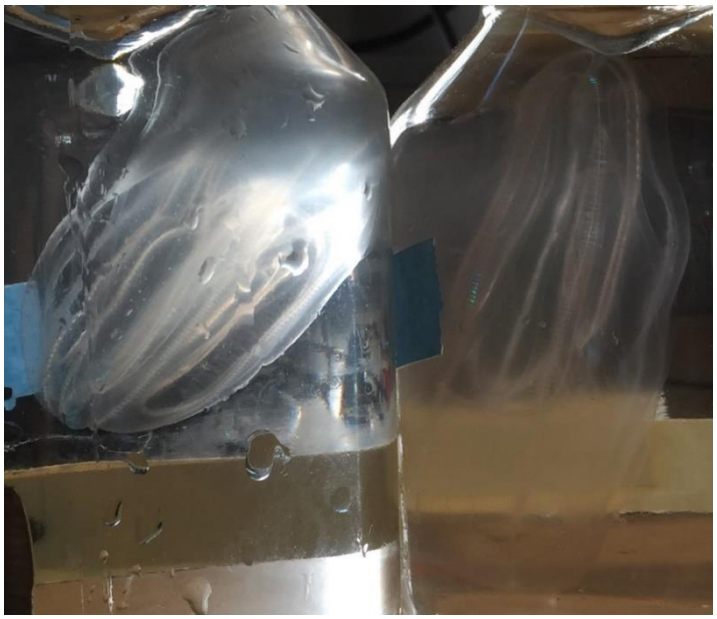
Respiration as a proxy for salinity stress in the invasive ctenophore *Mnemiopsis leidyi*: Do sub-populations display different adaptations to salinity stress?

Master Thesis

Alica Ohnesorge



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Academic Thesis: Declaration of Authorship

I, Alica Ohnesorge, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

“Respiration as a proxy for salinity stress in the invasive ctenophore *Mnemiopsis leidyi*: Do sub-populations display different adaptations to salinity stress?”

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Summary

Translocation of species outside their natural dispersal ranges are of concern for biodiversity and food web functioning. Even though a large number of species are transported around the world on a daily basis, introduction events rarely lead to the permanent establishment of non-native species in their new habitat. Irrespectively, the few non-native species which achieve high population densities, and, hence become invasive, may have a large ecosystem impact and are suggested to lead to a homogenization of the earth's biota. An example of a potent invasive species is the comb jelly *Mnemiopsis leidyi*. This comb jelly has been shown to successfully colonize a large variety of aquatic ecosystems with salinities ranging from 3 to 38. Regardless, the assemblages occupying various locations (=sub-populations) seem to differ in their salinity tolerance. For example, salinity has been shown to restrict the range expansion of some invasive sub-populations as shown for the northern invasive sub-populations in low saline areas of the Baltic Sea. In contrast, the southern invasive sub-population present in the Black Sea thrives at low salinities. This suggests that sub-populations do not tolerate the entire salinity window but are rather adapted to a narrower range. This leads to the question whether salinity tolerance is based on adaptations which are fixed to their respective habitat origins or if sub-populations from the northern invasive sub-population have the potential to acclimatize or adapt to the extreme low salinities of the Baltic Sea. We measured respiration rates and used it as a proxy for salinity stress investigating two different invasive sub-populations from high saline northern and low saline southern Eurasia. Animals were raised over two generations at their home salinity as well as at the contrasting salinity level of the respective other population. This led to two different selection salinities i.e. a high and a low salinity selection regime for both sub-populations. At the end of the second generation, respiration rates were measured at three different assay salinities with high, low and extremely low levels.

We found lowest respiration rates in treatments where animals did not experience a salinity change. At the extremely low salinity (7.5) tested, respiration rates were significantly enhanced, especially the northern invasive sub-population where parents were selected and kept at the original high salinity level of 27. For this treatment, respiration rates were 2.5 times higher when exposed to the extremely low assay salinity, compared to unstressed conditions. In contrast, Black Sea sub-populations show similar intermediate values at all salinity stress treatments. In addition, respiration rates of sub-populations also depended on

the salinity at which their parental lines were selected over two generations. Our results demonstrate that at a salinity of 7.5, individuals from the southern invasive sub-population have lower respiration rates than the northern invasive sub-populations. Thus, our results suggest southern invasive sub-populations are able to cope with low salinity stress much better compared to northern invasive sub-populations. This highlights the translocation of southern sub-populations to the low saline areas of northern Europe should be prevented by all means.

Eine Umsiedlung von Arten aus ihrem natürlichen Verbreitungsgebiet ist bedenklich für die Biodiversität und ein funktionierendes Nahrungsnetz. Obwohl täglich viele Arten um die Welt transportiert werden, geschieht es nur in seltenen Fällen, dass Einschleppungsereignisse zu einer dauerhaften Etablierung von nicht heimischen Arten in ein neues Habitat führen. Allerdings können die wenigen nicht heimischen Arten, die eine hohe Populationsdichte erreichen und als invasive gelten, einen großen Einfluss auf das Ökosystem haben und wirken potentiell in Richtung einer Homogenisierung der weltweiten Biota. Ein Beispiel für eine einflussreiche invasive Art ist die Rippenqualle *Mnemiopsis leidyi*, welche nachweislich ein großes Spektrum an aquatischen Ökosystemen mit Salinitäten von 3-38 erfolgreich besiedelt. Allerdings unterscheiden sich die Gruppen, die an verschiedenen Orten vorkommen (= Sub-Populationen) bezüglich ihrer Toleranz gegenüber Salinität. Wie beispielsweise nachweislich in Ostseegebieten mit niedriger Salinität gezeigt, kann Salinität die Ausbreitung einiger invasiver Sub-Populationen begrenzen. Im Gegensatz dazu gedeihen südlich invasive Sub-Populationen im Schwarzen Meer bei niedriger Salinität. Dies lässt darauf schließen, dass Sub-Populationen nicht das gesamte Salinitätsfenster tolerieren, sondern eher an einen kleineren Umfang angepasst sind. Dies wirft die Frage auf ob Salinitätstoleranz auf Adaptationen beruht, welche auf den jeweiligen Habitatsursprung festgelegt sind oder ob nördlich invasive Sub-Population das Potential besitzen, sich an extrem niedrige Salinitäten der Ostsee anzupassen. Wir bestimmten Respirationsraten als Proxy für Salinitätsstress von zwei verschiedenen invasiven Sub-Populationen aus dem hoch salinen nördlichen und niedrig salinen südlichen Eurasien. Tiere wurden über zwei Generationen auf ihrer jeweiligen Ursprungssälinität gehältert sowie auch auf dem

Salzgehalt der jeweils anderen Sub-Population. Dies ergab zwei verschiedene Selektions-Salinitäten – hoher und niedriger Salzgehalt für beide Sub-Populationen. Am Ende der zweiten Generation wurden Respirationsraten auf drei verschiedenen Test-Salinitäten gemessen (hoch, niedrig und extrem niedrig).

Die niedrigsten Respirationsraten fanden wir in den Versuchsgruppen, in denen Tiere keiner Veränderung der Salinität ausgesetzt waren. In der getesteten extrem niedrigen Salinität (7,5) waren Respirationsraten signifikant erhöht, besonders bei der nördlich invasiven Sub-Population mit Eltern welche auf der hohen Ausgangssalinität von 27 selektiert und gehalten wurden. Für diese Gruppe, getestet in der extrem niedrigen Salinität, waren Respirationsraten 2,5-mal höher als unter nicht-gestressten Bedingungen. Im Gegensatz dazu zeigten Sub-Populationen aus dem Schwarzen Meer mittlere Werte in allen getesteten Bedingungen mit Salinitätsstress. Des Weiteren waren die Respirationsraten von Sub-Populationen ebenfalls abhängig von der Salinität auf welcher die jeweiligen Eltern über zwei Generationen selektiert wurden.

Unsere Ergebnisse zeigen, dass bei einer Salinität von 7,5 Individuen der südlichen invasiven Sub-Population niedrigere Respirationsraten haben. Daher lassen unsere Ergebnisse darauf schließen, dass südlich invasive Sub-Populationen besser mit dem niedrigen Salzgehaltsstress zurechtkommen als nördlich invasive Sub-Populationen. Dies verdeutlicht, dass eine Umsiedlung der südlichen Sub-Population in die niedrig-salinen Gebiete Nordeuropas unter allen Umständen vermieden werden sollte.

I. Introduction- Non-indigenous species

Species establishing outside their natural habitat, which have a large ecosystem impact indeed, are steadily increasing (Seebens et al. 2017) apprehending concerns of biodiversity conservation and ecosystem functioning (Walsh et al. 2016). Non-indigenous (or non-native) species may alter the new, incorporating ecosystems where they appear to different extends, depending on their establishment success (Williamson & Fitter 1996). Thus, an imported species may get 'introduced' i.e. appears in its new habitat and establishes (Richardson et al. 2000). Eventually, during the process of establishment, colonization and naturalization these non-indigenous species reproduce and may establish self-sustaining populations that become part of the resident ecosystem and thus turn 'invasive' (Richardson et al. 2000). Since the terminology remained confusing due inconsistent usage terms mentioned above, Colautti & MacIsaac (2004) propose a new framework combining previous models and summarizes invasion stages. In detail, it distinguishes between non-indigenous species that are either A) 'non-invasive', which ought to be further subdivided into 'localized and numerically rare' (stage 3) and 'localized but dominant' (stage 4b) versus B) 'invasive' including 'widespread but rare' (stage 4a) and 'widespread and dominant' (stage 5). Thus, the term 'invasive' refers to spatial spread but does not to abundance (Colautti & MacIsaac 2004).

The term 'pest' refers to invasive species which appear in large quantities and have an ecosystem impact or negatively affect economy (Williamson & Fitter 1996). Anyways, the likeliness of an introduced species becoming an effective threat to the local ecosystem is rather small.

Each additional transformation from appearance to establishment, formation of self-sustaining populations and spread happens with an average likelihood of only about 10 % (Williamson & Fitter 1996). Thus, this phenomenon is followingly called the "Tens rule" (Williamson & Fitter 1996).

I.I. Determining factors for invasiveness

Many factors determine the success or failure of a non-indigenous species. Two filters are mainly responsible namely 'environment and community suitability' as well as 'local dispersal'. The latter implies propagule pressure (= the number of individuals plus the number of introduction events) (Colautti & MacIsaac 2004) which appears to be very crucial for the establishment success of a non-indigenous species (Williamson & Fitter 1996; Kolar & Lodge 2001; Colautti & MacIsaac 2004). On the other hand, the 'environment and community suitability' includes factors such as physio-chemical requirements of the species in question. Resource availability and community interaction also have the potential to prevent a non-indigenous species to successfully establish in its new environment (Colautti & MacIsaac 2004), while it has been suggested that areas of low species richness are particularly susceptible to successful establishment of non-indigenous species (Paavola et al. 2005).

Non-indigenous species which were able to tackle these challenges and managed to become invasive usually have certain life history strategies in common at least during a part of the invasion process. These characteristics can be summarized as r-selected life history-strategies in successful invasive species (Sakai et al. 2001). This r-selection includes traits related to high reproductive output (high fecundity, short generation time, early maturing), fast growth rates (Sakai et al. 2001; Willmer et al. 2005) and usually large environmental envelope which allows these generalists to deal with a large range of factors including abiotic stress and low environmental stability (Willmer et al. 2005; Solan & Whiteley 2016; Sakai et al. 2001).

I.I.I. The invader *Mnemiopsis leidyi* (A. Agassiz)

The gelatinous macrozooplanktonic comb jelly *Mnemiopsis leidyi* is a successful invasive species, to which most of the above-mentioned characteristics of a successful non-indigenous species apply. Additionally, this species is capable of reproduction in the larval stage (Martindale 1987) and is a simultaneous, self-fertilizing hermaphrodite (Jaspers et al. 2015) which allows it to circumvent the Allee effect (= fitness is positively correlated to population size/ density) potentially limiting non-indigenous species during an early colonization period (Courchamp et al. 2008).

Under favorable conditions the spherical cydippid larvae hatch in less than a day (Purcell et al. 2001) and can reach specific daily growth rates of > 0.8 (Reeve et al. 1989). The larvae gradually grow and start lobe formation at > 2 mm oral-aboral length (Jaspers et al. 2013). Depending on temperature they can reach adulthood within two to three weeks including daily egg production. 23-days old animals have been shown to have average reproduction rates of > 1000 eggs per 24 hours (Baker & Reeve 1974). Maximum per capita egg reproduction rates of more than 10,000 eggs per day have been reported (Jaspers et al. 2015; Baker & Reeve 1974).

The impact of feeding by *M. leidyi* may be very distinct. For instance, it was shown that the metabolic demand of the Black Sea population in autumn 1996 was twice as high as the mesozooplankton production could sustain (Anninsky et al. 1998). A similar scenario was revealed in the Caspian Sea ecosystem where predation impact on zooplankton exceeded the daily zooplankton biomass during the warm seasons (Finenko et al. 2006). This is reflected by the fact that *M. leidyi* occurrence can decimate other invertebrates of lower trophic levels (Anninsky et al. 2005).

In the Limfjorden area *M. leidyi* together with the native jellyfish *Aurelia aurita* have been shown to substantially decrease zooplankton biomass and cause shifts in the zooplankton community structure (Riisgård et al. 2012).

Therefore, its large ecosystem impacts have led to the fact that *M. leidyi* is ranked among the 100 worst invasive species worldwide (Lowe et al. 2000).

M. leidyi demonstrably appears to have a large environmental envelope as it tolerates a wide range of abiotic conditions. For instance, *M. leidyi* can survive periods of hypoxia and has been shown to have the ability to oxyregulate even at low oxygen partial pressures (Thuesen et al. 2005). In experiments all individuals survived four days in [O_2] 0.5 mg L^{-1} at 22.4-24.2 °C (Breitburg et al. 2003). This advantage allows the species to occupy niches where other taxa cannot survive. With decreasing oxygen partial pressure of the surrounding seawater, the O_2 content of the lobe gel also declines (Thuesen et al. 2005). When encountering hypoxia, oxygen can be released from intragel region to more important tissues (Thuesen et al. 2005). This can be seen as a survival strategy (Thuesen et al. 2005). Yet, *M. leidyi* does not survive anoxic conditions ([O_2] < 0.5 % air saturation; 25 °C). When oxygen drops to these levels, animals die, even when transferred into oxygenated water immediately afterwards (Thuesen et al. 2005).

The established temperature frame tolerance is 1.3 to 32 °C (Ivanov et al. 2000). Additionally, very low salinities of 3.4, common for estuaries in *M. leidyi*'s native habitat (Miller 1974), as well as extremely high salinities of up to 38 (Fuentes et al. 2010) were reported to be tolerated. However, in invaded habitats of Northern Europe, low salinities have been demonstrated to negatively impact reproduction rates and low salinity levels are suggested to set limits to its range expansion (Jaspers et al. 2011a).

I.II. Invasion success of *M. leidyi*

Possessing these optimal prerequisites for invasiveness *M. leidyi* managed to have exemplary success as a key stone invasive species (Purcell et al. 2001). Being native to the west Atlantic coastline of North and South America (Purcell et al. 2001) *M. leidyi* was recently reported to have reached a global distribution (Costello et al. 2012).

During two independent invasion events it has successfully colonized southern and northern areas of western Eurasia (Reusch et al. 2010). *M. leidyi*'*s* range expansion success started in 1982 when it was detected in the Black Sea for the first time (Shiganova 1998). In 1989, it reached its first peak abundance in the Black Sea with a lag time of seven years (Shiganova 1998).

In 1999, *M. leidyi*'s appearance in the Caspian Sea was recorded for the first time (Purcell et al. 2001), again most likely due to shipping traffic and subsequent ballast water release (Ivanov et al. 2000). One year later, it already appeared in large quantities and managed to spread over most of the Caspian Sea (Ivanov et al. 2000). Even though fresh water influences northern areas of the Caspian Sea which remained uncolonized (Shiganova et al. 2003) it was sighted close to the Volga delta with extremely low salinities of 2-4 (Ivanov et al. 2000).

A second release of *M. leidyi* took place in the northern part of Europe (Reusch et al. 2010). During 2005, it was discovered in the North Sea area for the first time offshore the Netherlands (Faasse & Bayha 2006), the English Channel (Antajan et al. 2014) as well as in the Skagerrak Oslofjorden (Oliveira 2007). The first verified appearance of *M. leidyi* in the Limfjorden was in 2007 (Tendal et al. 2007; Jaspers et al. 2018).
In late summer 2006, *M. leidyi*'s presence was noticed in the Baltic Sea where it was sighted in the south-western areas around the German coastline (Kube 2007).
These secondary spread patterns for *M. leidyi* are closely linked to ocean currents which is especially the case for northern European waters which are highly interconnected by currents (Jaspers et al. 2018).

I.III. What controls *Mnemiopsis leidyi* abundance and spread?

Shortage of food is being multiply discussed as one crucial factor capable of restricting fecundity, somatic growth and biomass of *M. leidyi* (Kremer 1994; Reeve et al. 1989). Nevertheless, latest research results reveal that *M. leidyi* is able to withstand short term food shortage via shrinking and continuous egg production without food for up to 12 days (Jaspers et al. 2015).

For marine invertebrates low salinity is one of the most important factors limiting range expansion and could even be used in risk assessment to predict possible future invasions in brackish water bodies (Paavola et al. 2005).

Especially the abiotic characteristics of the Baltic Sea are not suitable to the physiological tolerances of *M. leidyi*. This applies in particular to salinity that is low in the north-eastern Baltic Sea with surface water salinities typically below 6-7 (Leppäkoski et al. 2002). It has been shown egg production rates are heavily impacted below salinities of 10 almost vanishing at a salinity of 6 (Jaspers et al. 2011a), assuming a probable limitation of *M. leidyi* establishment in central and northern areas of the Baltic Sea (Haraldsson et al. 2013; Jaspers et al. 2013).

In contrast to native populations in the US (e.g. Narragansett Bay, Long Island Sound) where *M. leidyi* is most abundant from June to September (temperature > 20 °C, salinity 21-32) (Kremer 1994), northern invaded populations show peak abundances in autumn (Schaber et al. 2011; Riisgård et al. 2012; Haraldsson et al. 2013). In the Baltic Sea, seasonal population developments of *M. leidyi* (Schaber et al. 2011; Haraldsson et al. 2013) lead to the suggestion the central Baltic *M. leidyi* population is a non-self-sustaining sink-population (Haraldsson et al. 2013). Similar source-sink dynamics have been reported for native habitats along the North-East US-coast (Costello et al. 2012). Certainly those were related to retention times instead of salinity effects (Costello et al. 2012).

Further research on *M. leidyi* from North Europe revealed a vast reduction of reproduction capacities correlated with reduced salinity (Jaspers et al. 2011a). Neither the effect of temperature nor food availability could explain higher reproduction rates in the higher saline Kattegat region (salinity: 25± 3) compared to the low saline central Baltic (salinity: 7.8± 0.3) via a study conducted in October 2009 (Jaspers et al. 2011a). Both per capita as well as

volume-specific production rates were significantly higher in animals originating from the Kattegat by the approximate factors 10 and 7 respectively (Jaspers et al. 2011a). This *in situ* trend could be confirmed by subsequent laboratory experiments where food appliance, temperature level and animal sizes were constant and therefore designating salinity as a response variable (6-33) representative for different hydrographic regimes of the Baltic Sea (Jaspers et al. 2011a). At the lowest salinity tested (6), which is characteristic for the central Baltic, reproduction capability almost completely vanished (Jaspers et al. 2011a). Hence, the low salinity in the north-eastern regions of the Baltic seem to be the decisive factor in limiting the expansion of *M. leidyi* populations in the Baltic Sea (Jaspers et al. 2011a). This fact is consistent with ensuing *in situ* observations that abundance of adult *M. leidyi* > 6.5 mm (minimum size of continuous egg production), their larvae and eggs are overall negatively correlated to salinity in the Baltic Sea. At the lowest-salinity station examined, characterized by a surface salinity of 6, eggs were not detectable at all (Jaspers et al. 2013).

Also the Black and Caspian Sea are brackish habitats which have surface salinities in their central areas of 17-18 and 12-13, respectively (Paavola et al. 2005). Irrespective of these rather low salinities *M. leidyi* appears highly abundant in these water bodies (Shiganova 1998; Ivanov et al. 2000). Yet, detailed salinity effects on reproduction rates in this southern invasive sub-populations remain to be investigated.

II. Importance of salinity – current status

II.I. Physiological relevance of salinity

Cells and extracellular fluids of almost all marine invertebrates are isosmotic (in osmotic equilibrium) to the surrounding seawater with a stable salinity. These animals are called 'osmoconformers' (Solan & Whiteley 2016). However, usually their cells are still slightly hyperosmotic towards seawater due to intracellular proteins and other metabolites ('Donnan effect') (Willmer et al. 2005).

The opposite term 'osmoregulators' refers to animals maintaining constant concentrations within their body fluids, independent on the ambient salinity (Willmer et al. 2005).

For both classes long-term exposure to unfavorable salinity conditions is often lethal or affects growth and reproduction (Solan & Whiteley 2016).

Moreover, ion-composition of the extracellular fluids in most marine species is very similar to seawater. Species with this characteristic are termed 'ionconformers' (Willmer et al. 2005).

Almost all marine taxa have a very limited conforming tolerance range – defining them as 'stenohaline' (Willmer et al. 2005). This applies to many full marine taxa which usually do not encounter huge salinity changes requiring a large regulating ability (Solan & Whiteley 2016). In contrast, 'euryhaline' species have the ability to withstand huge salinity ranges (Willmer et al. 2005) and are mostly comprised of osmoconformers (Solan & Whiteley 2016).

Besides, as to improving osmoregulation the ability to tolerate changing salinity is best at the species' temperature optimum (Solan & Whiteley 2016). Higher temperatures may impose an additional stress on the organism since seawater oxygen solubility is lower and increasing the animal's metabolic rate on the same time demanding more oxygen (Solan & Whiteley 2016).

With changes in ambient salinity both osmoregulators as well as osmoconformers have to adjust to the new environmental osmolarity as to changes of intracellular ionic concentrations affect correct cell functioning (Solan & Whiteley 2016). *M. leidyi* is classified as an hyper-osmoconformer, thus keeping its mesoglea osmolarity slightly above the osmolarity of the ambient water (Yazdani Foshtomi et al. 2007). Moreover, it specifically

regulates the concentrations of certain ions such as Ca^{2+} and SO_4^{2-} (Yazdani Foshtomi et al. 2007).

In connection with salinity perturbation two response stages can be identified (Solan & Whiteley 2016). The first rather unspecific cellular processes are results of macromolecular damage for instance as an effect of altered cell volume due to a change of ambient salinity and is referred to as 'cell stress response (CSR)' (Solan & Whiteley 2016). These processes are very conserved and therefore existent across many taxa (Solan & Whiteley 2016). Heat shock proteins (HSP) play a major role in the CSR and are frequently reported in the context of salinity stress (Solan & Whiteley 2016). For instance, they are involved in fixing damaged proteins (Solan & Whiteley 2016).
Besides, harmful reactive oxygen species (ROS) could increase due to salinity stress (Chainy et al. 2016). Thus, proteins and enzymes acting in antioxidant pathways are involved to mitigate harmful effects of ROS (Solan & Whiteley 2016).

'Cellular homeostatic response (CHR)' is the second response stage. CHR in contrast to CSR is rather plastic and can be unique across different species, cells or stressors (Solan & Whiteley 2016). This response terminates when conditions normalize again (Solan & Whiteley 2016).
CHR includes cell volume regulation (=iso-osmotic intracellular regulation) and osmoregulation (= iso-osmotic extracellular regulation) (Henry 2001; Solan & Whiteley 2016).
Furthermore, the cells' membranes may have mechanisms which could regulate permeability with respect to water (Willmer et al. 2005). On the one hand, the membranes' inner and outer side can be differentially permeable to water flow; on the other hand, the cell's permeability itself can be regulated (Willmer et al. 2005).

Indeed, all processes necessary to balance salinity changes require additional energy. As a consequence, energy might not be sufficiently available for other processes (Solan & Whiteley 2016). Thus, the enhanced energy demand affects the 'scope for growth' meaning there is not enough energy anymore to sustain processes like reproduction and somatic growth which can be reduced to secure crucial vital cellular processes (Solan & Whiteley

2016). Regardless, energy demands exceeding a certain limit over time usually turn out lethal (Solan & Whiteley 2016).

II.II. Stenohaline versus Euryhaline- Examples

Almost all full marine invertebrates are considered stenohaline (Willmer et al. 2005). Especially echinoderms are known for being very stenohaline that often do not show any osmotic regulation (Willmer et al. 2005). Also tunicates, sipunculans and scyphozoan cnidarians usually do not tolerate larger fluctuations of salinity (Willmer et al. 2005). Still, in many stenohaline taxa a slight osmotic regulation can be achieved by excluding common neutral amino acids like alanine and glycine during hypoosmotic stress (Willmer et al. 2005). When changes of cellular concentrations cannot be prevented, neural related problems do occur such as a rapid hyperpolarization and a reduced action potential (Willmer et al. 2005)

There are only few exceptions of marine invertebrates that are not isosmotic to their environment (Willmer et al. 2005). Among those are some crustaceans and especially decapods (Henry 2001) like several grapsid crabs and palaeomonid shrimps which are always substantially hypoosmotic with respect to the ambient water (often approx. 80-90 % concentration) (Willmer et al. 2005). These taxa are remarkably euryhaline (Willmer et al. 2005). Also the blue crab *Callinectes* and the mitten crab *Eriocheir* belong to the genera showing the biggest osmolarity regulation capabilities (Willmer et al. 2005) which is a common characteristic among topmost euryhaline invertebrates (Henry 2001).

Manuscript draft

Abstract

Invasive species are of increasing concern for food web structure and biodiversity conservation. An example for a notorious marine invasive species is the comb jelly *Mnemiopsis leidyi* native to the American east coast. Two independent introduction events led its establishment in northern and southern water bodies of western Eurasia. So far, low salinity is shown to limit the range expansion for northern invasive populations, whereas southern populations thrive at low salinities. We tested animals from two invasive sub-populations namely from the southern (Black Sea) and northern (Limfjorden) areas for tolerance and adaptation potential towards low salinity. Both populations were selected at high and low salinities (27 and 14) over two generations. At the end of the F2 generation, respiration rates were measured as a fitness proxy at high, low and an extremely low salinity (7.5) levels. We found respiration rates were significantly increased at the extremely low salinity ($p < 0.0001$) compared to other tested salinities. In particular, the increased respiration rates were distinct for the Limfjorden sub-populations, highest rates shown in the sub-population with parents selected and kept at the highest salinity revealing respiration rates 2.5 times higher than the respective sub-population with non-stressing treatment which never experienced a salinity change. Moreover, respiration rates were dependent on the salinity at which their parental lines were raised indicating high adaptational potential. Thus, since Black Sea sub-populations perform superiorly to Limfjorden at extremely low salinities and *M. leidyi* shows a high adaptational potential, translocation of the southern invasive sub-population into northern sub-populations should be avoided by all means.

1. Introduction

The translocation of species outside their natural dispersal ranges is steadily increasing without signs of saturation (Seebens et al. 2017) and their establishment in new, recipient ecosystems could have large impacts on ecosystem functioning (Walsh et al. 2016). However, the extent to which non-native or non-indigenous species may impact ecosystem functioning largely depends on their establishment success which is rather low compared to the number of species being translocated around the world on a daily basis (Williamson & Fitter 1996). The 'Tens rule' states that each further transformation from appearance to establishment, formation of self-sustaining populations, spreading and finally becoming an invasive pest species happens with an average likelihood of just about 10 % (Williamson & Fitter 1996).

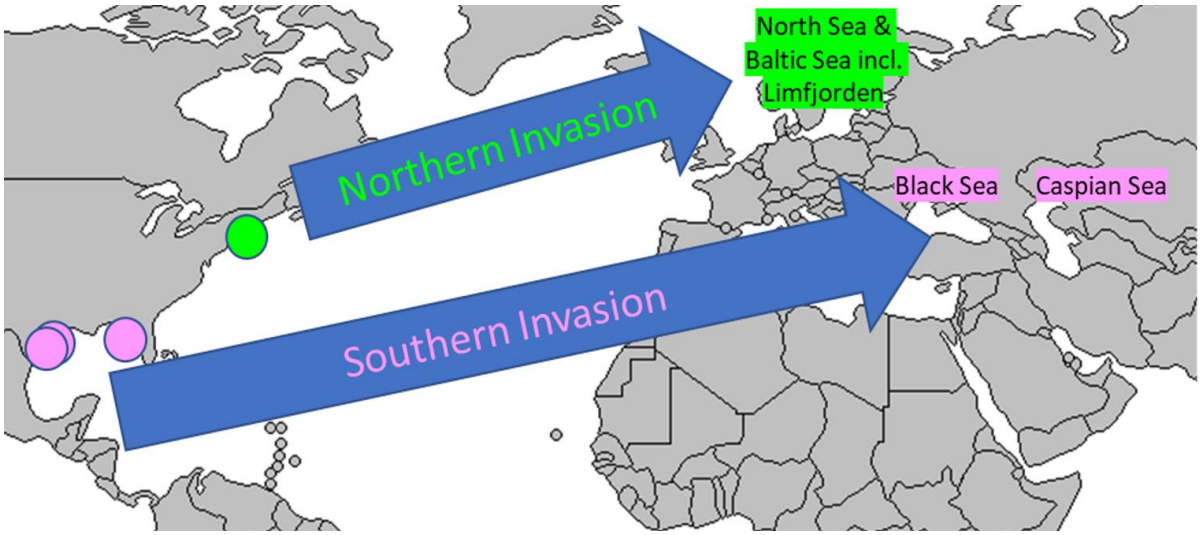

Figure 1: The two distinct introduction pathways to Eurasia of *Mnemiopsis leidyi*. Northern invasive sub-population (e.g. North and Baltic Sea) invaded from New England, while Southern invasive sub-populations (e.g. Black Sea and Caspian Sea) can be traced back to the Gulf of Mexico region. Data is based on microsatellite analyses by Reusch et al. 2010; © map: wikipedia.org.

The holoplanktonic comb jelly *Mnemiopsis leidyi* is an example of a successful non-indigenous species which has reached a global distribution range (Costello et al. 2012). It is native to the eastern coastline of North and South America (Purcell et al. 2001). During two independent invasion events it has successfully colonized southern and northern areas of western Eurasia (Reusch et al. 2010) (Figure 1). Its range expansion in western Eurasia is of particular concern since it has a high predation impact and substantially decreases mesozooplankton standing stocks (Anninsky et al. 1998; Finenko et al. 2006; Anninsky et al.

2005; Riisgård et al. 2012). The invasion success is inter alia based on its large reproduction potential whereas during early colonization *M. leidyi* is able to circumvent the Allee effect. It has per capita egg reproduction rates of more than 10,000 eggs per day (Jaspers et al. 2015; Baker & Reeve 1974) and is capable of simultaneous self-fertilization (Jaspers et al. 2015).
Besides, the species is characterized by a large environmental envelope which allows for tolerating a wide range of abiotic conditions (Ivanov et al. 2000; Purcell et al. 2001). For instance, very low salinities of 3.4 common for estuaries in its native habitat (Miller 1974) as well as extreme salinities of up to 38 (Fuentes et al. 2010) have been noted to be tolerated.
Nevertheless, in invaded habitats of Northern Europe, low salinities have been reported to negatively impact reproduction rates thereby setting limits to its range expansion. In detail, it has been shown egg production rates are dramatically reduced at salinities below 10 (Jaspers et al. 2011a). Therefore, it is suggested the abiotic regime of the central and northern Baltic Sea characterized by low salinities may be considered as an unsuitable habitat for *M. leidyi* (Jaspers et al. 2011a). In support, field investigations revealed the ratio between adults and upgrowing animals is very low, indicating a lack of active recruitment in the Baltic Sea (Jaspers et al. 2013). Additionally, investigating *in situ* egg abundances of *M. leidyi* along an environmental salinity gradient showed eggs were not detectable at a surface salinity of 6 (Jaspers et al. 2013). Similarly, laboratory experiments confirmed that egg production at this salinity almost vanished (Jaspers et al. 2011a).

Similar to the Baltic Sea, the Black Sea and Caspian Sea are considered as brackish water bodies characterized by surface salinities of 17-18 and 12-13 in their central areas, respectively (Paavola et al. 2005) where *M. leidyi* thrives (Shiganova 1998; Ivanov et al. 2000). Regardless, it remains to be elucidated if the southern sub-population displays a different salinity tolerance and is therefore more prone to thrive in very low saline environments such as the Sea of Azov characterized by a surface salinity of 5 (Paavola et al. 2005). Moreover, it is still unknown if the northern invaded sub-population has the potential to partially adapt to lower salinities.
Thus, we tested two invasive populations from the Limfjorden and Black Sea for their adaptive potential to low salinity environments. In order to deduce whether different *M. leidyi* sub-populations show pre-adaptation to low salinity stress, respiration rates were used

as a general proxy for metabolic activity and energy consumption. Assuming an animal is not starving its energy allocation can be divided into

$C = P + R + U + F \leftrightarrow R = C - P - U - F$

with C being energy derived from food consumption; P as the energy for instance incorporated into storage tissues; U and F as energy excreted as waste-products from metabolism; and R as respiration including activity, reproduction, growth and somatic maintenance (Sokolova et al. 2012). The latter can be measured as Standard metabolic rate (SMR) and increases when an organism is exposed to environmental stress (e.g. salinity stress) (Sokolova et al. 2012). As to SMR is a direct measure of respiration (Rosenfeld et al. 2015) salinity stress can be estimated from oxygen consumption rates. Anyways, aforementioned energy demanding processes are functionally linked together (Sokolova et al. 2012). Thus, to ensure sufficient energy supply for crucial maintenance processes required for survival during stress situations, energy is balanced and other processes like growth and reproduction lose priority and can be reduced (Sokolova et al. 2012). For instance, the copepod *Arcatia clausi* acclimatized to a salinity of 33 showed rather balanced energy expenditure (% of carbon ingested) for the production (25 %), respiration (36 %) and egestion (24 %) (Calliari et al. 2006). When exposed to a salinity of 16 over a period of about two days respiration and egestion increased to 43 % and 56 % respectively while production dropped to a value of 20 % (Calliari et al. 2006). As ingestion decreased while egestion increased, the overall energy balance was negative at this lower salinity which in turn leads to an absence of *A. clausi* in low saline areas (Calliari et al. 2006).

Indeed, many previous respiration studies on marine invertebrates confirm enhanced oxygen consumption with changes in ambient salinity in either direction (Allan et al. 2006; Calliari et al. 2006; Cheung & Lam 1995; Guerin & Stickle 1992; Gyllenberg & Lundqvist 1978; Gyllenberg & Greve 1979; Rivera-Ingraham et al. 2016; Sarà et al. 2008).

However, to our knowledge this is the first study on ctenophores explicitly examining the effect of salinity on respiration rates while previous publications only focused on other factors. For instance, a paper which considers the ctenophores *Pleurobranchia pileus*, *Beroe gracilis* and *Bolinopsis infundibulum* found metabolic activity to increase in respect to size, temperature, feeding and the animals' general condition (Gyllenberg & Greve 1979).

Respiration surveys on *M. leidyi* sub-populations of natives as well as from regions where it was introduced generally covered similar topics. For instance, the effect of size was evaluated for individuals from the Caspian (Finenko et al. 2006), the Black (Anninsky et al. 2005) and Mediterranean Sea (Lilley et al. 2014) generally showing an increase in respiration rate with body size but a wet-weight specific decrease in respiration (Anninsky et al. 2005).
Increasing respiration rate with temperature was surveyed in several studies. Q_{10}-values (= the factorial increase in metabolic activity for a 10 °C temperature increase) were determined to be 2.6 for the Mediterranean sub-population in a temperature range between 8.5-30 °C (Lilley et al. 2014), while a value of 3.7 was found for *M. leidyi* from the U.S. East coast at a temperature range from 10.3-24.5 °C (Kremer 1977). However, the high Q_{10} of 3.7 has to be particularly reconsidered since examined temperature range was large and two previous studies found values of only about 2 (Kremer 1977) which rather support the finding of a Q_{10}= 2.6 by Lilley et al. (2014) and are rather in agreement by research by Hansen et al. (1997) who found an average Q_{10} of 2.8 for a variety of zooplankton taxa within the temperature range of 5 to 25 °C.

An additional study examined respiration rates in hypoxic environments and revealed that *M. leidyi* can still oxyregulate at low O_2 partial pressures but does not tolerate anoxia and barely does endure severe hypoxia (< 2.1 % air saturation) at 25 °C (Thuesen et al. 2005).

Some studies aimed for the effect of food availability. In native sub-populations the effect of food on metabolism by using different food concentrations revealed *M. leidyi* respired gradually more with increasing food concentration at 21 °C (Kremer 1982).
On the contrary, another paper did not focus on food concentrations but the absence of prey namely respiration rates after a certain starvation period (Anninsky et al. 2005). They found respiration rates were reduced by about 50 % after 10 days of starvation but with an unsteady decrease (Anninsky et al. 2005).
The increase of respiration with food concentration can be explained by the Standard dynamic action (SDA) (Willmer et al. 2005). It is the additional energy expenditure above the standard metabolic rate (respectively heat produced) for processes of food processing (ingestion, digestion, absorption) and finally nutrient storage (Willmer et al. 2005; Secor 2009) and has a strong effect on respiration rate (Secor 2009). For invertebrates, metabolism is usually 2-3 times enhanced at an SDA peak (highest postprandial metabolic rate within

SDA period) compared to pre-feeding conditions (Secor 2009). Its duration is dependent on temperature, food amount and composition (Willmer et al. 2005). For other ctenophore species, namely *Beroe gracilis* kept at 8 °C, oxygen consumption is shown to be enhanced for about two days after feeding (Gyllenberg & Greve 1979). This is in agreement to studies performed on *M. leidyi*. After being caught in the field (Sevastopol coast, Ukraine) and two subsequent days of starvation in the laboratory Anninsky et al. (2005) showed a decrease of 28 % from 0.18 to 0.14 µmol O_2/ h/ g WW at 12 °C compared to the starting point right after animal collection. For the same period of starvation Kremer (1982) found a much steeper decrease as rates remained only about half (0.09 µmol O_2/ h/ g WW) of the initial value (0.16 µmol O_2/ h/ g WW) obtained from animals previously fed with a food concentration of 200 copepods L^{-1} for 24 h at 21 °C. In the same paper animals were additionally examined at a temperature of 22 °C 3 hours after feeding and compared to unfed individuals (measured ~ 11 hours after collection from the sea). Those animals that had been starved for about 11 hours showed a 31 % lower respiration rate than the fed ones investigated 3 hours after feeding (Kremer 1982).

These results demonstrate the sensitivity of respiration towards the animals' SDA. The variability of sensitivity mirrored in the unequal percentage values among the studies is likely the result of different temperatures as well as various food intake. For *M. leidyi* at 21 °C oxygen consumption doubles comparing unfed individuals to a food density of 50 copepods L^{-1} (200 µg C L^{-1}) and is increased 4-foldly at 500 copepods L^{-1} (2000 µg C L^{-1}) (Kremer 1982).

Hence, the respiration rate is strongly coupled to the SDA.

Certainly, salinities varied among aforementioned *M. leidyi* studies from 12 (Thuesen et al. 2005) to 31 (Kremer 1977) and were never explicitly considered as a determining factor for respiration in a study.

The aim of our study is to

i) investigate whether respiration rates differ in relation to salinity changes,

ii) whether pre-adaptation to salinity regimes impacts respiration rates and

iii) whether origin of *M. leidyi* from different sub-populations has an impact on respiration rates in particular at extremely low salinities

2. Materials and Methods

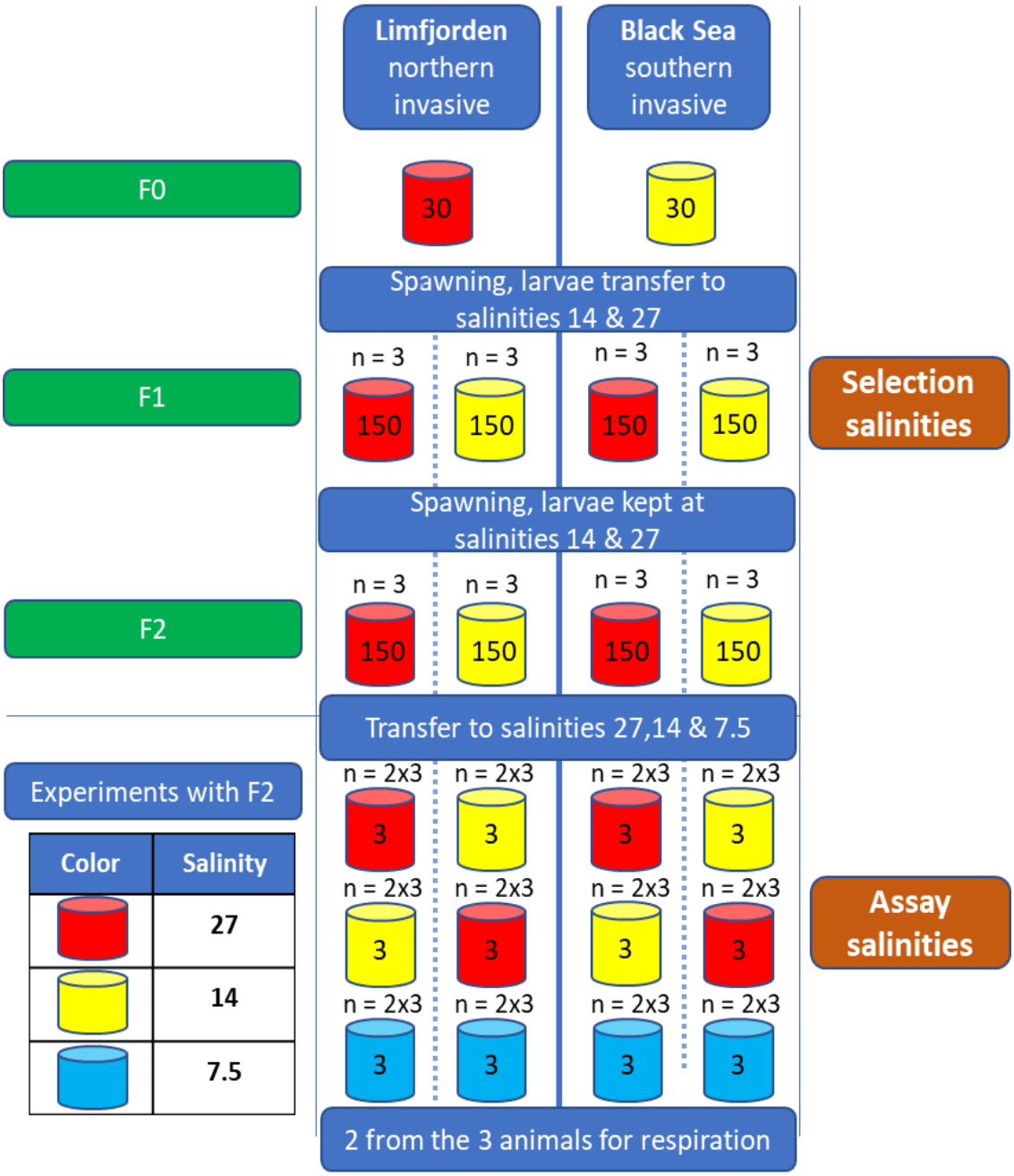

Figure 2: Experimental set-up. Limfjorden and Black Sea sub-populations of the F1 generation were both reared at Selection salinities 27 (red) and 14 (yellow). The following F2 offspring was further split into Assay salinities 27, 14 and 7.5 (blue). Digits above cylinders illustrate the amount of replicates per treatment; digits inside cylinders represent the amount of animals per container.

To test whether the northern and southern invasive sub-populations of the comb jelly *Mnemiopsis leidyi* display a difference in their salinity tolerances, animals were raised under common garden conditions throughout two generations and their respiration rates were measured in response to varying salinity levels.

The parental generation consisted of adult *Mnemiopsis leidyi* originating from the northern invasive sub-population present in Limfjorden (Denmark) (56°79' N 8°88' E) as well as adults from the southern invasive sub-population present in the Black Sea, Varna Bulgaria (43°12' N 27°55' E). Animals were caught alive, transported to the laboratory and acclimatized for several days before initiation of the F1 generation. Common garden experiments were performed at the 'Danish Shellfish Centre', DTU Aqua in Nykøbing Mors, Denmark (56°79' N 8°88' E) in the period from September to December 2016.
The overall experimental set-up is displayed in Figure 2.

M. leidyi from the Black Sea and northern European origin were cultured in 40 L containers at their respective habitat salinity of 14 and 27 and fed with an *ad libitum* diet of the copepod species *Arcatia tonsa*.

To initiate the F1 generations 30 healthy individuals of each population were transferred into 40 L containers and acclimatized for 1 day. After further 24 hours, the adults were removed from the tanks. For both populations 150 eggs/ larvae were transferred to 50 L containers with salinities of 14 and 27 (n=3 for each treatment) resulting in two different selection salinities for both Limfjorden and Black Sea populations. *M. leidyi* were raised on a diet of *A. tonsa* nauplii and subsequently fed with increasing size classes as the animals grew in size.
To initiate the F2 generation 30 adults from each replicate were transferred to a new container with seawater of the respective salinity and the adults were allowed to spawn for 24 hours before they were removed. The F2 generation was similarly raised on a diet of *A. tonsa* where increasing size fractions were offered as food as the animals grew in size.

As animals reached adulthood salinity response via respiration rates was investigated in a fully-crossed experiment testing a total of three assay salinities. The assay salinities consisted of high (27), low (14) and extremely low (7.5), whereas one of the assay salinities of 14 or 27 did not represent a change in salinity level as *M. leidyi* was raised at that respective salinity over two generations and is here referred to as selection salinity.
At the end of the F2 generation approximately 20 randomly picked *M. leidyi* were adjusted to the different assay salinities over a period of 1 week by dropwise addition of salt or

freshwater to meet the assay salinity. Animals were allowed to acclimatize to those salinities for at least one week before commencement of experiments.

During cultivation *M. leidyi* were fed *ad libitum A. tonsa*. Meanwhile, to ensure that all animals had the same food history experimental animals were fed 100 µg C L^{-1} for 48 hours before start of the respiration rate experiments. To do so animal size and incubation volume was set so that animals cleared max. 30 % of the container volume to ensure constant prey concentration over time (Jaspers et al. 2011b). Animal size ranged between 9 and 13.5 mm oral-aboral which corresponds to 0.46-1.46 gWW (Jaspers et al. 2015).

Three animals were placed into 20 L containers and each salinity level was replicated thrice leading to a total of twelve containers per tested salinity. During food acclimatization containers were covered with opaque foil to ensure uniform distribution of light-sensitive copepods.

Out of those twelve containers the second replicate containers were chosen every time. Of these containing three *M. leidyi* two animals each were randomly picked for respiration experiments. This yielded in six animals per sub-population.

2.1. Respiration measurements

To investigate whether the sub-populations showed differing response to salinity stress respiration was measured using an optical measuring unit by PreSens Precision Sensing GmbH (Regensburg, Germany). Respiration rates were measured as the decrease of oxygen saturation over time using airtight glass respiration chambers. Two multi-channel oxygen *Oxy-4 mini* meters with optical fibers and sensor spots (PreSens Precision Sensing GmbH, Germany) glued into respiration chambers provided the non-invasive measuring unit. Re-calibration of oxygen spots was rerun daily for the 100 % saturation points per definition using air-saturated water (via bubbling) and at least weekly for the 0 % per definition using a minimum of 10 g dehydrated Na_2SO_3 L^{-1}.

To substantially reduce the amount of bacteria in the water that might account for background respiration we aimed for abacterial conditions. Thus, the water used was 0.22 µm sterile-filtered seawater additionally treated with UV-C light. This procedure was done

just before starting experiments to minimize time for regrowth of bacteria. Distilled water was used for dilution.

Since temperature fluctuations affect air saturation capability of water and might consequently falsify results of oxygen saturation decline measurements used to measure respiration, a stable temperature is crucial. Thus, to ensure a stable temperature of 18 °C which is approximately the same at which animals were raised water was brought to the desired temperature using a water bath and filled chambers were placed into temperature-controlled wine coolers.

Animals were gently placed into the chambers which were subsequently closed allowing an overflow to prevent air bubbles. Each of the 12 chambers were populated with one animal. Subsequently, respiration chambers were placed back into the coolers and oxygen decrease was measured over time.
To verify sterilization of water a further run included the measurement of the same water also used for respiration chambers.
The chambers' volume ranged between 19.3 and 63 ml and was chosen depending on *M. leidyi* sizes in order to allow the animals moving undisturbedly but also to ensure that respiration signal was high enough to be detected, whereas the control bottle had a volume of 613 ml.

Measurements were performed behind light barriers (in darkness) similar to experiments conducted from Thuesen et al. (2005) and Lilley et al. (2014).
Each of the two oxygen measuring devices had four channels allowing a simultaneous data recording of maximum eight chambers. To increase the data yield, five chambers were replaced with a second chamber set after each 45 min of recording and their corresponding calibration data was transferred into the software (OXY 4 v2.30 mini). Again, after further 45 min the initial chamber set was reinstalled. This procedure was repeated at least two times.

In total, respiration was measured over a period of at least 2.5 hours. Measurements for each chamber set started approximately 40 minutes after taking the comb jellies out of the 20 L containers. Oxygen saturation was determined within a measuring interval of 15 sec in a repeated manner.

In order to gain the actual water volume for O_2 concentration calculations the animals were removed from the chambers and the remaining water volume was determined using a graduated cylinder.

To account for bacterial-background respiration chambers with the initial water from the comb jelly respiration measurements were topped up with sterile seawater and were incubated for at least 30 min after the comb jellies were removed from the chambers. A 70 % ethanol solution was used to clean the graduated cylinder and respiration chambers after each measurement to avoid bacteria contamination and growth.

2.2. Data processing and statistics

The first 20 min of each data series were neglected for acclimatization of the animals and to minimize temperature effects since we recognized curvature in the measurements during this time period. Heating of the small chamber volumes was probably caused by handling at a different room temperature when placing the animals into the chambers.

We consistently used data solely from the first 2 hours in order to minimize bacteria growth and starvation effects of *M. leidyi*. Respiration controls were recorded for 60 minutes. In case of apparent temperature effects due to handling the first minutes were cut off similarly to respiration measurements. Finally, bacterial respiration was subtracted from the effect size (i.e. recording with *M. leidyi*).

Data was recorded in % O_2 saturation and converted into µmol O_2/ h/ g wet weight (gWW). On average, respiration accounted for 70.5 % of the measured oxygen decline. Yet, if bacterial respiration exceeded *M. leidyi* respiration, data was excluded.

For statistical analysis, the program R studio (version 3.2.5) was used with a significance level of 0.05. To meet assumptions of parametric tests (ANOVA) data was power transformed by equations I-III listed below (Dubcovsky & Davis 2017).

I. Plotting log (s_i^2) versus log($\overline{y}_i$) ; with s_i^2 = Variance and $\overline{y}_i$ = Mean

The log (variance) and log (mean) of each treatment group were calculated and the log (variance) plotted against the log (sample means).

To find the slope m that best satisfies the linear equation log (s_i^2) = m * log ($\overline{y}_i$) + b the slope m of the resulting regression line was determined. Consequently, it was used to calculate the exponent for the power transformation:

II. Solving a= 1-m/2 with m= best-fit slope of Equation I.

In our case we calculated m= 3.8883 and followingly a= -0.94415.

Finally, the data was power transformed by using the factor a as an exponent for all Y data:

III. $Y_{transformed} = (Y_{original})^a$

For statistical analysis, a 3-way ANOVA was performed using the three categorical covariates Population Origin (Black Sea / Limfjorden), Selection Salinity (14/27) and Assay Salinity (7.5/14/27). Successively, a Post-Hoc Analysis was performed using Tukey HSD Test (see Appendix, Table 6-10).

Within the following breakdown of the interactions revealed by ANOVA probability-values (p-values) of multiple comparisons were corrected using Benjamini-Hochberg procedure in R studio (see Appendix, Table 4).

3. Results

We found lowest respiration rates in those two treatments at which the Black Sea and Limfjorden sub-populations did not experience a change in salinity at all (Figure 3 and Appendix Table 3). In contrast, highest respiration rates were present at the extreme low experimental salinity (=assay salinity) (Figure 4 and Appendix Table 3) tested with highly significant enhanced respiration rates ($p < 0.001$) with Limfjorden sub-populations at extreme low assay salinity being statistically different from all other sub-populations at low and high assay salinities ($p = 0.006$).

All the same, parental selection salinity also had an effect as a single factor ($p= 0.0001$) as well as the interactions selection salinity x assay salinity ($p= 0.01$) and the three-way interaction ($p= 0.008$) were significant. For instance, comparing Limfjorden sub-populations at the extreme low assay salinity the treatment with the corresponding parental line already acclimatized to the low salinity showed substantially lower respiration rates compared to the treatment where parents remained at high salinity (= selection salinity) (Figure 4).

Table 1: 3-way ANOVA table

Factor	Df	Sum Squares	F value	P adj
Population origin	1	1.05	1.564	0.21672
Selection Salinity	1	11.82	17.574	0.00011 **
Assay Salinity	2	21.90	16.284	< 0.0001 ***
Population x Selection Salinity	1	4.74	7.048	0.01056 *
Population x Assay Salinity	2	3.43	2.549	0.08807 .
Selection Salinity x Assay Salinity	2	6.18	4.593	0.01465 *
Population x Selection Salinity x Assay Salinity	2	7.13	5.302	0.00809 **

We found the 3-way interaction of populations origin, selection salinity and assay salinity highly significant ($p= 0.008$) (Table 1; for details see Appendix, Table 4) but only for the lowest assay salinity of 7.5. Thus, the interaction between population and selection salinity depends on a third factor namely the lowest assay salinity. Followingly, although we found the factors selection salinity and assay salinity to be both highly significant, their effect is varying among different sub-populations. For instance, the main effect of extremely low assay salinity was especially pronounced at sub-populations from the Limfjorden. Besides, in

sub-populations with parental lines acclimatized to the lower selection salinity respiration rates at extremely low assay salinity might be lower compared to the corresponding sub-population were parents remained at the high selection salinity.

The significant two-way interaction population x selection salinity is dependent on what selection salinity is present and derives from Black Sea sub-populations with assay salinities of 27 and 14 only. For instance, the most significant difference (p= 0.0008) among pairwise comparisons was found between Black Sea sub-populations with an assay salinity of 14. Depending on their level of selection salinity the respiration rates of the sub-population with the selection salinity of 27 were significantly higher (p = 0.001) than those from the corresponding sub-population at the selection salinity of 14.

The second significant two-way interaction of selection salinity x assay salinity is due to the level of assay salinity for Black Sea sub-populations at a selection salinity of 14 and Limfjorden sub-populations with a selection salinity of 27 which were p= 0.002 for both. For these Limfjorden sub-populations respiration increased with decreasing assay salinities whereas for Black Sea sub-populations with a selection salinity of 14 respiration rates were also highest at the lowest assay salinity tested and intermediate at the highest assay salinity of 27.

These interactions show that the outcome of the respiration rates is not dependent on a single factor like assay salinity alone but the combination of population, level of selection salinity and assay salinity is rather decisive for their outcome.

3.1. Assay salinities 14 & 27

We did not find any overall difference between low (14) and high (27) final experimental salinity (=assay salinity) among sub-populations (p= 0.97). Only the Black Sea sub-population which remained at the salinity of 14 was significantly different from almost all other treatments showing extraordinary low respiration rates.

Indeed, as already stated before salinity to which the corresponding parental lines were acclimatized (=selection salinity) and assay salinity as well as their interaction (p= 0.015) were decisive for the outcome of respiration rates.

Followingly, there is still a clear pattern indicating respiration was generally higher in treatments that experienced a change in salinity in both selection salinity as well as in assay salinity (Figure 3).

Selection salinity on its own ($p < 0.001$) as well as assay salinity ($p < 0.0001$) have been shown to be highly significant. On average, sub-populations raised at a selection salinity of 27 showed 1.3 times higher respiration rates than those raised at a selection salinity of 14. The significant effect of factor assay salinity is due to the extremely low assay salinity (Figure 4). As already mentioned, the average respiration rates of high and low assay salinities are almost equal (0.27 ± 0.12 and 0.26 ± 0.08 µmol O_2/ h/ g WW respectively). In contrast, the extremely low assay salinity treatments demonstrated respiration rates being enhanced by a factor of more than 1.5 in comparison to the high and low assay salinity treatments.

Further, lowest respiration rates were measured in the sub-populations were both selection salinity and assay salinity resembled their original habitat namely a salinity of 14 for Black Sea and 27 for Limfjorden (0.16 ± 0.02 and 0.22 ± 0.03 µmol O_2/ h/ g WW respectively) (Figure 3). Especially the Black Sea treatment which did not experience a change in salinity regimes was exceptionally low, thus being statistically different from almost all other treatments except from the aforementioned unstressed Limfjorden treatment which remained at a salinity of 27 throughout all generations. The second sub-population that did not differ significantly from the unstressed Black Sea sub-population was the Black Sea treatment which also remained at a salinity of selection salinity of 14 but was exposed to an assay salinity of 27.

The similarity among respiration rates of sub-populations tested at high and low assay salinity is most distinct in the Limfjorden treatments. Out of these, the highest respiration rate was measured in the treatment which remained at a selection salinity of 27 and an assay salinity of 14 with 0.30 ± 0.05 µmol O_2/ h/ g WW. Even so, the comparison of this sub-population and the lowest Limfjorden respiration rate present in the non-salinity stressed treatment only resembles a difference of 34.2 %. To summarize, all four Limfjorden sub-populations with assay salinities of 14 and 27 did not differ significantly from each other. Especially the sub-populations of the low selection salinity (14) were very similar showing only a difference of 3.5 % and reflect intermediate respiration rates. Although treatments

did not differ significantly from each other the aforementioned pattern caused by the effect of selection salinity, assay salinity and its interaction are clearly recognizable.

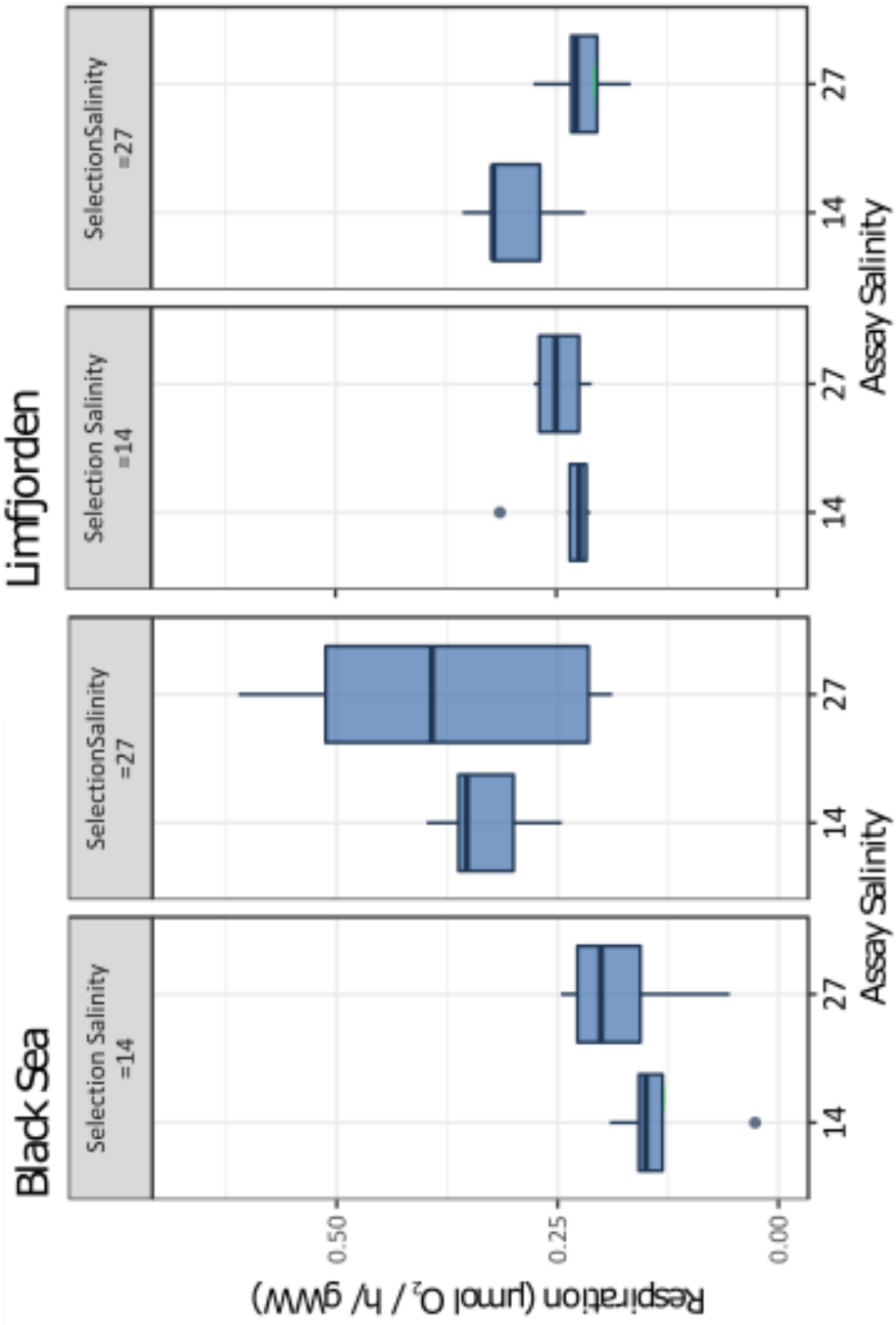

Figure 3: Respiration rates (µmol O_2/ h / g WW) of different sub-populations of the comb jelly *Mnemiopsis leidyi* in response to differing salinity levels. Sub-populations originating from the Black Sea (left) and Limfjorden (right) were raised at salinities of 14 and 27 (=Selection Salinity) over two generations before their respiration rates were measured with a fully crossed salinity assay composed of Assay Salinities of 14 and 27 at constant feeding condition of 100 µg C L^{-1}.
Respiration rates are given in µmol O_2/ h / g WW.
ANOVA revealed a highly significant 3-way interaction of population x selection salinity x assay salinity with p = 0.008 for all data.

In contrast, Black Sea sub-populations at high and low assay salinities showed a much greater heterogeneity. The difference between the lowest non-salinity stressed treatment to the highest values found in the sub-population kept at a selection and assay salinity of 27 (0.38 ± 0.17 µmol O_2/ h/ g WW) displayed 142.4 %.

The two remaining sub-populations exposed to a change in salinity with either the selection or assay salinity were characterized with intermediate respiration rates.

To summarize, the respiration of the Black Sea sub-populations showed two statistically highly significantly distinctive groups ($p < 0.0001$) with high rates found in the selection salinity of 27 and low rates found in the treatments of the selection salinity being 14. The latter group is significantly different from all other groups ($p = 0.03$) (Table 7).

3.2. Low Assay Salinity 7.5

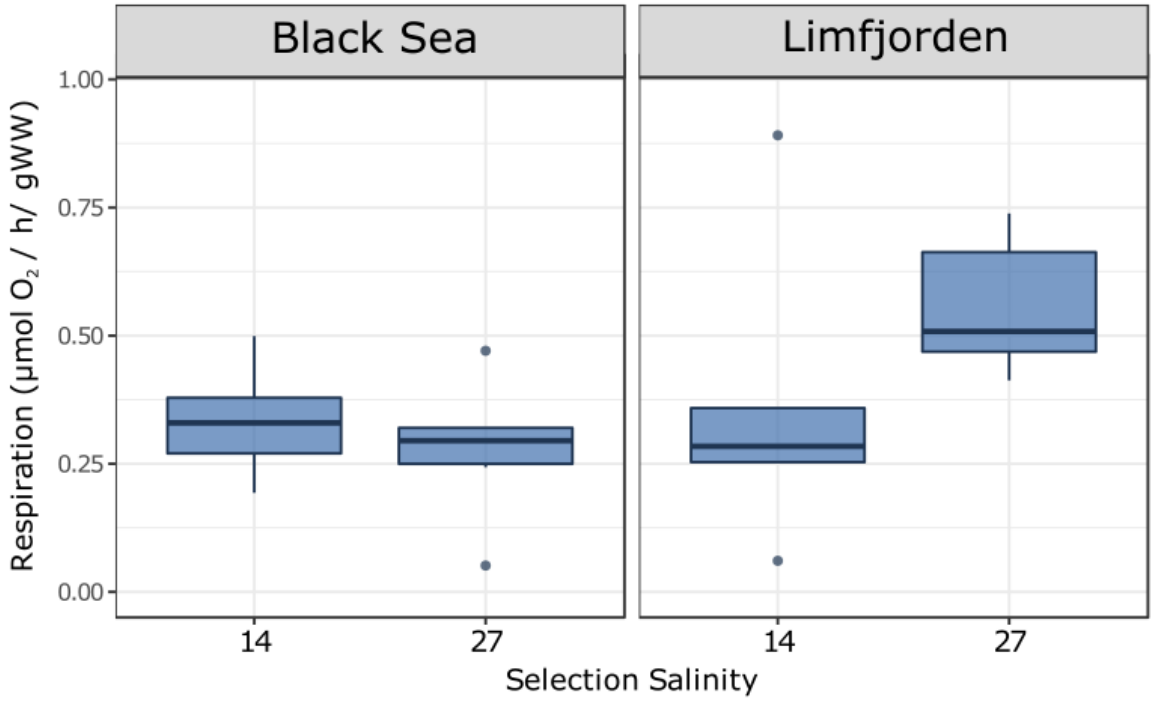

Figure 4 : Respiration rates at final experimental salinity (=assay salinities) of 7.5. Individuals from the same replicates at Assay Salinities 14 and 27 were further acclimatized to those extremely low salinity conditions. Sub-populations originating from the Black Sea (left) and Limfjorden (right). Respiration rates are given in µmol O_2/ h / g WW.

Both Black Sea sub-populations showed similar intermediate values. In contrast, Limfjorden sub-populations were particularly increased. This especially applies to Limfjorden animals at selection salinity = 27 which experienced diluted treatment conditions solely in the F2 generation.

We found sub-populations with an assay salinity of 7.5 differed significantly from those with a low assay salinity of 14 ($p < 0.0001$) and from sub-populations with a high assay salinity of 27 ($p < 0.0001$) (Figures 4 and 3).

Considering the sub-populations which did not experience a change in salinity regime, neither during the selection phase nor in the assay, showed considerable lower respiration rates and responded with increasing respiration the further the assay salinity deviated from both their origin salinity as well as from the respective selection salinity. Hence, Limfjorden treatments had increasing respiration rates from the assay salinity of 27 to finally 7.5.

All Black Sea salinity-stressed sub-populations showed an increased respiration compared to the non-salinity stressed control group which remained at a salinity of 14 throughout all generations. Interestingly, when comparing treatments where selection salinity remained 14 and only assay salinity was changed the lowest assay salinity of 7.5 revealed considerably higher respiration rates (0.33 µmol O_2 / h / gWW) in comparison to the highest assay salinity of 27 (0.21 µmol O_2 / h / gWW). Hence, in comparison to the Black Sea sub-population which remained at a salinity of 14 throughout all generations, the two other Black Sea sub-populations at a selection salinity of 14 showed higher oxygen consumption rates of factors 2.1 and 1.3 for assay salinities of 7.5 and 14 respectively; even though salinity difference from their original habitat salinity is actually larger in the latter case. Thus, Black Sea sub-populations have increased respiration rates at both higher and lower assay salinities, but the effect is especially pronounced at the extremely low assay salinity (Figure 4).

The high significance ($p < 0.0001$) of sub-populations at an extremely low assay salinity of 7.5 is mainly due to the corresponding Limfjorden treatments. Oxygen consumption of the Limfjorden treatments at the lowest assay salinity of 7.5 were statistically highly significant from all low and high assay salinity sub-populations ($p= 0.006$) and showed exceptionally high respiration rates. Considering the not salinity-stressed Limfjorden sub-population which remained at a salinity of 27 as a baseline, the treatment where parents already experienced a decrease in salinity with the selection salinity of 14 and were finally tested at the extreme low assay salinity of 7.5 was twice as high. The second Limfjorden treatment with an assay salinity of 7.5 but a selection salinity of 27 had respiration rates being even 2.5 times higher than the baseline (Figure 4). Moreover, variation is much higher in these treatments (SD=

0.12 and 0.26) in contrast to the corresponding low and high assay salinity treatments of Limfjorden where the variation was very low and equally distributed (SD=0.03-0.05).

In contrast, corresponding Black Sea sub-populations tested at the assay salinity of 7.5, with assigned selection salinities of 27 and 14 showed similar respiration rates that were statistically equal (p= 1) (Figure 4; Appendix Table 10) (0.32 ± 0.08 and 0.33 ± 0.10 µmol O_2/ h/ g WW respectively).
Besides, these values are especially comparable to the Black Sea treatments with a selection salinity of 27 all showing respiration rates that were about twice as high as the unstressed control treatment which remained at its original habitat salinity of 14.

However, Black Sea sub-populations at the extremely low and high assay salinities were not significantly different (p= 0.3). Regardless, there is a significance between the extremely low and low assay salinity sub-populations (p= 0.03) (Table 8).

Interestingly, the respiration rates of the Black Sea sub-populations exposed to the extreme low salinity of 7.5 were much lower compared to the respective Limfjorden sub-populations. This matches our hypothesis individuals originating from the Black Sea are used to lower salinities and potential physiological adaptations to these conditions might facilitate survival in extremely low salinities.

4. Discussion

4.1. Literature comparison

M. leidyi respiration measurements of comparable previous studies (similar temperature and feeding status if applicable) are summarized in Table 2. A more detailed table can be found in the Appendix, Table 11.

Table 2: Literature comparisons of *M. leidyi* respiration rates. Values were converted to µmol/ gWW / h at 18 °C using a Q_{10} of 2.8 as recommended by Hansen et al. (1997). Copepod concentrations were converted to µg C L^{-1} assuming a carbon content of 4 µg C per copepod (Berggreen et al. 1988). We show both respiration rates of all animals tested in the respective study and in addition respiration rates of solely size class of 0.46-1.46 gWW which was the size used in our experiments as well.

Reference	**Population origin**	**°C in study**	**Salinity**	**Feeding status**	**n (all/ 0.46-1.46 gWW)**	**µmol/gWW/ h all animals**	**µmol/gWW/ h size range 0.46-1.46 gWW**
Anninsky et *al*. (2005) Tbl 2+ Fig 8	Black Sea, Ukraine	12.5	16-18	Starved (< 1 day)	67	0.18	
Finenko et *al.* (2006), Fig 4	Caspian Sea, Iran	24	12.6-13	?	15/6	0.12 ± 0.06	0.10 ± 0.04
Kremer (1977) Fig 2/ PhD	Narragansett Bay, USA	18.0	31	Unfed for few h	26	0.16 ± 0.04	
Kremer (1982); Tbl1	Florida, USA	22		fed (3h starved after feeding)	11	0.11	
Kremer (1982); Fig 1	Florida, USA	21		50 copepods /L 200 µg C/L	10/2	0.20 ± 0.04	0.26 ± 0.06
Lilley et *al.* (2014); Fig 1A	Mediter-ranean Sea	15- 20	20-26	Starved overnight	36/5	0.11 ± 0.04	0.15 ± 0.07
Lilley et *al.* (2014); Fig 1C	Mediter-ranean Sea	20	20-26	Starved overnight	135/22	0.13 ± 0.07	0.19 ± 0.1
Thuesen et *al.* (2005); Tbl 1	Chesapeake Bay/ Solomons, Maryland, USA	25	12	?	12	0.13 ± 0.01	

Values were all converted to µmol O_2/ h/ g WW at 18 °C. To correct for temperature a Q_{10} of 2.8 valid for a broad range of zooplankton taxa (Hansen et al. 1997) was used.

The lowest value of 0.10 µmol O_2/ h/ g WW is found in the publication of Kremer (1982). Anyways, although animals were starved for about 3 hours this value is much lower compared to an earlier publication in which individuals were also starved for a few hours and still showed respiration rates of 0.16 ± 0.04 µmol O_2/ h/ g WW (Kremer 1977). Temperatures in these experiments were quite similar (22 ° and 18 °C respectively) and with the first value corrected for temperature, the animals' WW differed substantially between these two set-ups. While animals in Kremer (1982) were large, ranging from 1.5-28 g WW, those used in her earlier publication were smaller and size range was much narrower as these animals had a WW of 0.081-1.08 g (Kremer 1977).

When moderately fed (50 copepods L^{-1}) respiration rates were twice as high as the already mentioned lowest literature value found being 0.20 ± 0.04 µmol O_2/ h/ g WW and even as high as 0.26 ± 0.06 µmol O_2/ h/ g WW when considering only ctenophores of the size class 0.46-1.46 g WW as in our experiment (Kremer 1982) representing the highest values.

For our non-salinity stressed Limfjorden and Black Sea treatments we found respiration rates of 0.22 ± 0.03 and 0.16 ± 0.02 µmol O_2/ h/ g WW respectively. Thus, our results are in accordance with literature values (Table 2).

Certainly, respiration comparison among different studies is hampered by differing experimental set-ups as well as handling procedures which are likely to influence respiration rates similar to the condition of the experimental animals.

For instance, the aforementioned difference of about 30 % in respiration rates among size classes ranging from 0.5 to 9.4 gWW in the study focusing on food concentration from Kremer (1982) are not surprising since specific respiration rates in *M. leidyi* have been demonstrated to be negatively correlated with wet weight (Anninsky et al. 2005; Finenko et al. 2006; Lilley et al. 2014). Followingly, using animals of similar size is appropriate (Kremer 1982). Yet, the 30 % deviation between the size class of 0.46-1.46 gWW and >1.46 gWW is the most extreme example and in some studies the smaller size class even had lower respiration rates. However, these display a difference of only 19 % to respiration rates of the higher size class (Anninsky et al. 2005; Finenko et al. 2006).

If considering only these biases due to size effects our respiration rates fit the literature values but can be regarded as slightly above literature values as to we used animals comparably small (Table 2).

Perhaps the biggest impact on results is the discordancy on how to treat bacterial background respiration.

Via excretion and mucus production *M. leidyi* releases large quantities of colloidal and dissolved organic matter ('jelly-DOM') that is rich in labile carbon and thus highly bioavailable for heterotrophic bacteria (Condon et al. 2011). This is shown to fuel bacterial metabolism and respiration (Condon et al. 2011).

Thus, a very critical part of the *M. leidyi* respiration measurement process is the control of bacterial background respiration and to subtract it from the effect size. The authors used different methods to decrease the bacterial impact such as previous water filtration (Anninsky et al. 2005; Lilley et al. 2014; Thuesen et al. 2005) or antibiotic treatment (Thuesen et al. 2005). In our experiments, we abandoned the usage of antibiotics since we observed antibiotics affected respiration rates (Appendix, Table 6) concerning pilot runs with the same antibiotic concentrations used as in Thuesen et al. (2005).

In almost all previous studies, controls were run simultaneously. Nevertheless, to our knowledge none of these did consider control water which had been in contact with the ctenophores in their calculations as we did. Thus, in order to draw comparisons with our study one would either have to re-add background respiration to our results or certain reference results have to be assumed lower where bacterial respiration was not subtracted from the effect size. This is especially the case for those references that did not pre-treat the water (Kremer 1977, 1982; Finenko et al. 2006) or used rather coarse filtration (112 µm) as Anninsky et al. (2005).

But not only food amount and starvation prior to respiration rates varied substantially among studies, but also the incubation time of the animals in the respiration chambers. For instance, Kremer (1982) used a rather short incubation period of 3-4 hours, Anninsky et al. (2005) had a variable incubation period of 10-24 hours quite similar to Kremer (1977) (12-26 hours); Finenko et al. (2006) 14-15 hours; Lilley et al. (2014) measured for 17 hours on average whereas Thuesen et al. (2005) waited until conditions became almost anoxic.

To sum up, all these studies measured respiration at different time frames of the Standard dynamic action (SDA). This variable is quite an unknown factor and makes results overall hardly comparable. Even so, we gained an estimate of the SDA of *M. leidyi* at 18 °C.

Within our experimental measurements of the unstressed sub-populations fed with a known food concentration of 100 µg C L^{-1} over a period of 2 days we found respiration being about 3 times on average increased at the beginning of measurements in comparison to the ending of the recording about four hours later. Furthermore, we also measured respiration of some individuals of the Limfjorden F0 generation over a period of at least six hours after feeding them *ad libitum* for one hour (Figure 5). They showed the steepest decrease of respiration rates within the first two hours. In this comb jelly shown in Figure 5, which is exemplary for all animals tested, the respiratory rate within this time frame dropped to less than a half of the starting point. Afterwards, the respiration rates were fairly stable with a much flatter descent rate of 4.7 % per hour. After six hours of measurement respiration rates were found to be 2.6 times lower than the starting point.

Hence, one would possibly expect results of Anninsky et al. (2005) and Lilley et al. (2014) at least 3 times higher when incubation period was shorter and animals had not been starved. In that case, their measured respiration rates would exceed ours by about a factor of two.

Indeed, as the SDA factor might also vary, we strongly recommend using similar food ratios, starvation periods and incubation periods to gain comparable results even more so as to different sub-populations might include an uncertainty in the results. Yet, temperature differences between studies need to be considered and in best case respiration rates estimated at differing temperature ranges to gain a Q_{10} for the population in question. We did not do this, because even parental animals were acclimatized to room temperature (~18 °C) several weeks prior to starting the experiments already.

Summing up, as to many studies used animals which had been starved several hours prior to respiration measurements the corresponding results should actually be assessed by at least a factor of 3 higher when comparing them to studies performed with fed animals such as in our study. Certainly, as to these studies did only marginally consider bacterial background respiration the effect of decreased SDA in starved animals is partially eliminated.

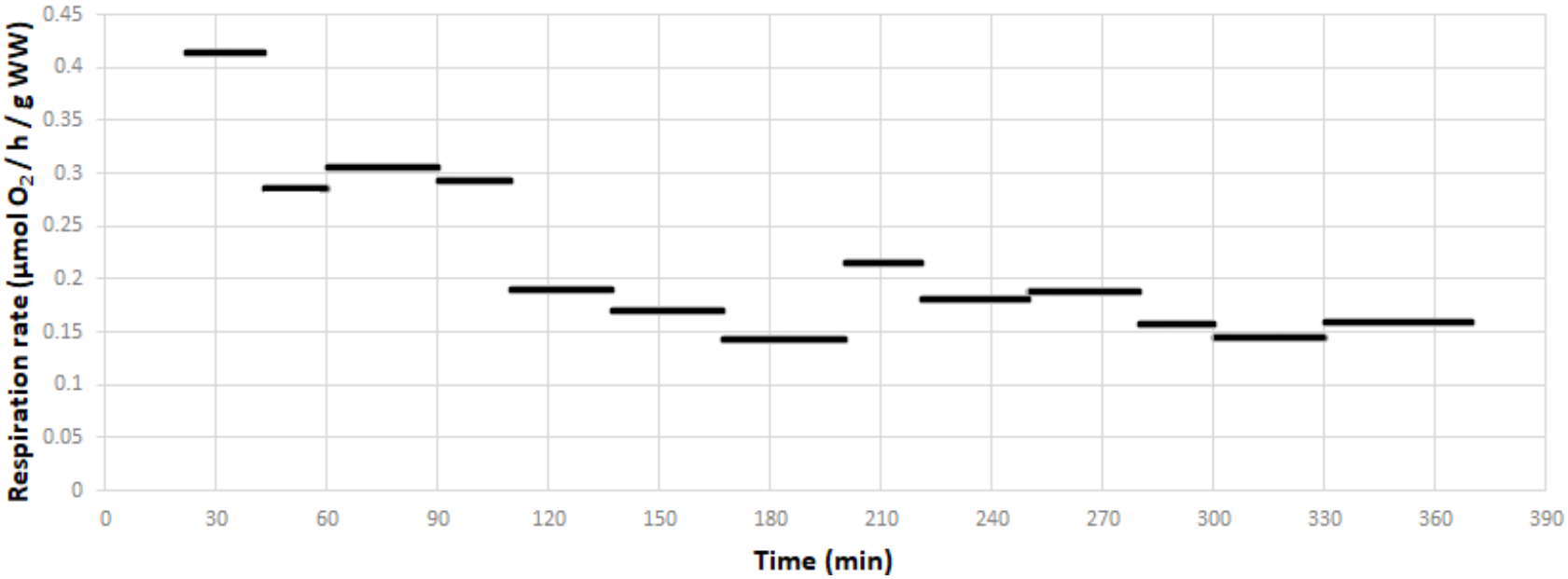

Figure 5: Standard Dynamic Action (SDA) development at 18 °C of *M. leidyi* (oral-aboral size: 13 mm) previously fed *ad libitum* for over 6 hours. Slope sections were averaged and start and ending of interval were cut off after approximately 30 min or if slope obviously changed. R^2 ranged from 0.76 to 0.97.
Respiration decreased by a factor of ~2.6 over 6 hours with steepest decrease within the first 2 hours and once again a subsequent small increase after about 3.5-4 hours. Followingly, respiration values remained at a more stable level.

4.2. Changes of respiration rate with salinity and habitat origin

To be certain that maternal effects were excluded and therefore to look for genotypic, hence adaptational rather than acclimatization effects, experiments were performed with individuals of the F2 generation. Similarly, it has been pointed out (Dam 2013) that common garden experiments should be conducted with animals previously raised at least over two generations (F2).

Moreover, we tested for the possible effect of pseudoreplication since always a pair of comb jellies of each three replicates per sub-population treatment was kept in the same container. However, we did not expect any effect due to pseudoreplication since the food concentration we worked with has been demonstrated to be high enough to sustain maximum egg production and growth (Jaspers et al. 2015). Besides, animals used in this study were very similar in size. Thus, we do not expect pronounced differences in feeding rates or interferences among animals of the same treatment. Further, statistical analyses confirmed including 'replicate' as a factor in an additional 4-way ANOVA was insignificant ($p= 0.66$).

In addition, we conducted a 3-way ANOVA with the means of each replicate ($n= 3$) to remove potential effects of pseudoreplication (as recommended by M. Lenz, pers. communication). The results were similar to the 3-way ANOVA based on all six replicates (as summarized in the Appendix 1, Table 5). Therefore, no effect of pseudoreplication could be detected.

The observed respiration rates generally confirm our prior expectations namely respiration rates may be increased if final experimental salinity (=assay salinity) was different from salinity of their habitat origin. We also expected salinity to which their parental lines were acclimatized (= selection salinity) may also be decisive. Thus, lowest respiration rates were found in those sub-populations which remained in the salinities matching their original habitats, hence Limfjorden at a salinity of 27 and Black Sea at 14. The further the salinities deviated from their original salinities the higher respiration turned out to be.

Even though sub-populations from both origins were raised over two generations at both high and low salinity levels, animals originating from the higher saline Limfjorden showed highest average oxygen consumption rates at the lowest salinity level throughout all

treatments. This result was expected since it reflects the largest change in salinity from habitat salinity towards assay salinity from 27 to 7.5.

The lowest oxygen consumption in the unstressed Limfjorden sub-population reflects a comparably low energy demand. These animals remained at a salinity of 27 for their entire lives just as their parental lines for at least two generations. Thus, this sub-population never experienced any kind of salinity stress resulting in a low metabolic rate and followingly a low respiration rate. In contrast, enhanced respiration rates as found for the lowest assay salinity indicate increased energy supply for processes to compensate for osmotic stress.

Also, Limfjorden sub-populations do show slightly lower respiration rates though with considerable scatter if selected at the lower salinity range (14) over two generations. Regardless, the significant population x selection salinity interaction indicates Black Sea sub-populations originating from a habitat characterized with a salinity of 14 have a lower respiration rate at the lowest assay salinity tested than the corresponding Limfjorden sub-populations. Moreover, Black Sea sub-populations showed moderate and quite equivalent oxygen consumptions at all treatments which included a change in salinity even at extremely low salinity (7.5).

Therefore, one might assume the southern invasive sub-population shows a lower oxygen demand and can channel more, surplus energy, into other factors such as reproduction.

Recently, reproduction rates of the northern invasive sub-populations have been shown to dramatically reduce within low salinity levels (Jaspers et al. 2011a). Similarly, the ratio between upgrowing and adult *M. leidyi* is very low in the low saline central Baltic Sea indicating a lack of active recruitment in low saline areas of northern Europe (Jaspers et al. 2013). The southern invasive Black Sea sub-population on the other hand could be expected to circumvent salinity constraints on reproduction rates as to we showed animals which have been exposed to constant food conditions (100 µg C L^{-1}) for 48 hours show much lower respiration rates at extreme salinity compared to the northern invasive sub-population. Hence, even though animals were kept at similar feeding conditions for 48 hours the lower respiration rate might indicate that more energy is available for reproduction.

Nevertheless, the Black Sea treatment with both selection and assay salinity being 27 has to be interpreted with care as to no bacterial background respiration was measured. Instead, we subtracted an average value of 0.16 µmol O_2/ h/ g WW which is the average bacterial

background respiration of the non-salinity stressed Black Sea sub-population. All the same, this procedure resulted in a high standard deviation of 0.17 for the Black Sea sub-population at high selection and assay salinity. Although the calculated values themselves are not correct the relative trend possibly still is. Even when the comb jellies' respiration rates could not be calculated the effect size still remained the largest of all Black Sea sub-populations. This either means a high ctenophore or bacterial respiration rate or both.

Previous studies examining the effect of salinity on a broad variety of marine invertebrate taxa generally confirm the trend of enhanced oxygen consumption with both increase and decrease in ambient salinity. Yet, some also showed increasing respiration from low to high salinity regardless of acclimatization salinity. This was for instance the case for the caridean shrimp *Palaemon peringueyi* which was caught and kept at a salinity of 35 and acclimatized to the experimental salinities (5, 15, 25, 35 and 45) overnight (Allan et al. 2006) and the gastropod *Nassarius festivus* where the effect was still present after two weeks of acclimatization from a salinity of 35 to 10, 15, 20, 25 and 30 (Cheung & Lam 1995).
Thus, acclimatization overnight probably is far too short especially for the treatment which experienced a drop of salinity by 30 units. When exposed to a sudden salinity shock animals may fall into a resting stage or may die (Sokolova et al. 2012), thus a too short acclimatization time might not deliver informative results. For this purpose, we slowly acclimatized our comb jellies to the lowest assay salinity over a period of one week.

Certainly, many taxa show an increase in respiration from high to low salinity. Six hours after being exposed to a salinity of 5 the flatworm *Macrostomum lignano* previously kept at a salinity of 35, it additionally showed reduced activity and other indicators for metabolic arrest (Rivera-Ingraham et al. 2016) which might be the case for all aforementioned taxa and is a common temporary survival strategy compensating for energy deficiency due to extreme stress situations (Sokolova et al. 2012). This explanation might also apply to the mussel *Brachiodontes pharaonis* which experienced an increase in respiration from acclimatized (45) to graduate acclimatization at lower salinity over a period of seven days until at a salinity of 15. Respiration rates at this low salinity steeply dropped to values below rates at the unstressed control condition at a salinity of 45 and 20 °C (10.3 and 7.6 µmol/ h/ g respectively) (Sarà et al. 2008) which is likely the result of extreme salinity stress often leading to a slow-down of activity (Sokolova et al. 2012).

Lowest respiration at acclimatized salinity supporting our hypothesis was for instance shown for two sub-populations of blue crab juveniles (*Callinectes sapidus*). Salinity at collection sites fluctuated between 20-30 and 30-45. Acclimatization to final salinities was reached by a change of 2-3 salinity units per day. Respiration rates were especially enhanced in extreme low salinity (2.5) namely 33 % for the sub-population from the lower salinity habitat and even more than twice as high for the sub-population from the hyper-saline environment (Guerin & Stickle 1992). In contrast, respiration rates at the highest salinity tested (50) differed by only 15 and 6 % respectively (Guerin & Stickle 1992).

Thus, in the experiment of Guerin & Stickle (1992) deviation from the acclimatization salinity towards extremely low salinities caused much higher respiration rates than exposure to higher salinities which is in general agreement to our experiments.

Copepods (*Acartia tonsa* and *A. clausi*) initially acclimatized to a salinity of 33 experienced a change of maximum 12 salinity units per day and were kept at the experimental salinity for a further day prior to experiments (Calliari et al. 2006). The respiration rates of *A. tonsa* showed a dome-shape pattern with lowest respiration at the acclimatization salinity and a steep increase at a salinity of 20 with respiration rates being more than two times higher than at the initial salinity (Calliari et al. 2006). Rates steadily decreased again approximating freshwater conditions (5) where rates were still almost 1.8 times higher compared to animals kept at a salinity of 33 (Calliari et al. 2006). *A. clausi* had lowest consumption at a salinity of 24 and increased both in higher (33) and lower (16) salinities with factors of about 2.3 and 1.4 respectively (Calliari et al. 2006). The decrease for *A. tonsa* at extreme low saline conditions once more suggests a probable high energy expenditure forcing the individuals into a resting stage. Also the copepod species *Eurytemora hirundoides* responded with increased oxygen consumptions by factors of about 1.2 to both higher (salinity of 9) and lower salinity (1.5) in comparison to their isosmotic range (salinity of 3) even after one week of acclimatization at 11 °C (Gyllenberg & Lundqvist 1978, 1979).

Similar to many marine invertebrates *M. leidyi* also has an internal osmotic pressure rather similar to the ambient seawater keeping their internal osmolarity slightly above external osmotic pressure (2-22 mOsmol L^{-1}) and thus classify as hyper-osmoconformer (Yazdani Foshtomi et al. 2007). After salinity changes they need to adjust to the new environmental osmolarity as changes of intracellular ionic concentrations of certain ions affect correct cell

functioning such as enzymes (Solan & Whiteley 2016), DNA-histone interactions and neuronal processes (Willmer et al. 2005).

Followingly, although *M. leidyi* only barely regulates mesoglea osmolarity, certain ion concentrations are still being regulated (Yazdani Foshtomi et al. 2007). For instance, animals from the Caspian Sea used to a salinity of 12.5 slightly hyperregulated their body fluid Ca^{2+} concentration at a salinity of 8, whereas body fluid SO_4^{2-} concentration was slightly hyperregulated at a salinity of 13 (Yazdani Foshtomi et al. 2007). The latter was discussed in the context of buoyancy regulation, while Ca^{2+} acts as second messenger and therefore is essential for correct cell functioning (Yazdani Foshtomi et al. 2007). Its active uptake in hypoosmotic environment is thought to compensate for its loss due to removal of extra water coming along with ion loss (Yazdani Foshtomi et al. 2007).
Thus, *M. leidyi* is classified an ion-regulator (Yazdani Foshtomi et al. 2007).
Maintaining a slightly increased hemolymph osmolality might be required for optimal cell functioning in freshwater conditions (Lee et al. 2012). Meanwhile, hyper-osmoconformers need extra energy to concentrate ions within their internal fluids when exposed to almost freshwater conditions (Lee et al. 2012).

Invertebrate species occurring in fluctuating salinities tend to hyperregulate their hemolymph osmolality at lower salinities while hyporegulating at higher salinities (Lee et al. 2012). Thus, as deviation from acclimatized salinity always requires additional energy we expected increased respiration rates at higher and lower salinities compared to the respective acclimatization salinity.

A way to counteract osmotic differences and therefore cell volume is achieved by adjusting the concentration of the cellular free amino acid pool (FAAP) and ion exchange (Solan & Whiteley 2016). The latter concept includes a variety of secondary transport systems. To counteract cellular shrinkage the $Na^+/K^+/2Cl^-$ symport into the cell is facilitated whilst during swelling and K^+ and Cl^- channels are activated to gate these ions outwards. Osmotic homeostases and cell volume regulation are also substantially related to the active exchange of cations by the Na^+/K^+- ATPase (Willmer et al. 2005). During the acclimation to dilute water, crabs of the species *Callinectes sapidus* acclimated to a salinity of 32 were transferred to a salinity of 10 and followingly showed a 300 % increase of Na^+/K^+- ATPase and a 150 %

increase of Na^+/K^+- ATPase α-subunit mRNA after 8 days in their gill tissue (Lovett et al. 2006).

Anyways, all processes counteracting salinity perturbation require additional energy making osmoregulation an energetically costly process. When encountering decreased salinity environment, ions and organic osmolytes are excreted and proteins are degraded and subsequently re-synthesized to regulate cellular osmolarity (Sokolova et al. 2012). Especially the enhanced gene expression due to protein-synthesis and ion pumping consume vast amounts of energy and therefore their rates may be decreased if required (Sokolova et al. 2012).

The synthesis of ATP is coupled to the oxidative metabolism which in turn increases the demand of substrates of the glycolysis and tricarboxylic acid cycle consuming energy reserves. Besides, an increased supply of oxygen is necessary as this is the ultimate electron acceptor used within the oxidation processes (Willmer et al. 2005). As a consequence of elevated oxygen demand, the animal shows an increased respiration rate (Solan & Whiteley 2016). The increased energy expenditure reduces the 'scope for growth' meaning less available energy for non-life-sustaining processes like reproduction and somatic growth (Solan & Whiteley 2016). This can be seen as a survival strategy which includes rebalancing of energy consuming processes usually prioritizing maintenance costs to fuel key cellular processes (= standard metabolic rate) over reproduction, somatic growth and activity (Sokolova et al. 2012).

The highest respiration rates found in the Limfjorden sub-population at a selection salinity of 27 and assay salinity of 7.5 resemble a carbon demand of approximately 4 µg C / h / animal. Assuming a filtration rate of 0.3 L /h / animal (Colin et al. 2010) and a carbon concentration of 80 µg C L^{-1} common for the Limfjorden area in summer (Zervoudaki et al. 2009), animals would be able to filter about 24 µg C/ h and might therefore likely have an excess of carbon irrespective of the enhanced carbon demand. Thus, carbon limitation in the natural environment of the Limfjorden is followingly very likely not the ultimate reason for limitation of range expansion.

Even so, in areas with less mesozooplankton abundance such as the Aarhus Bight with a concentration of 20 µg C L^{-1} (Zervoudaki et al. 2009) theoretically only yield 6 µg C / h/ animal which indeed might not be sufficient to sustain the carbon demand for the scope for growth or overall survival.

4.3. Differences among sub-populations

Restriction of gene flow among different populations promotes adaptation or even speciation for different environmental tolerances for instance with respect to salinity. When populations are separated the extent of exposure differs with respect to mutational input, genetic drift and selective regimes which is the prerequisite for undergoing independent evolution (Burton 1986). For instance, the copepod *Tigriopus californicus* populations experiencing highest fluctuations and maximum salinities have higher Gpt^F frequencies which is an allele associated with osmotic stress. Furthermore, the different populations showed many unique alleles (Burton 1986).

Since the northern and southern invasive populations of *M. leidyi* are geographically separated, we expect the development of allopatric speciation which leads to adaptive differentiation among distinct populations with respect to salinity. As *M. leidyi* was established in the Black Sea more than 35 years ago (Shiganova 1998) there should have been enough time to develop other genotypes that are adapted to the lower salinity environment.

Indeed, our results do show differences between sub-populations which can be traced back to their origin (Black Sea and Limfjorden). As aforementioned, the most striking finding was the apparent difference between Black Sea and Limfjorden sub-populations at the final experimental salinity of 7.5. Since these Limfjorden sub-populations had by far the highest oxygen consumption rates, we suggest them having a severe issue with extremely low salinities in contrast to Black Sea sub-populations which might have evolved mechanisms allowing them to better cope with these conditions.

Interestingly, invasive sub-populations from the Black-Azov Sea are considered to be the founder population for colonization of the high saline Mediterranean (Ghabooli et al. 2013) where *M. leidyi* thrives in salinities of almost 38 (Fuentes et al. 2010). Hence, southern invasive sub-populations seem to have the potential to tolerate both extremely low and high salinities. Indeed, genetic analyses suggests multiple introductions from the invasive Black Sea and native sub-populations from the Gulf of Mexico (Ghabooli et al. 2013).

4.4. Adaptation potential

Besides, as to differences were not solely evident due to population origin but also among sub-populations originating from the same habitat but also dependent on the salinities to which their parental-lines were selected over two generations (27 or 14) we suggest heritable shifts towards salinity tolerance (adaptation). Statistical results strongly support the findings of differences in respiration due to differences in selection salinity as this factor was highly significant (p= 0.0001) as well as the interaction between selection salinity and assay salinity (p= 0.01). Additionally, the three-way interaction between Population, selection salinity and assay salinity was significant (p= 0.008) which indicates the two-way interaction is dependent on a third factor namely the extremely low assay salinity.

Previous studies on the invasive euryhaline copepod *Eurytemora affinis* already demonstrated the effect of developmental acclimation towards salinity tolerance at adulthood (Lee & Petersen 2003). Adults from clutches of different habitats reared at a salinity of 27 survived significantly more at high-salinity stress of 40 compared to the clutches reared at lower salinities (10 and 5) (Lee & Petersen 2003). The inverse effect of low developmental salinity of 5 on freshwater tolerance was not significant (Lee & Petersen 2003).

Regardless, some clutches showed a significantly higher survival rate under freshwater conditions suggesting genetically based differences (Lee & Petersen 2003).

Moreover, these experiments led to the conclusion that although a certain species might have a wide tolerance range towards salinity, populations eventually differ with respect to this range and may only tolerate or be able to acclimatize to a subsection of the species' full range (Lee 2002).

When invading other habitats and facing new environmental conditions such as salinity changes strong selection and heritable shifts within respective tolerance windows can happen. Adaptation, acclimation or acclimatization may shift tolerance windows of sub-populations to enhance energy supply for fitness-related processes such as reproduction and growth (Lee 2002; Sokolova et al. 2012).

The potential to acclimatize and finally adapt to the new conditions is based on the concept of genotype-by-environment interaction (Via & Lande 1985). Genetic variation among

individuals within a population is likely the prerequisite for a change in the average phenotype when encountering new conditions (= phenotypic plasticity) upon which natural selection may happen (Lee & Petersen 2002). The resulting set of phenotypes caused by different conditions is termed the 'reaction norm' of the corresponding genotype (Lee & Petersen 2002). When testing heritable adaptation rather than acclimation towards freshwater tolerance in the copepod *E. affinis* some of the offspring of one population already reached the state of metamorphosed juveniles which was not the case for their parental line where all individuals died (Lee 1999). More generation cycles and longer acclimation times probably might have resulted in a higher yield since this copepod species is known to have adapted from saline to freshwater conditions in natural environment in about six years (Lee 1999).

Long-term acclimatization towards moderate changes in salinity in marine invertebrates usually implies metabolic modulations in order to decrease standard metabolic rate and maintain aerobic metabolism (Sokolova et al. 2012).
On the cellular level this might lead to an adjustment of the number of ion channels or an alteration of membrane permeability by changing the lipids or membrane surface structure whereas at the whole-organism level, an enhanced oxygen uptake and feeding behavior may counteract the increased energy demand (Willmer et al. 2005).

For the copepod *E. affinis* saline populations reared at freshwater conditions demonstrated rapid evolution on their ion transport mechanism by increased activity and expression of V-type H^+ ATPase (Lee et al. 2011) and increased hemolymph osmolality (especially below a salinity of 15) (Lee et al. 2012) in freshwater environments relative to their saline ancestors suggesting a superior ion uptake capacity. Besides, these evolutionary shifts towards dilute environments could be demonstrated to happen parallel in two genetically distinct clades suggesting labile mechanisms resulting in a rapid evolutionary response when salinity changes (Lee et al. 2011, 2012).
At a salinity of 15 both ancestral saline populations adapted to freshwater conditions for 11-12 generations and populations which invaded freshwater environments more than 100 generations ago showed a decline of V-type H^+ ATPase compared to the saline populations (Lee & Petersen 2003). This indicates a shift in salinity tolerance to a lower range as there might be trade-offs between low-and high-salinity tolerance, possibly contributing to higher

fitness and increased survival rates (Lee & Petersen 2003). Moreover, freshwater invaded populations showed increased plasticity towards changing salinity in contrast to the ancestral saline populations (Lee et al. 2011).

Since V-type H^+ ATPase is very conserved and also present in *M. leidyi* (Blaxter et al. unpublished) a similar adaptation mechanism in this species is likely .

4.5. Conclusion and future perspective

In my Master of Science thesis, I was able to show invasive sub-populations of *M. leidyi* have different salinity tolerances. Southern invasive sub-populations from the Black Sea showed lowest respiration rates at low (14) salinity and intermediate rates at both high (27) and extremely low salinity (7.5). In comparison, the northern invasive Limfjorden sub-populations were adapted to high salinities showing increasing respiration rates from high to extremely low salinity with much higher respiration rates at the lowest salinity tested than the respective Black Sea sub-populations.

Nevertheless, we also found the potential of *M. leidyi* invasive sub-populations to partially adapt to new salinity regimes after two generations already. This could be shown via the fact selection salinity was highly significant ($p = 0.0001$) and moreover, due to the significant interactions of selection salinity x assay salinity ($p = 0.01$) and the highly significant 3-way interaction of population x selections salinity x assay salinity ($p= 0.008$).

Genetic analyses of invasive *M. leidyi* from the high saline Mediterranean suggest the initial population originated from the Black Sea region, but its native habitat namely the Gulf of Mexico is likely to have acted as a second introduction source for the Mediterranean populations (Ghabooli et al. 2013). Since we could show sub-populations from the Back Sea are adapted to low salinities (14), the potential of *M. leidyi* to also thrive in the high saline Mediterranean with salinities of about 38 (Fuentes et al. 2010) might be facilitated by hybridization of the two sub-populations from the Black Sea and the Gulf of Mexico region. Hybrids can inherit abilities superior to their respective parental lines (=heterosis) (Yan et al. 2017). This effect towards salinity extremes is for instance a common phenomenon in hybrid oysters (Yan et al. 2017).

To test whether this could also be the case for hybrids of *M. leidyi* sub-populations we tested hybrids of the two invasive sub-populations used in the prior experiments (Limfjorden x Black Sea) but could neither confirm nor exclude these hybrids to be superior to their parents at extremely low salinities (Appendix, Figure 7). However, we solely raised the F1 generation and animals were not tested at constant food concentration. Thus, further research might focus on the adaptational potential of hybrid lines. We further recommend prevention of translocation of southern invasive sub-populations to the North of Europe by all means.

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7. Appendix

For reasons of clarity, we report treatments using the following notation:

XX_YY_ZZ with
XX being the Population: LF= Limfjorden; BS=Black Sea; Hy= Hybrids,
YY being the Selection Salinity: either 27 or 14 (or 20.5 in case of Hybrids) and
ZZ being the Assay Salinity: either 27, 14 or 7.5 (or 20.5 in case of Hybrids).

7.1. Overview respiration rates

Table 3: Respiration rates of all treatments in µmol O_2 /h/ gWW

ID	**N**	**µmol O_2 /h/ gWW ± SD**
LF_27_27	6	0.2214 ± 0.03
LF_14_14	6	0.2376 ± 0.04
LF_27_14	5	0.2972 ± 0.05
LF_14_27	5	0.2460 ± 0.03
LF_27_7.5	6	0.5561 ± 0.12
LF_14_7.5	4	0.4466 ± 0.26
BS_27_27	6	0.3821 ± 0.17
BS_14_14	4	0.1576 ± 0.02
BS_27_14	6	0.3328 ± 0.05
BS_14_27	4	0.2077 ± 0.03
BS_27_7.5	5	0.3247 ± 0.08
BS_14_7.5	6	0.3328 ± 0.10
Hy_20.5_20.5	16	0.8654 ± 0.46
Hy_20.5_7.5	6	0.5395 ± 0.40

7.2. Statistical Analyses

7.2.1. 3-way ANOVA results including interaction analyses

Table 4: ANOVA table. To make an analysis we used a 3-way ANOVA with the 1st factor being 'Population origin' which had two levels (Black Sea and Limfjorden), the 2nd factor was 'Selection Salinity' with two levels (27 and 14) and the 3rd factor 'Assay Salinity' with three levels (27, 14, 7.5).
Please note the highly significant 3-way interaction. Thus, some two-way interactions (Population x Selection Salinity and Selection Salinity x Assay Salinity) differ across levels of an additional factor and single factors have to be interpreted in this context. In order to analyze the source of interactions and to show how they change across levels of the additional factor, extra ANOVAs were manually performed. The effect of the additional factor not mentioned in the respective two-way interaction and how the interaction changes across the different levels of this factor were initially analyzed. Subsequently, the analysis was performed on how the interaction changes across levels of another fixed factor. Results are given below the usual ANOVA table (below black bar) and p-levels were adjusted using the Benjamini & Hochberg procedure.
Results showed the significant two-way interaction of Population x Selection Salinity dependent on Black Sea sub-populations with assay salinities of 27 and 14 and moreover, what level of selection salinity was present.
The second significant two-way interaction of Selection Salinity x Assay Salinity is based on Black Sea and Limfjorden sub-populations at their original habitat salinity (14 and 27 respectively) and respiration rates depend on what level of assay salinity is present (27, 14 or 7.5).
Besides, the post hoc Tukey HSD test (not shown here) indicated the 3-way interaction depends on the level of Assay Salinity and was solely significant at level 7.5.

Factor	**Df**	**Sum Squares**	**F value**	**P adj**
Population origin	1	1.05	1.564	0.21672
Selection Salinity	1	11.82	17.574	0.00011 **
Assay Salinity	2	21.90	16.284	$3.4*10^{-6}$ ***
Population x Selection Salinity	1	4.74	7.048	0.01056 *
Population x Assay Salinity	2	3.43	2.549	0.08807 .
Selection Salinity x Assay Salinity	2	6.18	4.593	0.01465 *
Population x Selection Salinity x Assay Salinity	2	7.13	5.302	0.00809 **

Factor	Df	Sum Squares	F value	P adj (Benjamini & Hochberg)
Interaction Population x Selection Salinity				
Population x Selection Salinity @ Assay Salinity				
Population x Selection Salinity @ Assay Salinity = 27	1	4.81	7.158	0.28435
Population x Selection Salinity @ Assay Salinity = 14	1	6.20	9.226	0.03097 *
Population x Selection Salinity @ Assay Salinity = 7.5	1	0.844	1.256	0.28435
Selection Salinity @ Population = Black Sea (Assay Salinity)				
Selection Salinity @ Population = Black Sea (Assay Salinity = 27)	1	5.322	7.920	0.04688 *
Selection Salinity @ Population = Black Sea (Assay Salinity = 14)	1	20.458	30.443	0.00079 ***
Selection Salinity @ Population = Black Sea (Assay Salinity = 7.5)	1	0.005	0.008	0.93069
Selection Salinity @ Population = Limfjorden (Assay Salinity)				
Selection Salinity @ Population = Limfjorden (Assay Salinity = 27)	1	0.557	0.829	0.45663
Selection Salinity @ Population = Limfjorden (Assay Salinity = 14)	1	1.398	2.080	0.26225
Selection Salinity @ Population = Limfjorden (Assay Salinity = 7.5)	1	1.764	2.625	0.26224
Interaction Population x Assay Salinity				
Population x Assay Salinity @ Selection Salinity				
Population x Assay Salinity @ Selection Salinity = 27	2	8.523	0.137	0.8734
Population x Assay Salinity @ Selection Salinity = 14	2	2.830	2.106	0.32901
Population @ Assay Salinity Selection Salinity =27				
Population @ Assay Salinity = 27 (Selection Salinity = 27)	1	4.374	6.509	0.06165
Population @ Assay Salinity = 14 (Selection Salinity = 27)	1	0.3057	0.455	0.51280
Population @ Assay Salinity = 7.5	1	4.02	5.982	0.06165

(Selection Salinity = 27)				
Population @ Assay Salinity Selection Salinity =14				
Population @ Assay Salinity = 27 (Selection Salinity = 14)	1	1.196	1.780	0.31042
Population @ Assay Salinity = 14 (Selection Salinity = 14)	1	8.383	12.475	0.02479 *
Population @ Assay Salinity = 7.5 (Selection Salinity = 14)	1	0.386	0.575	0.51280
Interaction Selection Salinity x Assay Salinity				
Selection Salinity x Assay Salinity @ Population				
Selection Salinity x Assay Salinity @ Black Sea	2	10.57	7.865	0.01314 *
Selection Salinity x Assay Salinity @ Limfjorden	2	2.74	0.607	0.60714
Assay Salinity @ Selection Salinity (Population = Black Sea)				
Assay Salinity @ Selection Salinity = 14 (Population = Black Sea)	2	18.44	13.719	0.00180 **
Assay Salinity @ Selection Salinity = 27 (Population = Black Sea)	2	0.06	0.0901	0.91383
Assay Salinity @ Selection Salinity (Population = Limfjorden)				
Assay Salinity @ Selection Salinity = 14 (Population = Limfjorden)	2	4.34	3.2262	0.10086
Assay Salinity @ Selection Salinity = 27 (Population = Limfjorden)	2	17.90	13.3170	0.00180 **

Table 5: Alternative ANOVA table with n = 3 (mean values of each replicate). Significance levels are equal to ANOVA with n = 6 (Table 4).

Factor	Df	Sum Squares	F value	P adj
Population origin	1	0.68	2.180	0.15339
Selection Salinity	1	8.16	26.017	$3.64 * 10^{-5}$ ***
Assay Salinity	2	12.32	19.643	$1.06 * 10^{-5}$ ***
Population x Selection Salinity	1	2.11	6.724	0.01626 *
Population x Assay Salinity	2	1.27	2.017	0.15598
Selection Salinity x Assay Salinity	2	2.31	3.678	0.04113 *
Population x Selection Salinity x Assay Salinity	2	4.46	7.109	0.00395 **

7.2.2. Post Hoc Results– Multiple comparisons

Post hoc results by Tukey HSD procedure.

Table 6: Assay Salinities comparison

x	27	14	7.5
27	-		
14	0.966	-	
7.5	< 0.0001	< 0.0001	-

Table 7: Population_Selection salinity comparison

x	BS_27_X	BS_14_X	LF_27_X	LF_14_X
BS_27_X	-			
BS_14_X	0.0001	-		
LF_27_X	0.870	0.001	-	
LF_14_X	0.341	0.027	0.772	-

Table 8: Population_Assay salinity comparison

x	BS_X_27	BS_X_14	BS_X_7.5	LF_X_27	LF_X_14	LF_X_7.5
BS_X_27	-					
BS_X_14	0.868	-				
BS_X_7.5	0.341	0.029	-			
LF_X_27	0.938	1.000	0.043	-		
LF_X_14	0.996	0.590	0.651	0.708	-	
LF_X_7.5	0.003	< 0.0001	0.321	< 0.0001	0.012	-

Table 9: Selection_Assay salinity comparison

x	27_27	27_14	14_14	14_27	27_7.5	14_7.5
27_27	-					
27_14	0.465	-				
14_14	0.031	0.0002	-			
14_27	0.722	0.038	0.579	-		
27_7.5	0.011	0.548	< 0.0001	0.0003	-	
14_7.5	0.239	0.997	< 0.0001	0.014	0.842	-

Table 10: Single comparison

x	BS_27_27	BS_27_14	BS_14_14	BS_14_27	BS_27_7.5	BS_14_7.5	LF_27_27	LF_27_14	LF_14_14	LF_14_27	LF_27_7.5	LF_14_7.5
BS_27_27	-											
BS_27_14	1.000	-										
BS_14_14	0.0002	< 0.0001	-									
BS_14_27	0.214	0.122	0.554	-								
BS_27_7.5	1.000	1.000	0.0004	0.253	-							
BS_14_7.5	1.000	1.000	0.0003	0.241	1.000	-						
LF_27_27	0.345	0.199	0.157	1.000	0.402	0.386	-					
LF_27_14	1.000	1.000	0.001	0.463	1.000	1.000	0.672	-				
LF_14_14	0.876	0.724	0.040	0.988	0.896	0.902	1.000	0.984	-			
LF_14_27	0.931	0.813	0.028	0.972	0.943	0.948	0.999	0.994	1.000	-		
LF_27_7.5	0.324	0.510	< 0.0001	0.0003	0.408	0.287	0.0003	0.198	0.007	0.011	-	
LF_14_7.5	1.000	1.000	0.0001	0.092	1.000	1.000	0.153	0.997	0.581	0.673	0.898	-

7.3. Antibiotic treatment

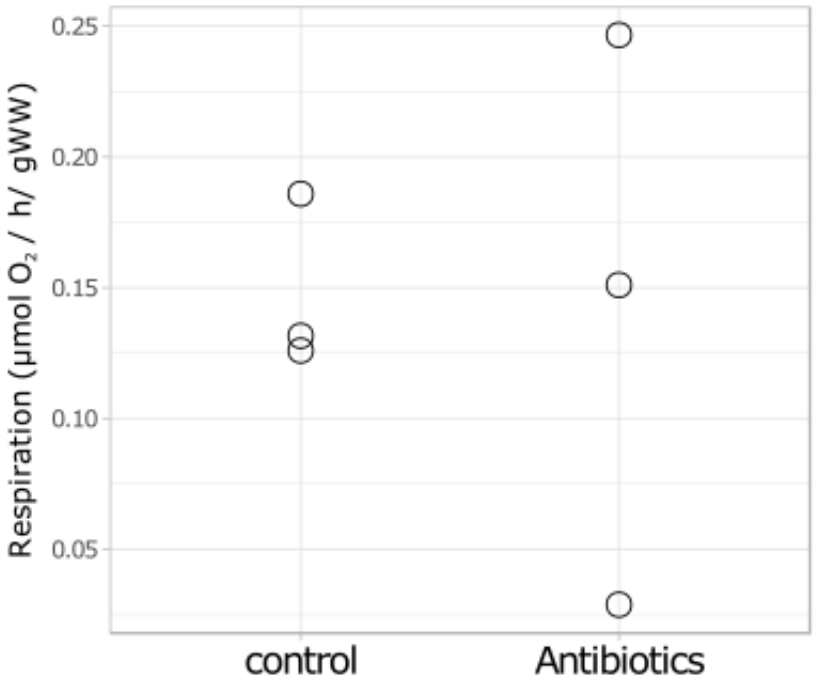

Figure 6: Effect of antibiotics (0.1 g L^{-1} of each streptomycin sulphate and sodium ampicillin) as used by Thuesen et al. 2005. Animals were taken from the F0 Limfjorden population at a salinity of 27 and fed *ad libitum* prior to respiration measurements. Size ranged from 15-21 mm oral-aboral.
Please note the large scatter with extremely low and high respiration rates for the antibiotic treatment in comparison to the untreated control group. Moreover, one animal of the antibiotic treatment died shortly after completing the experiment.

7.4. Hybrids

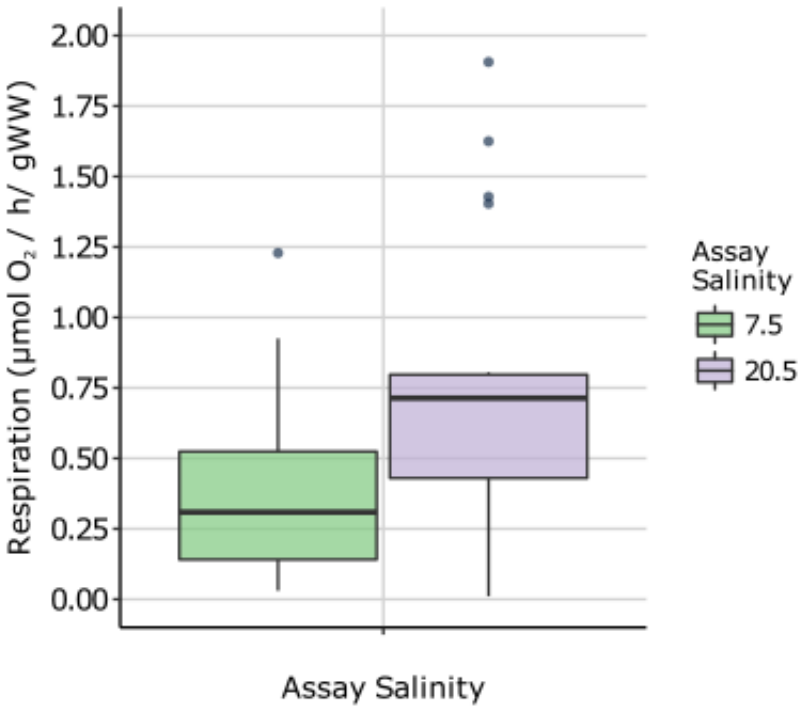

Figure 7: Respiration rates of hybrids (Limfjorden x Black Sea) in µmol O_2 / h /gWW. Animals were selected at a salinity of 20.5 and tested at assay salinities of 20.5 and extremely low salinity of 7.5.

An additional artificial hybrid line was raised by crossing 1 Baltic Sea x 1 Limfjorden individual (n=8). Parents were acclimatized to the new water conditions for four days prior to spawning. For the resulting offspring, water conditions had a salinity of 20.5, which is the arithmetical mean of the parents' salinity origin. The rate of actual hybrids from the spawning event will be analyzed by microsatellite analysis.

Respiration rates and standard deviation of the Hybrid treatments (Figure 7; Table 3) were both very high. While the sub-population at the extremely low salinity of 7.5 had a respiration rate of 0.54 ± 0.40 µmol O_2/ h/ g WW, and thus being comparable to the corresponding Limfjorden sub-population, the control Hybrid sub-population which remained at the salinity of 20.5 exceeded all other treatments by far (0.8654 ± 0.46 µmol O_2/ h/ g WW). However, these Hybrid sub-populations cannot be reliably compared to the Limfjorden and Black Sea treatments as they were not exposed to constant food concentrations which might explain the high variance. Besides, individuals were smaller

(0.11- 0.33 g WW) which might overvalue the results as respiration rates in *M. leidyi* have been demonstrated to decrease with increasing wet weight (Anninsky et al. 2005; Finenko et al. 2006; Lilley et al. 2014).

It is still surprising that the sub-population exposed to an Assay salinity of 20.5 had by far the highest values of all measured treatments and were even higher than the corresponding sub-population exposed to extreme low salinity.

This result is against our expectations that hybrids would show higher fitness at both high and low salinities since we expected differences in the environmental envelope of both sub-populations due to high genetic diversity.

In general, individuals that are the product of crossing distinct populations can either be superior or have a reduced fitness in comparison to their parental lines (Willett 2012).

However, breakdown of adaptations by 'outbreeding depression' is the one possible outcome. This is for instance demonstrated in copepod *Tigriopus californicus* hybrids crossed from individuals originating from different regions characterized by distinct salinity regimes. The result was reduced viability with respect to osmotic stress (Burton 1986). However, this outcome us usually apparent only in the F2 generation and likely the result of mitochondrial malfunction (Burton et al. 2006)

Further investigation should consider the potential for hybridization.

Table 11: Literature comparison of respiration rates of *M. leidyi* (in greater detail)

Reference	**Table /Fig**	**Population origin**	**°C orig**	**Salinity**	**Feeding status**	**n (all/ 0.46-1.46 g)**	**µmol/g WW/h at 18 °C all**	**µmol/g WW/h at 18 °C (0.46-1.46 g)**
Anninsky et *al*. (2005)	Tbl 2	Sevastopol coasts, Black Sea, Ukraine	12.5	16-18	Starved (< 1 day)	52	0.18	
Anninsky et *al*. (2005)	Fig 7	Sevastopol coasts, Black Sea, Ukraine	12.5	16-18	Starved (1 day)	40/15	0.23 ± 0.11	0.20±0.08

Anninsky et *al*. (2005)	Fig 8	Sevastopol coasts, Black Sea, Ukraine	12.5	16-18	Starved (0.05 d =1.2 h)	15	0.18	
Anninsky et *al*. (2005)	Fig 8	Sevastopol coasts, Black Sea, Ukraine	12.5	16-18	Starved (1 d)	15	0.23	
Anninsky et *al*. (2005)	Fig 8	Sevastopol coasts, Black Sea, Ukraine	12.5	16-18	Starved (2 d)	15	0.14	
Anninsky et *al*. (2005)	Fig 8	Sevastopol coasts, Black Sea, Ukraine	12.5	16-18	Starved (3 d)	15	0.26	
Anninsky et *al*. (2005)	Fig 8	Sevastopol coasts, Black Sea, Ukraine	12.5	16-18	Starved (5 d)	15	0.11	
Anninsky et *al*. (2005)	Fig 8	Sevastopol coasts, Black Sea, Ukraine	12.5	16-18	Starved (8 d)	15	0.14	
Anninsky et *al*. (2005)	Fig 8	Sevastopol coasts, Black Sea, Ukraine	12.5	16-18	Starved (10 d)	15	0.09	
Finenko et *al.* (2006)	Fig 4	Caspian Sea, Iran	24	12.6-13	?	15/6	0.12 ± 0.0591	0.10 ± 0.04
Kremer (1977)/ PhD	Fig 2	Narragansett Bay, USA	10.3	31	Unfed for few h	19/8	0.26 ± 0.11	0.28 ± 0.15
Kremer (1977)/ PhD	Fig 2	Narragansett Bay, USA	15.8	31	Unfed for few h	25/2	0.12 ± 0.03	0.13± 0.05
Kremer (1977)/ PhD	Fig 2	Narragansett Bay, USA	18.0	31	Unfed for few h	26	0.16 ± 0.04	
Kremer (1977)/ PhD	Fig 2	Narragansett Bay, USA	20.0	31	Unfed for few h	39/3	0.13 ± 0.04	0.16 ± 0.02
Kremer (1977) / PhD	Fig 2	Narragansett Bay, USA	21.8	31	Unfed for few h	24	0.14 ± 0.03	
Kremer (1977) / PhD	Fig 2	Narragansett Bay, USA	24.5	31	Unfed for few h	21/3	0.18 ± 0.03	0.13 ± 0.03
Kremer (1982)	Tbl 1	Florida, USA	22		Freshly collected	11	0.10	
Kremer (1982)	Tbl 1	Florida, USA	22		fed (3h starved after feeding)	11	0.10	
Kremer	Tbl 1	Florida, USA	22		unfed	11	0.08	

(1982)					(8+3 = 11 h)			
Kremer (1982)	Fig 1	Florida, USA	21		0 copepods/L = 0 µg C/L	10/1	0.09 ± 0.01	0.12
Kremer (1982)	Fig 1	Florida, USA	21		5 copepods/L = 20 µg C/L	10/5	0.23 ± 0.12	0.26 ± 0.15
Kremer (1982)	Fig 1	Florida, USA	21		50 copepods/L 200 µg C/L	10/2	0.20 ± 0.04	0.26 ± 0.06
Kremer (1982)	Fig 1	Florida, USA	21		500 copepods/L 2000 µg C/L	8/3	0.36 ± 0.05	0.48 ± 0.05
Kremer (1982)	Tbl 4	Florida, USA			Freshly fed (200 cop/L for 24h)	17	0.16	
Kremer (1982)	Tbl 4	Florida, USA			1 day starved	22	0.11	
Kremer (1982)	Tbl 4	Florida, USA			2 days starved	18	0.09	
Kremer (1982)	Tbl 4	Florida, USA			5 days starved	6	0.06	
Lilley et *al.* (2014)	Fig 1A	Mediterranean (France)	15-20	20-26	Starved overnight	36/5	0.10 ± 0.04	0.15 ± 0.07
Lilley et *al.* (2014)	Fig 1A	Mediterranean (France)	20-25	20-26	Starved overnight	38/6	0.1 ± 0.05	0.15 ± 0.06
Lilley et *al.* (2014)	Fig 1C	Mediterranean (France)	20	20-26	Starved overnight	135/22	0.13 ± 0.07	0.19 ± 0.1
Lilley et *al.* (2014)	Fig 1D	Mediterranean (France)	15-23	20-26	Starved overnight	51	0.14 ± 0.06	
Thuesen et *al.* (2005)	Tbl 1	Chesapeake Bay/ Solomons, Maryland, USA	25	12	?	12	0.13 ± 0.01	