *Isochrysis galbana* at a final concentration of 15000 cells ml⁻¹ due to their small cell sizes (ca. 4 µm). After 16 dpf larvae were fed larger *Rhosomonas* sp. (ca. 7 µm) for the remainder of the experiment at a final concentration of 3000 cells ml⁻¹.

Larval survival and growth was measured in triplicate 5 ml water samples from each tank every 3rd day. After adding a drop of 4 % para-formaldehyde (PFA, pH 8, corresponding salinity), larvae were counted under a stereo microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a Micro Publisher 3.3 RTV camera (QImaging, Surrey, BC, Canada). Larval survival was expressed as the ratio of larvae ml⁻¹ to the initial number of larvae ml⁻¹ (in %). Larval size was determined for ca. 15 larvae per replicate by measuring shell length using ImageJ 1.x (Schneider et al. 2012). At the end of the experiment (33 dpf), settlement success was quantified by gently removing settled larvae from the sides and bottom of the culture tanks with a 5 x 5 cm synthetic sponge and rinsing them onto a 65 µm mesh. Collected larvae were counted under a light microscope (Leica MDG41) and settlement success was expressed as the ratio of settled larvae to initial absolute number of larvae (in %).

Simulated desalination experiment

Fertilisation, larval maintenance and larval performance measurements were conducted as described in the population comparison experiment with the following modifications: spawning was induced in Ahrenshoop mussels placed individually in 100 ml beakers contained in a water bath. Gametes of six females and six males were used to generate six families. Embryos from all six families were pooled in equal ratios. Family pools were cultured at three different salinities (11, 9 and 7 psu) and two temperatures (12°C, 15°C) with 5 replicates each (30 replicates total). 2 L Duran bottles (Schott) were aerated using 10 ml plastic pipettes at a rate of 3 bubbles per second and placed in temperature-controlled water baths (Haake SWB25, Thermo Scientific) until the end of the experiment (69 days).

Statistics

Statistical analyses and graphical illustration were performed using Rstudio v.1.0.44 (Rstudio Team, 2015) and R v. 3.3.2 (R Core Team, 2014). Changes in larval concentrations were analysed using Two-way ANCOVA (factors: population + salinity or salinity + temperature) as a measurement of mortality rates using larval age (dpf) as a co-variate. Shell length growth rates (increases in shell length over time) were also analysed using ANCOVA (factors: population + salinity or salinity + temperature) with days post fertilisation (dpf) as a co-variate. Settlement success was statistically analysed using ANOVA in the simulated desalination experiment (factors: temperature + salinity) with Tukey HSD *post hoc* analyses between salinities. Due to deviations from gaussian distribution settlement success in the population comparison experiment was analysed using a GLM. A *p* value of < 0.05 was chosen as the significance level for all analyses and statistical results are given in Table S4 and S5

Testing for allele shifts in larval populations grown under simulated

DNA extraction and genotyping of broodstock was performed as described in Stuckas et al. (2017). DNA extraction from single settled larvae was performed following Zhan et al. (2008) with the following modifications: Spat from each replicate were rinsed with modified isotonic PBS, transferred separately into 200 µl PCR tubes, and stored at -20 °C until 20 µl of LoTEPA lysis buffer was added before proceeding as shown in Table S2. Genotyping was performed as described in Stuckas et al. (2017) with modifications to address the low volumes and quality of

DNA samples. Modified PCR reactions and programs are shown in supplementary Table S2 and S3, respectively. The MAL-1 locus had to be excluded due to lack of sufficient PCR products.

Hypothetical multilocus F1-offspring genotypes of larvae populations under expectations of neutral genetic drift were simulated from the parent mussel's genetic information for the four loci (excluding MAL-1) using the software Hybrid Lab v.1.0 (Nielsen et al. 2006). Simulated genotypes were joint and randomly selected afterwards in order to resemble the experiment as close as possible: A population of 4,000 F1-hybrids for every cross was computed, pooled to one large population of 24,000 hybrids, randomly mixed, and finally split into 10 replicates (2,400 hybrids). This was repeated two more times. Random mortality at approximately the same degree as in experimental treatments (ca. 99 %) was simulated by random draws of 20 hybrids from every replicate.

The genetic information of parent mussels, experimental and simulated settled larvae was implemented in the data set of Stuckas et al. (2017) with 22 Baltic *Mytilus* populations and two genetically pure, allopatric reference populations (*M. edulis*, Heligoland; *M. trossulus*, Penn Cove) and Bayesian inference was performed as described in Stuckas et al. (2017) excluding the parameter settings using *locprior* since they would have been contrary to the larval drift approach. Individuals with more than one locus information missing were excluded as well as the locus MAL-1.

Pairwise F_{ST} values were calculated with Arlequin v. 3.5.2.2 (Excoffier & Lischer 2010) using the same genetic data set as for the Bayesian inference computations. The significance level of $p < 0.05$ changed to $p < 0.000009$ after Bonferroni correction.

Results

Population comparison experiment

Low salinity proved to impair a multitude of fitness related parameters in both low salinity Baltic *M. trossulus* and high salinity Baltic *M. edulis* larvae. Development was delayed in both populations at 7 psu with the formation of the complete D veliger shell being delayed by ~ 12 hours compared to at 16 psu. This developmental delay was accompanied by significantly slower growth rates (shell length increase over time) at 7 psu in both populations (ANCOVA,

$F_{1,1745} = 1636, p < 0.001$, Figure 2A) with final shell lengths in the 7 psu treatment being ~ 25 % smaller than the 16 psu treatment. Low salinity also negatively impacted both larval settlement success (GLM, $p = 0.007$, figure 2C) and larval survival in Baltic *M. edulis* (ANCOVA, $F_{(1,235)} = 7, p = 0.009$, Figure 2B), observed by steeper declines in Baltic *M. edulis* larvae concentration over time in the 7 psu treatment. It was also observed that Baltic *M. trossulus* larvae exhibit comparatively better performance at low salinity than Baltic *M. edulis* larvae. This was demonstrated by lower mortality of Baltic *M. trossulus* larvae at 7 psu compared to Baltic *M. edulis* larvae (ANCOVA interaction (salinity:population), $F_{(1,1)} = 24.1, p < 0.001$, Figure 2C) and higher shell length growth rates in Baltic *M. trossulus* at 7 psu compared to Baltic *M. edulis* (ANCOVA interaction (salinity:population), $F_{(1,1745)} = 21.6, p < 0.001$). The same pattern was also observed regarding settlement where Baltic *M. trossulus* larvae exhibit higher settlement success than Baltic *M. edulis* larvae at 7 psu (GLM interaction (salinity:population), $p < 0.004$, Figure 2C). At 16 psu, settlement and growth were similar between both populations, however mortality rates were high in Baltic *M. trossulus* being similar to mortality rates of Baltic *M. edulis* at 7 psu (Table 1). These findings suggest minor negative impacts of high salinity on Baltic *M. trossulus* but major reductions in fitness related parameters in Baltic *M. edulis* larvae at low salinities. This demonstrates local adaptation for the first time in Baltic *M. trossulus* larvae to extremely low salinities in the Baltic Sea.

Simulated desalination experiment

Exposing a genetically admixed population (Ahrenshoop, 11 psu) to simulated desalination and temperature changes revealed major impacts on *Mytilus* larvae performance and length of planktonic phases. Interestingly, the length of the pelagic larval phase exceeded 65 days in the 7 psu treatment at low temperatures which has potentially major implications for both drift models and predation risk to pelagic predators. Exposure to desalination negatively impacted shell length growth (increase in shell length over time) in Ahrenshoop larvae (ANCOVA, $F_{(2,6016)} = 1364, p < 0.001$, figure 2D) especially at 7 psu with final shell lengths reaching only 70 % of larvae in the 9 and 11 psu treatments (Tukey HSD, $p < 0.01$, Figure 2B). Mortality rate (decrease in larvae concentration over time) was also higher at low salinity (ANCOVA, $F_{(2,558)} = 8.9, p < 0.001$, Figure 2E) with mortality being highest in the 7 psu treatment compared to the 9 and 11 psu treatments (Tukey HSD, $p < 0.001$, Table S5). Settlement success was severely impacted by both desalination treatments and temperature (ANCOVA, $F_{(1,16)} = 7.0, p < 0.001$, Table S4) with larval settlement being ~ 4-fold lower in all treatments compared to the 11 psu 15 °C treatment (ANCOVA interaction (salinity:temperature), $F_{(1,16)} = 7, p < 0.001$, Figure 2F).

Interaction effects between salinity and temperature were also observed for mortality, settlement success and shell length growth (Table S5). This demonstrates the interactive role of both temperature and salinity in influencing fitness related parameters in Baltic *Mytilus* larvae.

Allele shifts in larvae under simulated desalination

The 12 Ahrenshoop broodstock animals and 296 settled larvae from the six treatments in the desalination simulation were genotyped at four single copy nuclear loci (Glu-5', EFbis, M7 lysin, mac-1). Cluster analysis on experimental larvae from all replicates exhibited Q values ranging from 0.088 to 0.591, with mean Q values ranging from 0.323 to 0.336 between different model assumptions (Figure 3B, Table S6). In contrast, Q values from cluster analysis of simulated offspring allele frequencies ranged between 0.307 and 0.740 with mean Q values ranging from 0.551 to 0.555 between different model assumptions (Figure 3C, Table S6). These results show that simulated allele frequencies based on neutral genetic effects produced on average higher proportion of the Baltic *M. edulis* related cluster compared to experimental larval allele frequencies. This indicates significant allele frequency shifts towards higher proportions of Baltic *M. trossulus* alleles and subsequently points towards natural selection by low salinity against Baltic *M. edulis* alleles.

Pairwise F_{ST} values were calculated for the same data set as used in the Bayesian inference. The original table (Table S7) has been reduced for clarity and shows only the parental mussels from Ahrenshoop (this study), the experimental and simulated populations from this study, *M. edulis* and *M. trossulus* reference populations (Helgoland, Penn Cove) and finally, the Ahrenshoop population sampled in Stuckas et al. 2017 (Table 2).

Genetic difference between experimental larvae was minimal (mean $F_{ST} = 0.06$, range = 0.06 – 0.11) as was the genetic difference between simulated larvae (mean $F_{ST} = 0.00$, range = -0.02 - 0.08). However, allele frequencies between experimental larvae and simulated larvae, showed genetic differences > 3-fold higher (mean $F_{ST} = 0.06$, range = -0.02 – 0.20) Experimental larvae also showed higher genetic difference to parent mussels (mean $F_{ST} = 0.02$, range = -0.04 – 0.07) than simulated offspring did to parent mussels (mean $F_{ST} = -0.01$, range = -0.04 – 0.07). These patterns of high genetic differences in experimental larvae support the idea of non-neutral selection by low salinity driving genetic differences in Baltic *Mytilus* populations. This provides support to the idea that salinity maintains the genetic make-up of the Baltic Sea *Mytilus* hybrid zone.

Discussion

Baltic *M. trossulus* are locally adapted to the low salinities of Eastern Baltic Sea

The overall objective of this study was to address to which level phenotypic plasticity and adaptive evolution contribute to Baltic *Mytilus* population structure. In laboratory experiments, we 1) compared larval fitness at low and high salinity in Western Baltic *M. edulis* and Eastern Baltic *M. trossulus* populations and 2) investigated the effects of simulated future desalination on larval performance and selection in a highly admixed population from the centre of the Baltic Sea genetic transition zone (Figure 1A, B). This allowed clarification of the effects of desalination on fitness proxies and frequency changes of species specific alleles in experimental populations for the first time. Our findings lead us to the conclusion that, 1) Baltic *M. trossulus* population are locally adapted to extremely low salinities and 2) low salinity conditions cause selection in favour of Baltic *M. trossulus* alleles. This provides one explanation how the genetic structure of Baltic *Mytilus* species is shaped and sheds light into mechanisms driving local adaptation of Baltic *M. trossulus* populations in the Eastern Baltic Sea.

The evidence from this study exhibiting that selection drives an allele shift towards Baltic *M. trossulus* specific alleles under low salinity conditions suggests a phenotype-genotype association with salinity tolerance, i.e., the frequency of Baltic *M. trossulus* alleles in Baltic mussels is associated with better low salinity tolerance. In the population comparison experiment, Baltic *M. trossulus* larvae exhibited lower rates of mortality at 7 psu compared to Baltic *M. edulis*. This indicates that Baltic *M. trossulus* have better tolerance to low salinity than Baltic *M. edulis*. Marine euryhaline molluscs are osmoconformers meaning they adjust their intracellular osmotic pressure in line with changes in seawater osmotic pressure. This is maintained primarily through adjustments in intracellular concentrations of compatible organic osmolytes, but also slight changes in intracellular cation concentrations (Berger & Kharazova 1997; Yancey 2005). Minimum salinity thresholds may therefore be a result of depletion of intracellular cations, organic osmolytes, or both, to critical concentrations subsequently impeding cellular function. (Podbielski et al. 2016). It may be that Baltic *M. trossulus* have better low salinity tolerance than Baltic *M. edulis* due to adaptive changes in their intracellular osmolyte pools. This would also explain the selection for Baltic *M. trossulus* alleles at low salinity in the simulated desalination experiment. The high mortality in Baltic *M. edulis* larvae at 7 psu is subsequently a likely reason for the very low rates of settlement success in this

treatment. Local adaptation to low salinity has also been demonstrated by Thomsen et al. 2018 where a low salinity Baltic *M. trossulus* population exhibited better tolerance to low seawater calcium (vital for shell growth) than a high salinity Baltic *M. edulis* (Thomsen et al. 2018). As $[Ca^{2+}]$ decreases with salinity, this may be a potential mechanism of low salinity tolerance in Baltic *M. trossulus* larvae. Limiting levels of seawater calcium may also be a potential explanation for the slow growth rates at low salinity in both experiments.

If there is a strong genotype-phenotype association with salinity tolerance, Baltic *M. edulis* and Baltic *M. trossulus* should differentially perform under different salinity conditions. This way of thinking includes the hypothesis that the frequency of species specific alleles also determines the performance under high salinity. It is interesting that both Baltic *M. trossulus* and Baltic *M. edulis* larvae exhibited comparable growth and settlement success rates in the 16 psu treatment, however we must be cautious in concluding that fitness is comparable between both genotypes at higher salinities. Mortality for example was higher (despite high variation) in Baltic *M. trossulus* at 16 psu compared to Baltic *M. edulis* and when compared to Baltic *M. trossulus* at 7 psu (Table 1) suggesting a potential fitness disadvantage for Baltic *M. trossulus* at high salinity. Additionally, although highly variable and not statistically significant, we observed a slight trend towards higher settlement success in Baltic *M. edulis* at high salinity. It is possible that if this experiment was repeated at slightly higher salinities (e.g. 20 psu), Baltic *M. trossulus* larvae may begin to exhibit more detectable reductions in fitness. The same may also be said for the simulated desalination experiment. There were no clear changes in allele frequencies compared to simulated predictions in the 11 psu treatment. However, if higher treatment salinities were used, we might have observed a shift in allele frequencies towards Baltic *M. edulis* due to comparatively higher mortality of Baltic *M. trossulus* genotypes. Therefore, we cannot rule out that Baltic *M. trossulus* does indeed suffer reduced fitness at high salinities and that high salinity also acts as a natural selection pressure shaping the genetic patterns in the Baltic hybrid zone.

At present, there are no studies directly linking genetic factors with the performance of adult Baltic mussels to different salinity conditions. However, there are indirect lines of evidence suggesting that specimen and/or population specific frequencies of Baltic *M. edulis* and Baltic *M. trossulus* specific alleles are associated with local adaptation to specific salinity regimes. This information mainly come from reciprocal transplantation experiments between adult Western Baltic *M. edulis* and Eastern Baltic *M. trossulus* These analyses documented negative

impacts of low salinities on juvenile and adult Baltic *M. edulis*, e.g., reduced filtration and growth rates (Riisgård et al. 2012; 2013; 2014; Kossak 2006), and extremely high mortalities (Kautsky et al. 1990; Kossak 2006). However, growth, survival and feeding rates of Eastern Baltic *M. trossulus* populations were comparable to Western Baltic *M. edulis* populations at high salinities (25 – 30 psu) (Kautsky et al. 1990; Tedengren et al. 1990; Riisgård et al. 2014). This suggests that mortality at low salinities may be genotype specific but further work on larvae at higher salinities (25-33 psu) is required to fully understand potential fitness implications of high salinity exposure in Baltic *M. trossulus*

Additional support for an association between species specific alleles and salinity tolerance comes from investigations on *Mytilus* populations outside the Baltic. Previous work investigating the frequencies of *M. trossulus* vs. *M. edulis* alleles at different salinities has produced conflicting results with some studies showing higher frequencies of *M. trossulus* alleles at low salinities (Ridgeway & Nævdal 2004; Braby & Somero 2005) and others showing no changes in *M. trossulus* alleles with salinity (Moreau et al. 2005). These studies have all looked at adults and it may be that selection during the larval or post-larval period is strongest and drives allele frequencies and local adaptation to low salinity. Beaumont et al. 1988 found ontogenetic differences in selection in *M. edulis* with low salinity selection against the *Lap*⁹⁴ allele only in the post-larval phase rather than the larval phase. Ontogenetic differences in performance have also been shown by Qiu et al. 2002 where *M. trossulus* larvae outperformed *M. edulis* larvae at low salinity but not during the juvenile or adult stage.

Overall, the review of existing experimental data shows a trend that adult Baltic *M. trossulus* or adult hybrids with high Baltic *M. trossulus* allele frequencies have a higher fitness under low salinity. This suggests that, salinity driven post-settlement selection can act on adult Baltic *Mytilus* species and their hybrids. The study presented here experimentally demonstrated salinity driven selection in pelagic early life stages of Baltic *Mytilus* mussels. We argue that this is one mechanism of pre-settlement selection that can significantly shape the distribution of Baltic *Mytilus* species and their hybrids in the absence of strong barriers to larval drift.

What mechanisms underlie salinity driven selection in Baltic *Mytilus*?

By identifying salinity as one factor shaping the current genetic structure of Baltic *Mytilus* populations, the question arises what phenotypes are selected for and what mechanisms underlie their evolution. Adaptation to different salinity conditions has been a focus of ecophysiological

studies. Several physiological characteristics can explain differences in performances of adults at different salinities such as increased osmoregulatory costs (Maar et al. 2015) or reduced efficiency of protein turnover (Tedengren & Kautsky 1986). Furthermore, processes related to physiological energetics such as feeding rates, absorption efficiency and metabolism are related to environmental factors and highly plastic (Tedengren et al. 1990, Riisgård et al. 2013; Landes et al. 2015). Finally, recent research highlighted processes related to bio-mineralization as key factors. This is indicated in reciprocal transplantation experiments where adult Baltic *M. edulis* showed reduced ability to biomineralize skeletons under low salinity conditions (Riisgård et al. 2014). Salinities below 11 psu have been shown to invoke higher costs of calcification in juvenile Baltic *Mytilus* with these costs likely being attributed to limiting $[Ca^{2+}]$ of $< 3 \text{ mmol kg}^{-1}$ corresponding to ~ 11 psu (Sanders et al 2018; Thomsen et al. 2018). Overall, we propose that alleles at genetic loci associated with key physiological processes (e.g., biomineralisation) are targets of salinity driven selection and cause an indirect genome wide allele shift of species diagnostic markers. In fact, adaptive phenotypes related to physiological processes are multigenic traits (e.g., more than 100 genetic loci are currently associated with biomineralisation; Hüning et al. 2013; 2016; Liao et al. 2015; Gao et al. 2015; Arivalagan et al. 2016) and complex selection regimes are expected to affect the entire genome.

Explaining genome wide allele shifts seen in our desalination experiment with selection is not in contradiction to the overall high level of interspecific gene flow, i.e., pervasive introgression leading to Baltic hybrid swarm formation. Selection regimes can act as selective filters that allow some alleles to pass the transition zone while others cannot. This pattern of differential introgression has been previously demonstrated by showing strong allele frequency clines along the Baltic coast in case of genetic loci related to reproductive traits (Stuckas et al., 2009; 2017). Under the assumption that introgressed alleles are under negative selection, genetic loci related to key physiological processes should show strong allele frequency clines maintaining divergence between Baltic *M. edulis* and Baltic *M. trossulus*. In contrast, if introgression is adaptive, positive or balancing, selection is expected to shape smooth allele frequency clines with long introgression tails. Both assumptions provide testable hypotheses for genome wide comparative analyses of candidate genetic loci.

Finally, by focussing on ecophysiology alone to explain salinity driven selection will not cover the complexity of natural systems. For instance, our experiments do not show any significant effects of increased salinity on Baltic *M. trossulus* regarding growth and settlement (although

minor trends suggest this might not necessarily be true). This is in line with previous studies on adults showing that low salinity adapted Eastern Baltic *M. trossulus* populations exhibit comparable growth rates to native Western Baltic *M. edulis* populations at high salinities (25 – 30 psu) while the latter perform much worse at low salinities (7 psu) compared to native populations (Kautsky et al. 1990; Tedengren et al. 1990; Riisgård et al. 2014). Consequently, although Baltic *M. trossulus* are superiorly adapted to low salinity, they appear to show a large plasticity window under high salinity conditions. However, the frequency of Baltic *M. trossulus* alleles in Baltic high salinity areas (>10 psu) is low and specimens representing Eastern Baltic *M. trossulus* are almost completely absent from these areas. This becomes explainable in an extended context that considers interspecific competition between *Mytilus* species as well as predators. Shell shape and morphology exhibits large differences between Baltic *M. edulis* and Baltic *M. trossulus* genotypes with the latter possessing thinner and weaker shells. (Kautsky et al. 1990; Kossak 2006). Thinner and weaker shells have been attributed to Baltic *M. trossulus* being a preferred target for predators (such as sea stars and crabs) over thicker shelled Baltic *M. edulis*, as shown in reciprocal transplantation experiments (Kautsky et al. 1990; Reimer and Harms-Ringdahl 2001). This scenario describes an indirect relationship between salinity, processes related to shell phenotype and allele frequency clines and may potentially explain the current distribution of Baltic *Mytilus* species along the salinity gradient. It shows that an in-depth analysis of selection regimes acting on Baltic mussels requires the analyses of genetically determined phenotypes in both laboratory and field experimental settings.

Future Baltic scenarios and consequences for *Mytilus* abundance and distribution

Following our experimental findings, the Baltic *M. edulis* – Baltic *M. trossulus* distribution range and genetic transition zone will most likely move westwards with future desalination of the Baltic Sea (Meier et al. 2006; Gräwe et al. 2013). Concurrently, Baltic *M. trossulus* in the northeast Baltic basins are living at their hyposaline tolerance edge of 4.5 psu today (Westerbom et al. 2002) and the rapid expansion of low saline waters will most likely leave these regions inhabitable for marine bivalves. In the simulated warming and desalination experiment we observed potentially positive effects of warming on growth and settlement success and also potential negative effects such as increased mortality. Previous studies support the positive effect of higher temperatures on larval growth and developmental pace in *Mytilus* (Sprung et al. 1984; Beaumont et al. 2004; Sanchez-Lazo & Martinez-Pita 2012). It should be pointed out however that the warming treatment used in this experiment is well within the range of natural field temperatures due to annual variations and heat waves (Pansch et al. 2018). These

heatwaves also provide another potential source of thermal stress and are similarly expected to increase in frequency and duration (Oliver et al. 2018) imposing an additional threat for larval recruitment. We stress that the potential compensatory interactions between hyposaline stress and warming likely only apply as long as temperatures do not surpass the thermal tolerance range of Baltic *Mytilus*, otherwise lowered performance is expected.

As well as salinity and thermal stress, Baltic *Mytilus* populations will have to face further various challenges of anthropogenic origin in the upcoming century. Ocean acidification will affect shell formation (Gazeau et al. 2013; Waldbusser et al. 2014; Thomsen et al. 2015) and byssal attachment strength (O'Donnell et al. 2013), increasing their potential risk of predation. Potentially opened niches may soon be occupied again by recently introduced invasive species that can survive beyond the 12 psu threshold of current main predators (e.g. Forsström et al. 2015; Wójcik et al. 2015; Nurkse et al. 2016; Schrandt et al. 2016). It is not currently known how genotype-phenotype relationships (as recorded in this study) may dictate the ability of Baltic *Mytilus*, or indeed other species, to cope with predicted changes in other abiotic and biotic conditions.

Conclusions

Our findings demonstrate for the first time clear evidence of local adaptation in Baltic *M. trossulus* larvae to extremely low salinities. We also demonstrate for the first time, the strong selection pressure imposed by salinity and its role in shaping the genetic patterns of the Baltic *M. edulis*, Baltic *M. trossulus* hybrid zone along the Baltic Sea salinity gradient. This environmental system provides a fascinating and valuable model to investigate the role of environmental factors in shaping genetic differences between populations. High genetic divergence and unique hybridisation patterns of multiple species enable the investigation into potential mechanisms of adaptive evolution to extreme environments. Future work should utilise these systems to investigate key regions of the genome under selection to identify the pathways of adaptation evolution in a rapidly changing ocean.

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Authors contribution

Loreen Knöbel (LK), Jennifer Nascimento-Schulze (JNS) and Trystan Sanders (TS) share first authorship of this manuscript. Frank Melzner (FM) and Heiko Stuckas (HS) developed the hypothetical framework for the study. TS designed and conducted the population comparison experiment together with Claas Hiebenthal. JNS, Jörn Thomsen (JT), and LK and JNS conducted the fertilizations for the desalination simulation experiment which was run by JNS and co-supervised by TS and JT. JNS and TS analysed the larval data and genotyping, modeling and genetic analysis was carried out by LK, Dominique Zeus and HS at Senckenberg Natural History Collection Dresden. Writing was coordinated by LK and TS, JNS, FM and HS wrote the first manuscript draft. All co-authors contributed significantly to manuscript revisions.

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Competing interests

We have no competing interests.

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Tables

Table 1: Daily mortality and growth rates, and final settlement success for all treatments in the population comparison and simulated desalination experiment. Given are means +/- standard error. Mean daily mortality rates are displayed as a percentage of initial experimental larvae concentrations.

Experiment	Treatment	Daily mortality [% larvae day-1]	Shell length growth [μm day-1]	Settlement success [%]
Population comparison	Kiel- 7psu	1.43 ± 0.28	1.30 ± 0.56	0.37 ± 0.09
	Kiel -16 psu	1.07 ± 0.36	3.60 ± 1.10	2.00 ± 0.70
	Usedom - 7 psu	1.28 ± 0.31	2.90 ± 0.78	1.16 ± 0.37
	Usedom -16 psu	1.40 ± 0.32	4.10 ± 1.13	1.44 ± 0.58
Simulated desalination	7 psu- 12°C	0.93 ± 0.29	1.67 ± 0.42	0.39 ± 0.09
	9 psu- 12°C	1.14 ± 0.23	2.91 ± 0.60	0.53 ± 0.13
	11 psu - 12°C	1.09 ± 0.22	3.09 ± 0.67	0.93 ± 0.33
	7 psu - 15°C	0.86 ± 0.27	2.35 ± 0.53	0.44 ± 0.12
	9 psu - 15°C	0.87 ± 0.29	3.87 ± 0.77	0.64 ± 0.27
	11 psu - 15°C	0.94 ± 0.26	3.59 ± 0.82	4.62 ± 0.98

Table 2: Summary of pairwise F_{ST} values for parent mussels from the simulated desalination experiment with their experimental larval offspring and their simulated offspring assuming genetic drift, embedded in reference populations from Stuckas et al. 2017. Significant F_{ST} 's after Bonferroni correction were marked with an asterisk. Genetic data from this study: eAHP = broodstock from Ahrenshoop; experimental larvae = treatments 11-7 psu at 12 or 15 °C; Simulations = offspring assuming genetic drift. References from Stuckas et al. 2017: HLG = Helgoland; AHP = Ahrenshoop; PEN = Penn Cove. Summarised F_{ST} values for technical replicates of experimental larval treatments and for simulated offspring show averages with minimum to maximum values. See supplementary Table S11 for complete data.

	HLG	AHP	eAHP	11 psu 12 °C	11 psu 15 °C	9 psu 12 °C	9 psu 15 °C	7 psu 12 °C	7 psu 15 °C	Simulations	PEN
HLG	-										
AHP	0.57*	-									
eAHP	0.47*	0.08	-								
11 psu 12 °C	0.50 (0.44 - 0.55)*	0.04 (0.00 - 0.08)	0.02 (0.00 - 0.05)	0.01 (-0.03 - 0.05)							
11 psu 15 °C	0.43 (0.39 - 0.45)	0.06 (0.03 - 0.13)	0.01 (-0.01 - 0.02)	0.01 (-0.01 - 0.06)	0.01 (-0.01 - 0.04)						
9 psu 12 °C	0.51 (0.46 - 0.57)*	0.10 (0.05 - 0.14)	0.02 (0.00 - 0.05)	0.01 (-0.06 - 0.08)	0.00 (-0.04 - 0.05)	0.03					
9 psu 15 °C	0.47 (0.40 - 0.53)*	0.06 (0.01 - 0.09)	0.01 (-0.04 - 0.07)	0.01 (-0.03 - 0.07)	0.00 (-0.03 - 0.08)	0.00 (-0.06 - 0.07)	0.01 (-0.03 - 0.06)				
7 psu 12 °C	0.53	0.02 (0.01 - 0.03)	0.02 (0.00 - 0.04)	0.00	0.01	0.00	0.00	-0.01			

	(0.49 - 0.55)*			(-0.05 - 0.04)	(-0.04 - 0.07)	(-0.03 - 0.04)	(-0.04 - 0.06)	(-0.04 - 0.00)			
7 psu 15 °C	0.42 (0.38 - 0.52)*	0.09 (0.00 - 0.14)	0.03 (0.00 - 0.06)	0.02 (-0.03 - 0.08)	0.02 (-0.02 - 0.05)	0.02 (-0.06 - 0.07)	0.02 (-0.02 - 0.11)	0.02 (-0.04 - 0.06)	0.03 (-0.01 - 0.05)		
Simulations	0.46 (0.40 - 0.52)*	0.13 (0.05 - 0.19)	-0.01 (-0.03 - 0.01)	0.06 (0.00 - 0.14)	0.04 (-0.01 - 0.11)	0.08 (0.01 - 0.17)	0.06 (-0.02 - 0.20)	0.07 (0.00 - 0.16)	0.07 (0.00 - 0.14)	0.00 (-0.02 - 0.08)	
PEN	0.83*	0.50*	0.55*	0.52 (0.43 - 0.55)	0.47 (0.42 - 0.53)*	0.49 (0.48 - 0.50)	0.47 (0.34 - 0.54)*	0.53 (0.52 - 0.55)*	0.52 (0.45 - 0.62)*	0.58 (0.51 - 0.65)	-

Figure legends

Figure 1: The *Mytilus* population structure and correlations to the salinity (white numbers, in psu) gradient in the Baltic Sea. **A)** The *Mytilus* population structure in the Baltic Sea according to Stuckas et al. 2017. The Western Baltic Sea, including Kattegat and the Belt Sea, are populated by Baltic *M. edulis*, while the Eastern Baltic Sea is populated by Baltic *M. trossulus*. **B)** Broodstock was sampled from Kiel (K), Ahrenshoop (A) and Usedom (U).

Figure 2: Measurements of fitness related parameters in both larval experiments. The top three panels (A, B and C) show measurements from the population comparison experiment: shell length (log scale) increase over time (growth) (**A**), changes in larval concentration over time (mortality) (**B**) and settlement success (square-rooted) as a percentage of the total number of initial larvae (**C**). The bottom three panels show the same measurements for the simulated desalination experiment: shell length growth (**A**), mortality (**E**) and settlement success.

Figure 3: Population structure comparison of settled larvae from a simulated desalination experiment indicates an allele shift towards *M. trossulus* different from assumptions under neutral genetic drift. Shown are extracted parts of model assumption combination (STR 1) from the Bayesian inference (full data: Figure S2) plotting Q value frequencies against samples of different populations. A) Rerun of data from Stuckas et al. 2017. B) Settled larvae from the desalination experiment (treatments: 12 - 15 °C, 11 - 7 psu) together with *M. edulis* (Helgoland, HLG) and *M. trossulus* (Penn Cove, PEN) references as well as the Ahrenshoop population from Stuckas et al. 2017 (AHP) and used in this study (eAHP = AHP parents used in this experiment). C) Simulation of settled larvae under the assumption of neutral genetic drift together with *M. edulis* (Helgoland, HLG) and *M. trossulus* (Penn Cove, PEN) references as well as the Ahrenshoop population from Stuckas et al. 2017 (AHP) and used in this study (parents). References from Stuckas et al. 2017: HLG = Helgoland; TJÄ = Tjärnö; AAR = Aarhus; FLE = Flensburg; GEL = Gelting; MAA = Maasholm; KAP = Kappeln; ECK = Eckernförde; 9 = Kiel; 10 = Fehmarnsund; 11 = Grömitz; 12 = Steinbeck; 14 = Gollwitz; 14 = Warnemünde; 15 = Ahrenshoop; 70 = Barhöft; 71 = Dranske; 72 = Usedom; 73 = Hel; 74 = Askö; 75 = Penn Cove.

Figure 1

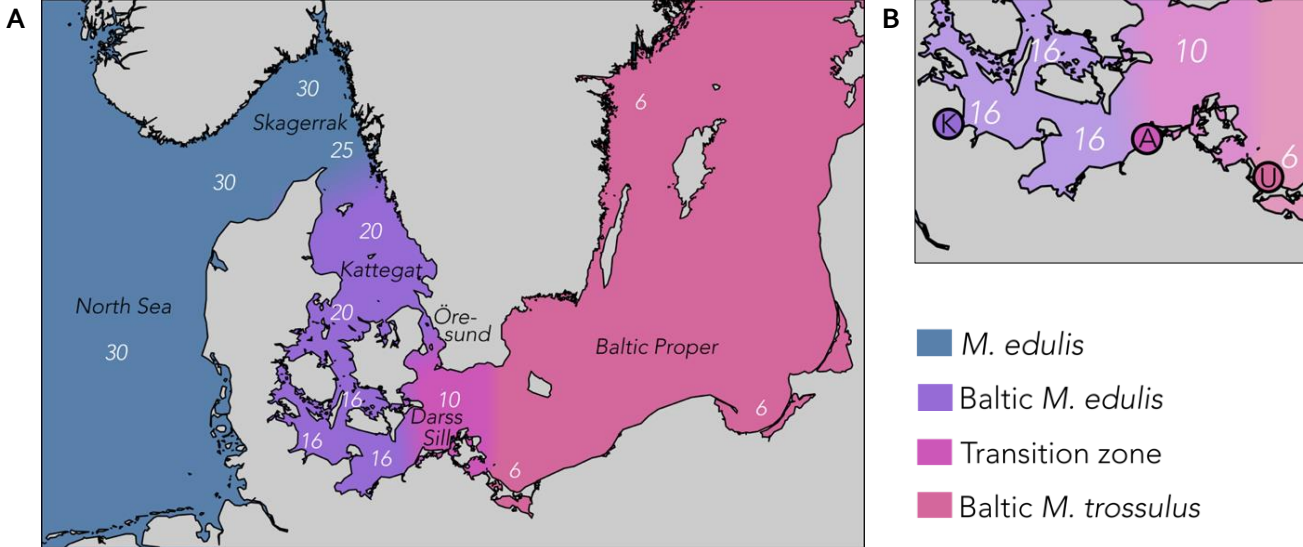


Figure 2

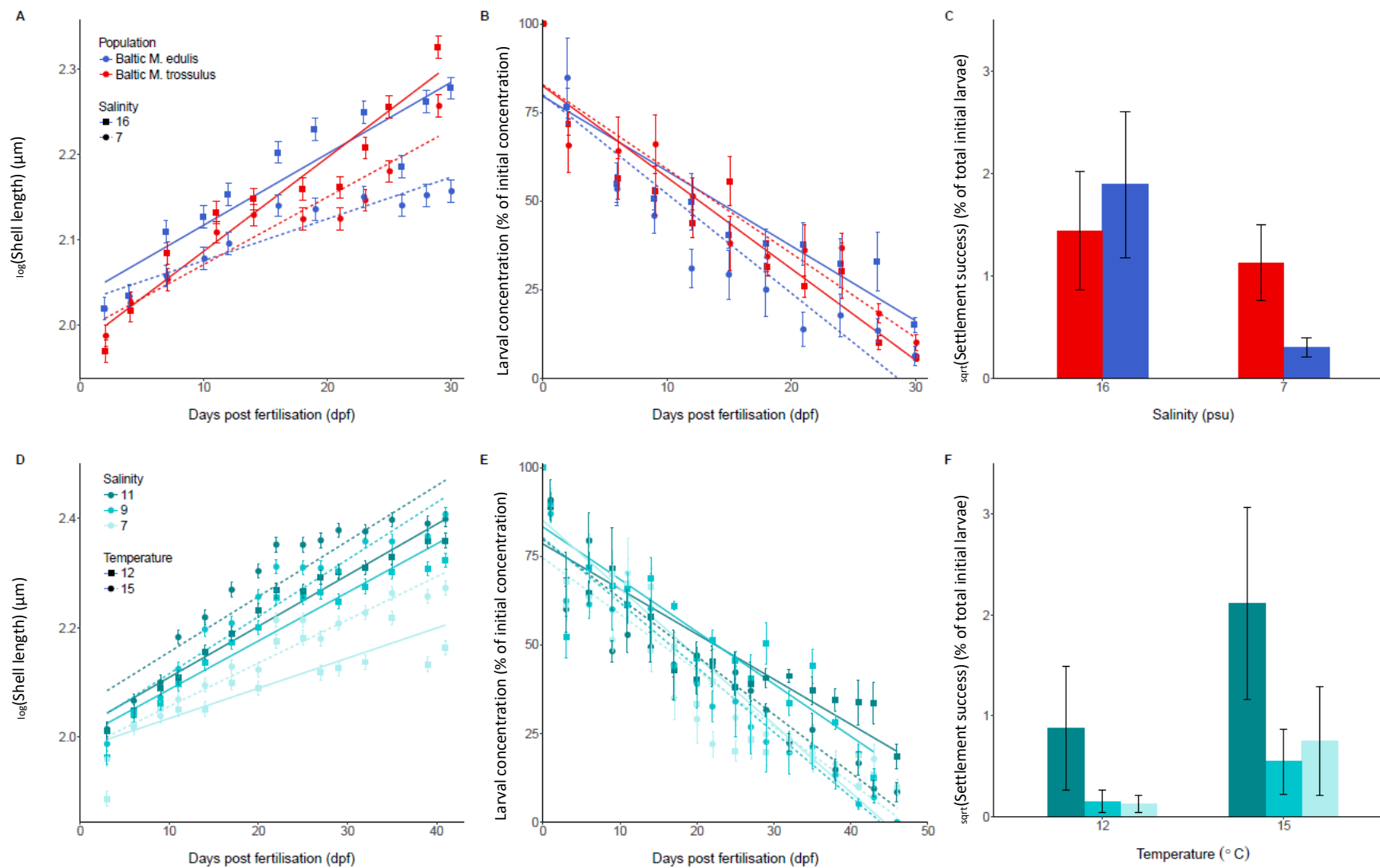
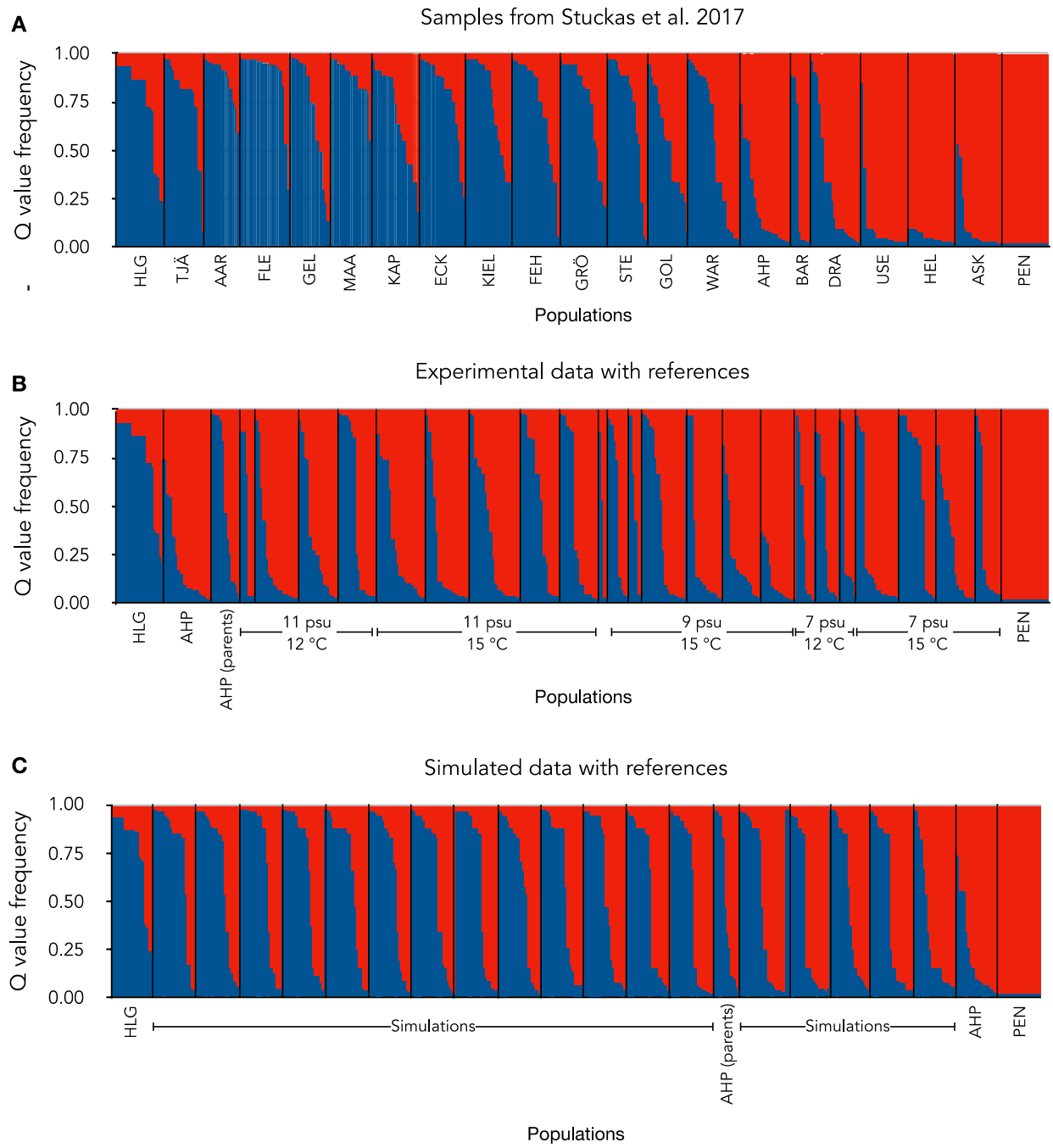


Figure 3



Supplementary**Table S1:** Sampling details and lab conditions. S = salinity in psu, T = temperature in °C, A_T = Alkalinity.

Sampling location	Coordinates	Collection date	Field conditions				Spawning date	Lab conditions		
			S	T	pH	A _T		S	T	pH
Kiel	54°19'45.7", 10°8'55.5"	20-04-2016	15.5	16.5	7.82	1972.76	16-05-2016	16	10	
Ahrens- hoop	54°38.6'N, 12°42.7'E	03-05-2016	10.5	18.5	7.93	1811.72	12-05-2016	11	10	
Usedom	54°3'17.8", 14°0'43.8"	14-04-2016	7.1	17.9	7.83	1790.41	16-05-2016	7	10	

Table S2: PCR conditions for DNA extraction and genotyping of larvae. The *M. edulis* and *M. trossulus* M7 lysin primer pairs were combined in a multiplex PCR and the forward primer of the EFbis locus got 6-Fam fluorescence labelled.

PCR-Assay	DNA extraction		Multiplex Glu-5', mac-1		Multiplex M7 lysin		EFbis	
	T [°C]	t [h:m:s]	T [°C]	t [h:m:s]	T [°C]	t [h:m:s]	T [°C]	t [h:m:s]
Initial								
Denaturation	-	-	95	00:05:00	95	00:05:00	95	00:10:00
Denaturation	56	1:30:00	95	00:00:30	95	00:00:30	95	00:00:15
Annealing	95	00:10:00	55	00:01:30	65	00:01:30	54	00:01:00
Extension	-	-	72	00:01:00	72	00:01:00	72	00:00:45
Final Extension	-	-	72	00:10:00	72	00:10:00	72	00:05:00
Cooling	4	00:10:00	8	∞	8	∞	8	∞
No Cycles	-		32		32		40	
Machine	FlexCycler block (Biozym, Block XXX assembly T48)							

Table S3: PCR reaction mix for larvae genotyping. Multiplex PCRs were performed following the instructions for “Multiplex PCR for Amplification of Microsatellite Loci (Subsequent Analysis on Sequencing Instruments)”.

PCR-Assay	Multiplex Glu-5', mac	Multiplex M7 lysin	EFbis		
PCR Kit	Type-It® Microsatellite PCR Kit (QIAGEN),		AmpliTaq Polymerase Biosystems	Gold® Kit,	DNA Applied
Reaction volume	7 µl	7 µl	10 µl		
Buffer	-	-	1x Gold Buffer		
MgCl ₂	-	-	3 mM		
dNTPs	-	-	0.2 mM		
Taq Polymerase	-	-	1.25 u		
Primer	Glu-5': 0.2µM Mac-1: 0.6 µM D-Loop: 0.2 µM	<i>M. edulis</i> : 0.2 µM <i>M. trossulus</i> : 0.2 µM	0.375 µM		
Reaction Mix			-		

Table S4: Statistical results of two-way ANCOVA (a), two-way ANOVA (b) and Generalized Linear Models (c) for comparison of shell length, larval concentration and settlement success in the population comparison and simulated desalination experiment.

Experiment	Parameter	Factor	Df	Sum sq	Mean Sq	F	Pr (>F)
Population comparison	*Larval concentration	Larval age (dpf)	1	607.7	607.7	382.4	<0.001*
		Salinity	1	11.1	11.1	7.0	0.009*
		Population	1	5.0	5.0	3.1	0.078
		Salinity : Population	1	38.3	38.3	24.12	<0.001*
		Residuals	235	373.4	1.6		
	Shell length	Larval age (dpf)	1	29.03	29.03	1636.2	<0.001*
		Salinity	1	4.63	4.63	260.92	<0.001*
		Population	1	0.19	0.19	10.6	0.001
		Salinity : Population	1	0.38	0.38	21.6	<0.001*
		Residuals	1745	30.96	0.02		
Settlement	Salinity		10005.0	22	44950		0.007
	Population		391.4	21	44559		0.166
	Salinity : Population		6963.4	20	37596		0.004*
Simulated desalination	*Larval concentration	Larval age (dpf)	1	304385	304385	1647.7	<0.001*
		Salinity	2	3276	1638	8.9	<0.001*
		Temperature	1	6753	6753	36.6	<0.001*
		Salinity : Temperature	2	1260	630	3.4	<0.034*
		Residuals	558	103079	185		
	Shell length	Larval age (dpf)	1	333.1	333.1	12131	<0.001*

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		Salinity	2	74.9	37.5	1364.	<0.001*
		Temperature	1	18.3	18.3	665.2	<0.001*
		Salinity:Temperature	2	0.3	0.2	5.6	<0.003*
		Residuals	6016				
	^b Settlement**	Salinity	1	1443.1	1443.1	43.1	<0.001*
		Temperature	1	813.2	813.2	24.3	<0.001*
		Salinity : Temperature	1	235.6	235.6	7.0	<0.001*
		Residuals	16	535.5	33.5		

*Log transformed data

** Squarerooted data

Table S5: Results of post-hoc analysis on the effects of salinity on shell length and larval concentration for the simulated desalination experiment.

Parameter	Factor	Level	Estimated	Std. error	t value	p-value
shell length	Salinity	9 - 7	28.859	1.223	23.603	< 0.001 *
		11 - 7	39.548	1.217	32.496	< 0.001 *
		11 - 9	10.68	1.146	9.329	< 0.001 *
larval concentration	Salinity	7 - 11	-7.621	1.972	-3.865	< 0.001 *
		9 - 11	0.081	1.999	0.041	0.999
		9 - 7	7.702	1.999	3.851	< 0.001 *
			difference	lower	upper	p-value
Settlement success	Salinity	7 - 11	-16.99	-23.04	-10.93	< 0.001
		9 - 11	-16.05	-22.10	-9.99	< 0.001
		9 - 7	-0.94	-7.00	5.11	0.920

Table S6: Mean population Q value for Bayesian Inference analyses using four different model assumption combinations (STR 1 – STR 4). Given are the 75 populations with their abbreviations (Abbr.) as used in Figure 3, their number within in the data set (#), number of specimen sampled for each population (N), and the academic mean Q value for one of the two genetic clusters (ME = *M. edulis*, reference Helgoland; MT = *M. trossulus*, reference Penn Cove) for each model assumption (STR 1 – STR 4).

Population	Abbr.	#	N	STR 1		STR 2		STR 3		STR 4	
				ME	MT	ME	MT	ME	MT	ME	MT
Helgoland	HGL	1	19	0.742	0.258	0.806	0.194	0.775	0.225	0.834	0.166
Tjärnö	TJÄ	2	16	0.749	0.251	0.777	0.223	0.792	0.208	0.801	0.199
Aarhus	AAR	3	14	0.875	0.125	0.888	0.112	0.909	0.091	0.913	0.087
Flensburg	FLE	4	20	0.895	0.105	0.902	0.098	0.922	0.078	0.924	0.076
Gelting	GEL	5	16	0.71	0.29	0.722	0.278	0.720	0.280	0.738	0.262
Maasholm	MAA	6	17	0.874	0.126	0.884	0.116	0.910	0.090	0.911	0.089
Kappeln	KAP	7	19	0.669	0.331	0.697	0.303	0.701	0.299	0.722	0.278
Eckernförde	ECK	8	18	0.793	0.207	0.794	0.206	0.815	0.185	0.814	0.186
Kiel	KIE	9	19	0.739	0.261	0.747	0.253	0.771	0.229	0.772	0.228
Fehmarnsund	FEH	10	19	0.726	0.274	0.728	0.272	0.742	0.258	0.743	0.257
Grömitz	GRÖ	11	19	0.724	0.276	0.728	0.272	0.755	0.245	0.750	0.250
Steinbeck	STE	12	16	0.705	0.295	0.721	0.279	0.728	0.272	0.736	0.264
Gollwitz	GOL	13	16	0.53	0.47	0.526	0.474	0.555	0.445	0.549	0.451
Warnemünde	WAR	14	21	0.523	0.477	0.522	0.478	0.534	0.466	0.532	0.468
Ahrenshoop	AHP	15	20	0.201	0.799	0.195	0.805	0.183	0.817	0.177	0.823
Parent mussels	eAHP	16	12	0.503	0.497	0.508	0.492	0.492	0.508	0.498	0.502
11 psu 12 °C replicate 1	11Ar1	17	6	0.422	0.578	0.42	0.58	0.42	0.58	0.42	0.58
11 psu 12 °C replicate 2	11Ar2	18	18	0.219	0.781	0.217	0.783	0.192	0.808	0.192	0.808
11 psu 12 °C replicate 4	11Ar4	19	16	0.318	0.682	0.313	0.687	0.305	0.695	0.300	0.700

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11 psu 12 °C replicate 5	11Ar5	20	16	0.457	0.543	0.457	0.543	0.446	0.554	0.445	0.555
11 psu 15 °C replicate 1	11HTr1	21	20	0.306	0.694	0.297	0.703	0.306	0.694	0.298	0.702
11 psu 15 °C replicate 2	11HTr2	22	18	0.239	0.761	0.235	0.765	0.218	0.782	0.217	0.783
11 psu 15 °C replicate 3	11HTr3	23	21	0.333	0.667	0.337	0.663	0.324	0.676	0.326	0.674
11 psu 15 °C replicate 4	11HTr4	24	16	0.5	0.5	0.503	0.497	0.505	0.495	0.506	0.494
11 psu 15 °C replicate 5	11HTr5	25	16	0.406	0.594	0.405	0.595	0.396	0.604	0.396	0.604
9 psu 12 °C replicate 2	9Ar2	26	4	0.364	0.636	0.366	0.634	0.361	0.639	0.364	0.636
9 psu 12 °C replicate 3	9Ar3	27	8	0.467	0.533	0.463	0.537	0.471	0.529	0.467	0.533
9 psu 15 °C replicate 1	9HTr1	28	6	0.519	0.481	0.517	0.483	0.513	0.487	0.514	0.486
9 psu 15 °C replicate 2	9HTr2	29	18	0.407	0.593	0.407	0.593	0.397	0.603	0.399	0.601
9 psu 15 °C replicate 3	9HTr3	30	15	0.275	0.725	0.274	0.726	0.248	0.752	0.248	0.752
9 psu 15 °C replicate 4	9HTr4	31	16	0.225	0.775	0.231	0.769	0.200	0.800	0.204	0.796
9 psu 15 °C replicate 5	9HTr5	32	14	0.118	0.882	0.115	0.885	0.089	0.911	0.088	0.912
7 psu 12 °C replicate 1	7ATr1	33	8	0.369	0.631	0.391	0.609	0.364	0.636	0.387	0.613
7 psu 12 °C replicate 3	7ATr3	34	10	0.361	0.639	0.367	0.633	0.351	0.649	0.358	0.642
7 psu 12 °C replicate 5	7ATr5	35	7	0.348	0.652	0.345	0.655	0.322	0.678	0.320	0.680
7 psu 15 °C replicate 1	7HTr1	36	18	0.219	0.781	0.221	0.779	0.196	0.804	0.197	0.803
7 psu 15 °C replicate 2	7HTr2	37	15	0.585	0.415	0.588	0.412	0.587	0.413	0.591	0.409
7 psu 15 °C replicate 4	7HTr4	38	16	0.275	0.725	0.294	0.706	0.260	0.740	0.277	0.723

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7 psu 15 °C replicate 5	7HTr5	39	11	0.297	0.703	0.298	0.702	0.276	0.724	0.276	0.724
Simulation of salinity temperature replicate 1	S1T1_vr1	40	20	0.614	0.386	0.614	0.386	0.622	0.378	0.621	0.379
Simulation of salinity temperature replicate 2	S1T1_vr2	41	20	0.697	0.303	0.699	0.301	0.706	0.294	0.708	0.292
Simulation of salinity temperature replicate 3	S1T1_vr3	42	20	0.663	0.337	0.667	0.333	0.670	0.330	0.674	0.326
Simulation of salinity temperature replicate 4	S1T1_vr4	43	20	0.575	0.425	0.585	0.415	0.567	0.433	0.580	0.420
Simulation of salinity temperature replicate 5	S1T1_vr5	44	20	0.633	0.367	0.638	0.362	0.631	0.369	0.638	0.362
Simulation of salinity temperature replicate 1	S1T2_vr1	45	20	0.471	0.529	0.477	0.523	0.456	0.544	0.467	0.533
Simulation of salinity temperature replicate 2	S1T2_vr2	46	20	0.331	0.669	0.33	0.67	0.31	0.69	0.31	0.69
Simulation of salinity temperature replicate 3	S1T2_vr3	47	20	0.652	0.348	0.653	0.347	0.656	0.344	0.657	0.343
Simulation of salinity temperature replicate 4	S1T2_vr4	48	20	0.603	0.397	0.61	0.39	0.60	0.40	0.61	0.39
Simulation of salinity temperature replicate 5	S1T2_vr5	49	20	0.602	0.398	0.605	0.395	0.602	0.398	0.606	0.394

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Simulation of salinity temperature replicate 1	of 2 1	S2T1_vr1	50	20	0.512	0.488	0.511	0.489	0.512	0.488	0.512	0.488
Simulation of salinity temperature replicate 2	of 2 1	S2T1_vr2	51	21	0.5	0.5	0.501	0.499	0.497	0.503	0.497	0.503
Simulation of salinity temperature replicate 3	of 2 1	S2T1_vr3	52	20	0.527	0.473	0.525	0.475	0.521	0.479	0.521	0.479
Simulation of salinity temperature replicate 4	of 2 1	S2T1_vr4	53	20	0.626	0.374	0.625	0.375	0.636	0.364	0.634	0.366
Simulation of salinity temperature replicate 5	of 2 1	S2T1_vr5	54	20	0.603	0.397	0.605	0.395	0.602	0.398	0.604	0.396
Simulation of salinity temperature replicate 1	of 2 2	S2T2_vr1	55	21	0.552	0.448	0.554	0.446	0.550	0.450	0.553	0.447
Simulation of salinity temperature replicate 2	of 2 2	S2T2_vr2	56	20	0.566	0.434	0.566	0.434	0.566	0.434	0.566	0.434
Simulation of salinity temperature replicate 3	of 2 2	S2T2_vr3	57	20	0.498	0.502	0.499	0.501	0.492	0.508	0.494	0.506
Simulation of salinity temperature replicate 4	of 2 2	S2T2_vr4	58	20	0.72	0.28	0.727	0.273	0.732	0.268	0.740	0.260
Simulation of salinity temperature replicate 5	of 2 2	S2T2_vr5	59	20	0.658	0.342	0.664	0.336	0.661	0.339	0.668	0.332
Simulation of salinity	of 3	S3T1_vr1	60	20	0.512	0.488	0.515	0.485	0.505	0.495	0.509	0.491

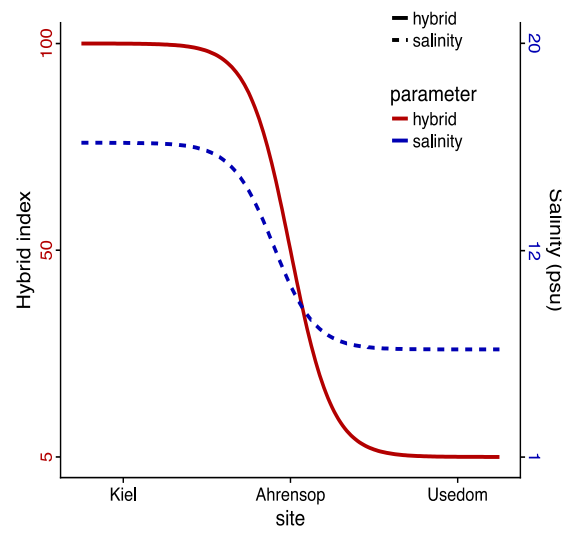
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temperature replicate 1	1											
Simulation of salinity temperature replicate 2	3 1	S3T1_vr2	61	20	0.713	0.287	0.714	0.286	0.725	0.275	0.725	0.275
Simulation of salinity temperature replicate 3	3 1	S3T1_vr3	62	20	0.603	0.397	0.613	0.387	0.601	0.399	0.613	0.387
Simulation of salinity temperature replicate 4	3 1	S3T1_vr4	63	20	0.528	0.472	0.53	0.47	0.52	0.48	0.52	0.48
Simulation of salinity temperature replicate 5	3 1	S3T1_vr5	64	20	0.597	0.403	0.597	0.403	0.598	0.402	0.597	0.403
Simulation of salinity temperature replicate 1	3 2	S3T2_vr1	65	20	0.544	0.456	0.545	0.455	0.534	0.466	0.537	0.463
Simulation of salinity temperature replicate 2	3 2	S3T2_vr2	66	20	0.514	0.486	0.515	0.485	0.507	0.493	0.511	0.489
Simulation of salinity temperature replicate 3	3 2	S3T2_vr3	67	20	0.451	0.549	0.451	0.549	0.439	0.561	0.440	0.560
Simulation of salinity temperature replicate 4	3 2	S3T2_vr4	68	20	0.463	0.537	0.467	0.533	0.449	0.551	0.455	0.545
Simulation of salinity temperature replicate 5	3 2	S3T2_vr5	69	20	0.593	0.407	0.596	0.404	0.598	0.402	0.601	0.399
Barhöft		BAR	70	8	0.338	0.662	0.332	0.668	0.342	0.658	0.339	0.661
Dranske		RÜG	71	20	0.301	0.699	0.298	0.702	0.292	0.708	0.290	0.710
Usedom		USE	72	19	0.108	0.892	0.103	0.897	0.081	0.919	0.080	0.920

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Hel	HEL	73	19	0.049	0.951	0.045	0.955	0.015	0.985	0.015	0.985
Askö	ASK	74	18	0.103	0.897	0.099	0.901	0.076	0.924	0.074	0.926
Penn Cove	PEN	75	19	0.016	0.984	0.015	0.985	0.000	1.000	0.000	1.000

Figure S1



Chapter 4 – Mechanisms of salinity adaptation

Intracellular organic and inorganic osmolyte modification facilitates local adaptation of mussels to low salinity

Intracellular organic and inorganic osmolyte modification facilitates local adaptation of mussels to low salinity

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This manuscript will be combined with complimentary transcriptomic data and prepared for submission within 6 months.

Abstract

It has been extensively documented that euryhaline osmoconforming marine invertebrates adjust their intracellular osmotic pressure in response to extracellular changes in osmolality. This mechanism involves the physiological adjustment of both intracellular concentrations of inorganic ions and compatible organic osmolytes. What is less known, is how these intracellular changes may ensue during multigenerational adaptation to low salinity habitats, which may shed light on our understanding of the mechanisms of adaptive evolution. In this context, the Baltic Sea salinity gradient provides an excellent natural system to investigate physiological adaptations to low salinity habitats. To investigate the adaptive changes in intracellular osmolytes, changes in intracellular organic and inorganic osmolytes with salinity were compared in two populations of Baltic mytilid mussels originating from different salinities (7 and 16 psu). By using flame photometry and ¹H-NMR spectroscopy, it was found that low salinity populations of Baltic mytilids exhibit decreased total concentrations of organic osmolytes and increased intracellular concentrations of cations across all experimental salinities. These changes were primarily due to lower levels of aspartate, betaine and taurine and higher Na⁺ content in the low salinity populations. Observed changes in osmolyte pools were facilitated by evidence of local adaptation in the low salinity population, demonstrated by better tolerance to low salinity than the high salinity population. These are the first documented adaptive changes in osmolytes in osmoconforming invertebrates which may have significant implications for cellular physiological mechanisms and ion regulation which require further investigation. These findings indicate a potential mechanism of adaptation to extremely low salinities in an ecologically important benthic invertebrate species in the Baltic Sea.

Introduction

Osmoconforming invertebrates such as euryhaline bivalve molluscs maintain their intracellular environment iso-osmotic to external seawater osmolality (Willmer 2006). This is accomplished through the accumulation of organic osmolytes (OO) such as free amino acids (FAA's) and derivatives, polyols and sugars (Yancey 2005). Exposure to osmotic stress, induces an array of short-term and long-term physiological mechanisms to maintain cell volume and prevent cell lysis and mortality in osmoconforming molluscs (Pierce 1982). Short term (hours) responses to hyposalinity stress involves the cellular excretion of cations (mainly K⁺) in order to maintain cell volume (Silva & Wright 1994; Neufeld & Wright 1996). Longer-term responses (days) to hyposalinity however, involve the loss of intracellular OO's which enables stable intracellular

concentrations of K^+ (Lange 1963; Yancey 2005). The OO's utilized by osmoconformers to adjust intracellular osmotic pressure are termed compatible osmolytes meaning they do not detrimentally interfere with intracellular molecules and cellular function. This is in contrast to intracellular K^+ and other cations, of which increasing or decreasing cellular concentrations negatively impact enzyme activities (Hochachka & Somero 2002). For these reasons, OO concentrations in marine osmoconformers exhibit higher magnitudes of change with seawater osmolality than intracellular cation concentrations. Evidence exists however, that intracellular cation concentrations are modulated during long term exposure to hypo- and hyper-salinity stress (Berger & Kharazova 1997) which has been shown to impact enzyme function and efficiency (Sarkissian 1974; Ballantyne & Berges 1990). The energetic costs of these physiological responses to osmotic stress are thought to be significant in osmoconforming bivalves and can have implications for metabolism and growth particularly in habitats with regular salinity fluctuations (Neufeld & Wright 1996; Hawkins & Hilbish 1992).

The Baltic Sea is a brackish water system with a salinity gradient extending from high salinity (> 25 practical salinity units, psu) in the Danish Belt Seas to < 6 psu in the Central and Eastern Baltic (Gräwe et al. 2013). Despite these extreme environmental conditions, marine calcifying molluscs are abundant along this salinity gradient and mussels within the genus *Mytilus* (from here on: Baltic *Mytilus*) dominate the benthos forming extensive benthic reefs and increasing benthic biodiversity (Norling & Kautsky 2008). The colonisation of marine organisms along this salinity gradient provides a valuable model to investigate biochemical adaptations to environmental change. Baltic *Mytilus* at salinities < 8 psu show extremely stunted growth and calcification rates with maximum sizes less than half of North Sea *Mytilus* populations (Kautsky et al. 1990). These extremely slow growth rates are thought to be a result of high protein turnover rates and/or diminished ability to biomineralize exoskeletons (Tedengren & Kautsky 1986; Thomsen et al. 2018).

Compared to fully marine populations, intracellular OO concentrations are substantially lower in both Baltic *Mytilus* and *Macoma balthica* with these reductions in *Mytilus* spp. primarily a result of decreased taurine and glycine concentrations (Kube et al. 2006). What is not clear, is whether these differences are driven primarily by habitat osmolality or whether Baltic populations have locally adapted their intracellular OO pools to the extremely low Baltic salinity. This has potential implications for energy metabolism as synthesis of amino acids such as glycine and taurine come at an energetic cost (Bishop et al. 1983; Welborn & Manahan 1995). Additionally, it is not known how, or if, intracellular cation concentrations are

modulated in populations adapted to different salinities. Acclimation of *M. edulis* for 14 days to 25 ‰ salinity has been shown to invoke a significant reduction in neuronal cell K^+ and Na^+ concentrations (Willmer 1978a). This may have major implications for enzyme function and cellular metabolism as changing intracellular $[K^+]$ have been found to alter the enzyme kinetics of metabolic enzymes and mitochondrial function in osmoconforming invertebrates (Sarkissian 1974; Ballantyne & Berges 1990).

The aim of this study is to investigate the main constituents of the organic and inorganic osmolyte pools in two populations of Baltic *Mytilus* that are locally adapted to different salinity regimes. Both populations (Kiel: 16 ‰ and Usedom: 7 ‰) were exposed to a range of salinities (4.5, 5, 6, 7 and 16 ‰) in a fully crossed laboratory experiment. Intracellular gill content of OOs were analysed using untargeted 1H -NMR-based metabolic profiling and inorganic cations (K^+ and Na^+) were analysed using flame photometry. The goal was to 1) identify whether qualitative and/or quantitative changes in OO pools are influenced by environmental conditions and local adaptation and 2) identify changes in intracellular cations with long term acclimation to low salinity.

Methods

Animal collection and experimental design

Adult Baltic *Mytilus* (shell length 31 ± 3 mm) were collected from Kiel Fjord ($54^\circ 19' 48.846''$ N, $10^\circ 8' 59.6436''$ E; 16 ‰) and Usedom island ($54^\circ 3' 20.5668''$ N, $14^\circ 0' 40.0572''$ E; 7 ‰) in September 2016 and transported in cooled boxes to GEOMAR climate chambers. Animals kept in 20 L plastic aquaria at $10^\circ C$ and fed twice daily with $30\,000$ cells ml^{-1} *Rhodomonas baltica* for 2 weeks prior to experimentation. The experimental design consisted of a common garden experiment with two populations (Kiel and Usedom) and 5 salinities (4.5, 5, 6, 7, and 16 ‰) covering mean environmental salinities of both populations as well as salinities down to the known physiological salinity limit of Baltic *Mytilus*. Stocking densities were 13 animals per 10 L aquaria and salinities were decreased from native salinities over the course of 5 days and water changes were done every 2 days. Animals were fed twice daily with $15\,000$ *Rhodomonas baltica* cells ml^{-1} and aquaria were checked daily for dead animals which were immediately removed.

The experiment was terminated after four weeks and gill, haemolymph and seawater samples were taken. Gill and inner mantle samples were rinsed 3×1 ml in iso-osmotic ion free artificial

seawater (Table S1) at the corresponding treatment osmolalities to remove external seawater ions, blotted 3 times on paper to remove excess water and weighed. Haemolymph samples were taken by gently opening the shell valves and draining all seawater. A syringe was then inserted into the posterior adductor muscle and haemolymph was removed and centrifuged for 3 minutes at 10000g to remove cell and tissue debris. Tissue samples were then frozen at -80°C for later osmolyte analysis. Haemolymph and seawater samples were stored at -20°C for later analysis.

Tissue and seawater cation measurements

Gill tissue was used for intracellular cation measurements by placing frozen tissue in 0.1 M nitric acid (1:10 ratio of tissue wet mass to volume) and homogenized for 60 s using an ULTRA-TURRAX attached to a VWR VDI 12 homogenisator (IKA, USA). Extracts were then freeze-thawed 3 times and spun at 1500 g for 5 mins to remove particulate material. Gill tissue extracts, haemolymph and seawater cation concentrations (Ca^{2+} , Na^{+} and K^{+}) were determined using a flame photometer (EFOX5053 Eppendorf, Germany) with urine standards (Biorapid).

Tissue water content and haemolymph osmolality

Total tissue water content was estimated based on the ratio between soft tissue dry mass and soft tissue wet mass which has been shown to be as accurate as using isotopic analysis ($^3\text{H}_2\text{O}$) (Neufeld & Wright 1996). Gill samples were rinsed, blotted dry and weighed for determination of wet mass (WM). Gills were then placed in pre-weighed tin foil cups and dried at 60°C for 12 hours and re-weighed to determine the dry weight (DW). Osmolality of seawater and haemolymph samples were determined using a freezing point osmometer (Osmomat 030 Gonotec, Germany).

Tissue extractions and metabolic profiling

For NMR based metabolic profiling, intracellular metabolites were extracted using a methanol extraction method similar to (Le Belle et al. 2002). Frozen gill tissue (~ 120 mg) was ground to a powder using a pestle and mortar under liquid nitrogen and then placed in an Eppendorf tube and weighed. 1.2 ml of < 4°C 70 % methanol was added to each tube and tissue was homogenized for 60 s using an ULTRA-TURRAX attached to a VWR VDI 12 Homogenisator (IKA, USA). Tubes were then centrifuged in quadruplets at 0 °C for 5 mins at 14,000 g after which the supernatant was immediately removed and frozen in 2 ml tubes at -20 °C. Samples

were desiccated overnight in an Eppendorf Rotary Concentrator 5301 (Hamburg, Germany) at 30 °C and placed in a glass desiccator until metabolic profiling.

Untargeted NMR based metabolic profiling was conducted using a 9.4T wide bore NMR spectrometer (Avance III HD 400 WB, Bruker-Biospin GmbH, Ettlingen, Germany) equipped with a high-resolution magic angle (HRMAS) triple tunable ^1H - ^{13}C - ^{31}P -probe at the NMR laboratory of the Alfred-Wegener-Institute, similar to (Schmidt et al. 2017). Desiccated tissue extracts were dissolved in D_2O (deuterium oxide) containing 0.05% trimethylsilyl propionate (TSP, Sigma Aldrich) as a reference standard, at a ratio of 1 μl D_2O to 1 mg tissue wet weight. The solution was placed in a 50 μl standard zirconium rotor for HRMAS one-dimensional (1D) ^1H -NMR spectroscopy. Spectra were obtained at 20 °C at a spinning rate of 3KHz using the following acquisition parameters: Bruker protocol cpmgpr1d, ns = 32, TD =70,656, SW = 8802Hz; acquisition time of 4 s and a relaxation delay of 4 s (Rebelein et al. 2018). Magnetic field homogeneity was optimized using a standard shimming protocol resulting in a typical line width of 2-4Hz. After Fourier transformation using an exponential multiplication of 0.5 Hz, baseline and phase were adjusted and the spectra were calibrated to the TSP standard at 0.0 ppm. Processed spectra were analysed using Chenomx NMR Suite 8.2 profiling software (Chenomx Inc., Alberta, Canada). Intracellular and membrane bound organic compounds were assigned and analysed from the compound library available from Chenomx. ATP concentrations were semi-quantified as a reference and showed no significant changes with salinity or population. Due to extremely low concentrations compared to more dominant osmolytes, ATP concentrations were multiplied by a factor of 20 and expressed as such (Fig. 3).

Intracellular cation content

For calculating intracellular cation content, raw values were corrected for tissue extraction solution volumes and for total tissue water content. Intracellular cation content (cation_I) was calculated separately for Na^+ and K^+ using the following equation:

$$\text{(equation1) } \text{cation}_I = [\text{cation}]_T - (V_E \times [\text{cation}]_E)$$

Where $[\text{cation}]_T$ is the total tissue cation concentration, V_E = the extracellular water volume and $[\text{cation}]_E$ is the extracellular osmolyte concentration. Intracellular OO content of the haemolymph is negligible and therefore this equation was not required to calculate the intracellular content of OO's (Zuburg 1981). A value of 23 % was used for the contribution of extracellular water to total tissue water were taken from published literature (Wilmer 1978a;

Wright et al. 1989, Silva & Wright 1994; Neufeld & Wright 1996). Intracellular water volumes were not quantified (see discussion) therefore intracellular osmolyte (ions and organic osmolytes) contents were corrected for tissue dry mass and expressed in $\mu\text{mol g}^{-1}$ DW (dry weight).

Statistics

All statistical analysis was performed on R software (version 3.1.0). As all intracellular osmolyte and osmotic changes have been found to show linear relationships with salinity, impacts of salinity and populations were analysed using a series of ANCOVA's with salinity as a co-factor and population as a factor. ANCOVA's were conducted individually on the 8 most abundant organic osmolytes, Na^+ , K^+ as well as the total sum of the organic osmolytes and the total sum of the 3 measured cations (Na^+ , K^+ and Ca^{2+}). Haemolymph osmolality was statistically compared to respective seawater osmolality using an ANCOVA with salinity as a co-factor and salinity and media (seawater or haemolymph) as factors. Mortality and DW/WW ratios were analysed using an ANOVA and a Tukey post-hoc test was performed on significant factors.

Results

Our study revealed clear changes in intracellular osmolytes with salinity as well as distinct population differences between a low salinity adapted population (Usedom, 7 psu) and a high salinity adapted population (Kiel, 16 psu). Mortality rates for Usedom mussels were $\sim 20\%$ at all salinities compared to very low mortality rates in Kiel mussels at 16 psu ($\sim 2\%$) but very high mortality rates of $\sim 68\%$ below 6 psu in Kiel mussels from high salinity (ANOVA, $F_{(4,30)} = 8.3$, $p < 0.001$). These differences suggest the Usedom population has a higher tolerance to low salinity than Kiel mussels (Fig. 1). Haemolymph osmolality decreased linearly with seawater osmolality down to 4.5 psu in both populations with no deviations from iso-osmolality (ANCOVA, $F_{(1,76)} = 0.04$, $p = 0.843$; Fig. 2) demonstrating Baltic *Mytilus* to be true osmoconformers.

We identified 21 organic compound signals from NMR-spectra including: alanine, arginine, aspartate, betaine, glutamate, glutamine, glycine, guanidine succinate, isoleucine, leucine, lysine, methionine, methylamine, O-phosphocholine, ornithine, proline, serine, succinate, taurine, threonine and valine. Of these, the OO's making up the highest proportion of the total intracellular OO pool were taurine (16 %), glycine (16 %), aspartate (12 %) and betaine (4 %)

(Fig. 3). Taurine and glycine showed the largest magnitude of change with salinity and were the main drivers of the linear decrease in total OO content with decreasing salinity (ANCOVA, $F_{(4,30)} = 71.8$, $p < 0.001$, Fig 3). Intracellular content of alanine, betaine, glutamate and aspartate were also higher in Kiel mussels than Usedom mussels and this was largely responsible for the higher total OO content in Kiel mussels (ANCOVA, $F_{(1,30)} = 111.6$, $p < 0.001$, Fig. 3). High salinity Kiel mussels also exhibited higher tissue DW/WW ratios than low salinity Usedom mussels (ANCOVA, $F_{(1,36)} = 6.2$, $p = 0.018$, Fig. S1) indicating lower tissue water content in Kiel animals. Visual inspection of tissue samples during sampling also revealed a paler, more dilute appearance of Usedom tissues. There was also a significant interaction effect between population and salinity (ANCOVA, $F_{(4,30)} = 3.0$, $p < 0.035$) caused by the large increase (10 - 15-fold) in tissue glycine content in Usedom mussels at 16 psu.

Both populations showed linear decreases in total cation content with salinity (ANCOVA, $F_{(4,30)} = 71.8$, $p < 0.001$), although the linear intercept was much higher than for the OO's suggesting Baltic mussels keep their intracellular cation content relatively more stable than OO's (Fig. 4). The low salinity Usedom population showed a more significant change in cations with salinity than Kiel (ANCOVA interaction, $F_{(4,30)} = 3.6$, $p = 0.016$). These changes in cations were primarily driven by a large change in $[\text{Na}^+]$ (ANCOVA, $F_{(4,30)} = 37.1$, $p < 0.001$) with salinity (Fig. 5). K^+ content did not change with salinity and was not different between populations, although variability was high within treatments (ANCOVA, $F_{(4,30)} = 0.6$, $p = 0.67$, Fig. 5). Regarding total cation content, Usedom mussels had a significantly higher content of intracellular cations than Kiel across all salinities (ANCOVA, $F_{(4,30)} = 12.8$, $p < 0.001$, Fig. 4).

Discussion

This is the first study to identify modulations in the organic and inorganic osmolyte pools in a marine bivalve population locally adapted to low salinity. We found low salinity adapted populations have both lower total intracellular organic osmolytes (mostly due to decreased taurine content) and higher cation contents (mostly due to Na^+) than high salinity adapted populations. Coupled with evidence of low salinity adaptation in the population originating from lower salinities (lower mortality) suggests these quantitative changes in osmolyte content might be adaptive.

We show that decreasing contents of intracellular OO's with salinity are driven mainly by changes in taurine, alanine, aspartate and glycine. This was also found by Shumway et al. 1977

in eight marine bivalve species and by Kube et al. 2006 when comparing Baltic and non-Baltic *Mytilus* populations. The lower total OO pool in the Usedom 7 psu population compared to the 16 psu Kiel population can also be explained by lower concentrations of these same compounds. Developing lower taurine biosynthesis rates may be an energy saving mechanism of these low salinity mussels as this population never experiences salinities over 11 psu and thus, never has to accumulate high cellular concentrations of taurine. Taurine biosynthesis rates are thought to be very low in bivalves and a large proportion of the intracellular taurine pool is probably derived from dietary supplementation (Bishop et al. 1983). This could explain why Usedom mussels exposed to 16 psu exhibited a disproportionately large increase in glycine content compared to other osmolytes. Taurine synthesis and accumulation may be a slow process and not possible in the time-frame of this experiment (4 weeks) whereas glycine (the most abundant amino acid) can be quickly accumulated through the catabolism of endogenous protein reserves (Hawkins & Hilbish 1992).

Recent findings on salinity tolerance in the sea anemone (*Diadumene lineata*) by Podbielski et al. 2016 led the authors to suggest salinity tolerance is dictated by the salinity at which the OO pool is depleted (S_{crit}). This concept however, does not appear to support the findings in this study as mussels from Usedom that exhibited better tolerance to low salinity also possessed lower amounts of intracellular osmolytes. Therefore, Usedom mussels will likely deplete their intracellular osmolyte pools at a higher salinity than Kiel mussels, despite having a lower salinity tolerance limit than Kiel mussels. The differences between our findings and those of Podbielski et al. 2016 likely reflect differences between species either in the relative constituents of their organic osmolyte pools or, other factors which govern their salinity tolerance limit.

Traditionally, it is believed that osmoconforming bivalves maintain relatively stable intracellular cation concentrations and only majorly adjust these in response to short term (hours) osmotic stress to maintain cell volume (Silva & Wright 1994). There is evidence from *M. edulis* that intracellular Na^+ concentrations increase linearly with salinity and K^+ concentrations decrease below 8 psu (reviewed by Berger and Kharazova 1997; Shakhmatova et al. 2005). Although, the magnitude of these cation concentration changes is less severe than that of the OO. Salinity induced changes in intracellular cation concentrations in bivalve's correlate with changes in enzyme activity (Wickes & Morgan II 1976; Ballantyne & Berges 1990). Pyruvate kinase for example, an enzyme involved in glycolysis, functions at an optimum $[K^+]$ concentration with enzyme function being severely impeded above and below this

optimum concentration (Bowlus & Somero 1979). Similarly, rates of protein synthesis and electron transport also exhibit maximum rates at an optimum $[K^+]$ and $[Na^+]$ (Ballantyne & Moyes 1987; Hochachka & Somero 2002). As well as enzyme function, intracellular cation concentrations are vital for other cellular functions such as transmembrane transport of taurine (Silva & Wright 1992), calcification (Mackinder et al. 2011) and neuronal function (Willmer 1978b). It therefore seems likely that a critical minimum intracellular cation concentration may dictate the salinity tolerance of marine bivalves. Increased tissue cation content in the low salinity adapted population may be a cellular adaptation to maintain cellular function at extremely low salinities.

Baltic *Mytilus* are classified as a “hybrid swarm” between *Mytilus edulis*-like and *Mytilus trossulus*-like specimens (Riginos & Cunningham 2005). A genetic gradient in allele frequencies persists with *M. edulis*-like individuals being predominant at higher salinities (> 13 psu) and *M. trossulus*-like individuals being predominant at lower salinities (< 9 psu) with the highest admixture of both species being found near the island of Zingst on the German Baltic Sea coast (Stuckas et al. 2017). These genetic differences may be responsible for the differences in organic osmolyte pools between our experimental populations as FAA pools are related to the specific amino acid metabolism of the species (Bishop 1976). Analysis of the *M. trossulus* proteome reveals this species employs more energetically efficient metabolic pathways during hypo-osmotic stress when compared to the more stenohaline *M. galloprovincialis* (Tomanek et al. 2012). This may suggest that *M. trossulus* genotypes are better adapted to low salinities than the more stenohaline, marine species *M. edulis* and *M. galloprovincialis* which consequently may explain the population differences in osmolyte pools in this study.

The water content of marine invertebrate tissues is comprised of intracellular water and extracellular water (haemolymph) the latter of which has an ionic composition similar to that of the ambient seawater. This has implications for calculations of intracellular ion contents as cation measurements of tissue extracts will inadvertently contain ions from the intracellular and extracellular compartments (Willmer 1978a; Silva & Wright 1994). We minimised this effect by rinsing tissues in ion free iso-osmotic solution before ion measurements. Additionally, evidence suggests that two-week acclimation to a range of osmolalities does not significantly change the volume of extracellular water in *Mytilus edulis* but rather the volume of intracellular water (Willmer 1978a; Gainey Jr 1994; Neufeld & Wright 1996). This implies that the higher tissue water content in low salinity mussels observed in our study is likely due to increased

intracellular water rather than extracellular water volume (Fig. S1). However, as intracellular water volume was not quantified in this study, we chose to express intracellular osmolyte contents in this study corrected for dry mass ($\mu\text{mol g}^{-1}$ DW). Further work should aim to accurately quantify changes in intracellular and extracellular water content with changing salinity to get a clearer picture of the mechanisms of cell volume control during long term exposures to very low salinities.

Conclusions

This study is the first to identify potential adaptive changes in osmolyte pools at low salinities and provides a baseline for future work into studying the mechanisms of salinity tolerance in osmoconforming bivalves. Our results suggest Baltic *Mytilus* populations have adapted to tolerate extremely low salinities through a combination of decreased intracellular OO content (primarily due to decreased intracellular taurine content) and increased content of intracellular cations, primarily Na^+ . The significance of these intracellular changes requires further investigation regarding potential impacts on cellular functions such as enzyme synthesis and activity. Further comparisons with other species will enable a better understanding of how coastal desalination may impact species distributions in the future.

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Figures

Fig. 1 Total mortality rates per tank after the termination of the experiment expressed as a percentage of the initial number of animals per tank. Error bars represent standard deviation.

Fig. 2 Osmolality (mOsm kg⁻¹ solution) of seawater and haemolymph for Kiel (left) and Usedom (right) populations at the end of the experiment. Linear decreases of both haemolymph osmolality and seawater osmolality indicate Baltic *Mytilus* are true osmoconformers down to 4.5 psu.

Fig. 3 Changes in the content (μmol g⁻¹ dry weight) of the most abundant tissue organic osmolyte content with salinity and population analysed using ¹H-NMR-based metabolic profiling. Concentrations are expressed on a log scale and ATP concentrations were multiplied by a factor of 20 for comparative reasons (see M&M for details). Shaded areas around the linear regressions represent standard error

Fig. 4 Sum of all organic osmolyte contents (μmol g⁻¹ dry weight) (A) presented in Fig. 3 (excluding ATP) and intracellular cations (Na⁺ and K⁺) and their changes with salinity and population. The shaded area around the linear regressions represents the standard error.

Fig. 5 Changes in intracellular contents (μmol g⁻¹ dry weight) of Na⁺ (A) and K⁺ (B) with salinity and between populations. Shaded areas around the linear regressions represent standard error.

Figure 1

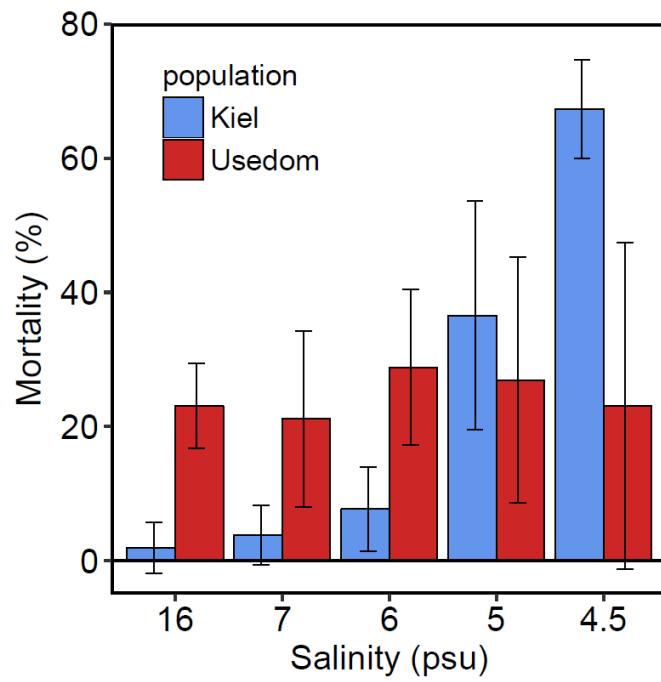


Figure 2

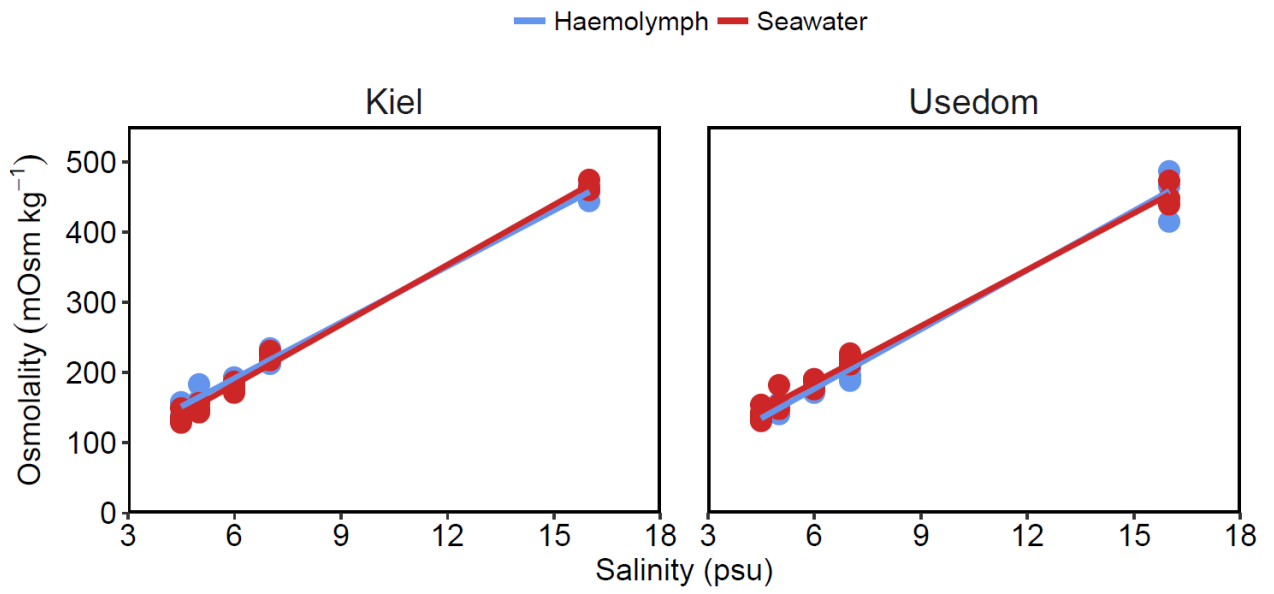


Figure 3.

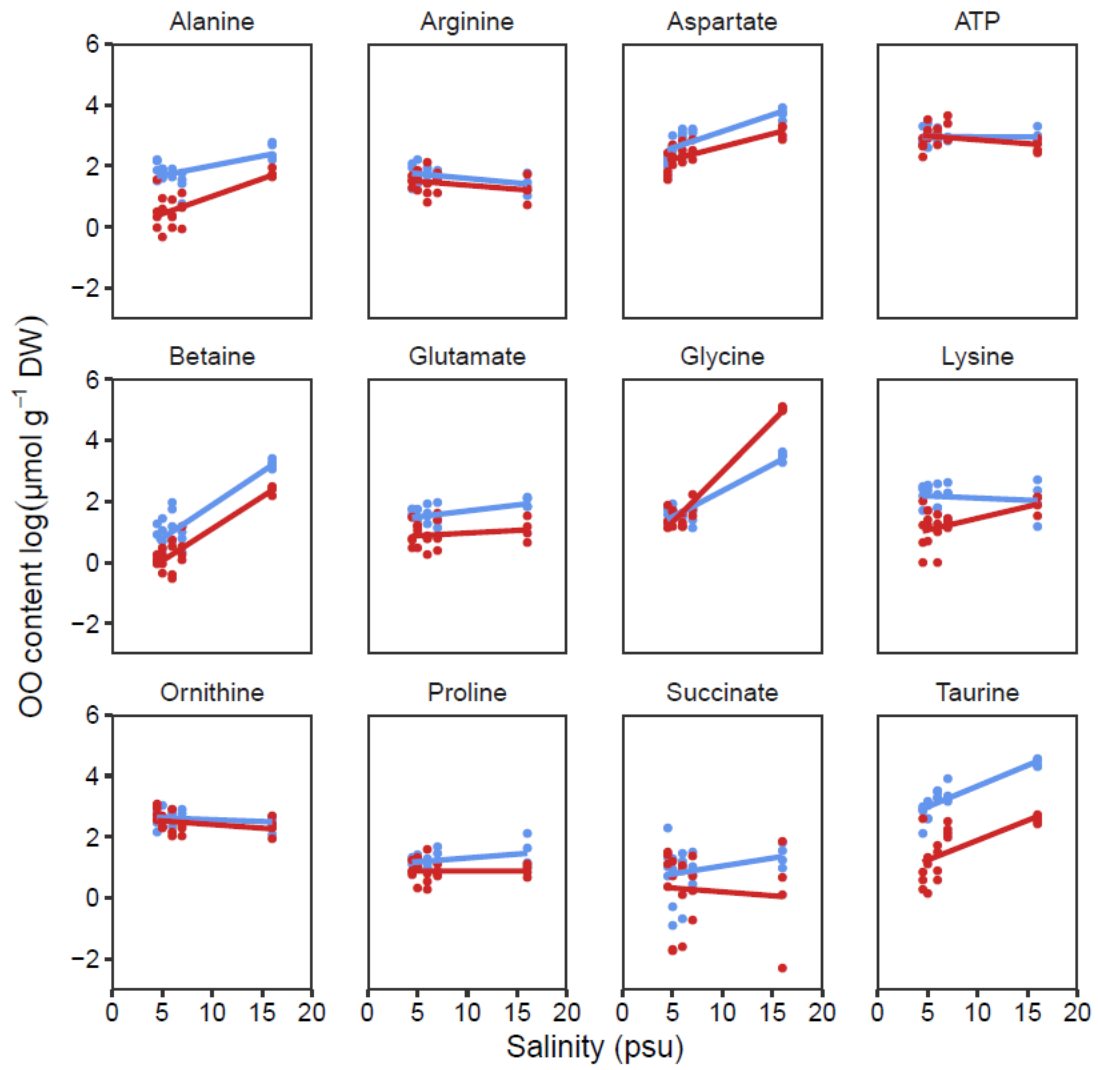


Figure 4.

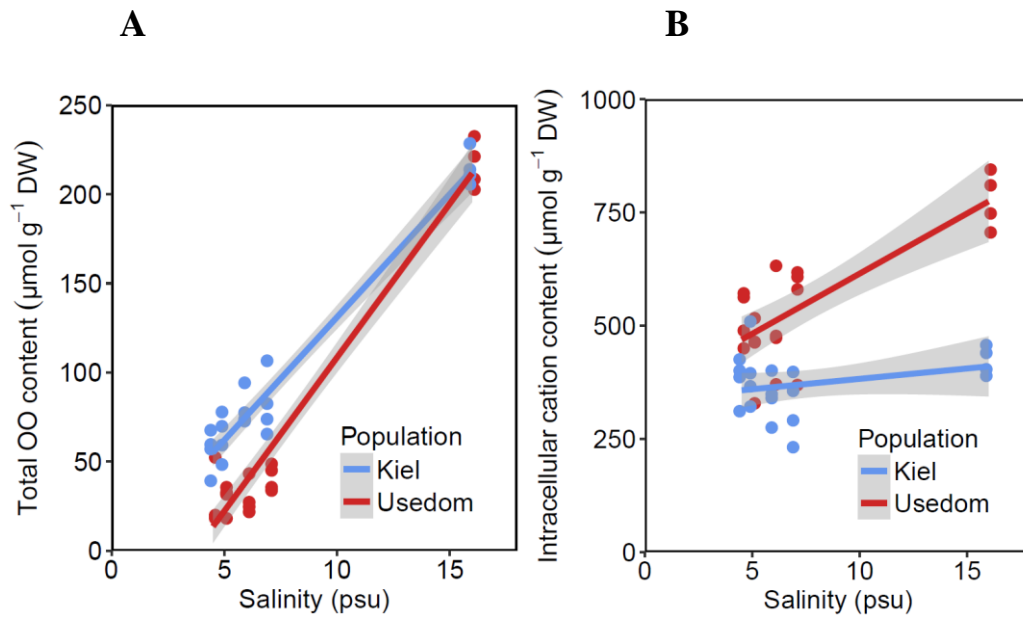
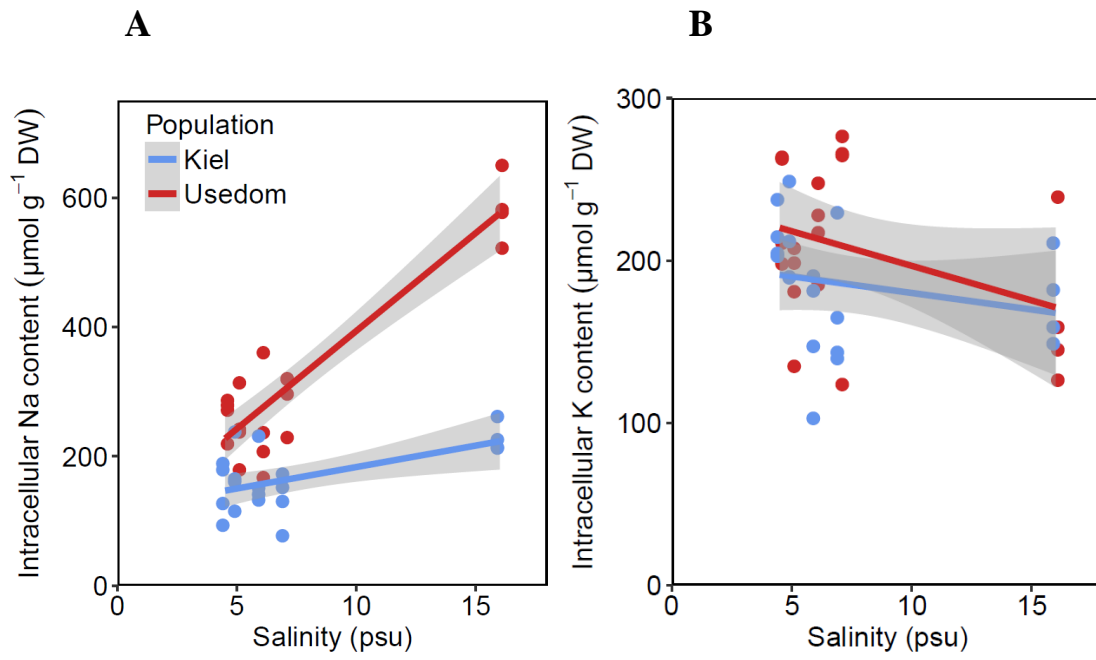


Figure 5.



Supplementary material

Table S1 Recipe for iso-osmotic solutions used for rinsing tissue samples to remove seawater before osmolyte and cation analyses. pH was adjusted using 5M NaOH and analysis of solutions using flame photometry revealed $[\text{Na}^+]$ and $[\text{K}^+]$ were negligible.

Salinity (psu)	Choline chloride		pH _{NBS}	Osmolality (mOsm/kg)
	concentration (mM)			
16	275		8.0	507
7	120		8.0	231
6	102		8.0	189
5	85		8.0	157
4.5	76		8.0	140

Figure S1

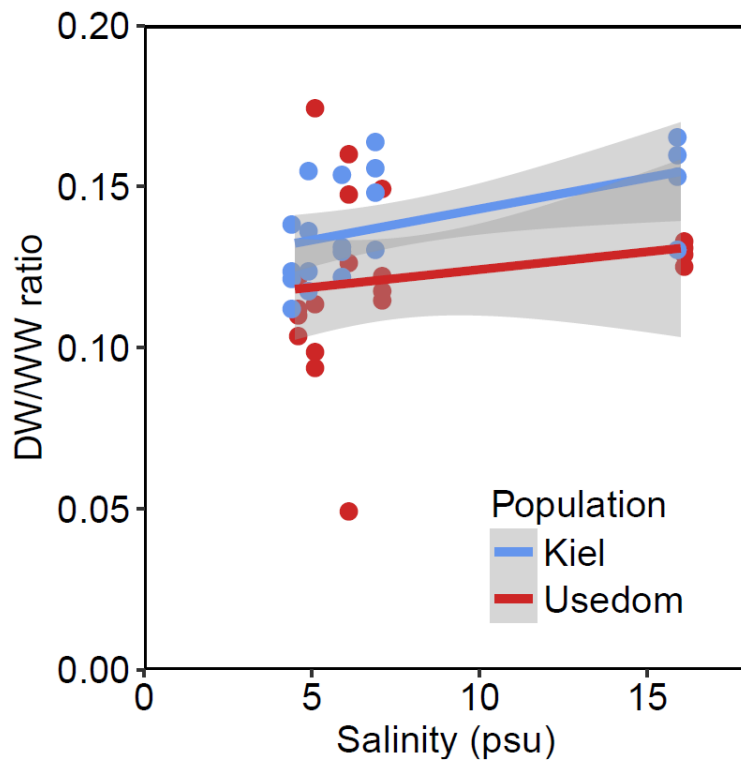


Fig. S1 Changes in mantle tissue dry weight-wet weight ratios with salinity and between populations. Grey shaded areas represent standard error around the linear regressions.

Table S2 Results of statistical analyses on experimental parameters analysed through ANOVA and ANCOVA.

Mortality ANOVA					
factor	df	Sum Sq	Mean Sq	F value	P value
salinity	4	3.032	0.758	4.874	0.004
population	1	1.156	1.156	7.434	0.011
population*salinity	4	5.172	1.293	8.314	<0.001
Residuals	30	4.665	0.156		
				Tukey post-hoc	
				Salinity	p-value
				5-4.5	1.000
				6-4.5	0.480
				7-4.5	0.056
				16-4.5	0.027
				6-5	0.371
				7-5	0.036
				16-5	0.017
				7-6	0.749
				16-6	0.557
				16-7	0.998

Osmolality ANCOVA					
factor	df	Sum Sq	Mean Sq	F value	P value
salinity	1	1082805	1082805	7068.3	<0.001
media	1	6	6	0.0395	0.843
salinity * media	1	20	20	0.1315	0.719
Residuals	76	11643	153		

ATP ANCOVA					
factor	df	Sum Sq	Mean Sq	F value	P value
salinity	4	0.51	0.13	1.91	0.14
population	1	0.02	0.02	0.26	0.61
population*salinity	4	0.65	0.16	2.46	0.07
Residuals	30	1.99	0.07		

Aspartate ANCOVA					
factor	df	Sum Sq	Mean Sq	F value	P value
salinity	4	13.34	3.34	31.516	<0.001
population	1	4.93	4.93	46.571	<0.001
population*salinity	4	0.36	0.09	0.859	0.5
Residuals	30	3.18	0.11		
Betaine ANCOVA					
factor	df	Sum Sq	Mean Sq	F value	P value
salinity	4	41.278	10.32	50.662	<0.001
population	1	10.54	10.54	51.757	<0.001
population*salinity	4	0.77	0.19	0.94	0.454
Residuals	30	6.11	0.20		
Glutamate ANCOVA					
factor	df	Sum Sq	Mean Sq	F value	P value
salinity	4	2.05	0.51	3.08	0.031
population	1	8.75	8.75	52.727	<0.001
population*salinity	4	0.48	0.12	0.718	0.5864
Residuals	30	4.98	0.17		
Glycine ANCOVA					
factor	df	Sum Sq	Mean Sq	F value	P value
salinity	4	55.15	13.79	163.839	<0.001
population	1	0.00	0.00	0.001	0.971
population*salinity	4	4.19	1.05	12.437	<0.001
Residuals	30	2.53	0.08		
Taurine ANCOVA					
factor	df	Sum Sq	Mean Sq	F value	P value
salinity	4	21.53	5.38	31.661	<0.001
population	1	41.22	41.22	242.494	<0.001
population*salinity	4	0.84	0.21	1.23	0.319
Residuals	30	5.10	0.17		

Lysine ANCOVA					
factor	df	Sum Sq	Mean Sq	F value	P value
salinity	4	2.75	0.69	2.911	0.038
population	1	12.56	12.56	53.205	<0.001
population*salinity	4	1.27	0.32	1.348	0.275
Residuals	30	7.08	0.24		
Alanine ANCOVA					
factor	df	Sum Sq	Mean Sq	F value	P value
salinity	4	9.29	2.32	18.114	<0.001
population	1	18.91	18.91	147.427	<0.001
population*salinity	4	0.57	0.14	1.111	0.37
Residuals	30	3.85	0.13		
Proline ANCOVA					
factor	df	Sum Sq	Mean Sq	F value	P value
salinity	4	1.33	0.33	3.211	0.026
population	1	4.07	4.08	39.34	<0.001
population*salinity	4	0.32	0.08	0.773	0.551
Residuals	30	3.11	0.10		
Total OO content ANCOVA					
factor	df	Sum Sq	Mean Sq	F value	P value
salinity	4	18.21	4.55	71.775	<0.001
population	1	7.08	7.08	111.629	<0.001
population*salinity	4	0.76	0.19	2.98	0.035
Residuals	30	1.90	0.06		
Na ANCOVA					
factor	df	Sum Sq	Mean Sq	F value	P value
salinity	4	4.76	1.19	37.109	<0.001
population	1	1.39	1.39	43.251	<0.001
population*salinity	4	0.30	0.08	2.325	0.079
Residuals	30	0.96	0.03		
K ANCOVA					

Chapter 4 – Mechanisms of salinity adaptation

factor	df	Sum Sq	Mean Sq	F value	P value
salinity	4	0.10	0.03	0.596	0.668
population	1	0.02	0.02	0.55	0.464
population*salinity	4	0.44	0.11	2.53	0.061
Residuals	30	1.30	0.04		
Total cation content ANCOVA					
factor	df	Sum Sq	Mean Sq	F value	P value
salinity	4	1.93	0.48	35.894	<0.001
population	1	0.17	0.17	12.778	0.001
population*salinity	4	0.19	0.05	3.607	0.016
Residuals	30	0.40	0.01		

3. Discussion

The first chapter of this thesis investigated whether salinity or calcification substrate limitation is responsible for reduced calcification rates in Baltic *Mytilus* at low salinity. It was found that Ca^{2+} availability reduces calcification rates and shell growth rates below $4 \text{ mmol Ca}^{2+} \text{ kg}^{-1}$ corresponding to salinities of ~ 11 psu. C_T availability was found to impede calcification rates only below $\sim 900 \text{ } \mu\text{mol HCO}_3^- \text{ kg}^{-1}$. Field monitoring revealed this is well below natural mean HCO_3^- concentrations of $1661\text{-}1871 \text{ } \mu\text{mol kg}^{-1}$ found at the three monitoring sites. Field monitoring further brought to light the high coastal A_T compared to published Baltic S- A_T relationships which suggests a high seawater buffering capacity to pH change despite the extremely low salinities. Field monitoring further highlighted the potential influences of food availability and environmental variability in governing calcification variability along the Baltic Sea salinity gradient.

The second chapter in this thesis aimed to estimate the energetic costs of calcification and investigate whether increased calcification costs at low salinity ultimately limit growth in Baltic *Mytilus*. Energy budgets for three populations of juvenile mussels adapted to different salinity regimes were calculated in laboratory experiments and the costs of calcification were found to constitute 30 – 60 % of available growth energy in Baltic *Mytilus*. It was also discovered that low salinity and temperatures synergistically act to increase the energetic costs of calcification by ~ 2 – 3-fold compared to warmer, saltier conditions. These findings suggest calcification is an energetically expensive process at low salinities and likely limits growth rates in Baltic *Mytilus* at low salinities.

The third chapter of this thesis further investigated physiological disparities between different *Mytilus* populations originating from different salinities, by investigating if salinity drives local adaptation and genetic divergence in low salinity *M. trossulus* populations. A larval selection experiment on a genetically diverse population demonstrated shifts in allele frequencies towards *M. trossulus* genotypes after selection by simulating desalination. These findings were complimented by evidence of local adaptation in *M. trossulus*-like genotypes demonstrated by higher survival and settlement success at low salinities compared to high salinity *M. edulis* genotypes.

The fourth and final chapter in this thesis investigated whether local adaptation of low salinity Baltic *Mytilus* populations is facilitated by adaptive changes in intracellular osmolyte pools. A population comparison of osmolyte changes in response to salinity revealed that low

populations adapted to low salinity have substantially reduced intracellular taurine content and subsequently overall organic osmolyte content, compared to high salinity adapted populations. This was accompanied by higher intracellular cation content and better tolerance to low salinity. This suggests that these cellular changes are adaptive and may underly the mechanisms of adaptation to extremely low salinities.

3.1 Salinity impacts calcification

3.1.1 Calcification substrate limitation

It has been shown by multiple studies that low salinity reduces calcification rates in marine bivalve juveniles and adults (Almada-Villela 1984; Malone & Dodd 1967; Dickinson et al. 2013). In many of these cases, decreases in growth rates have been attributed either to increased metabolic costs associated with osmotic stress (Dickinson et al. 2013) or metabolic depression (Hutchinson & Hawkins 1992) due to exposure to conditions outside the organism's plasticity window. In the case of Baltic Sea mussels, populations thrive at extremely low salinities showing high reproductive output (Kautsky 1982) and extensive benthic biomass (Norling & Kautsky 2008). There is also no evidence that metabolic rates are altered between different salinity adapted populations in the Baltic Sea thus suggesting local adaptation to low salinity environments (Tedengren et al. 1990; Landes et al. 2015; Maar et al. 2015). Consequently, the drastically reduced growth and calcification rates in Baltic mussels likely result from reduced efficiency of growth and/or calcification. Decreased growth efficiencies have been proposed by Tedengren & Kautsky 1986 caused by sub-optimal protein turnover rates, however, Riisgård et al. 2014 observed that body tissue growth rates are comparable between 25 and 7 psu acclimated animals suggesting limited calcification might be responsible for the reduced growth rates of Baltic *Mytilus*. The findings in chapter one demonstrate that calcium limitation (at least down to 0.5 mmol Ca²⁺ kg⁻¹) does not induce significant mortality in the time frame of this experiment, but does reduce calcification rates below 4 mmol Ca²⁺ kg⁻¹ seawater, in juvenile Baltic *Mytilus*. Similar results were found in larval *Mytilus* where [Ca²⁺] limited shell growth < 3 mmol kg⁻¹ (Thomsen et al. 2018). These results suggest limiting [Ca²⁺] occurs at salinities of ~ 11 psu which agrees with findings with previous work and those in chapter two where calcification rates appear to slow rapidly below 11 psu (Kossak 2006). Due to high mass specific rates of calcification, calcium limitation may be more of an issue in smaller life stages (larvae and post-settlement juveniles) (Thomsen et al. 2015). Calcification has also been found to be more negatively impacted during post-moult calcification in the blue crab *Callinectes*

sapidus, when calcification rates are highest (Cameron 1985). This highlights the importance of small life stages as potential bottlenecks when investigating the effects of environmentally limiting conditions on calcification.

In seawater, the majority (> 99.7 %) of dissolved inorganic carbon is present in three forms: CO_2 , HCO_3^- and CO_3^{2-} with 90 % being present as HCO_3^- (Zeebe & Wolf-Gladrow 2001). Together, both HCO_3^- and CO_3^{2-} carbon species make up the majority of marine A_T and are the primary source of inorganic carbon for calcification in marine calcifiers (McConnaughey & Gillikin 2008; Williams et al. 2009; Beldowski et al. 2010). The availability of inorganic carbon in seawater can therefore be altered in 2 ways: 1) Shifting pH (for example from atmospheric CO_2 absorption) which alters the relative concentrations of HCO_3^- and CO_3^{2-} (Fig 3.1) or 2) changes in A_T resulting from salinity fluctuations which alter the absolute availability of C_T (Lee et al. 2006). In this thesis inorganic carbon availability is expressed in terms of $[\text{HCO}_3^-]$ as this carbon species comprises > 90 % of C_T (Fig. 3.1) and is thus likely to be the main carbon source for calcification (Bach 2015; Thomsen et al. 2015). Additionally, CO_3^{2-} transporters have not been definitively observed in marine invertebrate calcifying epithelia, further suggesting HCO_3^- to be the primary carbon source for calcification. By manipulating only C_T

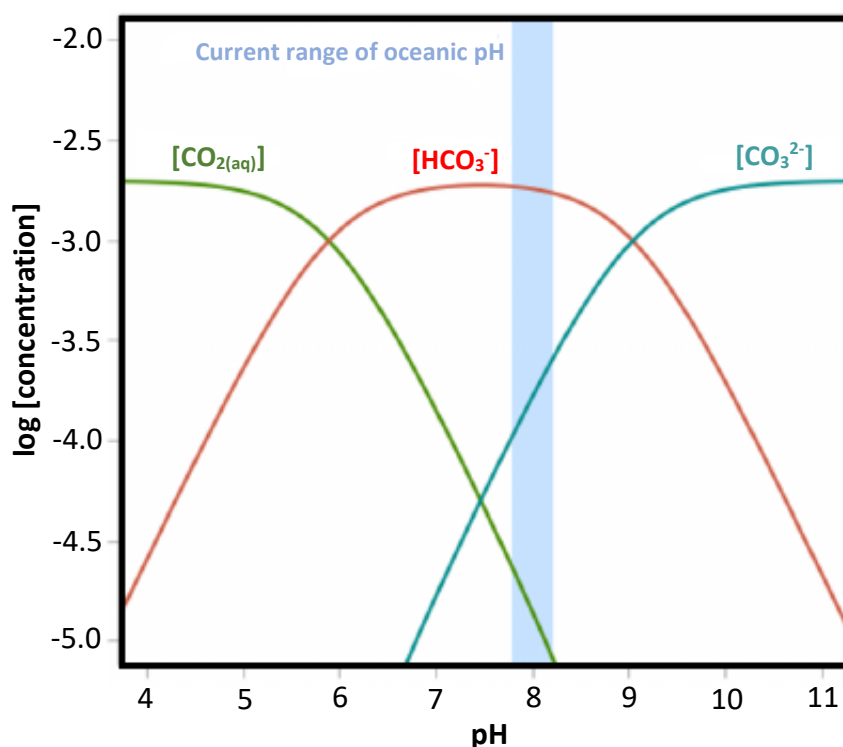


Fig. 3.1 The Bjerrum plot showing the relationship between $[\text{CO}_{2(\text{aq})}]$ in green, $[\text{HCO}_3^-]$ in red and $[\text{CO}_3^{2-}]$ in turquoise. The blue bar represents the range of current oceanic pH. It is evident from this plot that when pH decreases in seawater, $[\text{CO}_3^{2-}]$ decreases and $[\text{HCO}_3^-]$ initially increases. Figure is adjusted from Heinze et al. 2015 and was developed according to equations reviewed by Zeebe & Wolf-Gladrow 2001.

(and thus $[\text{HCO}_3^-]$) and keeping pCO_2 stable, it was found that $[\text{HCO}_3^-]$ impeded shell growth below $\sim 900 \mu\text{mol kg}^{-1}$, which is a similar threshold found in *Mytilus* larvae by Thomsen et al. 2015. There are 2 proposed mechanisms for reduced calcification rates with changes in carbonate chemistry parameters. The first is that decreasing pH or A_T reduces $[\text{CO}_3^{2-}]$ and thus, reducing the calcium carbonate saturation state (Ω) to below 1, subsequently imposing a kinetic constraint on calcification (Walsbusser et al. 2014; Waldbusser et al. 2016). These authors argue that calcification becomes impeded when $\Omega < 1$ due to kinetic constraints on CaCO_3 mineralisation and therefore, calcification becomes more energy demanding. This suggests, providing that more energy can be supplied, calcification rate can be maintained. However, some organisms (non-feeding marine bivalve trochophore larvae for example) do not have the ability to increase energy supply due to limited maternal energy reserves and are therefore sensitive to $\Omega > 1$. The second possible mechanism is that a sub-optimal substrate inhibitor ratio (SIR), which is the ratio of calcification substrate (HCO_3^-) to calcification inhibitor (H^+), causes reduced calcification rates at low pH and/or C_T (Jokiel 2013; Bach 2015; Thomsen et al. 2015; Cyronak et al. 2016). These authors argue calcification is a biological process and is related to the physiological conditions and ion concentration gradients within an organism calcifying tissue and space. This argument is based on the fact that animals can actively adjust the carbonate chemistry at the site of calcification (Venn et al. 2013; Ramesh et al. 2018) and that living organisms' shells are not a disorganised block of CaCO_3 , but biologically controlled and organised crystalline structures composed of organic materials capable of (to a certain extent) withstanding theoretically corrosive seawater chemistry. Evidence exists supporting both of these possible mechanisms however, definite answers are difficult to achieve due to the linear correlation between SIR and Ω in seawater. As calcium limitation was observed to impact calcification at low salinities (chapter one) the SIR can be extended to include $[\text{Ca}^{2+}]$ as a second substrate (chapter one: methods, Thomsen et al. 2018) which explains a higher percentage of the variance in calcification rate compared to Ω (chapter one: Fig S3). Although this does not indicate a definitive mechanism, it suggests SIR may be a better predictor of calcification rate in juvenile Baltic mytilid mussels.

3.1.2 Costs of calcification

In chapter two, calculations estimate the energetic cost of calcification to be 10 – 14 J mg CaCO_3 at 16 and 11 psu and 17 – 55 J mg CaCO_3 at 6 psu. Combined with the results from chapter one these findings suggest low calcification rates below 11 psu may result from high costs of calcification. Findings in chapter two also demonstrate that calcification demands a

considerable proportion of assimilated energy (30 – 60 %), highlighting it as a major energy consuming process in low salinity Baltic *Mytilus*. Earlier estimates for the calcification costs in marine gastropods suggest calcification costs to be 1 – 2 J mg CaCO₃ (Palmer 1992), ~ 10-fold lower than the estimates presented here. Previous estimates of the energy allocated to calcification in bivalves and gastropods (as a proportion of the total available energy), put this value at < 10 % (Watson et al. 2017), based on the published costs of calcification by Palmer 1992. These discrepancies may be attributed to methodological inaccuracies in Palmer's 1992 study and more accurate analyses presented here. Firstly, dry weight of both food, body tissue mass and shell mass were directly measured in chapter two, whereas these parameters were only estimated in Palmer 1992's study using pre-calculated linear regressions on barnacle size-mass relationships and indirect measurements of shell mass. Secondly, linear regressions of energy available for calcification vs calcification rate in chapter two exhibited mean R² values of 0.685 (± 0.206 standard deviation) while these linear relationships in Palmer's 1992 data displayed an R² value of 0.165 signifying huge variation in the data. Thirdly, the data used for calculating calcification costs in Palmer's 1992 study was obtained from three separate experiments involving a mix of populations, food sources and field and laboratory experiments. These factors likely contribute to variations in metabolic rates and may explain the variation of Palmers data, limiting the robustness of those estimates. For these reasons, it can be argued that the estimates presented in this thesis possess more rigour and are likely to be more accurate estimates of calcification in marine molluscs.

Of course, these disagreements between chapter two and Palmers 1992 study can also be attributed to ecological factors such as different experimental organisms (gastropods vs bivalves) or different salinities. All salinity treatments in chapter two (16, 11 and 6 psu) were well below fully marine salinities and Baltic *Mytilus* shells are thinner than marine populations even at Kiel salinities (Kautsky et al. 1990; Fig 3.2). Therefore, calcification at 16 psu in Kiel mussels may already be energetically challenged compared to fully marine salinities and this might explain the higher estimates of calcification costs compared to Palmer 1992. If the experiment in chapter two were to be repeated using a fully marine population of *M. edulis*, estimates of calcification costs may be lower and more in line with those of Palmer 1992. In spite of this, the estimates of calcification (on a per unit basis) presented in this thesis are still lower than the costs of organic soft tissue growth, in agreement with other studies (Palmer 1983; Anthony et al. 2002; Spalding et al. 2017). However due to the major energy investment

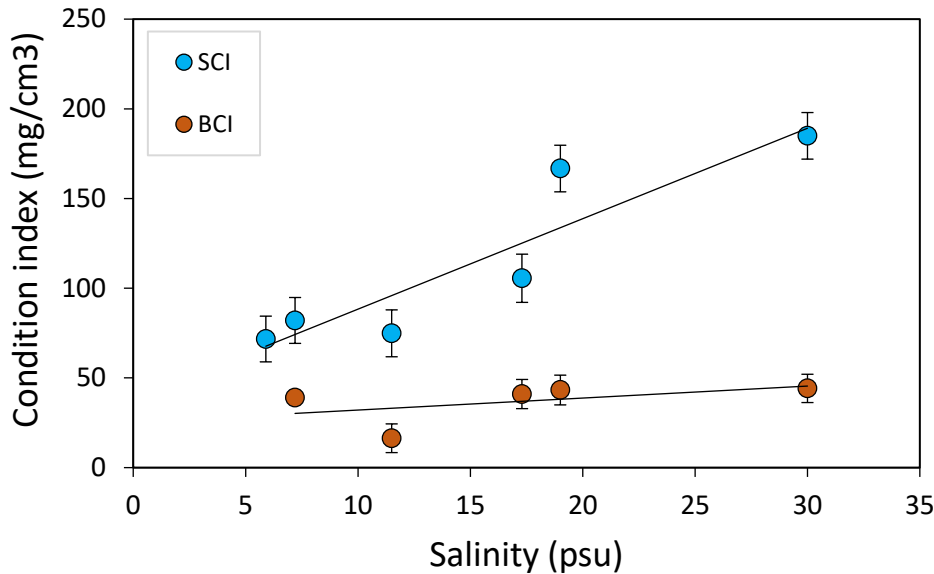


Fig. 3.2 Condition indices of 6 sub-tidal populations of *Mytilus* (mean shell length 31 ± 0.3 mm) along the Baltic Sea salinity gradient from the North Sea (Sylt, 30 psu), the Danish Belt Seas (Kerteminde, 19 psu), Kiel (17 psu), Ahrenshoop (11 psu), Usedom (7 psu) and the Swedish East coast (Nynashämn, 6 psu) sampled in July 2016. Shell condition index (SCI and body condition index (BCI) were calculated by: dry weight (shell or body tissue / maximum shell length³). Mean values are shown ($n = 20$) \pm standard deviation (Sanders unpublished data).

into calcification presented in chapter two (30 – 60 %), it is likely that this high energy demand is still a limiting factor in growth of bivalves at low salinities and potentially under other stressful environmental conditions.

The estimates for calcification costs in chapter two are based fixed conversion values of mass to energy and oxygen to energy across all treatments (Gnaiger & Bittrlich 1984). However, it could be possible that these conversions may in fact change with salinity and temperature. Low salinity adapted Baltic *Mytilus* have been shown to have ~ 3 x higher nitrogen excretion rates than North Sea populations suggesting higher rates of protein metabolism and potential lower protein turnover rates (Tedengren & Kautsky 1986). Extreme changes in protein turnover efficiency with environmental conditions have been documented in mytilid mussels with these changes having significant impacts on growth efficiencies and growth rates (Hawkins et al. 1986; Hawkins & Day 1996; Bayne & Hawkins 1997). It is therefore possible that salinity may have altered protein turnover efficiencies, resulting in different rates of protein accumulation and soft tissue growth efficiencies between salinity and temperature treatments. However, as mentioned previously, soft tissue growth rates in Baltic *Mytilus* have been shown not to be impacted by salinity (Riisgård et al. 2014) and any potential increased costs of soft tissue growth would be represented in the energy allocation pie chart (Chapter two: Fig. 4) as unaccounted for energy. Further work should aim to quantify changes in protein turnover

Discussion

efficiency and the impact of this on tissue growth rates as these factors have major implications for energy budget estimations.

The results from chapter two indicate that costs of calcification increased significantly at low salinities and temperatures in seawater conditions where $\Omega_{\text{aragonite}} < 1$. These increased costs could be attributed to higher energetic costs of mineral formation due to kinetic constraints in CaCO_3 mineralisation. Non-larval bivalves are poor at regulating the ion and acid-base composition of their extracellular fluids with respect to seawater conditions and pCO_2 in the extra pallial fluid (EPF) at the site of calcification is even higher than external seawater due to metabolic CO_2 excretion (Heinemann et al. 2012). Therefore, EPF Ω is lower than that of seawater, thus putting the internal shell surfaces more at risk to dissolution than external surfaces (Melzner et al. 2011). Interestingly, the 11 psu treatments in the chapter two experiment experienced $\Omega_{\text{aragonite}} < 1$ as well as the 6 psu treatment, despite no increased calcification costs and comparable calcification rates with the 16 psu treatment (where $\Omega_{\text{aragonite}} > 1$). This suggests that increased calcification costs at low salinity may not only be a product of high mineralisation costs when $\Omega < 1$, and other factors may be responsible for dramatic decreases in calcification rates at 6 psu.

Low calcification rates at 6 psu documented in chapter two agree with the findings from chapter one. Calcification rates were comparable between 16 and 11 psu in both experiments but significantly reduced only at 6 psu. The results from chapter one reveal this is likely due to limited availability of Ca^{2+} rather than C_T . Therefore, increased costs may arise from a larger energy investment into intracellular Ca^{2+} mobilisation either via plasma membrane Ca^{2+} -ATPases (PMCA's) or vesicular transport of calcium to the site of calcification (Carré et al. 2006). Although PMCA's have been identified in the calcifying epithelia of coral species (Zoccola et al. 2004; Barrott et al. 2015), and recently in bivalves (Wang et al. 2008), their active role in calcification is still not clearly demonstrated. Calcification rates may also be impeded by low $[\text{Ca}^{2+}]$ due to reduced paracellular supply of Ca^{2+} through reduced transmembrane Ca^{2+} gradients (McConnaughey & Whelan 1997). Reduced $[\text{Ca}^{2+}]$ and $[\text{CO}_3^{2-}]$ resulting from low salinity, lowers the SIR at the site of calcification and therefore more energy may be required to maintain a supply of calcification substrate to the site of calcification. Costs of calcification substrate uptake likely stem from Na^+/K^+ -ATPase (NKA) activity as these ATPases create a transmembrane Na^+ ion gradient which drives the uptake of HCO_3^- ($\text{Na}^+/\text{HCO}_3^-$ -co-transporters or Na^+ -driven $\text{Cl}^-/\text{HCO}_3^-$ -exchangers) and extrusion of H^+ (Na^+/H^+ exchangers - NHE's), both of which are important processes in calcification (Boron 2004;

Zoccola et al. 2015; Ip et al. 2015). Cellular costs of NKA activity have been shown to constitute a considerable proportion of cellular and organismic energy budgets (Rolfe & Brown 1997; Pan et al. 2015). Further physiological work is needed to investigate potential changes in ion transport activity in bivalve calcifying tissue in relation to salinity to understand the source of decreased calcification rates and increased calcification cost at low salinities.

3.2 Local adaptation to low salinity

3.2.1 Salinity and selection

The selection experiments in chapter three demonstrate for the first time the role of salinity in natural selection for *M. trossulus* genotypes in the Baltic Sea. It is also unveiled that these *M. trossulus* genotypes are locally adapted to low salinities suggesting the extreme salinities in the Baltic Sea drive both species segregation and subsequent physiological adaptation. Both field and laboratory experiments have previously shown that salinity is a powerful force in shaping *Mytilus* allozyme frequencies and adults at several nuclear loci (Beaumont et al. 1988; Johannesson et al. 1990). Population analyses along salinity gradients has also revealed strong correlations between salinity and allozyme frequency changes at the *Lap* locus, even over relatively short geographic distances (Gardner & Kathiravetpillai 1997; Gardner & Palmer 1998). This suggests salinity must be a strong selective force to maintain such genetic gradients in the presence of high gene flow. The enzymatic product of the *Lap* locus (an aminopeptidase involved in amino acid metabolism and turnover) is potentially involved in cell volume regulation and intracellular organic osmolyte adjustment in relation to salinity changes suggesting a possible physiological mechanism behind this selection (Koehn et al. 1980a; 1980b). This agrees with findings by Tedengren & Kautsky 1986 suggesting changes in amino acid metabolism at low salinity and the findings in chapter four regarding changes in intracellular free amino acid (FAA) pools with low salinity adaptation. However, it is almost impossible to infer a physiological mechanism behind salinity driven selection based on allozymes or a handful of genetic markers such as those utilised in chapter three. Although these findings clearly show the power of salinity as a selective force for certain genotypes, further work should utilise a genome wide approach to identify the targets of selection. This will enable more detailed physiological mechanisms to be linked to selection and provide insight into the environmental factors driving evolution.

In the Baltic Sea, selective forces are likely drivers of the genetic clines in alleles frequencies between both *M. edulis*-like and *M. trossulus*-like genotypes (Stuckas et al. 2017) and *Macoma*

balthica balthica and *M. balthica rubra* (Luttikhuisen et al. 2012). In Baltic *Mytilus* populations, it is possible that this selection for *M. trossulus* alleles, demonstrated in chapter three, results from better tolerance of *M. trossulus* to low salinities. Field studies have revealed that *M. trossulus* inhabits lower salinities than both *M. edulis* and *M. galloprovincialis* (Ridgeway & Nævdal 2004; Braby & Somero 2005). Laboratory experiments have also shown that *M. trossulus* larvae perform better at lower salinities than *M. edulis* (Qiu et al. 2002). Comparing proteomes of *M. trossulus* to the more stenohaline, high salinity *M. galloprovincialis*, Tomanek et al. 2012 suggest the better low salinity tolerance of *M. trossulus* results from differences in energy and protein metabolism during exposure to low salinity. This suggests a pre-disposition to low salinity tolerance in *M. trossulus* and may explain initial colonisation of the low saline Baltic Sea and the apparent local adaptation of Baltic *M. trossulus*-like genotypes to low salinity.

The experiment in chapter three revealed strong selection by low salinity during the larval stage of Baltic *Mytilus*. This is contradictory to findings by Beaumont et al. 1988a and 1988b where selection by salinity was found to be weak or non-existent during the larval phase and was strongest post-settlement. Other studies have found different allele frequencies between juveniles and adults in wild populations of *M. galloprovincialis* and *M. edulis* at different habitat salinities, suggesting selection by salinity also occurs in adults (Koehn et al. 1975; Gardner & Palmer 1998). The selection for *M. trossulus* alleles in larvae from chapter three likely results from a strong selection pressure against *M. edulis* alleles even in the larval phase, due to physiological stress of extremely low salinities. This selection is complimented by the high mortality (40 – 70 %) observed in adult *M. edulis* at salinities below 6 psu in chapter four. This higher tolerance coupled with low predator abundance likely allowed extensive colonisation of *M. trossulus*-like individuals in the low saline parts of the Baltic Sea. Further work should build on the findings presented in this thesis and investigate if other environmental stressors (warming, pH) may be powerful selective forces driving genetic divergence and local adaptation in other coastal habitats.

3.2.2 Shell phenotypes

The thinner shells of low salinity *M. trossulus*-like populations compared to high salinity *M. edulis*-like populations (Fig 3.2) can be attributed to either genetic differences, environmental factors (e.g. salinity) or likely, a combination of both. Comparisons of pure *M. edulis* and *M. trossulus* from the Canadian Atlantic coast demonstrate that pure *M. trossulus* specimens

exhibit thinner, narrower and more rounded shells than pure *M. edulis*, being responsible for significantly weaker shells in *M. trossulus* (Penney et al. 2007). Similar genotype-phenotype relationships between *M. edulis* and *M. trossulus* have also been found in Scotland (Beaumont et al. 2008), suggesting genetics plays a strong role in dictating shell phenotypes in pure *Mytilus* populations. Comparisons of North Sea and Baltic Sea populations have found a weaker relationship between genotype and shell morphology, with environmental variability being linked to high intra-population variability in shell morphology (Breusing 2012). Work by Kossak 2006 noted that shell stability decreased linearly with salinity being almost 8-fold lower in 6 psu *Mytilus* populations compared to 33 psu populations. Laboratory experiments further revealed that these differences in shell strength result from environmental salinity rather than genotype with shell strength significantly reduced below 20 psu (Kossak 2006). These findings highlight the powerful effect of low salinity on shell phenotypes in the Baltic Sea. Teasing apart the contributions of genotype and environmental conditions to shell phenotypes is difficult due to strong relationships between environmental conditions and genotype frequencies (Comesaña et al. 1999; Ridgeway & Nævdal 2004; Braby & Somero 2005; Tam et al. 2014). The transplant experiments by Kautsky et al. 1990 reveal that low salinity Baltic *M. trossulus*-like individuals have thinner shells than North Sea *Mytilus edulis* populations even after 2 years transplanted to the North Sea suggesting a link between genotype and shell morphology in the Baltic Sea. However, comparisons of both Baltic *Mytilus* genotypes and pure *Mytilus* genotypes must be done with caution due to the high genetic divergence of Baltic *Mytilus* populations compared to North Sea and North American *Mytilus* populations (Stuckas et al. 2017).

Although salinity is likely a powerful environmental driver impacting shell phenotypes of Baltic Mytilids, predation can also have a significant impact on bivalve shell morphology and strength (Leonard et al. 1999). The foremost benthic predators of North Sea *Mytilus edulis* (the starfish *Asterias rubens* and the crab *Carcinus maenus*) are completely absent in the Central and Eastern Baltic below ~ 12 psu (Kautsky 1981; Casties et al. 2015). Predation experiments with *A. rubens* comparing North Sea and Baltic Sea *Mytilus* populations revealed that Baltic mussels are the preferred prey and are opened significantly faster by *A. rubens* than North Sea mussels (Kautsky et al. 1990; Norberg & Tedengren 1995). Crab predation pressure is potentially a strong enough selection force against thin shelled *M. trossulus*-like genotypes to maintain the dominance of *M. edulis* alleles at high salinities. It has also been observed that the posterior adductor muscle of Baltic *M. trossulus* populations is ~ 40 % smaller in mass than

Baltic *M. edulis* populations, putting them more at risk to predation by *A. rubens* (Kautsky et al. 1990). This would provide a potential explanation for why *M. trossulus* allele frequencies are low in the high salinity parts of the Baltic. Thin, slow growing shells may have been an adaptive advantage in the low saline Baltic Sea where calcification is energetically challenging, and benthic predation is substantially lower than at higher salinities, for example the North Sea. Alternatively, local adaptation to these conditions may have led to the observed shell phenotypes of Baltic *Mytilus* populations. The findings in chapter three demonstrate salinity as a powerful driver in shaping spatial patterns of Baltic *Mytilus* genotypes, however further work is needed to decipher a cause-effect relationship between shell morphology and population structure of *Mytilus* in the Baltic Sea.

3.2.3 Intracellular osmolytes

In chapter four, potential intracellular adaptations to low salinity were observed in *M. trossulus* originating from very low salinities. It was documented that low salinity *M. trossulus* populations exhibit significantly smaller total organic osmolyte pools than high salinity *M. edulis* populations. It was also observed that taurine is the dominant organic osmolyte in high salinity *M. edulis* populations making up 28 % of the organic osmolyte pool however, in low salinity *M. trossulus* populations, taurine constitutes only 9% of the total organic osmolyte pool. Therefore, lower levels of intracellular taurine content are likely responsible for the overall decrease in total intracellular organic osmolytes in *Mytilus* populations from low salinity. This has been demonstrated in *Macoma balthica* and *Mytilus* spp. by Kube et al. 2006 comparing marine populations to Baltic populations and by Hosoi et al. 2003 when exposing the Pacific oyster, *Crassostrea gigas*, to hypersaline stress for 72 hours. Similar results were also found in *Crassostrea virginica* in Chesapeake Bay by Pierce et al. 1992 where populations originating from low salinity exhibited 3-fold lower taurine concentrations than high saline populations. None of these studies however, directly compared osmolyte pool composition in different populations at the same salinity, meaning potential adaptive changes in osmolyte pools cannot be inferred. Chapter four provides the first experiments to empirically test population differences in osmolyte pools in bivalve populations originating from habitats with different salinity regimes and potentially identify a link between these changes and salinity tolerance.

Changes in taurine content with long term salinity exposure may reflect alterations in metabolic pathways involved in taurine production during adaptation to low salinity. Although the costs

and rates of taurine metabolism in molluscs are not well understood (see Welborn & Manahan 1995), this could signify a potential adaptation of metabolic pathways during low salinity adaptation. If *Mytilus* populations in the Baltic Sea are never exposed to salinities above 10 psu maintenance of metabolic pathways for high levels of taurine synthesis and transport would be inefficient, in energetic terms. There may be further potential trade-offs associated with decreased intracellular taurine concentrations. Work by Sokolov & Sokolova 2018 has demonstrated that taurine stimulates mitochondrial function and ATP production suggesting that decreased intracellular concentrations of taurine may negatively impact ATP turnover in cells. The direct and indirect roles of taurine in marine osmoconformers still remains unclear and further work should aim to identify metabolic pathways and physiological implications of intracellular changes in taurine concentrations.

Further results in chapter four revealed that intracellular cation concentrations also decrease with salinity, however relative changes with salinity were not as severe as with organic osmolyte content. As mentioned in the introduction, intracellular cations in bivalves are expelled upon short term exposure to a hyposaline medium, however, they return to near control levels after several days (Silva & Wright 1994). Cation concentrations and cellular homeostasis is thus maintained and intracellular osmolalities are adjusted to the external media by the accumulation of compatible organic osmolytes (Yancey 2005). Stable intracellular cation concentrations are important for maintaining optimum enzyme function for cellular biochemical pathways (Hochachka & Somero 2002). Studies show however, that long term acclimation to different salinities results in altered intracellular cation concentrations (Willmer 1978a; Berger & Kharazova 1997), in agreement with the findings presented in this thesis. This suggests that cellular function may be impeded by altered intracellular cation concentrations (Hochachka & Somero 2002). In marine osmoconformers, deviations from this optimum concentration results in severely impeded rates of biochemical processes for example rates of electron transport (Fig. 3.3). Reduced enzyme activities and glutamate oxidation in mitochondria have also been observed in *Crassostrea virginica* at low salinity which could potentially have negative impacts of metabolic efficiency and cellular energy budgets (Ballantyne & Moyes 1987a; Ballantyne & Berges 1991). The results in chapter four reveal that at the same salinities, Baltic *M. trossulus* from low salinity consistently exhibit higher intracellular cation content than high salinity *M. edulis*. As intracellular cation concentrations decrease with salinity, this may be an adaptive response for marine osmoconforming invertebrates living at low salinity in an effort to maintain optimum cellular function. This

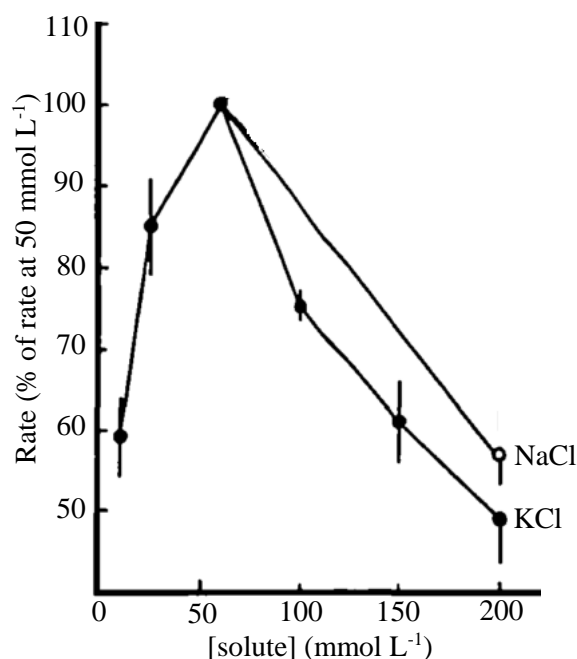


Fig. 3.3 Rates of electron transport in oyster (*Crassostrea virginica*) gill mitochondria, when exposed to different concentrations of $[K^+]$ (by adjusting KCL) and $[Na^+]$ (by adjusting NaCl). Rates of electron transport are expressed as a percentage of maximum rates observed at 50 mmol L⁻¹ solute concentrations. The figure as adapted from work by Ballantyne & Moyes 1978a.

implies that low salinity tolerance may be defined by a critical cation concentration and such an idea would support the better salinity tolerance observed in low salinity *M. trossulus* compared to high salinity *M. edulis* in chapters three and four. Indeed, other factors may also dictate salinity tolerance and hinder cellular biochemical processes under salinity stress such as reduced cellular osmolality (Ballantyne & Moyes 1987b) and critical organic osmolyte concentrations (proposed by Podbielski et al. 2016). Experimental work in the future should further investigate how salinity tolerance and local adaptation can be impacted by adaptive changes in cellular osmolyte pools by focusing on accurately measuring intracellular cation changes with salinity in different coastal marine invertebrates.

The experiments in chapter four also revealed that tissue water content of low salinity adapted *Mytilus* populations was higher than high saline populations, evident from lower dry weight/wet weight ratios of body tissue in the low salinity population (chapter four: Fig. S1). The water content of bivalve tissues is composed of both intracellular water and extracellular water (haemolymph) (Silva & Wright 1994). Therefore, these changes in tissue water content may result from either changes in intracellular water or extracellular water volume. Ion concentrations and organic osmolyte compositions of these water pools are drastically different (as haemolymph ion composition closely resembles that of seawater in marine

osmoconformers), therefore changes in the relative volume of both pools would significantly impact the calculation of intracellular cation content based on whole tissue measurements (see chapter four equation 1). Other studies have documented increased tissue water content upon exposure (weeks) to low salinities and have attributed this to increased intracellular water rather than extracellular water or haemolymph (Shumway 1977; Willmer 1978a; Gainey 1994; Neufeld & Wright 1996). Therefore, it may be that increased intracellular water is also be a factor explaining the results presented in this thesis. More dilute tissues in low salinity adapted populations of Baltic *Mytilus* may also simply be a result of reduced intracellular concentrations of FAA's and other molecularly heavy organic osmolytes, which would decrease tissue dry weight/wet weight ratios (chapter four, Fig. S1). Tissue water content changes should be investigated further by quantifying extracellular water in multiple tissues of different salinity adapted populations of osmoconforming marine invertebrates.

3.2.4 Energy expenditure and physiology

Adjustments of intracellular cation concentrations result from changes in ion regulation activity. As mentioned previously (6.1.2 Costs of calcification), the membrane bound NKA is vital in maintaining cellular gradients of Na^+ (Castillo et al. 2015) and demands a considerable amount (19 – 28 %) of available cellular metabolic energy (Leong & Manahan 1997; Rolfe & Brown 1997; Pan et al. 2015). It has been demonstrated that osmoregulating organisms such as decapod crustaceans and some species of bivalve mollusc increase their NKA activities as low salinities so that transmembrane ion gradients can be maintained in the face of cellular osmotic stress (Henry & Mangum 1980; Huong et al. 2010). However, since osmoconforming marine invertebrates do not regulate extracellular ion composition, it would be expected that NKA activities are not impacted by salinity in marine osmoconformers. Despite this, acclimation to low salinity has also been observed to induce an increase in NKA activity in osmoconforming bivalves such as *Mytilus edulis* and in *Perna perna* (Willmer 1978b; Rola et al. 2017). This may also be a physiological response employed by low salinity populations of Baltic *Mytilus* and would theoretically generate a significant increase in cellular energy demand to supply ATP for the NKA. However, because low salinity does not impact oxygen consumption rates in Baltic *Mytilus*, (See results in chapter two, Landes et al 2015; Maar et al. 2015), increased energy allocation to ion regulation processes would need to be compensated for by metabolic adjustment of energy supply to other energy demanding processes. Changes in ion regulatory activity due to low salinity may also interfere directly with the ion transport mechanisms of calcification and negatively impact calcification rates. Together, a combination

of low SIR (limiting calcification) and increased ion regulatory costs may act synergistically and bring about the drastic reduction in calcification rates and increases in calcification costs below 11 psu (chapters one and two). Further work investigating NKA activity in marine calcifiers at low salinity, particularly in the calcifying tissue, is needed to understand how ion transport costs are impacted by low salinity.

It has been demonstrated that feeding rates, food absorption efficiency and Scope for Growth are comparable at marine salinities between Baltic (6 psu adapted) and Kattegat (25 psu) mussels (Tedengren et al. 1990). Similarly, feeding rates of low saline Baltic *Mytilus* acclimated to 25 psu have been observed to be comparable to Kattegat mussels at 25 psu (Riisgård et al. 2013). This highlights relatively high physiological plasticity of Baltic *Mytilus* to a range of salinities as evident by the low mortality rates of low saline Baltic *Mytilus* across a range of salinities, compared to Baltic *Mytilus* from high salinities. Interestingly, Baltic *Mytilus* from the Kattegat (25 psu) and Kiel (16 psu) are not able to acclimate their energy budgets and physiology to maintain the same levels of growth as low saline Baltic *Mytilus* populations at 6-7 psu (Tedengren et al. 1990; Riisgård et al. 2013; Chapters three and four). From these patterns, two conclusions may be drawn: 1) low salinity Baltic *M. trossulus*-like populations exhibit higher plasticity to salinity than high salinity *M. edulis*-like populations and 2) low salinity Baltic *M. trossulus*-like populations exhibit local adaptation to extremely low salinities, outside of the physiological plasticity range of *M. edulis*-like populations. Caution must be given when making these conclusions however, as the highest salinity treatments in chapters three and four were 16 psu and higher salinities may unveil potentially better performance of high salinity *M. edulis* populations compared to low salinity *M. trossulus*. This might suggest that low salinity *M. trossulus* genotypes do not necessarily have a larger plasticity window than *M. edulis*, but rather just a shifted one. Several studies have shown that low salinity adapted Baltic *M. trossulus*-like populations consistently exhibit higher ammonia excretion rates and lower O:N ratios than high salinity populations, across a range of acclimated salinities (Tedengren & Kautsky 1986; Tedengren et al. 1990; Gilek et al. 1992). This implies differences in nitrogen metabolism may result from genetic or adaptive differences, rather than physiological plasticity in response to changing salinity. Nitrogen excretion is intrinsically linked to protein turnover and efficiency which is, in turn, linked to growth rates and growth efficiency (Hawkins 1985; Hawkins & Day 1996). Evidence reveals that nitrogen excretion rates increase during initial exposure to low salinities due to cellular excretion of FAA's during hypo-osmotic adjustment. However, rates return to control levels after ~ 4 days at low salinity

suggesting this is only a short-term plastic response and not a long term physiological change (Livingstone et al. 1979). It is therefore curious that low salinity Baltic mussels apparently exhibit permanently high nitrogen excretion rates at constantly stable low salinities, as these changes may potentially have severely negative impacts on growth efficiency. Further work is needed to identify the potential adaptive advantages of increased nitrogen excretion rates at low salinities and the potential trade-offs of these differences with growth and fitness.

3.3 Habitat monitoring and environmental fluctuations

Compared to the global oceans, low pH, ocean warming, and environmental variation are already prominent in the Baltic Sea enabling it, in many cases, to also be used as a model system for global change in coastal oceans (Reusch et al. 2018). The presence of the salinity gradient in the south-western Baltic Sea also provides an excellent model system for investigating the effects of salinity and other correlating parameters on physiology, selection and adaptation. Habitat monitoring data from chapter one suggests that environmental factors such as food availability and A_T do not correlate linearly with the salinity gradient and exhibit high spatial heterogeneity. Food availability for example, was ~ 2-fold lower at Ahrenshoop (11 psu site) compared to the other monitoring sites and this low food availability may have contributed to the low calcification rates at this site and the poor body condition (Fig. 3.2). Interestingly, patterns of shell length growth in Baltic mussels across a range of salinities was found to be remarkably similar between field and laboratory experiments (Kossak 2006), suggesting abiotic factors other than salinity have little impact on calcification in Baltic mussels. It seems likely therefore that food availability is the likely explanation for the extremely high growth rates in Kiel fjord. This has been previously observed where extremely high particulate organic carbon (POC) availability in Kiel fjord accompanies high growth rates, despite extended periods of high seawater pCO_2 (Thomsen et al. 2013). Further biotic factors such as predator risk may also be important in future Baltic ecosystems. Invasive species, for example the crab *Rhithropanopeus harrisi* and the round goby *Neogobius melanostomus*, are becoming more prominent in the Baltic Sea and consume large quantities of Baltic *Mytilus* and have led to major shifts in trophic interactions (Nurkse et al. 2016; Kotta et al. 2017). In rapidly changing regions such as the Baltic Sea, it is important not to neglect the potentially major biotic changes (predator-prey interactions, food availability) associated with abiotic environmental changes.

Monitoring of carbonate chemistry at the three near-shore coastal sites in chapter one revealed that A_T and C_T are relatively high compared to published Baltic S- A_T relationships (Beldowski et al. 2010; Müller et al. 2016). These higher values ($\sim 150 \mu\text{mol kg}^{-1}$ higher than predicted by equations obtained by Beldowski et al. 2010) probably arise from riverine input or submarine groundwater discharge of terrestrially derived C_T (Kuliński et al. 2017) and are likely beneficial to calcifying Baltic mussels. High A_T increases the buffering capacity of seawater, limiting the impacts of increased atmospheric pCO_2 on seawater pH changes and partly protecting these near shore coastal habitats from high levels of acidification. This buffering ability has also been documented in Baltic coastal macrophyte meadows where photosynthetic activity has been observed to mediate the negative impacts of simulated ocean acidification in marine calcifying mussels (Wahl et al. 2018). There is also the influence of dissolved organic carbon (DOC) on Baltic A_T which has been demonstrated to constitute $\sim 2.5 \%$ of A_T and lead to errors in pCO_2 and pH calculations of $\sim 42 \%$ and 0.4 pH units, respectively (Kuliński et al. 2017). This multifactorial influence on Baltic Sea A_T coupled with predicted increases in atmospheric CO_2 and precipitation in the Baltic Sea catchment area, makes it difficult to accurately predict changes in Baltic Sea carbonate chemistry in near-shore coastal zones (Wahl et al. 2016). It is vital to ensure continued monitoring in southern Baltic sites to acquire a better understanding of how freshwater input influences the carbonate chemistry of near-shore habitats.

Environmental variability is also a major feature of near-shore coastal zones where multiple environmental parameters can vary significantly in conjunction with each other (Melzner et al. 2013). These variations can have synergistic or antagonistic effects on organisms particularly with regards to acidification and carbonate chemistry fluctuations (Waldbusser & Salisbury 2014). In the south-western Baltic Sea, wind-driven upwelling of anoxic, hypercapnic water can lead to abrupt changes in carbonate chemistry parameters over the course of days and hours (Thomsen et al. 2010; Melzner et al. 2013; Saderne et al. 2013). Additionally, wind driven Baltic inflow events of high salinity North Sea water can cause substantial increases in salinity lasting for weeks (Mohrholz et al. 2015). The cost of cell volume regulation during salinity fluctuations has been shown to be significant in mytilid mussels demanding more than 30 % of metabolic energy (Hawkins et al. 1992; Neufeld & Wright 1996). Salinity monitoring data in chapter one reveals large variations in salinity ($> 50 \%$) on monthly and weekly time scales at Kiel and Ahrenshoop which likely come with a significant energetic cost to osmoconformers. Marine organisms possess the ability to exhibit phenotypic plasticity in response to environmental change, however, maintaining this plastic ability likely comes with its own costs

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(often not energetically quantifiable) with potential impacts on fitness related traits such as growth and reproduction (DeWitt et al. 1998; Hofmann & Todgham 2010). It is therefore important to understand how the energetic costs of environmental fluctuations and the ecological costs of phenotypic plasticity interact and ultimately lead to an organisms' ability to cope with environmental change. This is particularly prevalent in coastal and nearshore habitats such as the Baltic Sea where local habitat conditions are more variable and less predictable using regional and global scale models.

4. Conclusions and future outlook

The results presented in this thesis demonstrate that extremely slow growth rates of Baltic *Mytilus* living at low salinities results from a limited ability to biomineralize CaCO_3 rather than increased energetic costs associated with osmotic stress. Reduced rates of calcification are severely impeded by low salinities primarily due to limited calcium availability. Seawater carbon limitation however, does not impede calcification until $\sim 900 \mu\text{mol kg}^{-1} [\text{HCO}_3^-]$ and therefore, seems unlikely to be a limiting factor in the Baltic due to high coastal C_T . The effect of Ca^{2+} limitation is considerably pronounced below salinities of ~ 11 psu which coincides with significantly higher energetic costs of CaCO_3 deposition. This suggests high costs of calcification at low salinity may stem from increased costs of ion transport to supply calcification substrate (Ca^{2+}) for calcification, or kinetic constraints on mineral formation resulting from undersaturated CaCO_3 conditions ($\Omega < 1$). More energetically expensive calcification can impact energy allocation to body tissue growth and subsequently result in negative impacts on reproductive output and fitness (Palmer 1981). With climate models predicting severe desalination in the Central Baltic Sea over the next 80 years, the distribution boundary of Baltic *Mytilus* will likely shift westwards by several hundred kilometres. Due to the integral role mytilid mussels play in Baltic benthic ecosystems, this distribution shift will likely be accompanied by severe changes to Baltic Sea ecosystems. Field monitoring data from this thesis suggests however, that modelling potential distribution changes of Baltic *Mytilus* must consider potential changes in salinity variation, carbonate chemistry and food availability, particularly in near-shore reefs, as these factors strongly influence calcification rates in bivalves.

This thesis also provides first evidence of local adaptation to low salinity in adult and larval Baltic mussels. This was demonstrated in larvae reared at 7 psu where low salinity adapted *M. trossulus* exhibited lower mortality and higher settlement success than high salinity adapted *M. edulis*. The same was observed in adults where *M. trossulus* genotypes exhibited lower mortality rates below 5 psu compared to *M. edulis* genotypes. Both of these results were complemented by strong selection against *M. edulis* alleles at low salinity in larval experiments, suggesting salinity is a strong selective force in shaping the Baltic *Mytilus* hybrid zone. These findings highlight the potential role of genetic variation and hybridisation in facilitating local adaptation to extreme environmental condition. A possible mechanism facilitating this local adaptation appears to be an increase in intracellular concentrations of cations (and anions) at low salinities. Enzyme activity and cellular metabolic rates exhibit optimum performance

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within a narrow $[K^+]$ and $[Na^+]$ window, thus suggesting low salinity tolerance may be determined by a critical intracellular ion concentration below which cellular function is detrimentally impeded. Therefore, increasing intracellular concentrations of cations is a likely physiological adaptation to increase tolerance to low salinity by preventing detrimental impacts of decreasing intracellular ion concentrations on cellular biochemical processes. These results demonstrate the ability of marine bivalves to adapt to extreme environmental salinities, through fundamental changes in their cellular biochemistry.

The findings presented in this thesis raise several further questions: **1)** what is responsible for increased calcification costs at low salinities? **2)** what physiological mechanisms facilitate local adaptation to other environmental factors (e.g. low $[Ca^{2+}]$)? To precisely answer these questions, more in depth investigation is required into cellular physiology and genome wide analysis. Further work should investigate the ion regulatory pathways utilised in molluscan calcification and quantify the potential impacts of low salinity on these processes. Experiments challenging calcification and quantifying changes in key ion transport pathways (e.g. Ca^{2+} transport) can reveal the cellular mechanisms utilised by molluscs during calcification. In this sense, salinity provides an excellent environmental parameter to investigate the mechanisms of calcification. Furthermore, future experiments investigating the impact of predicted climate change on marine organisms should utilise environmental gradients and naturally occurring locally adapted populations. Comparing the physiological responses of different populations to environmental stress, coupled with transcriptomic and genome wide analysis, can reveal the physiological mechanisms involved in the process of evolution to a multitude of environmental conditions (salinity, temperature, pH). These approaches will allow the processes and mechanisms of physiological adaptation to be understood and allow us to better estimate a species resilience or sensitivity to environmental change

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Eidesstattliche Erklärung

Hiermit Versichere ich, dass ich die vorliegende Arbeit mit dem Titel:

Bioenergetics of calcification in mytilid bivalves along the Baltic Sea salinity gradient

selbständig geschrieben und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Die vorliegende Arbeit entspricht den von der Deutschen Forschungsgemeinschaft erklärten Regeln guter wissenschaftlicher Praxis und wurde nicht schon an anderer Stelle im Rahmen eines Prüfungsverfahrens vorgelegt. Veröffentlichte Manuskripte sind dementsprechend gekennzeichnet. Ich stimme zu, dass diese Arbeit der Bibliothek des GEOMAR Helmholtz-Zentrum für Ozeanforschung Kiel sowie der Universitätsbibliothek der Christian-Albrechts-Universität zu Kiel zugänglich gemacht wird.

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