The Physiological and Behavioural Response of *Mytilus* Mussels to Salinity in a Changing Ocean

Submitted by Louisa Jasmine Williams to the University of Exeter as a thesis for the degree of Masters by Research in Biological Sciences, September 2023

I certify that all material in this thesis which is not my own work has been identified and that any material that has previously been submitted and approved for the award of a degree by this or any other University has been acknowledged.

Abstract

Mytilus mussels are a widely distributed group which plays an important role in the structural development and maintenance of ecosystems, and represents an important aquaculture species. They live in both coastal and estuarine environments where they are exposed to wide ranging environmental conditions, particularly salinity fluctuations. They also form hybrid zones in the areas where species overlap, and in the Southwest of the UK there is a hybrid zone between *Mytilus edulis* and *Mytilus galloprovincialis*. Following an evaluation of current literature in chapter one, chapter two aimed to examine the physiological response of estuarine and coastal mussels from the Southwest hybrid zone to acute salinity changes, in addition to comparing the genetic differences between sites. Mussels exposed to salinity declines of 27 or 19 ppt had significantly lower metabolic rates than those exposed to 35 ppt, but there was no difference in metabolic rate between mussels collected from adjacent coastal and estuarine sites. Genetic analysis using a novel custom designed 60K mussel SNP array subsequently showed that all three sites had mainly *M. edulis* ancestry, although the upper estuarine site had a *M. galloprovincialis* frequency greater than that of the coastal site, contrary to previous analysis for this area. In chapter 3, valve movements of *M. edulis* were measured in response to different rates of salinity decline, using a custom-built hall sensor-based gape system. Mussels adducted their valves when external salinity reached 21 ppt in all rates of salinity change, but the salinity of retained pallial fluid was greater for faster rates, and was always higher than the salinity at which valves closed. Additionally, heart rate and metabolic rate were lower under declined salinities, with heart rate closely linked to gape, demonstrating periods of significantly reduced heart rate coinciding with periods of valve closure. This study is the first to simultaneously measure physiology and gaping behaviour of mussels in response to salinity changes, thus paving the way for future experiments on this subject. Climate change is predicted to cause an increase in flash flooding and ocean freshening, and therefore these results have significant implications for predicting the future ranges of *Mytilus* species.

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Mytilus Mussels in Variable Salinities: Hybrid Distribution, Physiological Performance, and Aquaculture in a Changing Climate

Mussels are an incredibly significant organism, both in terms of their ecological value as well as their importance for food production. They are ecosystem engineers, building beds which have been found to increase species richness (Borthagaray & Carranza, 2007). They have a mean filtration rate of 50 ml of seawater a minute (Beyer et al., 2017) and therefore perform the ecosystem service of removing excess algae from suspension, which can help to prevent eutrophication (Bergström et al., 2015). However, this also means that toxic substances present in the water can bioaccumulate inside the mussel; as a result of this they are frequently used as sentinel species for monitoring water quality (Barile et al., 2016; Guterres et al., 2020). In addition, mussels are an important aquaculture species, with 1.1m tonnes of *Mytilid* mussels produced in 2020, and bivalve aquaculture valued at 4.3 billion US dollars (FAO, 2022).

Despite their importance, there are still several gaps in our knowledge surrounding the fundamental physiology and behaviour of this key species, particularly when it comes to the effects of salinity changes. With organisms increasingly facing the effects of a changing climate, it is more important than ever to have a comprehensive understanding of how various environmental factors affect their physiological performance and behaviour, in order to better predict how this might change in the future. In this review, I will examine the distribution and speciation of *Mytilus* mussels and their hybrids, discuss the effects of living in highly changeable estuarine environments, and assess how a changing climate will impact this species in terms of its physiology, distribution and aquaculture production.

Hybridisation within the *Mytilus* species complex

Hybridisation occurs when viable offspring are produced from two genetically distinct species (Barton & Hewitt, 1985). Commonly observed in plants, animal

hybridisation is also a relatively common occurrence, with one analysis estimating around 10% of animals hybridise (Mallet, 2005). Although the study by Mallet was restricted to terrestrial animals, hybridisation has also been observed in many aquatic species (Hare & Avise, 1996; E. E. Nielsen et al., 2004; Rolán-Alvarez et al., 1997). Moreover, with larvae able to disperse across wide distances within aquatic environments, particularly for species which reproduce via broadcast spawning and have a pelagic larval phase, there is greater potential for larger hybrid zones in aquatic systems than on land.

Hybridisation is frequent between species of the *Mytilus* species complex, commonly known as blue mussels, which is comprised of three species: *Mytilus trossulus*, *Mytilus edulis*, and *Mytilus galloprovincialis*. *M. trossulus* is the ancestral species to the complex, appearing approximately 3.5 million years ago (Lockwood & Somero, 2011). Following the isolation of two separate populations, this then led to the emergence of *M. edulis* in the North Atlantic, which subsequently diverged into *M. galloprovincialis* in the Mediterranean around 2 million years ago (Lockwood & Somero, 2011). Several other related *Mytilus* species have also been defined within the genus, although debate remains as to whether these are all distinct species and which is the ancestral form. *M. chilensis* is thought to be the species found on the Pacific coast of South America, with *M. platensis* found on the Atlantic Coast (Zbawicka et al., 2018). However, it has been argued that these may be the same species (Gaitán-Espitia et al., 2016), or that both may be subspecies of *M. edulis* (Borsa et al., 2012; J. E. Toro, 1998). On the west coast of North America, the species *M. californianus* is widely accepted to be a separate species to others in the genus (Addison et al., 2008), and is even suggested to have had the earliest divergence from the other *Mytilus* mussels (Kenchington et al., 1995). The Kerguelen islands, a highly isolated archipelago in the Southern Indian Ocean, have *Mytilus* mussels which have previously been described as the endemic species *M. desolationis*, however it has later been defined as a sub-population of *M. edulis* (Blot et al., 1988; Koehn, 1991). *Mytilus* mussels in Australia and New Zealand were once considered to be introduced *M. galloprovincialis*, based on their allelic similarity (McDonald et al., 1991). However, fossil records indicate evidence of *Mytilus* mussels during the Pleistocene in Australia and New Zealand (Fleming & Suggate, 1964; Hope et al., 1977), and so mussels in this area are generally considered to be an

endemic species known as *Mytilus planulatus* (Popovic et al., 2019). *Mytilus coruscus* is a species found in China, Japan and Korea (Ye et al., 2012).

Although the species within the *Mytilus* complex are morphologically similar, they possess some key physiological differences. Of the three species, *M. trossulus* is typically found in the coldest waters, with distributions mainly in the Northern Pacific and Baltic Sea (Sarver & Foltz, 1993). As a result, this species is unable to withstand higher temperatures, with a LC_{50} (the temperature which results in 50% mortality) of 23.7 °C (Fly & Hilbish, 2013). In addition when scope for growth (SFG), a measure of physiological stress, was calculated, *M. trossulus* only had a positive SFG at temperatures lower than 17 °C (Fly & Hilbish, 2013). *M. edulis* is found at intermediate temperatures to *M. trossulus* and *M. galloprovincialis*, with a range which includes Eastern USA and Northern Europe (Sarver & Foltz, 1993). This species has a LC_{50} of 25.1 °C, and a positive SFG up to 23 °C (Fly & Hilbish, 2013).

Conversely, *M. galloprovincialis* is better adapted for warmer waters and has been shown to perform better than *M. edulis* at higher water temperatures (Hilbish et al., 1994). Temperature driven mortality of *M. galloprovincialis* only occurs at greatly elevated temperatures, with a LC_{50} of 28.7 °C, and a positive SFG at temperatures up to 30 °C (Fly & Hilbish, 2013). The distribution of *M. galloprovincialis* reflects this, with this species originating from the Mediterranean (Riginos & Cunningham, 2005). However, *M. galloprovincialis* has been classified as one of the world's 100 most invasive species (Lowe et al., 2000), and has since invaded North America, South Africa and East Asia via transport of larvae in ship ballast water (Han & Dong, 2020). This has had a particularly dramatic effect on the Californian coast, where it has largely replaced the native *M. trossulus*, which were found to be absent in size classes above 60 mm (Braby & Somero, 2006a). The higher thermal tolerance of *M. galloprovincialis* likely helps to explain its invasive ability (Schneider & Helmuth, 2007). The differences in physiological niche between these species are thus important to consider when understanding their distribution, and subsequently for predicting their response to climate change.

Table 1: A summary of some of the main global hybrid zones between *Mytilus* species and their key features. ME = *M. edulis*, MG = *M. galloprovincialis*, MT = *M. trossulus*.

Area	Species	Key features	References
Southwest UK	ME and MG	Cline from ME at Start Point, South Devon to MG in St Ives, North Cornwall Higher proportion of ME alleles in spat and small mussels, greater proportion of MG alleles in larger adults	(Hilbish et al., 2002) (Gilg & Hilbish, 2003a)
Ireland	ME and MG	Irish sea samples mostly ME Atlantic coast mostly MG or MG-ME hybrids	(Gosling et al., 2008)
France	ME and MG	Mosaic structure likely caused by barriers to gene flow	(Bierne et al., 2003)
California	MG and MT, also native *Mytilus californianus*	From mostly MG in the southern West Coast, to mostly MT north of central California MG more frequently found in larger size classes Mostly pure species or F_1 hybrids with limited introgression	(Dutton & Hofmann, 2008) (Wonham, 2004) (Saarman & Pogson, 2015)
Canada	MT and ME	Hudson Bay in Canada to Maine USA Larger size classes predominantly ME, smaller sizes predominantly MT Few F_1 hybrids, but many pure species and backcrosses	(Koehn et al., 1984) (Hayhurst & Rawson, 2009) (Comesaña et al., 1999) (J. Toro et al., 2004) (Comesaña et al., 1999)
Hokkaido Japan	MT and MG	South coast has cline from MG in the West to MT in the East, many F_1 mussels North coast has mosaic pattern of hybrids, few F_1 mussels Mainly MG on West coast Mainly MT on East coast	(Brannock et al., 2009)
Baltic Sea	ME and MT	Hybrid swarm with few pure species Cline from ME in the West to MT in the East Large amounts of introgression	(Stuckas et al., 2017) (T. Kijewski et al., 2019) (T. K. Kijewski et al., 2006; Stuckas et al., 2009)

Since their original divergence, the ranges of many of these species have expanded to overlap (Riginos & Cunningham, 2005), and where this has occurred

these organisms have the ability to form hybrids (Sarver & Foltz, 1993). This can result in introgression – the assimilation of alleles from one gene pool into that of another species (Harrison & Larson, 2014), a process which is being increasingly discovered in many species as genetic techniques become more sophisticated (Aguillon et al., 2022). In some areas, hybridisation of *Mytilus* species mainly occurs when two 'pure' species reproduce, creating a first-generation hybrid (known as an F_1 hybrid), but hybridisation over further generations is rare (Riginos & Cunningham, 2005). However, other areas have significant levels of hybridisation over multiple generations, resulting in a hybrid swarm in which the populations of mussels have high levels of introgression and backcrossing (Stuckas et al., 2017). Furthermore, hybrid zones may often involve a cline, defined as "a gradient or set of gradients in morphology or gene frequency, at one or more loci" (Barton & Hewitt, 1985), which may be as a result of environmental differences. These clines may also create a tension zone, where selection against hybrids and random dispersal result in the preservation of clines (Barton and Hewitt, 1989). Table 1 gives a summary of many of the *Mytilus* hybrid zones globally, and their key distinguishing features.

In the Southwest of England, *M. edulis* and *M. galloprovincialis* form a hybrid complex in which there are areas of hybrids, as well as areas which contain only one pure species (Skibinski et al., 1983; Gardner et al., 1993; Hilbish et al., 2002; Gilg and Hilbish, 2003). These areas frequently display a 'mosaic' structure, in which an area that contains only pure mussels of one species may be neighbouring another area which contains hybrid individuals (Bierne et al., 2002). In a study by Hilbish and colleagues (2002), mussels were collected from 33 sites around the coast of the Southwest, and genetically analysed using the Glu-5' genetic marker. They found that areas in South Devon tended to have mostly *M. edulis* alleles, whereas mussels in North Cornwall had mostly *M. galloprovincialis* alleles. Between these areas, there is a cline of hybrids along a stretch of around 180km, from more *edulis* like individuals from Start Point in Devon, to more *galloprovincialis* like individuals in St Ives, Cornwall (Hilbish et al., 2002). Figure 1 and Table 2 show the distribution of species around the Southwest, as well as some of the other areas in Europe.

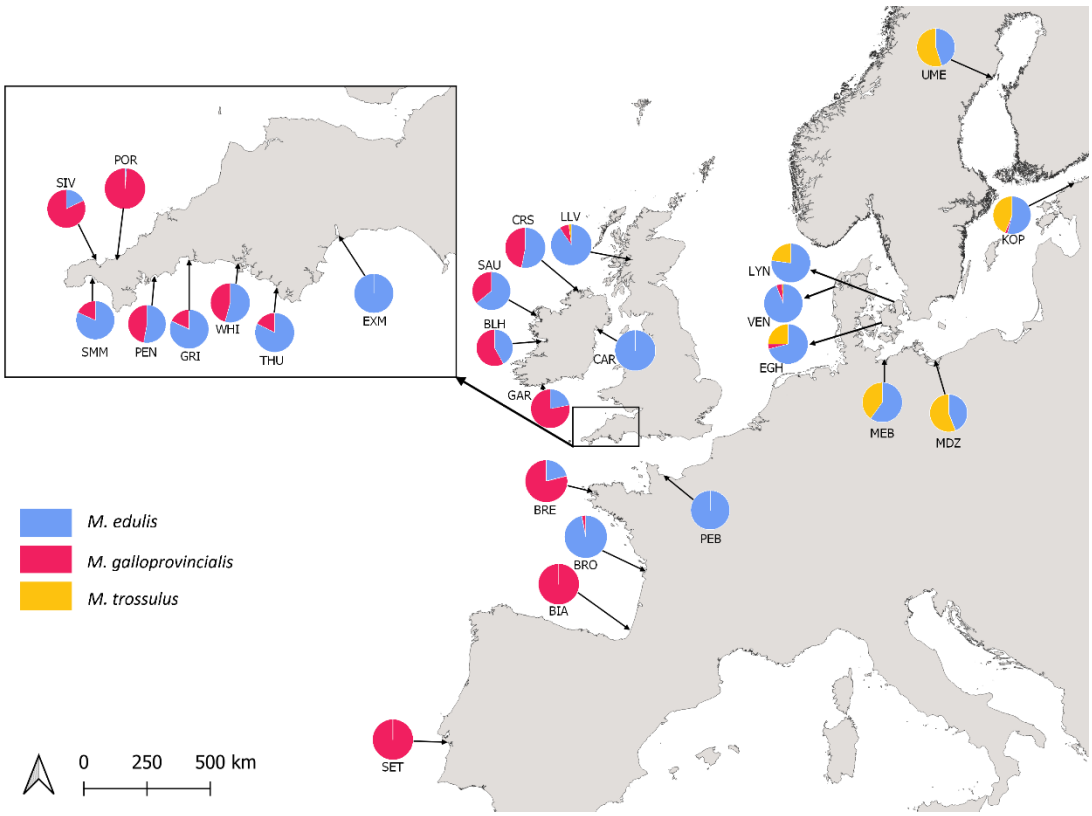


Figure 1: Map showing the allele frequency of *M. edulis* (blue), *M. galloprovincialis* (pink) and *M. trossulus* (yellow) at various locations (see Table 2) around the European coastline.

In addition to the genetic cline around the coast, Hilbish *et al* also observed a distinct variation in genetic composition between size classes and ages. Smaller size classes had a greater proportion of *M. edulis* alleles, whereas larger size class had a far greater proportion of *M. galloprovincialis* alleles. For example, at Whitsand Bay within the hybrid zone, the 10-15 mm size class had 85% *M. edulis* alleles, which gradually decreased as size class increased, with the 40-45 mm size class having only 25% *M. edulis* alleles (Hilbish et al., 2002). In a further study by Gilg and Hilbish (2003), similar results were found when the genetic composition of spat from around the Southwest of England was analysed. A far higher frequency of *M. edulis* alleles were found in mussel spat than in adults, suggesting that there is a selective pressure which affects later life stages of mussels rather than larvae (Gilg & Hilbish, 2003a).

This study also used a dispersal model to predict the movement of larvae and assess any possible dispersal barriers. They predicted that there is a barrier to dispersal at Start Point, where the hybrid zone begins, as East of this area larvae is comprised of more than 99% *M. edulis* alleles. In addition, they found that whilst there is some dispersal of larvae into the *M. galloprovincialis* zone in North Cornwall, there is very little dispersal from this area into the hybrid zone (Gilg &

Table 2: Allele frequency data for European *Mytilus* mussel populations taken from various studies. ME = *M. edulis*, MG = *M. galloprovincialis*, MT = *M. trossulus*. See Figure 1 for map.

Name	**Location**	**ME**	**MG**	**MT**	**Reference**
EXM	Exmouth	1	0	-	(Hilbish et al., 2002)
THU	Thurleston	0.82	0.18	-	(Hilbish et al., 2002)
WHI	Whitsand	0.54	0.46	-	(Hilbish et al., 2002)
GRI	Gribbin Head	0.82	0.18	-	(Hilbish et al., 2002)
PEN	Pendower Beach	0.53	0.47	-	(Hilbish et al., 2002)
SMM	St Michael's Mount	0.82	0.18	-	(Hilbish *et al.*, 2002)
SIV	St Ives	0.18	0.82	-	(Hilbish et al., 2002)
POR	Portreath	0.01	0.99	-	(Hilbish et al., 2002)
LLV	Loch Leven	0.9	0.08	0.02	(Michalek et al., 2021)
CAR	Carlingford	1	0	-	(Gosling et al., 2008)
GAR	Garrettstown	0.22	0.78	-	(Gosling et al., 2008)
BLH	Blackhead	0.42	0.58	-	(Gosling et al., 2008)
SAU	Saula	0.64	0.36	-	(Gosling et al., 2008)
CRS	Creeslough	0.53	0.47	-	(Gosling et al., 2008)
PEB	Port-en-Bessin	1	0	-	(Bierne et al., 2003)
BRE	Brest	0.21	0.79	-	(Bierne et al., 2003)
BRO	Brouage	0.97	0.03	-	(Bierne et al., 2003)
BIA	Biarritz	0	1	-	(Bierne et al., 2003)
SET	Setubal	0	1	-	(Bierne et al., 2003)
UME	Umeå	0.45	-	0.55	(Kijewski et al., 2019)
KOP	Kopli Bay	0.54	0.02	0.44	(Kijewski et al., 2019)
MDZ	Międzyzdroje	0.44	-	0.56	(Kijewski et al., 2019)
MEB	Mecklenburg	0.6	-	0.4	(Kijewski et al., 2019)
EGH	Egholm	0.71	0.04	0.25	(Kijewski et al., 2019)
VEN	Venø Bight	0.94	0.05	0.01	(Kijewski et al., 2019)
LYN	Lynetten	0.77	-	0.23	(Kijewski et al., 2019)

Hilbish, 2003a). Although we have a good understanding of the distribution within this zone, we still lack understanding of the physiological drivers of this mosaic distribution. Further research, combining genetic analysis with measurements of physiological performance, is key in order to identify their potential interactions, and predict future distribution changes.

The effects of living in intertidal and coastal environments

Living in temperate habitats, stretching from estuarine to fully marine conditions and spanning from the high intertidal to sublittoral, *Mytilus* mussels are frequently found in highly changeable habitats and are therefore exposed to a wide variation of conditions (Gosling, 2015). The intertidal zone exposes organisms to immersion and emersion cycles and mussels react to emersion by adducting their shell valves to protect from desiccation and predation (Widdows & Shick, 1985). During these periods, mussels are adapted to switch to anaerobic respiration by using a metabolic pathway which uses alternate electron acceptors instead of oxygen, allowing for anaerobic respiration which is much more efficient than that observed in many other animals (de Zwaan & Wijsman, 1976). This does, however, come at the cost of acidosis from a buildup of acidic waste products of respiration. Air exposure also means mussels experience far higher temperatures than when submerged. This can vary by 5-10 °C across acute spatial and temporal scales, depending on exposure to sun/shade (Schneider & Helmuth, 2007) and angle of exposure, which has been shown to be linked to mass mortality in California (Harley, 2008). This highly variable environment means that mussels are adapted to live in a wide range of conditions, and these interacting influences must be considered when attempting to predict the response to future environmental change.

Mytilus mussels are also commonly found within estuaries, where salinity fluctuation is impacted by a wide range of factors (see Figure 2) such as daily cycling between high and low tide, seasonal cycles of spring and neap tide (Robinson et al., 2007), and freshwater input from riverine discharges (see Figure 2b). In estuaries with a semi-diurnal tide, tidal volume changes by the rule of twelfths, where the change in volume follows a sine curve over the 6 hours of transition between high and low tide, or vice versa (Figure 2a) (Pearson, 2007). Estuarine salinity gradients are thus frequently complex, and greatly influenced by the fluid dynamics of water mixing. The varying conditions create a range of

estuary types (See Figure 2c) from highly stratified, where a halocline forms with the more dense salt water near the estuary bed and the less dense fresh water at the surface, to homogenous estuaries where the tidal force is sufficient to create consistent mixing throughout the estuary (Schubel & Pritchard, 1972). Stratification is often greater at neap tide, when tidal forcing is weaker and therefore less mixing occurs (Uncles & Stephens, 2011). Wind also has a significant effect on estuarine mixing, with speeds of only 5-7 m s^{-1} impacting the currents within the Tavy sub-estuary of the Tamar estuary in Plymouth, UK (Uncles & Stephens, 2011). Additionally, precipitation can cause varying degrees of change in estuarine salinity, with one study of an estuary in Florida finding that this varied greatly between areas, with precipitation only causing 4% of salinity change in high flow areas, but up to 61% of salinity change in restricted flow areas (Sumner & Belaineh, 2005). These factors all combine to mean that salinity can vary significantly across both acute and long-term time scales, as well as between different areas of the estuary. As a result, estuarine mussels are exposed to wide ranging and highly variable salinities, and this has implications for their distribution, physiological performance, and ability to tolerate other stressors. Given that the study of estuarine fluid dynamics and the physiology of their inhabitants tend to fall under two different research disciplines, there are few studies which thoroughly examine the ways in which these processes are interlinked; greater interdisciplinary collaborations are essential for better understanding these interactions.

Although mussels are osmoconformers, their ability to close their shell valves during air exposure is a mechanism often applied to cope with suboptimal salinities, with adduction allowing them to maintain a hyperosmotic internal environment with respect to the external aquatic environment. *M. edulis* retained a haemolymph osmolarity slightly above that of its surroundings when exposed to a six hour decline in salinity from 30 ppt to 15 ppt (Livingstone et al., 1979), with a similar response in noted in the Asian green mussel, *Perna viridis* (McFarland et al., 2013). Moreover, this protection mechanism may be species specific to mussels, having not been measured in *Crassostrea virginica* oysters (McFarland et al., 2013). When the valve gaping response of *M. galloprovincialis* was tested under an acute salinity decline, mussels exposed to a salinity of 20 ppt remained shut for a day, but then re-opened (Addis et al., 2021). However, under salinities of 5 ppt and 10 ppt mussels remained shut over the full 5-day

exposure, with mortalities noted in the 5 ppt treatment, indicating the extreme nature of this stressor. Despite this, surviving mussels were able to re-open when returned to 35 ppt (Addis *et al.*, 2021). This illustrates the longevity of this closure response, which allows mussels to survive periods of low salinity that they likely would not if they remained open. However, the mortalities show that this response still causes extensive physiological stress, especially over long periods of time.

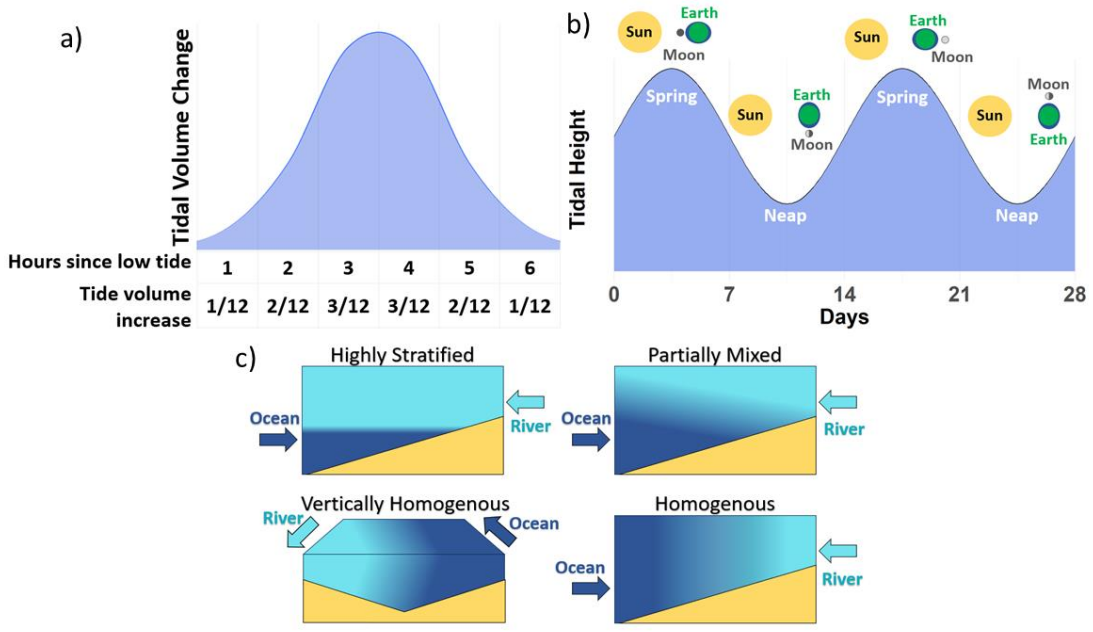


Figure 2: Factors affecting salinity within estuaries. a) The rule of 12ths describes the change in water volume over a semi-diurnal tidal cycle, with the largest change in volume at the middle of the cycle. b) The cycle between spring tide, when there is the greatest difference in high and low tide height, and neap tide, where there is the smallest difference in tide height, controlled by the position of the moon relative to the sun, and their combined gravitational pull. c) The different types of estuary mixing: Highly stratified, partially mixed, vertically homogenous (shown as a cross-section of the estuary) with longitudinal stratification, and homogenous.

This closure response was compared to rate of salinity change when Davenport (1979) exposed *M. edulis* mussels from the Menai Strait to varying salinity regimes, in which salinity was decreased to 0 ppt over periods ranging from 3.75 minutes to 4 days. He found that when salinity change was more rapid, the salinity of the fluid retained in the mantle was higher, however the external salinity at the point of valve closure was lower in longer salinity regimes. He observed that the retained mantle fluid corresponded to the external salinity at the point the exhalant siphon was observed to close, which was usually before the valve closure (Davenport, 1979). This suggests that the ability of mussels to remain temporarily hyperosmotic to their surroundings may be due to exhalant siphon closure more so than valve closure. In a similar study which exposed mussels to

declining salinity over a greater period of time, it was found that oxygen consumption was higher in mussels which had been acclimated to lower salinities (28 ppt and 18.5 ppt) than those at a normal seawater salinity of 37 ppt (Hamer et al., 2008). Despite these studies making it clear that the closure response has fundamental impacts on mussels' ability to cope with hypoosmotic stress, this is still a severely underutilised measurement in studies which examine the effects of salinity change.

Although the closure response can protect mussels from acute salinity changes, for the most part they are euryhaline osmoconformers, meaning that their internal salinity remains the same as the external environment, so long as the shell remains open (Shumway, 1977). When open, cell swelling caused by exposure to reduced salinity, and thus reduced internal osmolarity, is avoided in mussels via the use of free amino acids which break down osmolytes which usually adjust internal osmolality (Podbielski et al., 2022). These free amino acids are produced by an enzyme created by the *lap* gene, with the lap^{94} allele being the most efficient (Koehn et al., 1980). The lap^{94} allele has been found to decrease in areas of low salinity on the East Coast of the USA (Koehn et al., 1976), and also to decrease in frequency with *M. edulis* age (Koehn et al., 1980), suggesting that there is selection against the lap^{94} allele at low salinities where it is unnecessarily efficient and wastes energy (Riginos & Cunningham, 2005).

Despite their adaptations to cope with salinity change, reduced salinities can still have negative impacts on mussel health, physiology, and behaviour. One study examining four bivalve species exposed to a salinity ramp demonstrated that burrowing activity and scope for growth were reduced at lower salinities (Domínguez et al., 2020). Reduced salinity has also been shown to affect their immune system, which show reduced haemocyte concentrations, phagocytic activity and percentage of eosinophils after two days at 16 ppt (Bussell et al., 2008). Additionally, clearance rate, respiration rate and absorption efficiency were all lower with each subsequent salinity of 30, 25, 20 and 15 ppt when measured weekly over a period of four weeks, meaning that mussels at all salinities below 30 ppt had a negative scope for growth (Wang et al., 2011). However, negative scope for growth was only reached at salinities of 15 ppt when *Choromytilus chorus* mussels were exposed to a similar range of salinities for 15 days (Navarro, 1988). The differences in the results between these two studies may be explained

by the fact that the second study used mussels from an estuarine site with highly variable salinity, so they may have already acclimated to perform better under these conditions.

Similarly, several other studies suggest that mussels are able to acclimate to reduced salinities to some extent. An acute salinity drop was found to cause an initial decrease in the heart rate of *M. edulis*, but after 5 days this was recovered to normal, suggesting potential for acclimation to the decreased salinity (Bakhmet et al., 2005). Additionally, *C. virginica* oyster larvae originating from an area of low salinity were found to have a better survival when reared in low salinities in the laboratory than when reared in higher salinities, suggesting that the larvae acclimated to a narrower performance window (Eierman & Hare, 2013). Understanding the plasticity of mussel performance is key for predicting their ability to cope with a changing climate and how their distributions may change, and doing so across their entire life cycle is clearly vital to determine windows of sensitivity which may govern distribution and their ability to survive in any given habitat.

Salinity based differences in *Mytilus* distribution

Given that salinity has a significant effect on many physiological processes in *Mytilus* mussels, which vary amongst the different species, environmental salinity could be a key driver of the differing distributions of this group. For example, studies have shown that *M. edulis* is more likely to be found in sheltered areas, but is able to withstand salinity fluctuations, whilst *M. galloprovincialis* is frequently found in more exposed areas, but is less able to cope with a changing salinity (Gardner, 1996). In one study, sites of mussel collection along the European Atlantic shore were given an ecological score in which 0 represented sheltered areas of low salinity, whereas a score of 2 was given to exposed habitats with a high salinity, and 1 was given to those which were intermediate (either sheltered high salinity or exposed low salinity) (Bierne et al., 2002). A significant positive correlation was found between *M. galloprovincialis* allele frequency and ecological score, supporting the hypothesis that this species is more frequently found in exposed high salinity habitats, whilst the opposite was true for *M. edulis* (Bierne *et al.*, 2002). These factors may have an influence on local distributions, and cause the ‘mosaic’ pattern of species distribution demonstrated in some locations. Similar findings of salinity-based habitat

preferences have been found between *M. galloprovincialis* and *M. trossulus* within the Californian hybrid zone. When mussels were sampled from 28 locations along the North American Pacific coast, it was found that *M. galloprovincialis* was more frequently found in warmer areas with a stable salinity, whereas *M. trossulus* was found in cooler waters with a more variable salinity (Sarver & Foltz, 1993).

In a study conducted by Gardner and Thompson (2001), subtidal mussels were collected from an estuarine area within the Canadian hybrid zone, in which temperature and salinity were shown to be highly variable, in addition to collection from a coastal area which was shown to have more stable environmental conditions. Mussels from both habitats were then exposed to environmental conditions from the alternate collection site within the laboratory for a period of four months, with several factors including mortality, growth rate, clearance rate and absorption efficiency being measured. Whilst mortality was shown to be fairly low in coastal conditions, when mussels which originated from the coastal area were exposed to estuarine conditions, there was a significantly higher mortality of *M. trossulus* than *M. edulis* individuals. This difference was not observed for mussels exposed to estuarine conditions which originated from estuarine conditions (Gardner & Thompson, 2001). This was a surprising result, as *M. trossulus* are generally considered to be more tolerant to fluctuating salinity than *M. edulis* within this region, but the fact that mortality of *M. trossulus* was higher under simulated estuarine conditions suggests the opposite was true. This difference between species was not observed in mussels originating from the estuarine location, suggesting that these mussels were adapted or acclimated to these conditions. However, this study used morphological differences supported by allozyme analysis to distinguish between species. For accurate determination of the species, particularly for accurate determination of the allele composition of hybrids, modern genetic techniques are critical to determine the role of the environment on species, and hybrid, distribution.

The impact of reduced salinity on mussels has been studied extensively in the Baltic Sea, where hybrid *M. edulis-M. trossulus* mussels are found in brackish water which has a consistently low salinity, which has been found to account for 50% of genetic variability in this area (Kijewski et al., 2019). Mussels originating from areas of differing salinity in the Baltic were found to demonstrate local

adaptation, with more *trossulus*-like mussels showing improved growth and settlement success at salinities of 7 psu, whilst more *edulis*-like mussels performed better at 16 psu (Knöbel et al., 2021). These mussels are often dwarfed, and this has been hypothesised to be caused by low salinities which may create a greater cost of calcification (Sanders et al., 2018). It was also found that when the oxygen consumption of mussels living in areas across a salinity gradient were compared, there was no significant difference, suggesting these mussels were locally adapted to their respective environmental salinities (Landes et al., 2015). Filtration rate of mussels originating in the Great Belt near Denmark, where salinity is around 16.6 ppt, was only found to be lessened by exposure to a salinity of 5 ppt, with similar rates observed for salinities ranging from 10 to 30 ppt (Riisgård et al., 2013). However, mussels originating from the Central Baltic Sea, where salinity is typically 6-7 ppt, were able to recover their filtration rate after several days of exposure (Riisgård et al., 2013). These studies all suggest that the stable salinity gradient across the Baltic Sea may explain their unique hybrid swarm structure. However, whilst examining the effects of salinity on Baltic mussels is key for understanding their distribution, this is a system uniquely defined by a stable salinity cline across a large area, and therefore it is difficult to extrapolate these effects to mussels which live in highly dynamic estuarine systems. Despite this, studies into the effects of salinity are still largely dominated by those examining the Baltic system, meaning that we have many gaps when it comes to our understanding of estuarine mussels.

The effect of salinity changes on aquaculture

Mussel aquaculture is a globally expanding market, and farming of species lower on the food chain like bivalves presents an opportunity for a more sustainable food source than many other farmed animals (FAO, 2022). However, salinity changes can have a substantial impact on aquaculture. For example, Sardinian mussel farms experienced mass mortalities in both 2013 and 2018 as a result of flash flooding caused by cyclone conditions and excessive rain (Addis et al., 2021). Furthermore, the 2014 mass mortalities experienced in farmed *M. edulis* populations in the Breton Sound, France, have subsequently been linked to salinity as a potential stressor, which may have increased the sensitivity of mussels to the pathogen *Vibrio splendidus* (Polsenaere et al., 2017).

Even when the salinity changes are not severe enough to result in mortality, they are still often detrimental to aquaculture production. Several impacts of exposure to lower salinity, such as reduced growth rate (Domínguez et al., 2020; Landes et al., 2015), are undesirable for aquaculture productivity, making profitable aquaculture in low salinity zones like the Baltic Sea difficult (Buer et al., 2020). In Loch Etive in Scotland, the upper depths are brackish due to freshwater input and slow mixing, resulting in a halocline (Beaumont et al., 2008). Rope grown mussels in this area are found to have a more *trossulus*-like genotype closer to the surface, and more *edulis*-like as depth increases (Beaumont et al., 2008), which corresponds to the assumption that *M. trossulus* is more resistant to reduced salinities than the other *Mytilus* species. Interestingly, a phenotypic difference is also observed in this area, with the more *trossulus*-like genotypes having more fragile shells, a suboptimal trait for aquaculture processing and harvest (Beaumont et al., 2008). Around 37% of mussels in Loch Etive were found to be *M. trossulus*, with a further 23% being an *edulis-trossulus* hybrid, with a recent increase in *M. trossulus* abundance (Dias et al., 2009). A similar study conducted in Loch Leven found that although there was a much smaller proportion of *M. trossulus* (0.46%) and *edulis-trossulus* hybrids (3.42%), there was still a significant effect of reduced shell strength at lower depths (Michalek et al., 2021). Such was the severity of this impact that it resulted in significant losses to the industry, making production in the respective Lochs commercially non-viable (Gubbins et al., 2012).

M. trossulus is now stated to be a commercially damaging species by the Scottish Government (Scottish Government, 2014), and is sometimes referred to as the foolish mussel (Regan et al., 2021). Climate change is likely to increase water stratification, which will be less favourable for mussel aquaculture (Des et al., 2020), and may exacerbate the impact of invading *M. trossulus* on Scottish aquaculture. Aquaculture may also alter the genetic composition of mussel species, as the import of spat between aquaculture sites and subsequent spawning events can lead to increased gene flow and the emergence of hybrids (Michalek et al., 2016). However, a more recent evaluation which compared SNP analysis to the shell strength of *M. edulis* and *M. trossulus* from a mussel farm in Loch Fyne found that there was no significant difference in the shell strength of the species, suggesting hybridisation with *M. trossulus* may not be the sole cause of the observed mussel shell weakness (Carboni et al., 2021).

Although salinity changes pose a threat to aquaculture, as our understanding of the specific responses of different hybrid genotypes to a variety of stressors expands, there is potential for the selective breeding of specific genotypes which are best suited to survive environmental change. Hybrids of abalone have been found to be more resistant to thermal and immune stressors than the parental species, and hybrids are already used within abalone aquaculture for this reason (Lafarga de la Cruz & Gallardo-Escárate, 2011; Liang et al., 2014). Hybridisation has also been used in the aquaculture of several species of finfish to improve traits such as growth rate, disease tolerance and marketable appearance (Bartley et al., 2000). Whilst hatchery breeding of spat is used in some locations, UK mussel aquaculture currently relies mostly on wild spat collection, however future limits to wild spat supply may cause a shift to hatchery-based breeding techniques (Regan et al., 2021). There is potential for these techniques to be extended to improve their marketability and production rate, although the extent of the impact on wild mussel populations would need to be investigated. Given that we know that mussel adaptation to reduced salinities is possible (Eierman & Hare, 2013), advances in genetic techniques pose the option of breeding spat which have a better tolerance to salinity change. However, in order to achieve this we need to ensure we have a comprehensive understanding of the physiological effects of salinity.

Climate effects

In a changing ocean, mussels are likely to face increasing climate stressors on top of osmotic stress. According to the IPCC, anthropogenic CO_2 emissions have caused the warming of the oceans (IPCC et al., 2021). This has been shown to have widespread effects on the range, physiology, growth, reproduction, behaviour, phenology and immune response of many marine species (Gissi et al., 2021; Mackenzie et al., 2014; Pinsky et al., 2020). Many organisms are undergoing range shifts in an attempt to remain within a favourable environmental envelope despite warming, frequently leading to poleward shifts, which can have drastic effects on food webs (Edwards & Richardson, 2004; McQueen & Marshall, 2017; Perry et al., 2005; Pinsky et al., 2020; Sorte et al., 2010). Where organisms are unable to evade warming through redistribution, exposure to increasing temperature can have a profound effect on physiology, with many organisms experiencing altered metabolic rates under elevated temperatures (Deutsch et

al., 2015; Harianto et al., 2018; Marshall & McQuaid, 2011), in part due to the increase in enzymatic activity at elevated temperatures (Marañón et al., 2018). Increased ocean temperatures may also decrease growth (Pörtner et al., 2008) and abundance of marine species (Edwards et al., 2021).

In addition to ocean warming, oceans are acidifying as a result of increased levels of dissolved CO_2, which reacts with water to form carbonic acid. This in turn dissociates to form bicarbonate and then carbonate ions, releasing H^+ ions, making the water more acidic (Doney et al., 2009). As a result of future carbon emissions, the IPCC has projected a decrease in ocean seawater pH of 0.065-0.31 by 2100 (IPCC et al., 2021), with this decline having been predicted to have effects on many aspects of marine life (Doney et al., 2009). Calcifying organisms are thought to be particularly threatened by ocean acidification, given that it decreases the concentration of carbonate ions available for use in calcification, potentially causing dissolution of carbonate structures (Doney et al., 2009). This has been extensively studied in corals, with skeletal density decreasing under acidified conditions (Guo et al., 2020; Mollica et al., 2018; Williams et al., 2021). Acidification also causes dissolution of other carbonate structures, with vermetid shells and *Hydroides elegans* tubes showing dissolution at pH levels predicted for the end of the century (Chan et al., 2012; Milazzo et al., 2014).

Ocean acidification has also been found to impact metabolic rate of organisms. For example, embryos of the crab *Petrolisthes cinctipes* were found to have a metabolic rate which was reduced by 11% after 7-10 days exposure to a low pH of 7.58, when compared to those in an ambient control of 7.93, although this effect was not seen in larvae or juveniles (Carter et al., 2013). The effects of reduced pH on metabolic rate are wide-ranging, with a meta-analysis finding that whilst some studies showed that prolonged elevated CO_2 levels caused in increase in metabolic rate for various invertebrate and fish species, many other studies did not find a significant effect (Lefevre, 2016).

The above studies show that the effects of warming and acidification are diverse, and species, life stage and treatment specific. They also impact different biological processes via different mechanisms and thus elicit a range of contrasting impacts. As a result, this highlights the importance of measuring different endpoints, as assessing the impact of a stressor based on one effect is likely to create an incomplete representation of the holistic outcome of climate

change on animal performance, behaviour and survival. In addition to this, in dynamic systems like coastal and estuarine environments, pH can already fluctuate by large amounts (Hinga, 2002), and estuaries have been found to be warming and acidifying at a faster rate than other areas of the ocean (Scanes et al., 2020). This suggests that it may be difficult to extrapolate the general effects of climate change to organisms living in these systems, and therefore studying animals from these habitats is of utmost importance to predict their future performance.

Mussels from the *Mytilus* genus are a good model organism for understanding climate change impacts in coastal and estuarine environments, as they have a wide distribution and are common in many areas around the world, enabling a global comparison of effects (Beyer et al., 2017). The carbonate shell of bivalves is often negatively impacted by ocean acidification, with many studies showing that shell strength, calcification, organisation of crystal structure and growth all decline with increasing pCO_2 levels (Berge et al., 2006; Fitzer et al., 2014, 2015; Gazeau et al., 2007), which has found to be exacerbated by reduced food concentrations (Melzner et al., 2011). Similarly, acidification also impacts the calcification of larvae, with shells of *M. californianus* becoming 20% weaker and 15% thinner at increased CO_2 levels (Gaylord et al., 2011).

Acidification is also shown to cause a decrease in settlement and negatively impact metamorphosis in many marine invertebrate larvae, a phenomenon again predominantly studied in coral (Albright et al., 2010; Doropoulos & Diaz-Pulido, 2013; Yuan et al., 2018), but which has also been observed in molluscs (X. Guo et al., 2015; Jansson et al., 2016). In *M. edulis* for example, the proportion of deformed larvae has been shown to increase as seawater pH decreases (Kong, Jiang, et al., 2019). However, when the parents of larvae had also been exposed to acidification, larvae from acclimated parents demonstrated an adaptation to these conditions, with reductions in seawater pH shown to have less of a negative impact in comparison to acutely exposed conspecifics (Kong, Jiang, et al., 2019). These transgenerational effects were also found in *Saccostrea glomerata* larvae exposed to elevated pCO_2, where an improved rate of growth and development was measured in larvae spawned from adults conditioned to high pCO_2 compared to those from adults exposed to ambient pCO_2 (Parker et al., 2012). This suggests that as ocean pCO_2 levels increase, it may be possible for mussels to acclimatise

to the changing conditions. However, *Mytilus* metabolic rate does not appear to change significantly under hypothesised acidification scenarios, with several studies finding no impact of ocean acidification alone on the metabolic rate of *M. edulis* (Matoo et al., 2021), *M. galloprovincialis* (Gazeau et al., 2014), or *Mytilus edulis-trossulus* hybrids (Jakubowska and Normant, 2015).

The behaviour of organisms may also be affected by acidification, although findings have not been consistent across species; one study found that the feeding rate of *Perumytilus purpuratus* mussels decreased as pCO_2 increased (Vargas et al., 2015), but another found that the feeding rate of *M. galloprovincialis* increased (Lassoued et al., 2019). This difference may be explained by the fact that Lassoued et al., (2019) found that clearance rate was only affected by pCO_2 at optimal food conditions, so differences in food concentrations may explain these seemingly contradictory results. The effects of acidification on various forms of predator avoidance has also been examined in invertebrates, with mixed results. Valve closure responses to predator cues were not found to be affected by acidification in *M. galloprovincialis* mussels (Clements et al., 2020), but conversely when exposed to both a temperature increase and a pH decline, *Pecten maximus* exposed to 50 days of warmed conditions showed a reduced predator escape performance when compared to those which had been exposed to cooler water, suggesting predator responses of some bivalves may be affected by climate change (Schalkhausser et al., 2014).

Mussels attach to a substrate using byssal threads, a secretion of proteins which form a thread from the foot to an adhesive plaque which binds them to surfaces (Newcomb *et al.*, 2019). A decline in pH from 8 to 7.5 was found to reduce the overall attachment strength of *M. trossulus* by 35-41%, with interactions with temperature also found in *M. edulis* (Kong, Clements, et al., 2019). However, other studies have found that only temperature decreases attachment strength of *M. edulis*, with no effect measured for acidification (Clements *et al.*, 2018), although this effect may potentially be dependent on food availability (Lassoued et al., 2019). Whilst all of these studies provide us with a critical understanding of the potential effects of future climate change, they are frequently limited by the fact that they don't take into consideration the highly changeable environments that mussels inhabit, and the interacting effects that existing environmental variables may have on the consequences of climate change.

Combination effects of salinity, warming and acidification

In order to provide the most accurate predictions of how intertidal and estuarine environments are likely to be affected by climate change, it is essential to examine the combined effects of warming and acidification alongside salinity changes. Tidal air exposure was simulated in the laboratory by removing *M. edulis* mussels from water at a range of salinities for 90 minutes, every 12 hours, during which time they were exposed to differing air temperatures (Nielsen et al., 2021). A combination of a 30 °C air temperature and low salinities of 15 or 5 ppt significantly increased the mortality of mussels when compared to a control of 5 °C and 23 ppt, suggesting that increasing temperatures may have a synergistic effect on mussels which already have to cope with the stress of tidal air exposure and estuarine salinity changes. In addition, a study which examined the impact of temperature and salinity effects on the mortality of Sydney cockles (*Anadara trapezia*) over a period of 56 days found that temperature extremes of 10 °C or 30 °C caused a large increase in mortality when combined with salinities of 15 or 25 ppt (Taylor et al., 2017). When the transcriptome of *C. virginica* exposed to 4 weeks of various combinations of salinity and temperature were compared, it was found that many genes were differently expressed in the high temperature (30 °C) or low salinity (7 psu) treatments compared to controls of 20 °C and 25 psu, with synergistic effects in the combined high temperature low salinity treatment (Jones et al., 2019).

Low salinity may also have an interactive effect when combined with ocean acidification. For example, one study found that a combination of acidification and low salinity reduces the condition index of *Ruditapes philippinarum* clams (Velez et al., 2016). A further study exposed *M. edulis* to combinations of three different pH levels and two dissolved oxygen levels and calculated the scope for growth (Gu et al., 2019). They found that clearance rate, absorption rate and respiration rate decreased with decreasing pH, and was also decreased further at lower oxygen levels. These studies all seem to point to the conclusion that habitats with fluctuating environmental conditions may experience the negative impacts of climate change more acutely, and therefore the effects may be underestimated in studies which only assess them in stable conditions. Additionally, salinity has been found to alter *Mercenaria mercenaria* response to 21 weeks of exposure to high CO_2 levels, with ~800 µatm resulting in a reduced shell microhardness at 32 ppt, but not at 16 ppt (Dickinson et al., 2013). However, whilst ~800 µatm actually

reduced mortality when compared to ~ 380 µatm at 32 ppt, mortality was not reduced at 16 ppt (Dickinson et al., 2013). These complex interactions show that incorporating real life environmental variables like salinity is critical, as these have the potential to alter the predicted response to climate effects.

The effects of climate change on *Mytilus* distribution

The predicted impacts of ocean warming on the distribution of marine species has been widely studied, with many organisms expected to show a poleward range shift as their native habitats increase in temperature (Bates et al., 2014). This has already been observed to some extent in *M. edulis* along the Eastern coast of the US, the range of which has shifted around northwards by 350 km in the last 50 years (Jones et al., 2010). It has also been suggested that the ability of *M. galloprovincialis* to withstand higher temperatures will cause its continued range expansion (Han & Dong, 2020), and possibly increase its invasive spread. However, a study conducted in 2005-2007 which revisited sites in California, originally sampled by Rawson et al., (1999) in 1994-1995, found that the range of *M. galloprovincialis* had contracted significantly, with northern sites containing only 0-4% *M. galloprovincialis* where they previously had 53-80%, despite mussel abundance remaining high (Hilbish et al., 2010). This highlights the importance of examining the effects of other stressors on range shifts, including salinity.

Climate change is also predicted to cause an increase in freshening and flash flooding events, with many mid to high latitude areas showing a reduction in salinity (Durack & Wijffels, 2010; Seggel, A., De Young, C., and Soto, 2016). The IPCC predicts that there has already been a significant change in ocean salinity, with salinity increasing in many areas where it is high, due to evaporation, and decreasing in areas where it is already low, due to increased runoff, with the Arctic, North and South Pacific, South Indian, Bay of Bengal, and Southern oceans all predicted with to decrease in salinity with medium confidence (IPCC et al., 2021). One of the reasons for this salinity decrease is an increase in ice melt as a result of climate warming, which is already causing a significant freshening effect in the Arctic Ocean (Li & Fedorov, 2021). In addition, it was found that salinity of the sea water in the Gulf of Finland, in the Northern Baltic, has decreased over the last 40 years, with transects in this area showing that mussel abundance was lower in low salinity zones (Westerbom et al., 2019).

In the next century, the Baltic Sea has been predicted to face a salinity decline of 8-50% (Meier, 2006), which is likely to result in a range shift of many species, including *Mytilus spp* (Vuorinen et al., 2015). *M. trossulus* has been found to be more salinity tolerant than *M. edulis*, with only 47% of *M. edulis* developing into swimming embryos at 25 ppt, compared to more than 90% in *M. trossulus* (Qiu et al., 2002). Additionally, whilst extreme low salinities of 7 or 16 psu were found to cause developmental delay, *M. trossulus* had a higher settlement success at 7 psu than 16 psu whilst the opposite was true for *M. edulis*, suggesting *M. trossulus* are able to outcompete *M. edulis* at low salinities (Knöbel et al., 2021). This salinity-based selection suggests that distributions of *M. trossulus* may increase as a result of climate-induced salinity declines. However, this is likely to contrast against the selection effects of increased temperature, which *M. trossulus* is less tolerant to (Sarver & Foltz, 1993). Whilst temperature has generally been considered to be the greatest driving force of distribution changes under climate change, in some areas salinity might be causing a greater selection. Braby and Somero (2006) examined 21 locations within the *Mytilus* hybrid zone in California from 2000 to 2004, and found that the salinity effects were as expected, with *M. galloprovincialis* abundance positively correlated with salinity, and *M. trossulus* abundance negatively correlated with salinity. *M. trossulus* was also more likely to be found in areas with a wider salinity range, reinforcing the hypothesis that this species has a greater tolerance to salinity variations than *M. galloprovincialis* (Braby & Somero, 2006a). However, this study also found that the proportion of *M. galloprovincialis* was negatively correlated with temperature, whilst the opposite was true for *M. trossulus*. This is a surprising finding given that other work indicates that *M. galloprovincialis* is better adapted to warmer waters (Schneider & Helmuth, 2007), but one possible explanation is that within these study sites, temperature was negatively correlated with salinity (Braby & Somero, 2006a). This could suggest that salinity was a more important factor for defining species ranges than temperature. One possible explanation for the improved tolerance of *M. trossulus* to low salinities might be higher levels of MAPK-dependent phosphorylation, which is assoicated with dealing with osmotically stressful conditions (Evans & Somero, 2010). An increase in extreme weather events is also likely to lead to an increased frequency of flash flooding, which will increase runoff into coastal and estuarine environments, leading to temporary but drastic changes in salinity (Boyd et al., 2015). Evidently, salinity

tolerance plays an important role in the distribution of *Mytilus* species, the effects of which have often been overlooked when making predictions. This emphasizes the need to first have a comprehensive understanding of the fundamental physiological and behavioural responses of mussels to different salinities before we are able to predict future distribution changes resulting from climate change.

Conclusion

As the ocean continues to undergo unprecedented changes rising from anthropogenic climate change, the need to understand the impacts on ecosystems and the organisms within them is becoming increasingly important. Whilst there has been fairly extensive research conducted into the impacts of ocean warming and acidification, there has been comparatively few studies into the effects of salinity changes. This is especially relevant to coastal and estuarine species like mussels, which are likely to face an increase in frequency of sudden salinity changes from flash flooding and freshwater runoff. These salinity changes, in combination with the many other stressors mussels are facing, are expected to negatively impact their physiological functioning and additionally affect their distribution. With *M. galloprovincialis* being the best adapted *Mytilus* species to warming, it is predicted that this will continue to expand in range and invade areas currently dominated by other species. However*, M. trossulus* may also increase in prevelance in areas where freshening and salinity fluctuations are prevelant owing to its ability to better cope with osmotic stress. Such changes, both in performance in relation to changing environmental conditions, as well as distribution, are likely to have a major impact on future aquaculture production. It is thus critical that research investigates the physiological tolerance of different *Mytilus* species to changing salinity and temperature, and how this relates to current mussel distribution, in order to accurately project climate change impacts both for natural and farmed populations.

Thesis overview

The aim of this thesis is to understand the underlying physiological and behavioural mechanisms behind mussel response to salinity. In addition, it examines the differences in these responses between mussels originiating from coastal and estuarine habitats, and considers the genetic differences which may contribute to a difference in performance. In chapter 2, I examine the effects of different salinities on the metabolic rate of hybrid *M. edulis-galloprovincialis*

mussels originiating from several estuarine and coastal sites. In addition, I analyse the genetic composition of these species using a newly developed 60k mussel SNP array. The objective of this experiment was to test the hypothesis that the differing salinities experienced by mussels from coastal and estuarine habitats results in a different hybrid composition within these areas, as a result of the different physiological tolerances of the different mussel species. In chapter 3, I measure the gaping behviour of *M. edulis* exposed to differing rates of salinity change, originating from coastal and estuarine environments. Additionally, I take simultaneous measurements of gaping behaviour, heart rate and metabolic rate over a range of salinity exposures. This experiment aimed to understand the fundamental interactions between these physiological and behavioural mechanisms, which have yet to be recorded simultaneously in this species. Finally, in chapter 4, I discuss the implications of this research, placing it in the context of the wider field, and suggest possible routes of exploration for future studies.

The Metabolic Rate and Genetic Composition of *Mytilus* Mussels from Coastal and Estuarine Sites Within a Hybrid Zone

Introduction

The *Mytilus edulis* complex (comprised of *Mytilus edulis*, *Mytilus trossulus*, *Mytilus galloprovincialis* and their hybrids) are a group of mussels distributed across temperate habitats globally, and which, where these individual species ranges overlap, are able to form hybrids, frequently creating zones which contain a combination of both pure mussel species and hybrids. Around the Southwest coast of the UK, *M. edulis* and *M. galloprovincialis* are found both in areas of purely one species, in addition to forming parts of a complex hybrid zone, containing individuals of varying proportions of introgression and parental ancestry. When mussel samples were collected from 33 locations around the Devon and Cornwall coastlines, a hybrid zone was identified, stretching from Start Point in South Devon to St Ives in North Cornwall (Hilbish et al., 2002). In addition, this zone showed a cline from more *edulis*-like mussels in South Devon, to more *galloprovincialis*-like individuals in North Cornwall.

Despite these overall general patterns of distribution within the hybrid zone, considerable local differences in the species composition were shown to exist across a fine spatial scale. For example, St Michael's Mount was shown to mostly consist of individuals with *M. edulis* alleles, with an average allele frequency of 0.82 *M. edulis*. However, the nearby area of Porthcurno showed a much greater variation in hybrid frequency, ranging from 0.95 to 0.18 *M. edulis* alleles, depending on the size of the mussel. There have been several hypotheses suggested to explain these local differences in distribution. One suggestion is that *M. edulis* is more frequently found in sheltered habitats with a fluctuating salinity, whereas *M. galloprovincialis* prefers exposed areas with a consitently high salinity (Gardner, 1996). This appears to support the observed distribution; mussels at the coastal site of Whitsand Bay were found to be hybrids, whereas mussels from the nearby Tamar estuary were mostly pure *M. edulis* (Hilbish et

al., 2003). When sites of mussel collection along the European Atlantic shore were rated to characterise prevailing environmental conditions, there was a significant positive correlation found between *M. galloprovincialis* allele frequency and scores which indicated an exposed, high salinity site (Bierne et al., 2002). These studies determine genotype by using the Glu-5' marker, a sequence which varies in base pair length between *Mytilus* species (Rawson et al., 1996). Whilst this method provided an excellent insight into the hybrid structure of *Mytilus* mussels, more advanced genetic techniques have since been developed, such as the use of next-generation sequencing to identify many thousands of single nucleotide polymorphisms (SNP) (or single base differences in genetic code, representing the most common form of genetic variation), enabling the examination of genetic variation between individuals in far greater detail than possible with an individual or small number of targetted markers (Marth et al., 1999). Following the development on novel genetic resources for this species group, a novel 60K SNP array has been developed for the *Mytilus* species complex (Nascimento-Schulze et al., 2023), which allows for a much more detailed analysis of hybrid structure.

Mytilus species are frequently found in coastal and estuarine sites, which despite being neighbouring habitats differ greatly in their conditions. While coastal areas are often impacted by wave action, salinity is usually high and stable. In contrast while estuaries are sheltered, they are defined by salinity fluctuations, which can vary significantly both spatially and temporally (Sumner & Belaineh, 2005; Uncles & Stephens, 2011). Estuaries can be classified by the way that fresh river water and saline sea water mix, with highly stratified estuaries containing a sharp boundary of salinities, and homogenous estuaries containing thoroughly mixed brackish water (Schubel & Pritchard, 1972). At high tide, estuarine habitats are filled with high salinity sea water, but at low tide there is a greater proportion of river water making the estuary brackish. Over the tidal cycle, which lasts approximately 6 hours in regions experiencing semi-diurnal tides, the range of tidal height follows the 'rule of twelfths' (Pearson, 2007), which describes the sine curve of tidal change in water volume. This means that the smallest change in tide height happens during the first and sixth hours, whereas the largest change happens in the third and fourth hours, which likely means that salinity change over a tidal period is also not linear. These complex changes result in mussels distributed across these habitats experiencing highly dynamic and variable

environmental conditions, which change drastically both daily and seasonally, making prediciton of salinity exposure for different individuals or populations difficult to predict.

Mussels are facultative anaerobes, an adaptation which allows them to close and enter anaerobic respiration during periods of intertidal air exposure (de Zwaan & Wijsman, 1976). This response also occurs in acute exposures to low salinities, during which mussels close their shell valves to protect from osmotic stress (Davenport, 1979; Shumway, 1977), and often results in a significant decline in oxygen consumption and clearance rate, as they are no longer able to pump water around their body (Famme, 1980). Whilst several studies have examined the effects of longer periods of salinity decline (Hamer et al., 2008; Landes et al., 2015), limited research has focused on the oxygen consumption of mussels during acute salinity declines, despite the fact that this would have environmental relevance for mussels likely to experience rapid freshening during flash flooding events or increased freshwater runoff.

Various studies have examined other physiological effects of low salinity, which has been shown to cause reduced filtration rate, calcification rate and heart rate (Braby & Somero, 2006a; Riisgård et al., 2013; Sanders et al., 2018). Additionally, it causes an increase in upregulation of proteins related to stress response, and a decrease in haemocyte cells (Bussell et al., 2008; Evans & Somero, 2010). These studies demonstrate a wide range of negative effects caused by reduced salinity, which are likley to impact estuarine mussels. The physiological differences between coastal and estuarine mussels has also been directly compared, with *M. galloprovincialis* from an estuarine site in Croatia found to have a higher energy consumption than those from an adjacent coastal site, likely due to the energetic costs of dealing with a fluctuating salinity (Erk et al., 2011). Additionally, two separate studies found that both *Perumytilus pupuratus* and *Mytilus chilensis,* reciprocally transplanted between estuarine and open coastal sites, had a higher growth rate at the coastal site, both for those originating in coastal and estuarine environments (Osores et al., 2017; Ramajo et al., 2021). However, few studies have comapred these physiogical differences to the genetic distribution.

To date, probably the best comparison we have of physiology and genetics within a hybrid zone is that of Gardner and Thompson (2001), who examined differences

between *M. edulis* and *M. trossulus* collected from a coastal and estuarine site in the hybrid zone in Newfoundland, Canada (Gardner & Thompson, 2001). When exposed to fluctuating salinities in the lab, mussels originating from the coastal area had a higher mortality than those originating from the estuarine area, suggesting that the estuarine mussels were acclimated to the conditions. There were greater *M. trossulus* mortalities in estuarine conditions than *M. edulis*, suggesting that the environmental differences may have resulted in some species selection (Gardner & Thompson, 2001). Whilst this study provides excellent insight into how physiological differences might impact hybrid distribution, this is yet to be examined in other areas; for example, few studies have examined the physiological differences between *M. edulis* and *M. galloprovincialis* in estuarine and coastal environments in Southwest England. By better understanding the factors that affect their distribution, and also the physiological niches of the species, it will enable us to predict future changes in distribution and understand how environmental change may impact this key species. This study examines the effect of declining salinity regimes on the oxygen consumption of mussels from both coastal and estuarine environments.

The aim of this study was to assess if mussels from coastal and estuarine sites have a different physiological tolerance when exposed to a decline in salinity. Mussels were collected from two sites within the Tamar estuary in Plymouth, as well as a nearby coastal site, and exposed to an acute salinity ramp mimicking a theoretical tidal cycle, with salinity decreasing to either 27 ppt or 19 ppt in addition to a control condition in which no salinity reduction was experienced. Oxygen consumption was then measured using closed respirometry. Differences in oxygen consumption were then compared between locations of origin and salinity exposure. Additionally, genetic composition of the individuals used for physiology experiments was subsequently determined by using a novel *Mytilus*-specific SNP array (Nascimento-Schulze et al., 2023). It was hypothesised that closure of shell valves at lower salinities would result in a lower MR in mussels, and that individuals from the estuarine sites would have a different MR to those originating from the coastal site. Additionally, we predicted that the coastal mussels would be hybrids, whereas the estaurine sites would be mostly comprised of *M. edulis*-like individuals.

Materials and methods

Sample collection

The Tamar estuary is partially mixed, with highly variable salinity based on freshwater runoff (Uncles et al., 1983). This area is within the hybrid zone between *M. edulis* and *M. galloprovincialis* (see Figure 3a), and previous genetic analysis of mussels at Whitsand Bay has found them to be hybrids (Bignell et al., 2011; Gilg & Hilbish, 2003a; Hilbish et al., 2002, 2003). However, sites within the estuary, including Cremyll and the Tamar Bridge near Saltash, had a hybrid index which suggested they were almost entirely *M. edulis* (Bignell et al., 2011; Hilbish et al., 2003). Mussels were originally collected at spring low tide from two locations in Plymouth, United Kingdom on 04/11/21: Whitsand Bay (coastal habitat; 50.323,-4.221; mean whole animal weight 7.67±2.90 g), and Saltash (upper estuarine site; 50.409,-4.207; mean whole animal weight 15.71±10.14 g), within the higher area of the Tamar estuary (see Figure 3b). These were split into two experimental groups, A and B, each containing 10 Whitsand and 10 Saltash mussels. Mussels were also later collected from Cremyll (lower estuarine site; 50.361, -4.176; mean whole animal weight 9.68±4.93 g) within the lower area of the Tamar estuary (see Figure 3b) on 15/03/22, and 20 mussels from this location formed experimental group C.

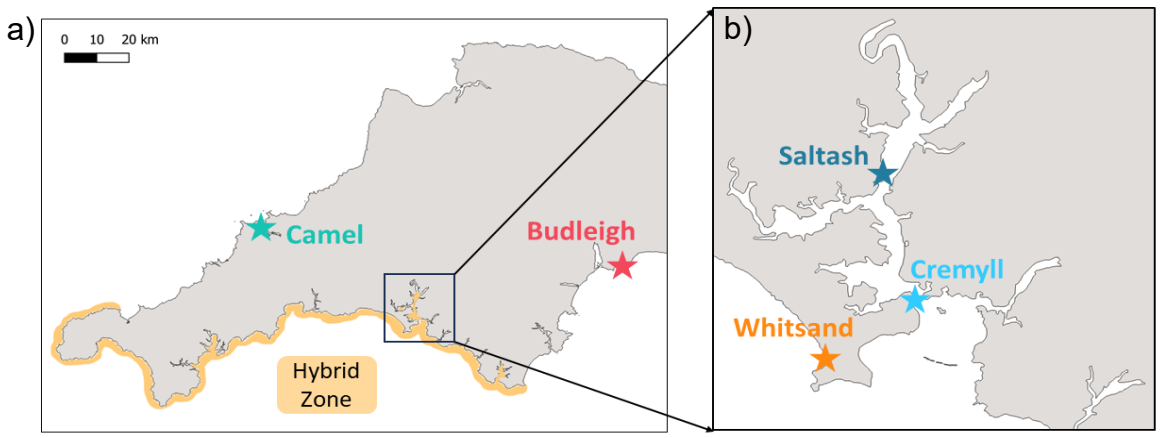


Figure 3: a) A map of the Southwest UK with the hybrid zone between *M. edulis* and *M. galloprovincialis* (as defined by Hilbish et al. (2002)) highlighted in yellow, and the known *M. edulis* Budleigh site and known *M. galloprovincialis* Camel site marked. b) The sites of experimental mussel collection in Plymouth: Whitsand, an open coastal site; Cremyll, within the lower part of the Tamar estuary; Saltash, within the upper part of the Tamar estuary.

Experimental procedure

Following collection, mussels were cleaned of epibionts and labelled with numbered bee tags adhered with Araldite rapid epoxy, and maintained within a 74 litre continuously recirculating system at The University of Exeter (34.13 (±0.76) ppt, 13.49 (±0.32) °C, 16:8 light:dark cycle). Experiments began 15/02/22

for groups A and B, and 06/05/22 for group C. Mussels were fed with 0.027 ml/L of Shellfish Diet (Reed Mariculture) the day prior to each experiment. During experiments, mussels were placed individually in 800 ml open glass jars within a larger stock tank of artificial seawater (see Figure 4a) at 34.39 (±0.84) ppt and 13.49 (±0.32) °C. Each jar also contained an air lift pump which created constant mixing between the jars and the larger stock tank, as well as aeration of the water (see Figure 4b). The tank included a blank jar which contained an air lift pump but not a mussel. Following a 30-minute acclimation period, mussels were then exposed to one of three salinity regimes (high, medium and low), before oxygen consumption was measured. This was repeated three times for each group as a technical replicate. These conditions attempted to mimic the acute salinity changes that estuarine mussels are exposed to over the course of a tidal cycle. Salinity was changed by adding fresh water to the tank on an hourly basis, which was quickly circulated by the air lift pumps.

In the low salinity regime, salinity was dropped hourly to a final salinity of 19 ppt. This salinity was chosen following several pilot tests in which it was found that mussels are usually still consuming oxygen at this salinity, but begin to stop below this threshold. The salinity regime was dropped using the rule of 12ths, which describes the sine curve of tidal height change. This meant that salinity was gradually dropped by different amounts each hour (to 33.7 ppt; 31 ppt; 27 ppt; 23 ppt; 20.37 ppt; 19 ppt) in order to replicate estuarine salinity change during a tidal cycle (see Figure 4c). Salinity was measured following each salinity drop using a Hach HQ 40d Digital Multi Meter with an Intellical CDC401 digital conductivity probe to ensure the target salinities had been achieved. Following the final salinity drop, mussels were given 30 minutes to acclimate before the air lift pumps were removed, the initial pO_2 was measured using a Firesting GO_2 pocket oxygen meter and probe (OXROB10), and the jars were sealed under water to avoid introduction of air bubbles. Jars remained inside the larger tank for the duration of the experiment, to avoid disturbing the mussel and also to help maintain a constant temperature. After three hours, the jars were mixed and opened and the pO_2 was tested again so that the oxygen consumed by each mussel over the three-hour period could be calculated.

In the medium salinity regime, mussels were again exposed to an hourly salinity drop, but this was stopped at the mid-point after the third drop to 27 ppt, as shown

in Figure 4c. Mussels were then given 30 minutes to acclimate before the air lift pumps were removed, the initial pO_2 was measured, and the jars were sealed. After three hours, the jars were opened and the pO_2 was tested again.

In the high salinity regime, the salinity was not dropped (see Figure 4c). Following the 30-minute acclimation period after mussels were put into jars, the air lift pumps were removed, the initial pO_2 was measured, and the jars were sealed. After three hours, the jars were opened and the pO_2 was tested again.

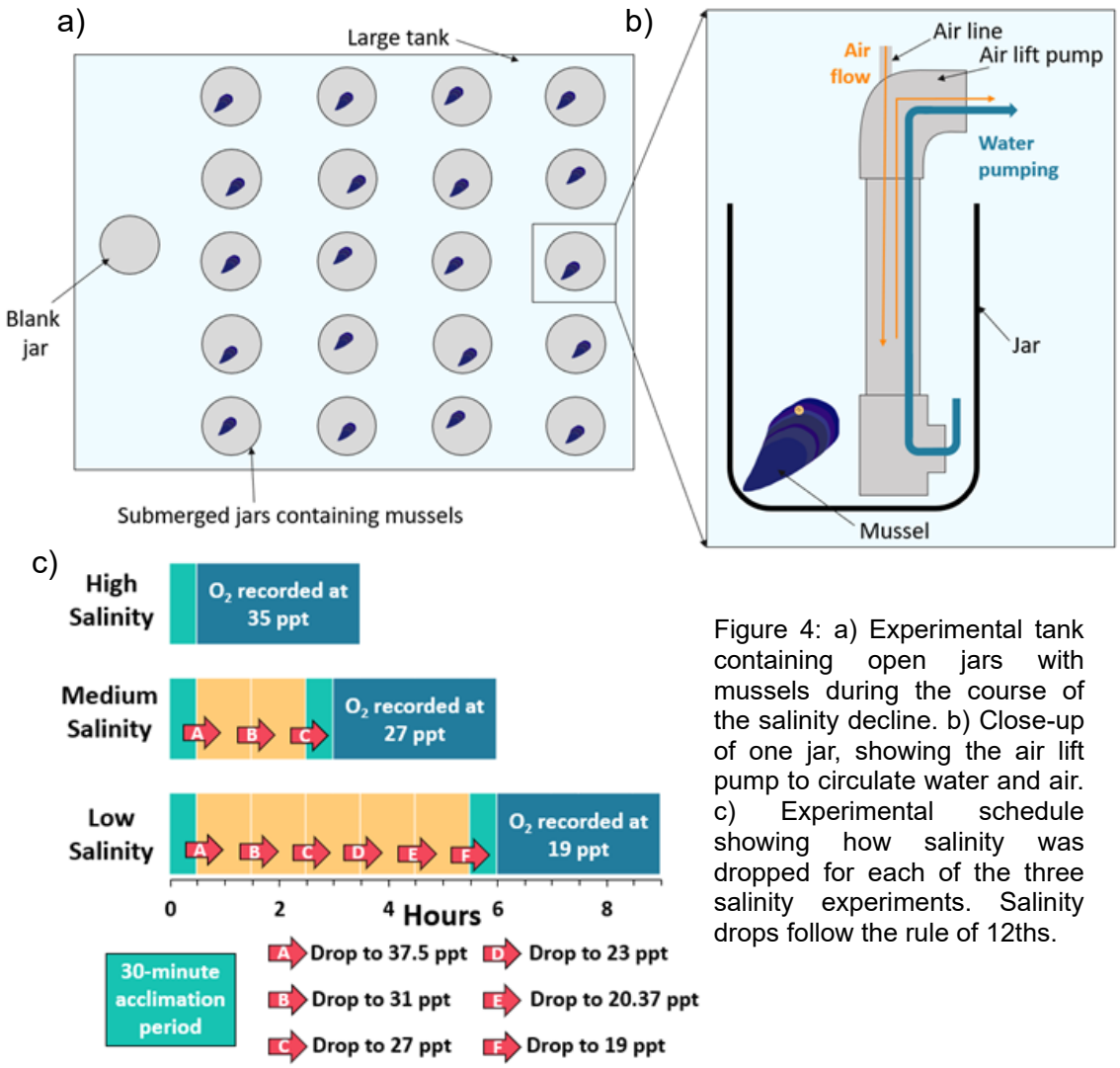


Figure 4: a) Experimental tank containing open jars with mussels during the course of the salinity decline. b) Close-up of one jar, showing the air lift pump to circulate water and air. c) Experimental schedule showing how salinity was dropped for each of the three salinity experiments. Salinity drops follow the rule of 12ths.

At the end of experiments, whole animal mass was measured by severing the posterior adductor muscle and blotting water from the mantle cavity, before the animal was weighed. Tissue was then removed from the shell and weighed to find the wet tissue mass, and then a sample of adductor mussel was removed, placed in a 1.5 ml microcentrifuge tube and preserved in 96% ethanol, before being stored at 4 °C prior to genetic testing.

Metabolic rate calculations

The difference between the initial and final oxygen concentration in each jar was calculated in Torr, before any drop in oxygen concentration in the blank jar was subtracted from this value (as a result of background microbial respiration). Using a method obtained from Green and Carritt (1967), salinity and temperature were used to find the solubility values of oxygen. These values were then used to calculate a value for metabolic rate per gram of wet tissue (µmol/g/h) using the formula:

O_2 Consumption Rate (MO_2; µmol/g/hour) =

($\Delta[O_2]$ (µmol/l) x Chamber Volume (l)) / (Body Mass (g) x Time period (h))

Metabolic rate was also calculated per gram of dry tissue, as measured following 72 hours in a drying oven after genetic sampling. Analysis found that using wet or dry mass did not affect the interpretation of the results, and therefore for simplicity only the metabolic rates calculated using the wet tissue mass are presented. In the low salinity regime, occasionally mussels did not consume oxygen and there was a slightly greater drop in oxygen in the blank jar, resulting in a negative value for the metabolic rate. In these cases, metabolic rate was assumed to be zero.

Genetic analysis

Adductor mussel samples were extracted and stored in 96% ethanol, before being sent to Identigen Ltd. in Ireland for genotyping using a custom designed Axiom Affymetrix my-design 60K mussel array as described by Nascimento-Schulze et al. (2023). Identigen Ltd completed DNA extraction, and QC, prior to analysing each sample on the array. Previously genotyped samples shown to consist of *M. edulis* from Budleigh Salterton and *M. galloprovincialis* from the Camel estuary were included in order to assist with subsequent genotype identification of unknown samples used in this experiment. Following genotyping, Axiom Analysis Suit software (Affymetrix, Thermo Fisher Scientific) was used to process sample data. Firstly, markers that had a call rate below 95% were filtered from the dataset within the software. Following this, EIGENVAL and EIGENVEC files were generated using PLINK software (Purcell et al., 2007), and R studio (RStudio Team, 2020) was used to calculate a principal component analysis (PCA), and plot the first two principal components using the ggplot2 package

(Wilkinson, 2011). Whilst a PCA analysis selects principal components with the greatest overall variance, discriminant analysis of principal components (DAPC) analysis selects principal components based on those which show the greatest separation between groups, and is therefore often better at showing the differences between them (Jombart et al., 2010). DAPC analysis was calculated using a genind dataset created from a vcf file using the vcfR package (Knaus & Grünwald, 2017). The adegenet package (Jombart, 2008) was then used to plot the first two linear discriminants showing each collection site as an individual cluster using the dapc function. Finally, Admixture software (Alexander et al., 2009) was used to find a value of K with the lowest cross-validation error. This value of K was used to plot an admixture in R to show the ancestry of each individual compared to origin population.

Statistical analysis

Data was analysed using R studio (RStudio Team, 2020). The mean was calculated for the metabolic rates of each mussel over the three technical replicates at each salinity. A Shapiro Wilk test found the data was not normally distributed, and a permuational ANOVA (permanova) was used to assess the effect of both salinity and location on the MR, followed by pairwise comparisons.

Results

There was a total of 10 mortalities out of the 60 mussels over the course of the experiments, 8 of which were mussels originating from the upper estuarine site (Saltash) and 2 mussels originating from the lower estuarine site (Cremyll). The mean salinity in each treatment were as follows; high salinity treatment, 34.13 (±0.76) ppt; medium salinity treatment, 27.01 (±0.06) ppt; low salinity treatment,18.99 (±0.06) ppt.

Metabolic rate

Metabolic rate was found to decline with salinity (Figure 5), with a permanova finding this effect to be significant (Pseudo-F=70.867, df=2, p=0.001). Pairwise comparisons also found significant differences between all pairs of salinity treatments (High:Low - t=11.823, df=2, p=0.001; High:Medium - t=3.291, df=2, p=0.004; Low:Medium – t=7.804, df=2, p=0.001). The highest MR was in the high salinity regime at 2.15 (±0.56) µmol/g/h, with the medium salinity exposure having

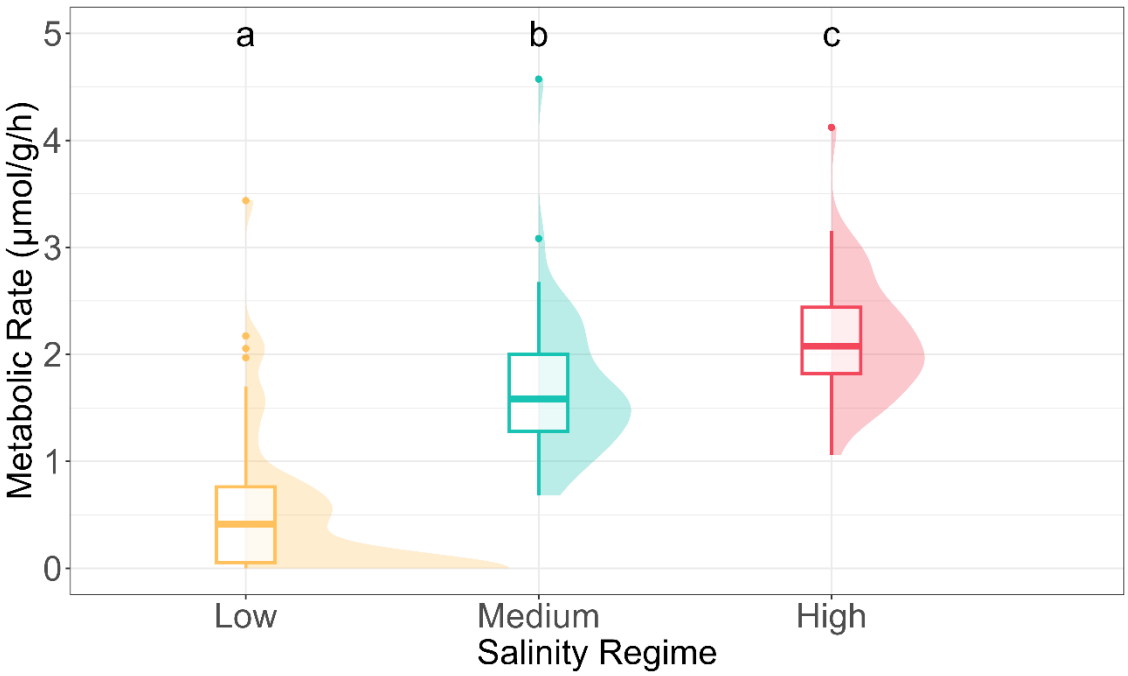


Figure 5: Box plots and density plots showing the metabolic rate of mussels in different salinity regimes ending in salinities: low (19 ppt), medium (27 ppt) and high (34 ppt). Different letters indicate significant differences between the groups.

a MR of 1.69 (±0.67) µmol/g/h, and the low salinity resulting in the lowest MR of 0.60 (±0.71) µmol/g/h. In the low salinity regime, many of the jars containing a mussel had a measured oxygen change which was the same as or slightly less than in the blank jar, meaning that MR was assumed to be zero. This happened at least once across the three technical replicates of the low salinity exposure for 30/50 mussels (60%), with 15 mussels doing this in only one replicate (30%), 9 mussels doing this in two of the replicates (18%), and 6 doing this in all three replicates (12%). When comparing this response by collection site, this happened in at least one replicate for 75% of coastal mussels, 44% of lower estuarine mussels, and 58% of upper estuarine mussels. This only occurred once in the medium salinity regime (in a mussel which also had an MR of zero in all three low salinity replicates), and did not occur in any of the high salinity replicates. The permanova found no significant differences in the MR between the coastal (Whitsand), lower estuary (Cremyll) and higher estuary (Saltash) sites (Pseudo-F=2.231, df=2, p=0.107), as shown in Figure 6. The permanova also found no significant interaction between the salinity and location (Pseudo-F=0.84, df=4, p=0.493).

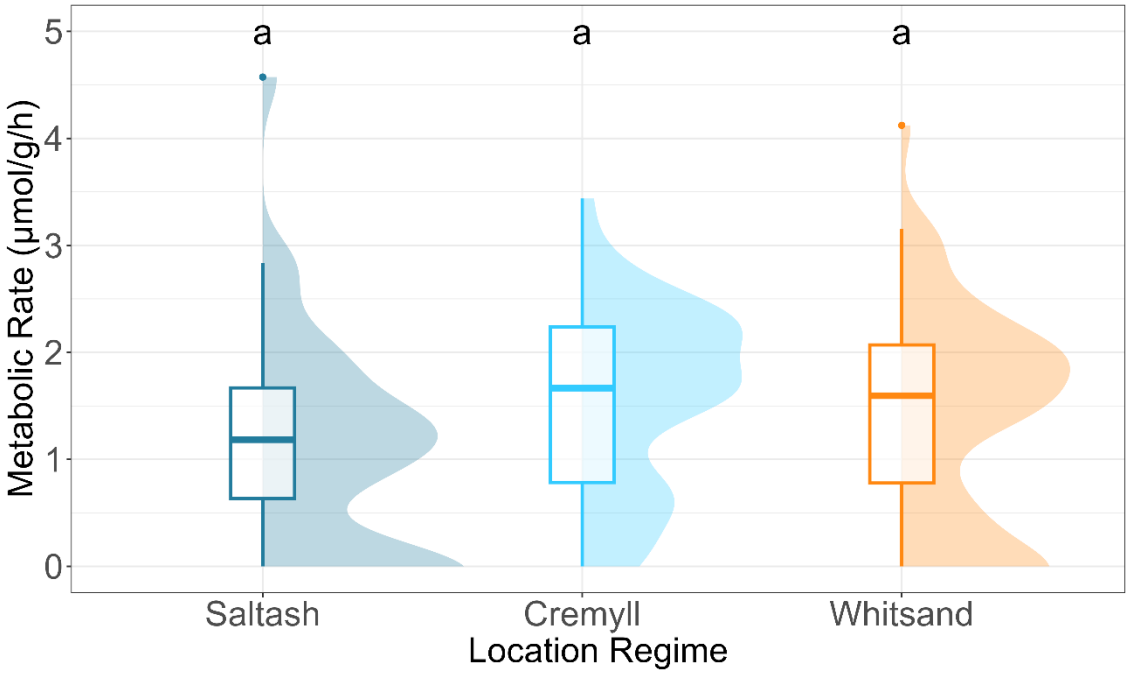


Figure 6: Box plots and density plots showing the metabolic rate of mussels collected at different sites in and around the Tamar estuary: Saltash (high estuary), Cremyll (low estuary) and Whitsand (coastal). Shared letters indicate a lack of significant difference between the groups.

Genetic analysis

After removing markers with a call rate less than 95%, 2,096 loci were retained. A principal component analysis (PCA) found that the amount of variance explained by the first two principal components was relatively low at 23.91% (PC1 explained 18.8% and PC2 5.11% of the variance). As shown in Figure 7, there was clear separation of known *M. galloprovincialis* samples from Camel, from all other sites, but no clear separation of experimental populations, or of the known *M. edulis* samples collected from at Budleigh Salterton. Following a discriminant analysis of principal components (DAPC), the first two linear discriminants were plotted, representing 85.7% and 10.9% of the total variance, compared to 2.2% and 1.2% for LD3 and LD4 respectively (Figure 8). In addition to the separation demonstrated between Camel and the remaining samples, as was shown with PCA, DAPC analysis also highlighted some separation of samples originating from Saltash from all other locations. However, there was no obvious separation between samples originating from Budleigh, Cremyll or Whitsand. Admixture was run to test K values, and the value of K with the least cross-validation error was found to be 2, which were inferred to be *M. edulis* and *M. galloprovincialis* (see table 3). When admixture coefficients were calculated and plotted, it was found that the majority of individuals at the coastal Whitsand site and the lower estuary

Cremyll site were almost entirely *M. edulis*, whereas those from Saltash (upper estuary) had a slightly higher frequency of *M. galloprovincialis* ancestry with a mean of 0.16, compared to 0.01 at Cremyll and 0.04 at Whitsand (Figure 9).

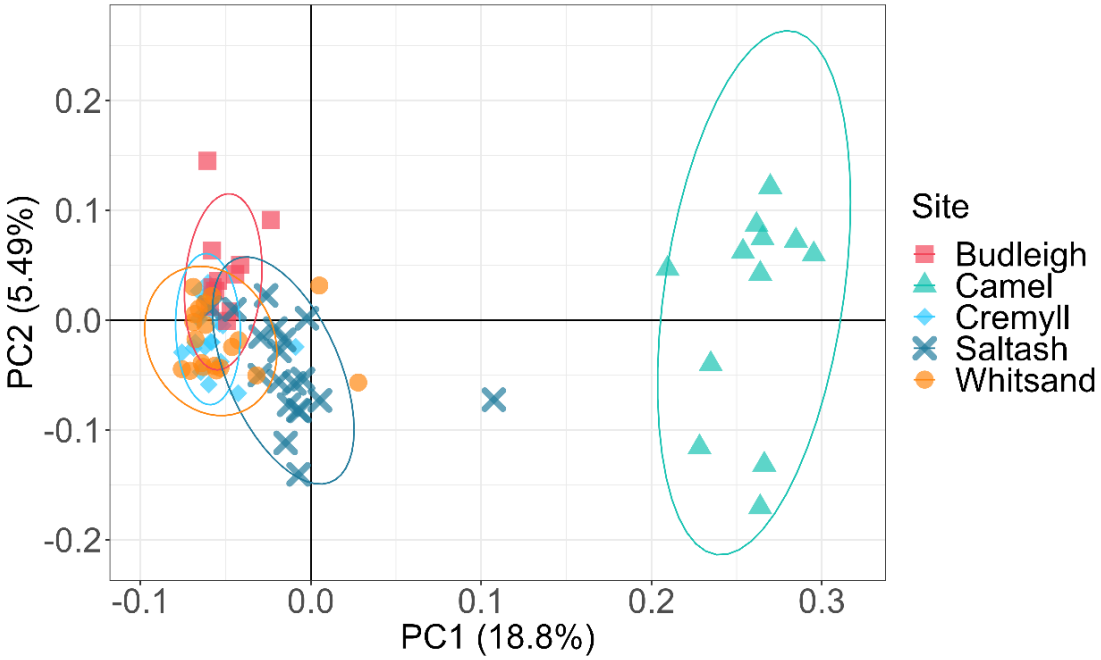


Figure 7: Scatter plot of the first two principal components when a PCA was calculated from the SNPs with a call rate > 0.95. This compares the variance between mussels from the three experimental sites, as well as from a known *M. edulis* site (Budleigh) and a known *M. galloprovincialis* site (Camel). Points represent individual mussels, colour and shape denotes the collection site. Ellipses show the 95% confidence interval of the points for each site.

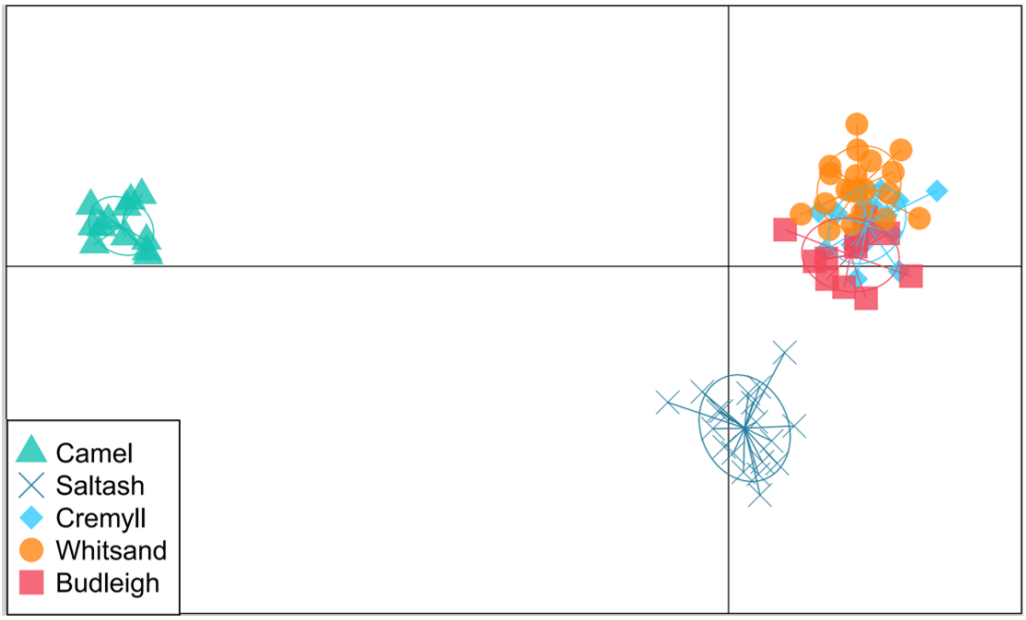


Figure 8: Scatter plot of the first two linear discriminants from a discriminant analysis of principal components calculated from the SNPs with a call rate > 0.95. This compares the variance between mussels from the three experimental sites, as well as from a known *M. edulis* site (Budleigh) and a known *M. galloprovincialis* site (Camel). Points represent individual mussels, colour and shape denotes the collection site, clustered within ellipses.

K clusters	Cross-validation error
2	0.347
3	0.365
4	0.385
5	0.413

Table 3: The results when admixture was run for each K value 2-5, and the subsequent cross-validation errors. 2 K clusters resulted in the lowest cross-validation error.

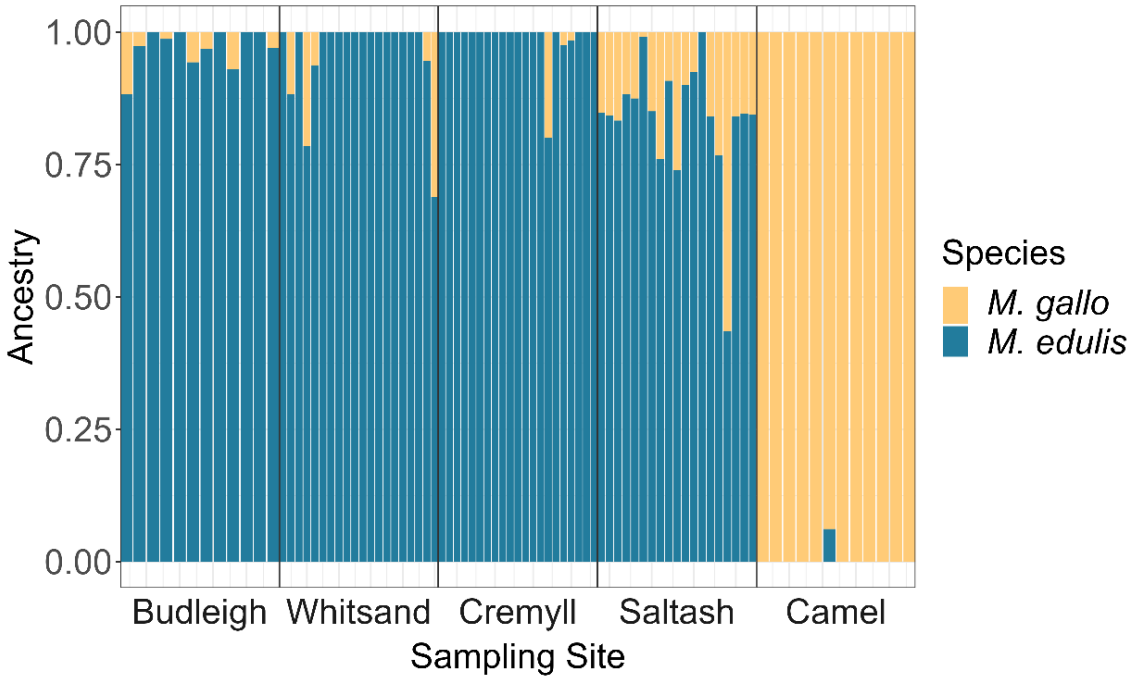


Figure 9: A bar plot of admixture analysis showing the frequency of *M. galloprovincialis* (*M. gallo*) and *M. edulis* ancestry for each of the three experimental sites, as well as the known *M. edulis* site (Budleigh) and the known *M. galloprovincialis* site (Camel).

Discussion

This study confirmed the hypothesis that acute salinity declines decrease the MR of mussels, however we did not observe the hypothesised differences between the MR at the estuarine and coastal sites. This may be linked to the fact that the genetic differences are also not what we expected; all three sites had a much greater level of *M. edulis* ancestry than *M. galloprovincialis* ancestry, and whilst we expected mussels from the coastal site to show a greater level of hybridisation than the estuarine sites, the opposite was true, with coastal and lower estuarine sites being mostly *M. edulis*, whereas the upper estuarine site had a slightly higher *M. galloprovincialis* ancestry. A combined understanding of genetics and physiological tolerance is key for helping us predict the effects of climate change and drivers of distribution.

Metabolic rate

There were significant differences in the MR between all salinities, suggesting that an acute salinity decline causes a reduction in MR. When mussels were examined at the end of each measurement period, it was observed that in the low salinity regime, mussels appeared to be shut, suggesting that the reduced MR is likely to be a result of mussels closing at low salinities to avoid osmotic stress. This probably explains the fact that 60% of mussels in the low salinity regime had a MR of 0 in at least one of the replicates, as they may have been shut for the entire measurement period, meaning that they did not consume oxygen. This is supported by a previous experiment in which *M. galloprovincialis* exposed to a salinity ramp down to 20 ppt, were shown to remain closed for the first day of exposure (Addis et al., 2021). Given that mussels in our experiment were exposed to an acute reduction in salinity, down to a slightly lower salinity of 19 ppt, it is not surprising that many mussels were observed to be closed and did not consume oxygen. It is difficult to disentangle MR and valve closure given that we measured the oxygen change over a three-hour period. This means it is possible that while open, the mussels had the same MR in all salinities, but were more likely to be closed for at least some of the time in lower salinities, and therefore the MR over the whole 3-hour measurement period is lower in lower salinities. A study measuring gape and MR simultaneously would allow us to see if the MR in acute salinity changes is entirely governed by gape, or if there is still an effect when the mussels are open. In *C. virginica* oysters, it was found that reduced salinity declined both oxygen consumption and valve opening during winter (17 °C) but not summer (27 °C) experiments (Casas et al., 2018), which supports the hypothesis that the reduction in MR may have been the result of valve closures. However, other mollusc species including *Nassarius festivus* and *Amphibola crenata* also show a reduced oxygen consumption in response to lower salinities, suggesting that it may not only be the closure reponse of bivalves which affects the MR (Cheung & Lam, 1995; Shumway & Marsden, 1982).

Conversely, there were no significant differences found in the MR between mussels originating from different locations. This is an interesting and surprising result, as it suggests that despite the mussels coming from locations with differing salinity profiles, they did not demonstrate differing physiological tolerance to low salinity exposure. This contrasts findings for *M. chilensis* in Chile, where mussels from a coastal site had a higher metabolic rate than those from an estuarine site

(Osores et al., 2017). Our results indicate that mussel MR has a high level of plasticity, and therefore they may be able to adapt to an acute change in salinity. Climate change is likely to lead to changes in salinity in many areas of the ocean, particularly within coastal and estuarine sites, which are more likely to receive freshwater runoff from land (IPCC et al., 2021). If mussels have a capacity to adapt to changing environmental conditions, this suggests that they may be able to cope with climate change induced shifts in salinity, to some extent.

Genetic analysis

The first two principal components did not show obvious clustering of mussel samples originating from the different collection sites, which suggests that the three experimental sites consist of individuals from a single shared ancestry. The fact these also clustered with individuals from Budleigh Salterton, known to be of *M. edulis* origin, indicates that mussels originating from Whitsand, Cremyll and Saltash are of predominantly *M. edulis* ancestry. DAPC analyses also further confirmed that the sites were separated from the known *M. galloprovincialis* population at Camel, but also suggested that there was additional separation between individuals originating from the upper estuarine site (Saltash) and the other sites. This is further supported by Admixture analysis, with visualisation of genotypes showing 2 genetic clusters, separating *M. edulis* (Budleigh Salterton) and *M. galloprovincialis* (Camel), with mussels from the three experimental sites consisting of individuals of predominantly *M. edulis* origin. Additionally, whilst the coastal Whitsand and lower estuary Cremyll sites appear to have very little *M. galloprovincialis* introgression, there is a greater proportion of *M. galloprovincialis* at the upper estuary Saltash site. This is a surprising result, as previous analysis has suggested the opposite, with Whitsand historically characterised by a high frequency of hybrid individuals and a high proportion of *M. galloprovincialis* like genotypes, whereas the estuarine sites have historically consisted of mainly pure *M. edulis*. When Tamar mussels were sampled using the Glu-5' marker between 1996 and 1999, including sites near Saltash and Cremyll, the frequency of the *M. edulis* allele was more than 83% in all size classes, and in the largest (>30 mm) size class, which all the mussels sampled in our experiment would fall into, this frequency was at least 91% at all estuarine sites (Hilbish et al., 2003). Contrastingly, Whitsand Bay had a decreasing *M. edulis* allele frequency with increasing size, with the >30 mm size class only containing 32% *M. edulis* alleles (Hilbish et al., 2003). Similarly, when mussels were sampled from Cremyll and

Whitsand in 2006 and analysed with the *Glu-5'* marker, it was found that Whitsand was comprised of 37% *M. edulis*, 7% *M. galloprovincialis* and 56% hybrids, whereas Cremyll had 100% *M. edulis*, with the other estuarine sites sampled also containing mainly (>93%) *M. edulis* (Bignell et al., 2011). Our findings for Cremyll are very close to these values, at 99% M. *edulis* ancestry. However, we found the average *M. edulis* ancestry to be 84% at Saltash, compared to 97% in the >30 mm size class when analysed by Hilbish et al. (2003), and 96% at Whitsand, compared to 32% in the >30 mm size class in Hilbish's data.

In the 15 years between the sampling by Bignell et al. (2011) and our study, it is possible that the hybrid frequencies have shifted. After a period of 18 years, a 25 km shift was found in the midpoint location of the Baltic hybrid cline (Strelkov et al., 2017), whilst in just a year (between 2005 and 2006) a significant change in the hybrid frequency was observed at sites within the Canadian hybrid zone (Shields et al., 2010). Modelling of larval dispersion has suggested that *Mytilus* larvae are able to regularly travel around 30-50 km at this location during their pelagic larval phase (Gilg & Hilbish, 2003b), meaning larvae should be able to disperse between Whitsand and Saltash, which are around 18 km apart by water. This suggests that any differences in species composition between these areas are likely to be a result of post-settlement selection based on prevailing environmental conditions.

Previously, it has been suggested that this selection occurs against *M. galloprovincialis* in low salinity areas, as this species is expected to be less tolerant to low salinity changes (Gardner, 1996). However, our data shows the opposite, with upper estuarine Saltash having the highest amount of *M. galloprovincialis* ancestry of the three sites studied. One possible explanation for this is that selection is occurring not as a result of salinity, but because of temperature. In the years since previous studies have examined the species compositions of these sites, ocean warming has occurred (IPCC et al., 2021), and this has been shown to affect estuarine areas more acutely (Scanes et al., 2020). It may be possible that upper estuarine mussels experience higher temperatures than the lower estuarine and coastal sites, and therefore there is greater selection towards *M. galloprovincialis*, which has been shown to be more temperature tolerant (Hilbish et al., 1994). However, the aforementioned temporal variability in distribution may mean that we would not find this effect if we sampled

the sites repeatedly over several years. Additionally, these differences may be explained by the fact that there may be differences in the genetic composition when analysed by the single Glu-5' marker compared to the many SNP markers.

Implications of genetic analysis on physiology

Mussels from all three sampling sites had a similar genetic composition, which may explain the reason they had no measurable difference in MR. Coastal mussels responded to lower salinities in the same way as estuarine mussels, with many not consuming oxygen under the low salinity regime, likely as a result of shell closure. This illustrates the plasticity of mussel physiology, as despite the fact that the coastal Whitsand mussels were unlikely to have experienced such low salinity exposure naturally, they performed similarly to the estuarine mussels. In a previous study, *M. chilensis* transplanted from a coastal to an estuarine site for 63 days showed an increase in MR, with the opposite being true for those transplanted from the estuarine to coastal site (Osores et al., 2017). This makes it evident that there is some plasticity in this trait, which is likely why our study showed no difference in performance between sites, although it should be noted that in the study by Osores and colleagues, mussels originating from the coastal site always had a higher MR than those from the estuarine site no matter whether they were transplanted or in their native habitat. The discrepancy between this study and ours may be explained by the fact that we only examined the short-term response to low salinity which is likely to be largely governed by valve closure, a response which the intertidal coastal mussels would still regularly perform in response to air exposure, even if not to salinity changes. Research examining the salinity at which mussels close would be useful to compare this effect with MR.

Limitations and future research

The Tamar is a partially mixed estuary (Uncles et al., 1983), which means it features a cline in salinity from high at the mouth to low in the upper estuary, but also by depth from the higher-density sea water near the estuary bed, to the lower density river water near the surface. In addition, semidiurnal and lunar tide cycles will affect the tidal force and therefore the penetration of salt water up the estuary. As long-term logging of salinity and fluid dynamics modelling of water mixing were beyond the scope of this study, the pattern of salinity decline based on tidal flow was designed to provide a non-linear change, which may be more equivalent to

the complex salinity changes than a linear decline. Ideally, future research should use long-term monitoring in order to design realistic regimes in the lab which reflect natural exposures.

There are currently few studies which compare analysis of genetic distribution and physiology. In order to predict how future climate change is likely to affect the distribution of this critical species, we must first gain a full understanding of how local differences in environmental factors are affecting the small-scale distributions. In order to do this, it is essential to compare the physiological performances of mussels from different ecological niches, but also analyse the genetic composition to understand if this could be resulting in different distributions. In this study, genetic analysis showed that both coastal and estuarine sites were mostly *M. edulis*, which helped to explain the lack of difference in performance between sites. This should be addressed further in future studies, potentially by measuring MR of mussels reciprocally transplanted between coastal and estuarine sites, which will allow us to understand where observed physiological differences are a result of different genetics, and where they are simply a plastic response to a different environmental variable.

Conclusions

This chapter has illustrated that short-term salinity changes have an impact on the metabolic rate of mussels, via an alteration in MR, with MR shown to be reduced in lower salinity. This highlights the need to examine the effects of stressors during both acute and long-term exposures, especially for an environmental factor like salinity which can vary significantly over short periods of time. No differences were found in the MR of mussels originating from coastal and estuarine habitats, which suggests that this trait has a high level of plasticity across our experimental populations. There were also very few genetic differences between the sites, which all had a much higher proportion of *M. edulis* ancestry than *M. galloprovincialis*. However, admixture analysis indicated that the upper estuarine site of Saltash had a higher proportion of *M. galloprovincialis* introgression, compared to the lower estuarine (Cremyll) and coastal (Whitsand) sites which were almost entirely *M. edulis* ancestry. This contradicts previous analyses, which suggest that estuarine sites were mostly *M. edulis*, whereas coastal sites were hybrids. A potential explanation for this is that recent ocean warming has meant that temperature now has a stronger effect on selection than

salinity. Future studies should combine repeated genetic analysis with logging of site conditions over time, combined with measurements of physiological performance, in order to understand how environmental conditions, genetics and physiology interact to determine the genetic composition of mussels in the face of changing environmental conditions.

The Behavioural Response of Mussels to Declining Salinities

Introduction

In order to filter feed, consume oxygen, and expel waste, mussels must open their shell valves to allow water to be drawn into their pallial cavity across their gills by the movement of cilia, via an inhalant siphon (Ward & Shumway, 2004). Given that gaping their shells allows for feeding, mussels show an increased gape under increasing algal concentrations (Lassoued et al., 2019; Robson et al., 2010), and *Arctica islandica* clams show an increased gape with Chl *a* availability (Ballesta-Artero et al., 2017). Mussel gape has been found to follow a circadian rhythm, and this is frequently linked to tidal cycles (Bertolini et al., 2021; Miller & Dowd, 2017). They have also been found to gape more during the night, both in situ on commercial rope grown sites, and within controlled laboratory experiments (Lassoued et al., 2019; Robson et al., 2007).

However, opening of the shell valves represents a trade-off, concurrently increasing the risk of predation and exposure to toxicants, in addition to making them more vulnerable to osmotic stress in changing salinities. Valve closure is a key defence mechanism against predation, with increased periods of mussel closure associated with exposure to cues from injured conspecifics (Robson et al., 2010). Additionally, mussels which gaped slightly wider than their neighbours during emersion are more likely to be targeted by black oystercatchers (Miller & Dowd, 2019), indicating the importance of this response for survival. Mussels also have a strong behavioural response to a wide range of pollutants, with valve closure being observed under exposure to copper, mercury, zinc, crude oil, and oil dispersants (Ait Fdil et al., 2006; Davenport, 1977; Durier et al., 2021; Redmond et al., 2017). Furthermore, bivalves show an increased rate of flapping behaviour (rapid opening and closing of valves) during exposure to toxic dinoflagellates, with *Pinctada fucata* and *Ruditapes philippinarum* showing this behaviour in response to *Heterocapsa circularisquama* exposure (Basti et al., 2009; Nagai et al., 2006), whilst *Mytilus galloprovincialis* and *Crassostrea gigas* demonstrate the same response when exposed to *Alexandrium minutum* (Comeau et al., 2019; Tran et al., 2010).

Often found in estuarine environments, marine mussels are frequently exposed to large fluctuations in salinity. In order to understand the differences between coastal and estuarine populations, common garden experiments are often used to measure the effects of reciprocal conditions. One study found that when *C. virginica* larvae originating from different coastal and estuarine habitats were reared in a range of salinities, oysters originating from an area where salinity ranges from 6.6-14.5 psu had the best survival when reared under low salinities, suggesting phenotypic acclimation to these conditions (Eierman & Hare, 2013). Additionally, phenotypic plasticity was shown in reciprocally transplanted coastal and estuarine *Mytilus chilensis* mussels, with metabolic rate being higher in mussels at the estuarine site, and clearance rate being higher in mussels at the coastal site, no matter their site of origin (Osores et al., 2017). This is supported by another study which found that *Mytilus edulis* and *Mytilus trossulus* from Newfoundland in Canada had significantly reduced filtration rates following a four-month exposure to simulated estuarine conditions between 11.4 and 29 ppt (Gardner & Thompson, 2001). These studies illustrate the ability of mussels to acclimate to a wide range of conditions across estuarine and coastal environments.

Being osmocoformers, extreme salinity fluctuations put mussels at risk of osmotic stress (Davenport, 1979), and this has been found to have a variety of negative effects. For example, *Perumytilus purpuratus* living in estuarine environments are shown to have a reduced growth rate and calcification, and an increased metabolic rate, when compared to their coastal neighbours (Ramajo et al., 2021). Similarly, a study in the Mediterranean mussel, *M. galloprovincialis,* showed that following a 14 day exposure to reduced salinities, mussels had a higher oxygen consumption at 28 and 18 psu compared to the 37 psu control (Hamer et al., 2008). Additionally, whilst no effect on condition index was shown, this study also showed increased mortality at 11 psu (Hamer et al., 2008). Across a range of salinity ramp designs, which gradually dropped salinity based on field measurements, *Ruditapes decussatus*, *Venerupis corrugata*, *Cerastoderma edule* and *R. philippinarum* were found to have reduced burrowing activity and scope for growth at lower salinities (Domínguez et al., 2020).

In addition to variation in salinity, as intertidal organisms mussels are often exposed to semidiurnal periods of emersion, and therefore have developed a

number of mechanisms to deal with this. They are facultative anaerobes, which means that they are able to switch to anaerobic respiration during periods of shell valve closure, allowing them to survive periods of aerial exposure without desiccation (de Zwaan & Wijsman, 1976). This involves the production of opines from pyruvate, which is less acidic than lactate and allows for survival during relatively long term periods (several days) of anoxia (Müller et al., 2012). Whilst this process is more efficient than anaerobic respiration in most other eukaryotes, this represents a trade-off, preventing feeding and waste excretion as well as causing acidosis during the closure period, with mussels required to balance this behavioural response to maximise benefit, and minimise adverse impacts (Müller et al., 2012).

When different areas of the *M. edulis* mantle were systematically isolated and supplied with fresh water while the rest of the mussel was exposed to sea water, it was found that mussels still gaped when their exhalant siphon or ventral part of the inhalant siphon was supplied with fresh water (Davenport, 1981). However, when the cilia of the inhalant siphon were supplied with fresh water, the mussel remained shut, suggesting that this area contains osmoreceptors (Davenport, 1981). Additionally, experiments which showed that the salinity of the internal pallial fluid remained consistent, combined with the fact that the internal structures remained free from dye when closed mussels were placed into fresh water dyed with methylene blue, both suggest that mussels do not gape to 'test' the external salinity, but some ions simply diffuse through the closed valves to osmoreceptors (Davenport, 1981). Exposure to solutions of differing ion concentrations suggested that *Mytilus* gape is reactive to Na^+ and Mg^{2+} concentrations, but not Ca^{2+} ions (Akberali & Davenport, 1982). This suggests therefore that mussel gape is closely linked to their ability to sense osmotic change, related to specific ions, and that this mechanism is fundamental in driving their behavioural response to low salinity exposure.

In addition to protecting mussels from desiccation during emersion, shell closure is used to protect individuals from the harmful effects of reduced salinity, and allows mussels to maintain stable mantle fluid osmolality and reduce osmotic stress (Shumway, 1977). This behavioural response to salinity is also impacted by the rate of salinity change, with *M. edulis* found to maintain fluid at a higher salinity in their pallial cavity during faster declines, suggesting that the mussels

respond more quickly when salinity changes rapidly, in order to protect themselves from osmotic stress (Davenport, 1979). Additionally, when *M. edulis* were exposed to a 12 hour cycle from 30 ppt to 15 ppt and then back up to 30 ppt, the osmolality of mussel haemolymph was found to be slightly higher than that of the external water during the decline (Livingstone et al., 1979). This is likely to be as a result of mussels no longer exchanging water with their external environment due to behavioural valve or siphon closure, which allows these osmoconformers to be slightly hyperosmotic to their surroundings. Similar conclusions were formed from experiments using the Asian green mussel, *Perna viridis,* where mussels placed into a range of static salinities demonstrated hyperosmotic haemolymph compared to the surrounding seawater at salinities below 15 ppt, as well as reduced clearance rates as a result of the observed valve closure (McFarland et al., 2013). Interestingly, *C. virginica* oysters did not show the same response, with a haemolymph concentration much closer to that of its surroundings, but with less of a reduction in clearance rate at reduced salinities (McFarland et al., 2013). A difference in this effect has been observed between coastal and estuarine *Perna perna* mussels, with estuarine mussels having hyperosmotic haemolymph after 4 and 14 days of exposure to 20 and 25 ppt, whereas coastal mussels were iso-osmotic with seawater (Rola et al., 2017). It was observed that valves remained open during this study, so this does not appear to be an effect of isolation by valve closure, however it is possible that closure of the exhalant siphon or reduced filtration contributed to this maintenance of a hyperosmotic internal environment (Rola et al., 2017). This provides an interesting insight into the adaptations between mussels living in coastal and estuarine environments.

One way of quantifying gape is by using hall sensors, which measure magnetic field strength. When a small magnet is placed at a short distance from a hall sensor, the magnetic field strength measured by the sensor decreases as the magnet moves further away from it (Wilson et al., 2005). This has been used for several applications in the study of animal physiology and behaviour (Hanuise et al., 2010; Myers & Hays, 2007; Wilson & Liebsch, 2003), including for studying the valvometry of bivalves (Wilson et al., 2005). The deployment of hall sensors can be used to provide highly detailed assessments of organism responses to contaminants (Borcherding, 2006). This has led to their integration into increasingly sophisticated, and commercially available, monitoring ‘tool kits’,

which are now being deployed in a range of environmental and industrial applications (Andrade et al., 2016; Barile et al., 2016; SJ & NG, 2015; Taylor et al., 2013).

Given the behavioural response of a range of bivalves species to changing environmental conditions, the use of hall sensors also has high potential for assessing bivalve responses to climate change. Whilst increased CO_2 concentrations have not been shown to impact gaping behaviour in *C. virginica or M. galloprovincialis* (Clements et al., 2018; Lassoued et al., 2019), climate induced freshening is projected to increase significantly in the coming years, with higher occurrences of flash flooding that will severely impact coastal ecosystems (IPCC et al., 2021). With salinity directly impacting bivalve gaping behaviour (Davenport, 1979), changing seawater salinity as a result of climate change will very likely impact the physiological response of bivalves to a suite of environmental conditions. Nonetheless, to date, only a very limited number of studies have examined the impact of salinity changes using hall sensors to measure gaping in bivalves. In one study, *M. galloprovincialis* were exposed to a reduction in salinity (4 hour ramp) down to salinities of 35, 20, 10 or 5 ppt, followed by a continuous 5 day exposure at that salinity, followed by a further 5 days of recovery at 35 ppt (Addis et al., 2021). Exposure to 5 ppt caused mortality of most mussels, whilst exposure to 10 ppt resulted in closure for the duration of the exposure, followed by reopening during the recovery period. In 20 ppt, mussels closed their valves for the first day, but then opened for the remainder of the exposure. Similarly, when using hall sensors to measure gape, oysters (*C. virginica)* were found to have a higher percentage of valve gape at 15 and 25 ppt compared to at 3, 6 or 9 ppt (Casas et al., 2018). These are two of the very few studies which have updated our understanding of the bivalve gaping response to salinity using modern technology, providing an interesting insight into the possible effects of climate change induced freshening on bivalve gape behaviour. However, the gaping response of mussels to changing salinities is still a severely under-researched area, and further studies using these technologies are therefore urgently required to help better understand the future implications of freshening.

Similarly to shell valve gape behaviour and metabolic rate, heart rate (HR) in mussels is also impacted by salinity, with HR decreasing concurrently with

reduced salinity in *M. edulis*, *M. trossulus* and *M. galloprovincialis* (Bakhmet et al., 2005; Stickle & Sabourin, 1979). However, this effect was not found in *P. viridis*, which demonstrated an increasing HR with temperature, but no impact of salinity between 15 and 35 ppt (Nicholson, 2002). Heart rate is a common measure of physiological performance, and has mainly been studied in mussels using impedance pneumography, and more recently infrared sensors (Vereycken & Aldridge, 2023). Mussel HR is unusual in that it commonly enters periods of bradycardia (significantly reduced heart rate), or stops completely, seemingly without a serious impact on mussel health (Bakhmet & Khalaman, 2006). Various external factors have been shown to impact heart rate. For example, subtidal but not intertidal *M. galloprovincialis* have been found to have a decreased HR with increasing temperatures while submerged, but neither intertidal nor subtidal mussels were affected by temperature during emersion (Collins et al., 2020). However, when examining the effects of temperature in the different mussel species *M. edulis*, *M. trossulus* and *M. galloprovincialis,* Braby and Somero (2006) found that HR increased with increasing temperatures for all species.

Alongside being affected by temperature and salinity, HR has been found to be closely related to gaping behaviour. When HR and gape were measured in response to copper in two separate studies, both were shown to decrease in response to contamination, with periods of valve closure also being closely followed by periods of bradycardia (Curtis et al., 2000; Shen & Nugegoda, 2022). Whilst HR is clearly linked to valve closure, understanding how it relates to other parameters is key for understanding the physiological response, and therefore tolerance, to different environmental conditions, particularly those relevant to climate change.

The aim of this study was to determine the behavioural response of the blue mussel, *M. edulis*, to reduced salinity, and determine if the rate of salinity change impacts the point at which mussels adduct their shell valves. This response was compared in three populations of mussels, originating from different estuarine or coastal sites, to ascertain whether estuarine mussels are better able to cope with low salinity exposure. To provide a detailed assessment of mussel gape behaviour, hall sensors were used to measure gape alongside pallial fluid osmolality, whilst gape, heart rate and oxygen consumption were also measured concurrently in a subset of mussels, providing the physiological context to mussel

gape behaviour. Based on previous research by Davenport (1979), it was hypothesised that mussels would close their valves at a lower salinity during faster salinity decreases, but that pallial fluid osmolality would be higher during faster salinity decreases. Additionally, it was hypothesised that gape, HR and MR would all be lower at reduced salinities compared to in full strength seawater.

Materials and methods

Sample collection

Mytilus edulis were collected at low tide on the 12/09/2022 from Orcombe Point, Exmouth (coastal site; 50.607,-3.387; mean weight 5.97±1.29 g, mean length 36.04±2.16 mm), and at low tide on the 06/02/2023 from Teignmouth beach (coastal site; 50.546,-3.492; mean weight 6.04±1.90 g, mean length 43±3.31 mm) and from Shaldon bridge, in the river Teign (estuarine site; 50.546,-3.511; mean weight 12.03±4.49 g, mean length 49.79±6.06 mm), see Figure 10a. Previous genetic testing has shown Exmouth mussel populations to be entirely *M. edulis* (Nascimento-Shulze, in publication), and Teignmouth mussels were assumed to also be *M. edulis* based on this location being outside of the previously described area of the *M. edulis-galloprovincialis* hybrid zone. Mussels were cleaned of all epibionts, before being placed inside holding tanks in the Aquatic Resource Centre at the University of Exeter, maintained for two to three months in a recirculating aquaculture system, using artificial seawater at a salinity of 34.3 ppt and 15 °C, with a 20:4 hour light:dark regime, and fed with 0.027 ml/L of Shellfish Diet (Reed Mariculture) three times per week for the duration of experiments.

Experimental design

a) Mussel gape behaviour

Hall sensors measure magnetic field strength and record a voltage which is proportional to the sensor's proximity to a magnet. A custom-built hall sensor system running on an Arduino Mega 2560 was designed and built to measure gaping behaviour in the mussels. Firstly, araldite adhesive was used to adhere a waterproofed hall sensor (Allegro A1319LUA-5-T) to one shell valve of the mussel, and a small neodymium magnet (1x12 mm) to the other shell valve (see Figure 10d), with mussels left air exposed for 3 hours to enable the adhesive to fully cure. Mussels were subsequently given at least 24 hours to recover from this air exposure prior to any experiments beginning. Given that mussels are frequently heavily covered in epibionts, such as barnacles, it was assumed that

the attachment of the sensor and magnet did not impact the normal mussel behaviour.

An initial pilot study was conducted where 8 mussels originating from the Exmouth population were exposed a reduction in salinity down to 12 ppt. This indicated that all mussels closed before salinity reached 18 ppt. As a result of this, the experimental salinity threshold for this experiment was set to 18 ppt to enable accurate quantification of gaping behaviour around this key point, both immediately before and during valve closure.

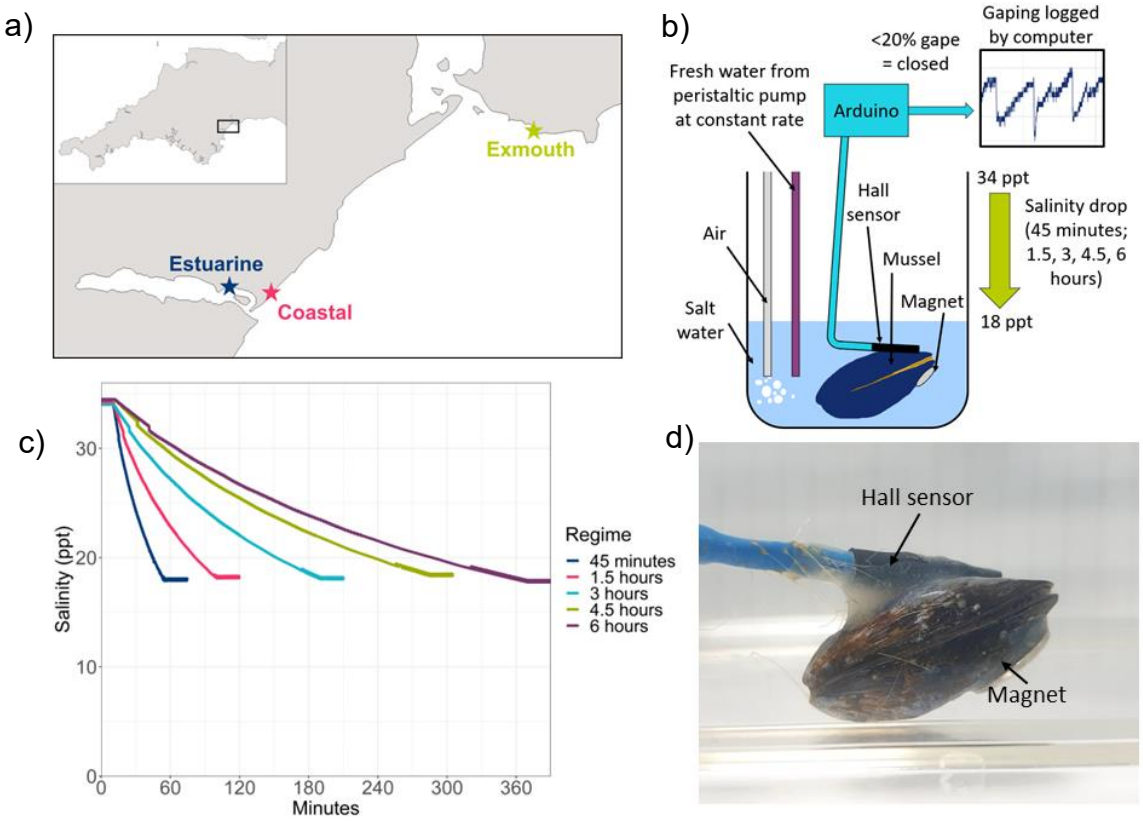


Figure 10: a) Map of the Southwest UK showing the three sampling sites, b) Diagram showing the experimental setup used for each mussel, c) line graph showing the salinity decline during each regime, and d) an image of a mussel with a hall sensor and magnet attached.

Subsequently, each group of experimental mussels (the first containing 8 Exmouth mussels, and the remaining two each containing 4 coastal and 4 estuarine Teignmouth mussels) were placed individually into 1 L PVC experimental chambers containing 300 ml of seawater at 34.05 (±0.19) ppt, maintained at 15.93 (±0.96) °C with continuous aeration. One experimental chamber also contained a salinity probe (Hach HQ 40d Digital Multi Meter with an Intellical CDC401 digital conductivity probe). Mussels were left undisturbed for 30 minutes after handling, to allow normal gaping behaviour to resume, before

recording of gaping, salinity and temperature commenced. Gaping was recorded using hall sensors every second for each mussel, and salinity and temperature were recorded every 10 seconds. After 10 minutes of recording normal gape behaviour of mussels under control sea water conditions, a peristaltic pump was used to pump 0 ppt reverse osmosis (RO) water at a steady rate (determined by the salinity regime) into each of the pots, with mixing ensured by aeration. Salinity was dropped from the initial 34.05 (±0.19) ppt to 18.05 (±0.68) ppt over a range of time periods (6, 4.5, 3, 1.5 hours, and 45 minutes), as shown in Figure 10c. A repeated measures design was used, with each mussel exposed to and measured under each salinity ramping regime, with at least 24 hours to recover from one experiment before being exposed to the next regime. As a result of the increasing total volume of water as RO was added, the salinity drop was not linear (see Figure 10c), but the five time periods corresponded to a mean rate of change of 2.67, 3.56, 5.33, 10.67, and 21.33 ppt/hour respectively. Following the salinity drop, gaping was recorded for a further 20 minutes at the final experimental salinity 18.05 (±0.68) ppt.

For the experiments conducted with the Teignmouth mussels, the osmolality of the pallial fluid was also measured. At the end of each experiment, mussels were removed from the water and then dried thoroughly before the shell was opened slightly by gently inserting a scalpel opposite the hinge and twisting to push the valves apart, allowing the pallial fluid to drain. These samples were collected and frozen. Osmolality of the samples was measured using an osmometer (Wescor 5520 Vapor Pressure Osmometer) and compared against a standard curve for samples at known salinities and osmolalities, to obtain the salinity of each sample.

To test for linearity of the voltage recorded by the hall sensors across the range of gaping measured, at the end of the experiments using Teignmouth mussels, the adductor muscle was cut, and voltage was recorded at fixed points, using wedges of increasing known thickness that were placed between the shell valves opposite the hinge.

b) Combined gaping, heart rate and oxygen consumption

In a separate set of experiments, coastal and estuarine mussels from Teignmouth were used to examine the impacts of reduced salinity on heart rate and metabolic rate, related to gaping behaviour. Hall sensors and magnets were attached to

opposite shell valves with araldite rapid adhesive as previously described, in addition to heart rate sensors (Vishay CNY70 infrared sensors, connected to ElectricBlue Pulse V2 heart rate monitor), which were adhered on the dorsal side of the shell near the hinge, directly above the heart (see Figure 11b).

During this trial, 6 mussels were placed singly into watertight 800 ml jars (Scott duran) with hall sensors, HR sensors and also an oxygen probe (OXROB10; connected to a 4 channel FireSting® - O2 meter) fitted through IP68 rated cable glands, with two of the jars also containing an additional temperature probe (pt100; FireSting®), as shown in Figure 11. Additionally, each jar contained a small magnetic stir bar, and was placed on a stir plate for the duration of experiments to ensure constant mixing. Mussels were suspended in the upper section of the jar, preventing the magnetic field of the stir plate from interfering with hall sensor measurements (see Figure 11a). Jars were filled with 35.09 (±0.04) ppt salinity water, when fully submerged, ensuring no air bubbles were trapped, before being placed on the stir plates. Following sealing inside the jars, mussels were given 30 minutes of recovery time before data was recorded for a

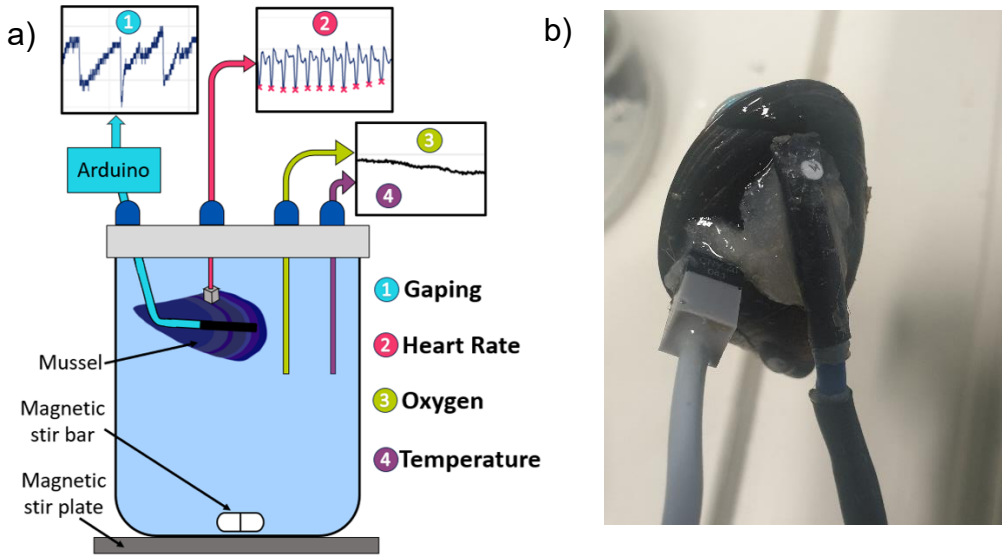


Figure 11: a) Diagram showing the experimental setup for each mussel. 1) Gaping recorded by a hall sensor running on an Arduino; 2) Cardiac frequency logged by ElectricBlue Pulse V2, beats identified in R to calculate beats per minute (bpm); 3) Oxygen measured by OXROB10 probe, converted to metabolic rate using formula (see text); 4) Temperature logged by pt100 probe. b) Photograph of a hall sensor and heart rate sensor adhered to a mussel.

period of three hours. Oxygen consumption, gape and temperature were recorded every second, whereas cardiac frequency (HR) was measured 10 times a second. Oxygen concentration was also measured in one additional jar, not containing a mussel, as a blank. This was repeated with the same mussels at

salinities of 27 (±0.08) ppt and 19 (±0.05) ppt. At the end of experiments, mussel tissue was removed from the shell and weighed to obtain the wet tissue weight.

Data analysis

a) Mussel gape behaviour

The correlation between voltage and gape distance was calculated independently for each sensor, with some sensors having increasing voltages and others decreasing as gape increased. Based on this, the correlation could be either positive or negative, so absolute values (without a sign) of these correlations were used to calculate a mean correlation. The percentage gape of the shell valves was subsequently calculated by using the minimum and maximum gape in voltage for each mussel, across the experiments, and converting this to a percentage representing 0% and 100% gape respectively. In order to allow for comparison between salinity regimes and individuals, a threshold of 20% was chosen below which mussels were considered to be closed, a value which has previously been used to represent closure in several bivalve studies (Ballesta-Artero et al., 2017; Jou et al., 2013; Miller & Dowd, 2017), and below which in this experiment mussel valve gape behaviour appeared stable, indicative of a closed steady state (see Figure 12).

Data was analysed using R studio (RStudio Team, 2020). The sensitivity and range of the sensor recordings varied, potentially influenced by factors such as sensor and magnet placement, as well as mussel size. As a result of this, some sensors gave a reading which fluctuated above the 20% threshold, despite the mean value being well below. In order to account for these differences, a 60 second rolling mean was calculated for the gape of every mussel using the rollmean function from the zoo package (Zeileis & Grothendieck, 2005). As mussels were observed to intermittently close, or partially close, their valves for short periods as part of their normal behaviour in full salinity sea water, the salinity at which mussels closed was measured as the point where the rolling mean of the gape drops below 20% and remains below 20% for the remainder of the experiment. This allowed the closure response of the mussels to be compared between salinity regimes and location of origin.

Statistical analysis was conducted on the mussels which closed during the experiments. One outlier was removed from the closure dataset where an Exmouth mussel closed permanently at an unusually high salinity (32.4 ppt) in

the 1.5 hour regime. A Shapiro Wilk test found that the closure salinities had a normal distribution (W=0.98, n=87, p=0.14), and a Levene test to check the homogeneity of variance found that there was no significant difference in the variance between the regimes (F=0.50, n=87, p=0.73), and also between the locations (F=0.17, n=87, p=0.85). A permutational anova (permanova) was calculated to model the effects of regime and location on the closure salinity.

For analysis of the pallial fluid salinity, one outlier was removed from the 45-minute regime where the retained pallial fluid salinity of a coastal mussel was unusually low (18.79 ppt), with gape appearing to increase but remaining below 20% suggesting the pallial fluid may not have remained isolated. A Shapiro Wilk test found that the pallial fluid salinity was normally distributed (W=0.98, n=57, p=0.30) and a Levene test found no significant difference in the variances between either regimes (F=1.73, n=57, p=0.16) or locations (F=0.09, n=57, p=0.77), so parametric tests were used. A permanova was calculated to model the effect of regime and location on the pallial fluid salinity.

A Pearson's Product-moment correlation test was used to see if there was a relationship between the closure salinity (measured via gape) and the retained pallial fluid salinity. In addition, a paired t-test was used to see if there was a significant difference between pallial fluid salinity and closure salinity. The difference between pallial fluid salinity and closure salinity was calculated (referred to as pallial-closure interval hereafter), and a Shapiro Wilk test found the data to be normally distributed (W=0.98, n=57, p=0.34), and there was no significant difference found in variance by a Levene test between either regimes (F=0.77, n=57, p=0.55) or locations (F=0.07, n=57, p=0.79). A permanova was calculated to model the effect of location and regime on the pallial-closure interval.

b) Combined gaping, heart rate and oxygen consumption

Gaping data was converted to percent gape and a 60-second rolling mean was calculated using the same method as described above.

For the heart rate data, beats per minute (bpm) was calculated from the cardiac frequency by using the stat_valleys function from the ggpmisc package to find the repeating patterns in the data (see Figure 11a). The heart beats were marked and visually examined, and parameters of the function were adjusted accordingly. The

intervals in between beats were calculated in seconds, and the formula 60÷interval time was used to calculate the bpm. The rolling mean of the heart rate was also calculated using the rollmean function from the zoo package (Zeileis & Grothendieck, 2005).

The solubility value of oxygen in seawater was determined based on the temperature and salinity, using an algorithm derived from Green and Carritt (1967). Metabolic rate was calculated using the formula:

O_2 Consumption Rate (MO_2; μmol/g/hour) =

($\Delta[O_2]$ (μmol/l) x Chamber Volume (l)) / (Body Mass (g) x Time period (h))

Metabolic rate was calculated based on the initial and final oxygen measurements across one-minute intervals within the total time period, and additionally during five-minute intervals for data smoothing. The overall metabolic rate for the whole three-hour measurement period was also calculated. The difference in oxygen in the blank experimental chamber during each of these measurement intervals was subtracted from the change in oxygen measurement in mussel experimental chambers, to account for background respiration. Metabolic rate was calculated based on the wet tissue mass of each animal.

A Student's t-test was used to compare the overall mean gape between salinities, and an LMM was used for both overall mean HR and MR between salinities, accounting for the mussel as an interacting variable and followed by pairwise comparisons.

Results

a) Mussel gape behaviour

General gape behaviour

The general gaping behaviour of mussels is shown in Figure 12, where percentage gape of a mussel is presented during each of the salinity regimes. It can be seen that individual mussels always close their shell valves at a similar salinity, with the pallial fluid being isolated from the external medium prior to this point. Many of the mussels exhibited "flapping" behaviour prior to the final closure (Figure 12a and c), where valves rapidly close, either partially or fully, and then reopen. This did not appear to be dependent on salinity regime, but some individuals were observed to conduct this behaviour more frequently.

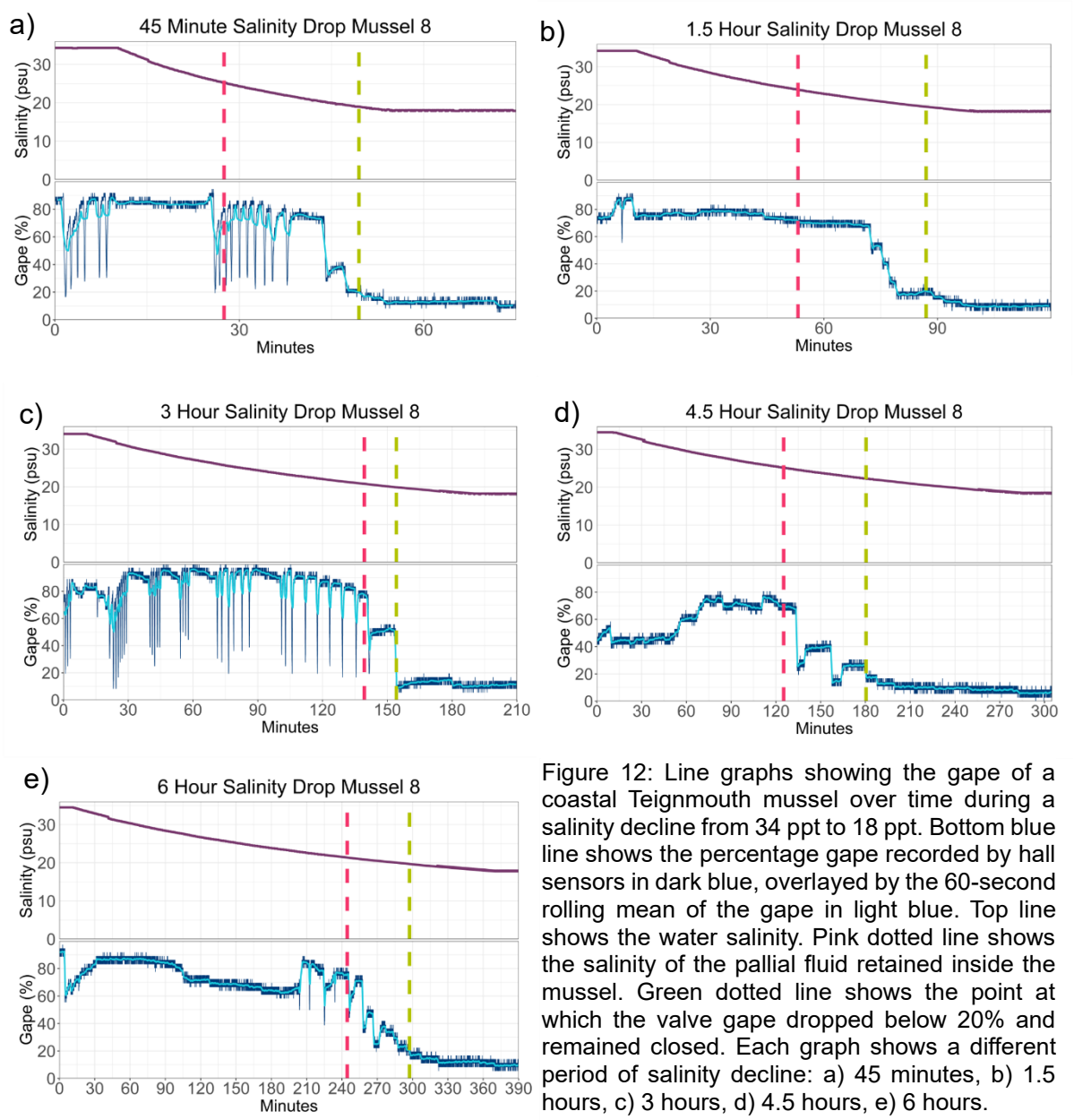


Figure 12: Line graphs showing the gape of a coastal Teignmouth mussel over time during a salinity decline from 34 ppt to 18 ppt. Bottom blue line shows the percentage gape recorded by hall sensors in dark blue, overlayed by the 60-second rolling mean of the gape in light blue. Top line shows the water salinity. Pink dotted line shows the salinity of the pallial fluid retained inside the mussel. Green dotted line shows the point at which the valve gape dropped below 20% and remained closed. Each graph shows a different period of salinity decline: a) 45 minutes, b) 1.5 hours, c) 3 hours, d) 4.5 hours, e) 6 hours.

Mussels rarely closed below 20% gape prior to final closure, and when they did this was typically for a short period of time. In the vast majority of cases (95%), mussels reached a salinity at which they shut their valves below 20% gape, and remained closed for the remainder of the exposure. The exceptions to this were as follows: one coastal mussel which closed during the 45 minute regime, but then reopened to around 56% gape during the 20 minute period after the salinity stopped dropping; a separate coastal mussel which did not close during the 3 hour regime, and gradually reopened after closing in the 1.5 hour regime; and one coastal mussel which opened slightly above 20% after closing in the 45 minute regime. In all three cases where the mussel closed for an extended period and then reopened, the salinity at which this closure period began was taken as the closure salinity for analysis. However, as reopening likely resulted in

exchange of pallial fluid with external seawater, data from these instances was not used in analysis. Exclusion of this data was further supported by the fact that these three points were the only outliers when plotting all data. In the single case where the mussel did not close, this data could not be included in any analysis.

Salinity at valve closure

A two-way permanova found no interaction between the regime and location (Pseudo-F=1.586, df=8, p=0.144). The mean salinity at which mussels closed was found to be 21.00 (±1.43) ppt, and whilst this value was slightly higher when the rate of salinity decline was slower (Figure 13), the permanova found no significant effect of salinity ramping rate on the salinity at valve closure (Pseudo-F=1.415, df=4, p=0.240).

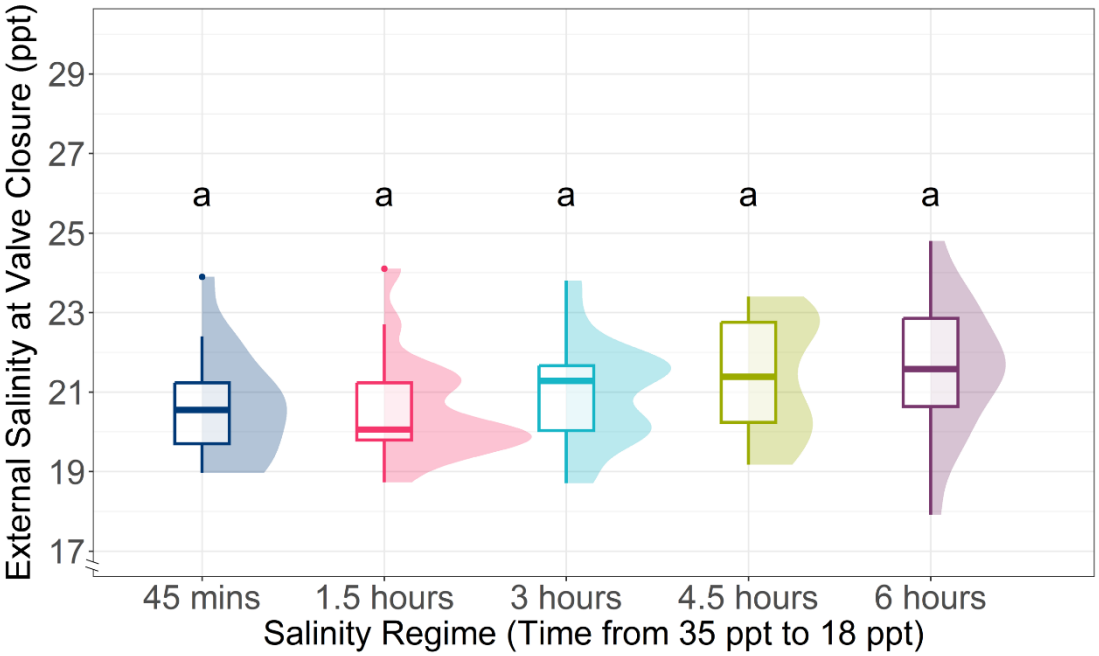


Figure 13: Boxplots and density plots showing the water salinity at the point at which mussels closed and remained below 20% in the different salinity drop regimes. Letters indicate a lack of significant difference between the regimes.

Conversely, as illustrated in Figure 14, the permanova found a significant difference in the salinity at which mussels from different populations were shown to close (Pseudo-F=5.118, df=2, p=0.007). Pairwise comparisons following the permanova demonstrated that whilst there was a significant difference between the salinity of closure between mussels from the Exmouth and coastal Teignmouth site (t=3.142, n=27, p=0.005), and between the coastal and estuarine Teignmouth sites (t=2.257, n=29, p=0.027), there was no significant difference between mussels from the Exmouth and estuarine Teignmouth sites (t=1.053, n=27, p=0.296). Exmouth mussels were shown to have a slightly higher mean

closure salinity of 21.54 (±1.31) ppt, whereas the closures for Teignmouth estuarine and coastal were 21.12 (±1.29) ppt and 20.41 (±1.48) ppt respectively.

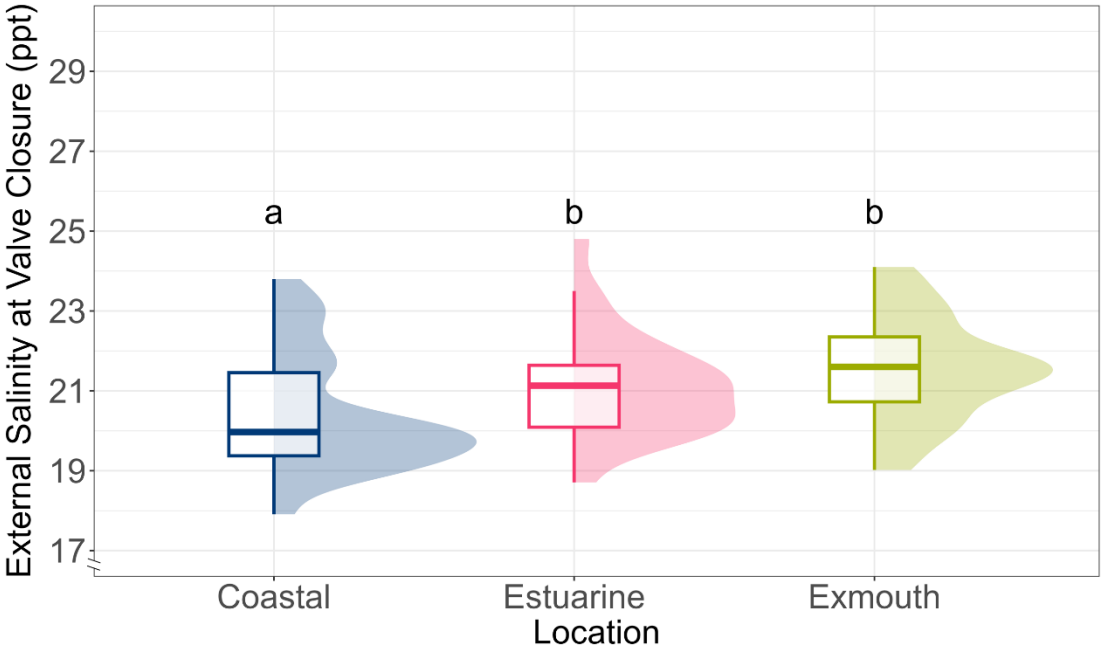


Figure 14: Boxplots and density plots showing the water salinity at the point at which mussels closed and remained below 20% in mussels collected from the coastal and estuarine Teignmouth sites, and coastal Exmouth site, when salinity was dropped from 34 ppt to 18 ppt. Different letters indicate a significant difference by pairwise comparison.

Salinity of retained pallial fluid

A permanova found no interaction between regime and location (Pseudo-F=0.281, df=4, p=0.875). The mean salinity of the retained pallial fluid was found to be 24.51 (±1.82) ppt. The permanova found that this was significantly affected by the regime of salinity change (Pseudo-F=6.850, df=4, p=0.002), as shown in Figure 16, with pairwise comparisons finding that the 45 minute and 1.5 hour regimes were significantly different to all other regimes, but there were no significant differences between the 3 hour, 4.5 hour and 6 hour regimes (see table 4). The means for each regime were as follows: 45 minutes = 26.44 (±1.36) ppt, 1.5 hours = 24.90 (±0.98) ppt, 3 hours = 23.86 (±1.36) ppt, 4.5 hours = 23.69 (±1.74) ppt, 6 hours = 23.48 (±2.07) ppt. The permanova also found that population significantly affected the salinity of retained pallial fluid at closure (Pseudo-F=12.035, df=1, p=0.004), as shown in Figure 15, with the estuarine mussels having a significantly higher pallial fluid salinity (estuarine = 25.29 (±1.63) ppt, coastal = 23.70 (±1.68) ppt). The retained pallial fluid was maintained consistently at a higher salinity than the minimum salinity of 18 ppt reached by

the external seawater during the experiment, with the lowest pallial fluid salinity recorded being 19.47 ppt.

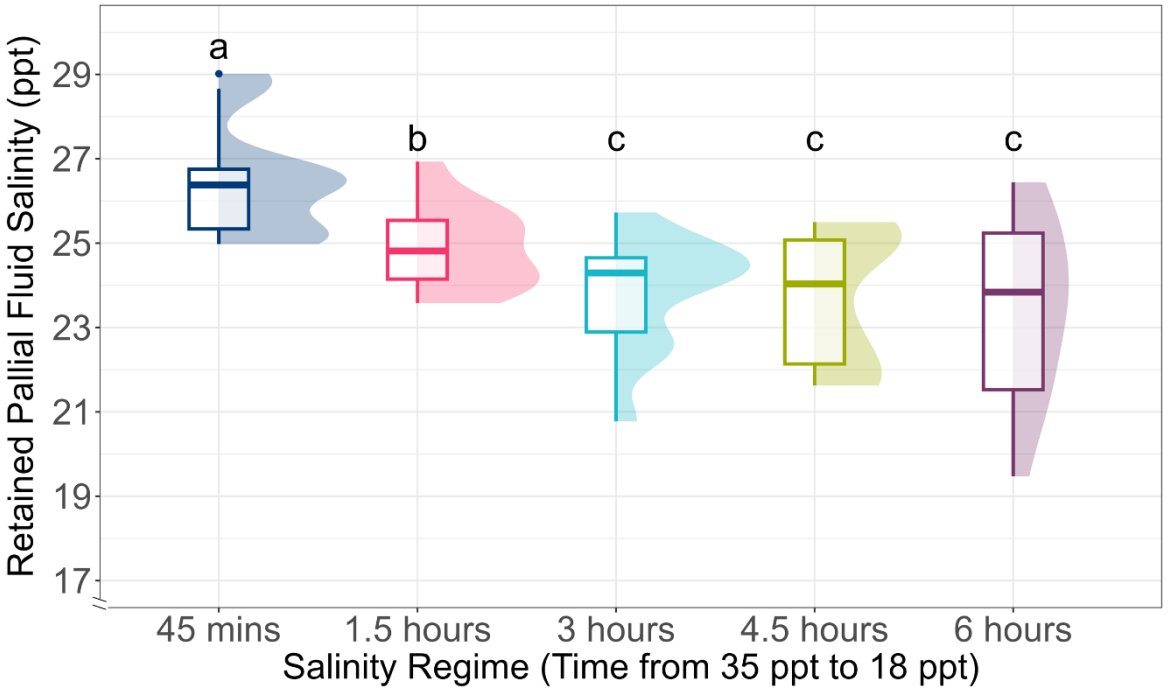


Figure 16: Boxplots and density plots showing the salinity of the pallial fluid retained by mussels in the different salinity regime time periods of declining sea water from 34 ppt to 18 ppt. Different letters indicate a significant difference by pairwise comparison.

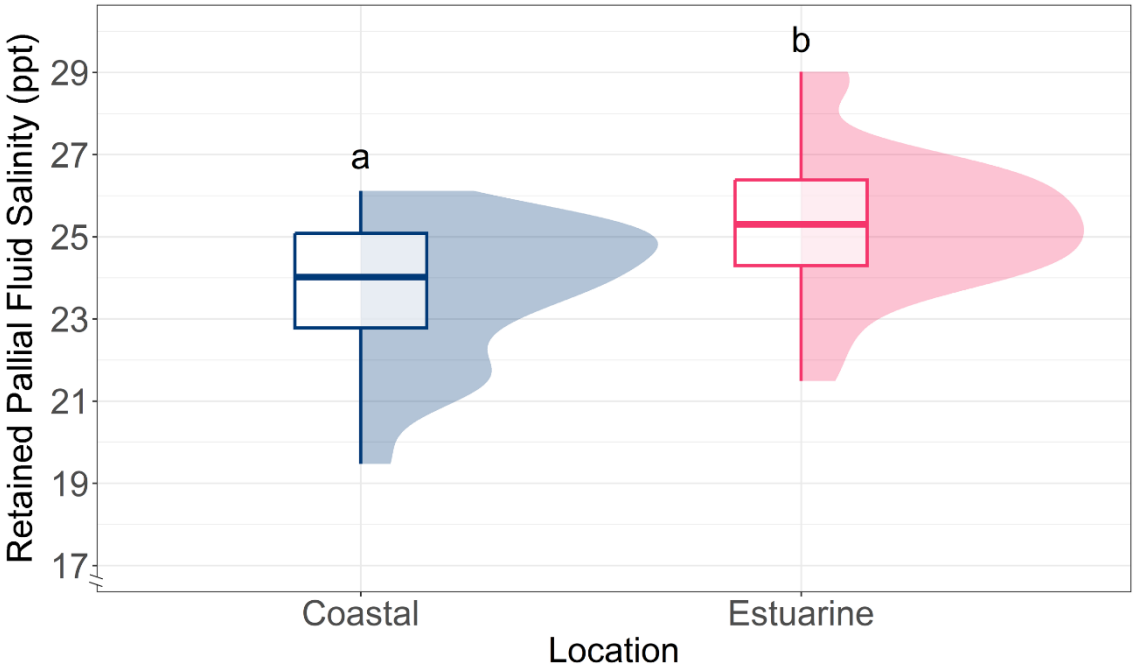


Figure 15: Boxplots and density plots showing the salinity of the pallial fluid retained by mussels collected from the coastal and estuarine Teignmouth sites when salinity was dropped from 34 ppt to 18 ppt. Different letters indicate a significant difference by pairwise comparison.

Comparing closure salinity and pallial fluid salinity

A permanova comparing the pallial-closure interval found no interaction between the regime and location (Pseudo-F=0.970, df=4, p=0.456). Figure 17 shows that

pallial fluid salinity was significantly higher than the salinity at valve closure (paired t-test, t=-14.218, df=56, $p=2.2x10^{-16}$). There was also a significant weak correlation of 0.30 between the two (Pearson's Product-moment correlation, t=2.300, df=55, p=0.025). The mean difference between the pallial fluid and the closure salinity was 3.66 (±1.95) ppt. The pallial fluid salinity was higher than the closure salinity in all but one case, and this outlier is not shown in the figures.

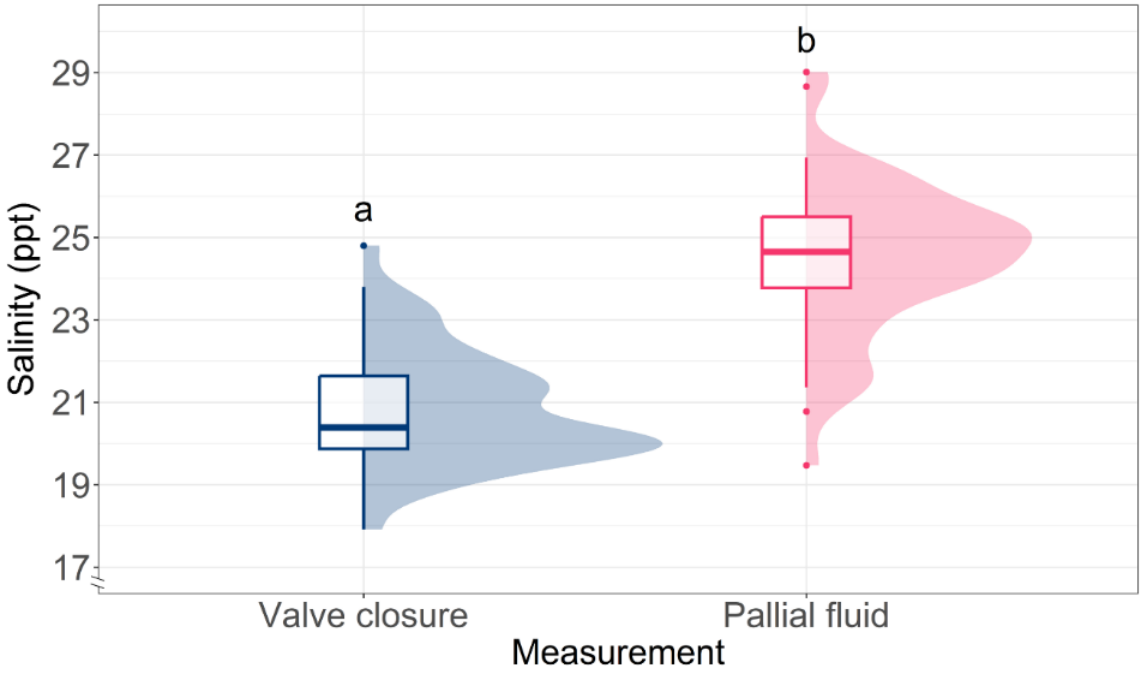


Figure 17: Boxplots and density plots showing the salinity at valve closure below 20% compared to the pallial fluid retained by mussels when salinity was dropped from 34 ppt to 18 ppt. Different letters indicate a significant difference by pairwise comparison.

The permanova found significant differences in the pallial-closure interval between regimes (Pseudo-F=21.266, df=4, p=0.001), with pairwise comparisons finding the 45 minute and 1.5 hour regimes to be significantly different to all other regimes, but no differences found between the 3, 4.5 and 6 hour regimes (see table 4). As shown in Figure 19, the 45 minute regime had the highest pallial-closure interval at 5.23 (±1.49) ppt, followed by the 1.5 hour regime at 4.69 (±0.67) ppt, the 3 hour regime at 2.89 (±1.35) ppt, the 4.5 hour regime at 2.64 (±1.02) ppt, and the 6 hour regime at 1.88 (±1.58) ppt. In addition, no significant difference was found in the pallial-closure interval between the two locations (Pseudo-F=3.157, df=1, p<0.078). The estuarine site had a mean pallial-closure interval of 4.16 (±1.78) ppt, and the coastal site had a mean pallial-closure interval of 3.15 (±2.01) ppt, as shown in Figure 18.

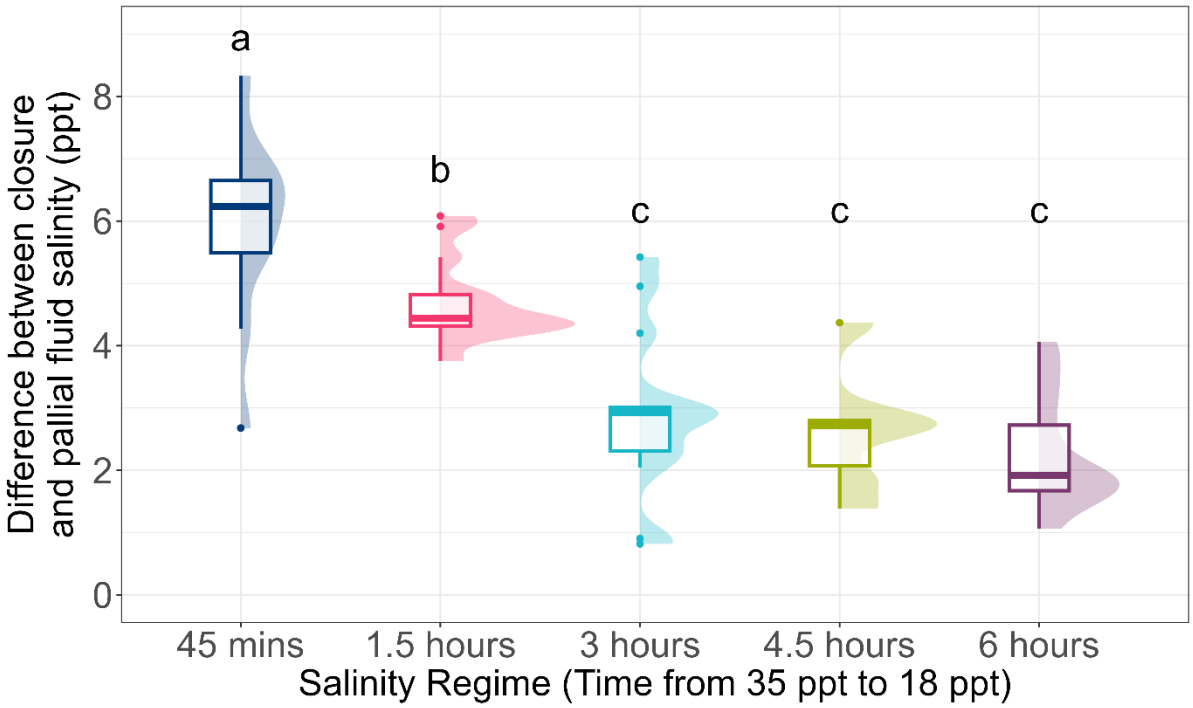


Figure 18: Boxplots and density plots showing the difference between the salinity of pallial fluid and the salinity of the external water at valve closure compared between the salinity regime time periods. Different letters indicate a significant difference between groups.

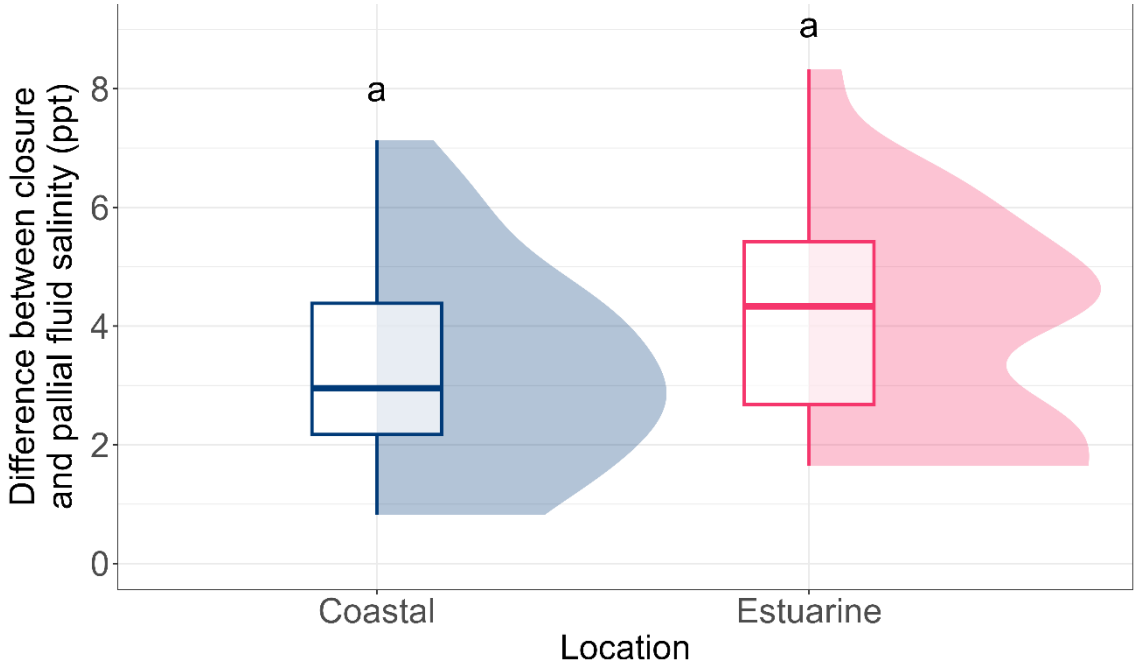


Figure 19: Boxplots and density plots showing the difference between the salinity of pallial fluid and the salinity of the external water at valve closure compared between the two locations. Different letters indicate a significant difference between groups.

b) Combined gaping, heart rate and oxygen consumption

Several technical issues with sensors resulted in less combined parameter (HR, MR and gape) data being recorded for than originally intended. Gape was recorded in 5 mussels exposed to 35 and 27 ppt, whereas HR and MR were recorded for 6 mussels in 35 and 27 ppt, and 5 mussels in 19 ppt.

Table 4: Results of a permanova showing pairwise comparisons between both pallial fluid salinity and the pallial-closure interval for each of the salinity regime time periods. 0.75 represents the 45 minute regime, and asterisks show the significance level.

Regime pairwise comparison	Pallial fluid salinity n=57		Pallial-closure interval n=57	
	t value	p value	t value	p value
1.5*0.75	3.325	0.003 **	2.582	0.016 *
3*0.75	4.358	0.001 ***	5.243	0.001 ***
4.5*0.75	3.703	0.003 **	4.438	0.003 **
6*0.75	3.905	0.001 ***	6.699	0.001 ***
3*1.5	2.384	0.025 *	4.854	0.001 ***
4.5*1.5	2.322	0.039 *	5.282	0.001 ***
6*1.5	2.186	0.046 *	7.569	0.001 ***
4.5*3	0.268	0.784	0.561	0.578
6*3	0.115	0.916	1.674	0.101
6*4.5	0.147	0.878	0.811	0.443

Gape

A Welch two sample t-test found no significant difference in the gape between 27 and 35 ppt (t=-0.603, df=7.607, p=0.564), with mean percentage gape of 57.57 (±18.27) % in 27 ppt and 51.28 (±14.50) % in 35 ppt, as shown in Figure 20. Gape was highly variable throughout the measurement period in both 35 ppt and 27 ppt, with mussels still frequently showing opening and closing behaviour even in full salinity sea water.

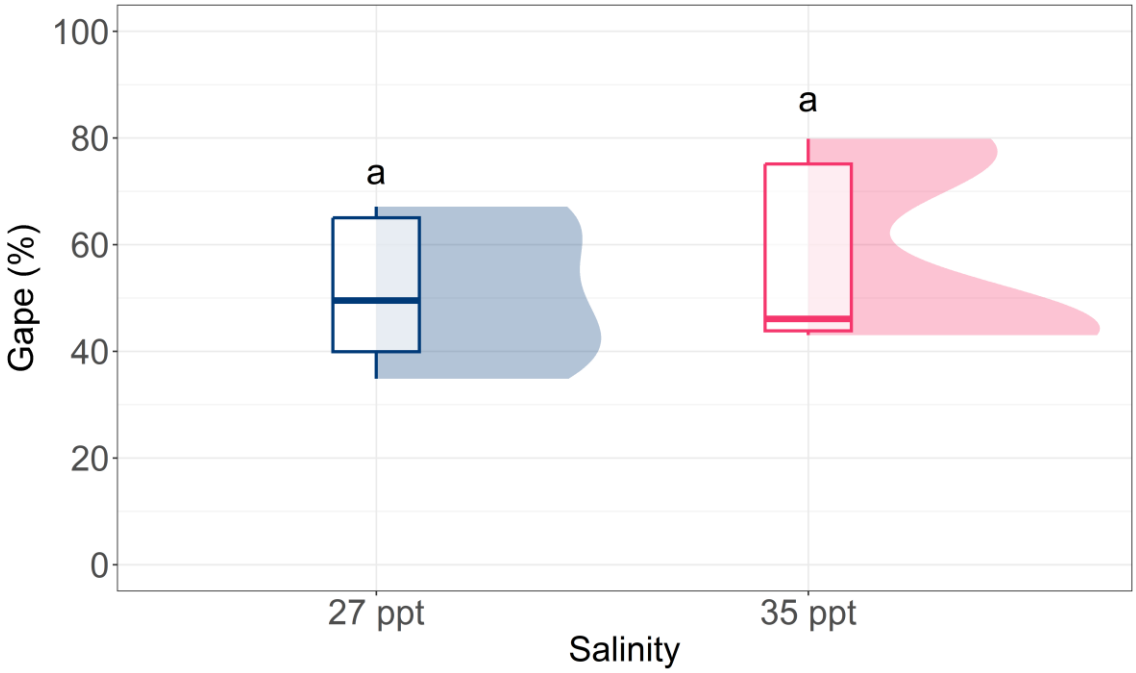


Figure 20: Boxplots and density plots showing the overall mean gape of mussels exposed to 27 ppt and 35 ppt for 3 hours. Letters represent a lack of significant difference between salinities.

Heart rate

An LMM followed by pairwise comparisons found significant differences between HR in all three salinities (between 19 and 27 ppt, z=2.380, n=5 p=0.031; between 27 and 35ppt, z=2.417, n=6, p=0.031; between 19 and 35 ppt, z=4.678, n=5, p=8.71x10^{-6}). The mean HR was 18.58 (±1.43) bpm at 35 ppt, 15.78 (±0.56) bpm at 27 ppt, and 12.11 (±3.95) bpm at 19 ppt. There was a clear decrease in HR in the lower salinities (Figure 21), and additionally patterns were observed in which prolonged valve closure resulted in a clear reduction in heart rate. In Figure 23, a mussel exposed to 35 ppt closed below 20% gape twice during the measurement period. At around 56 minutes, the valve closes for 27.65 minutes, and around 14 minutes into this closure period the HR begins to drop rapidly from 21.75 bpm down to a minimum of 12.91 bpm. Later at about 132 minutes, the valve closes for 16.06 minutes, and 6.69 minutes into this period the HR drops from 22.21bpm down to a minimum of 14.78 bpm.

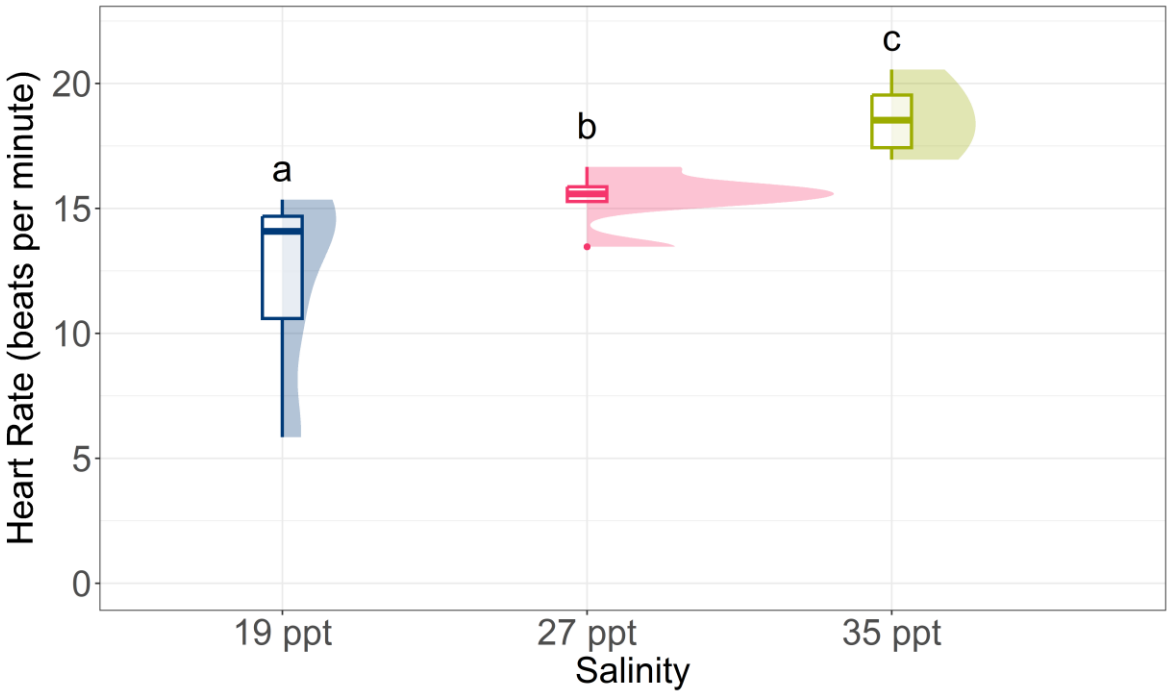


Figure 21: Boxplots and density plots showing the overall mean heart rate of mussels exposed to 19 ppt, 27 ppt and 35 ppt for 3 hours. Different letters indicate significant differences between salinities.

Metabolic rate

An LMM followed by pairwise comparisons found the 27 ppt and 19 ppt treatments to be significantly different (z=2.887, n=5, p=0.0117), but there was no significant difference between 27 ppt and 35 ppt (z=-2.176, n=6, p=0.0591), or between 19 ppt and 35 ppt (z=0.855, n=5, p=0.393) as shown in Figure 22. The mean MR was 3.88 (±1.58) µmol/g/hour at 35 ppt, 4.79 (±2.14) µmol/g/hour

at 27 ppt, and 2.91 (±2.01) µmol/g/hour at 19 ppt. MR was typically relatively consistent throughout the measurement periods with some fluctuation, and did not appear to increase or decrease during the experiments. However, where atypically large spikes in the MR occurred, these appear to closely correspond to rapid valve closures (see Figure 23).

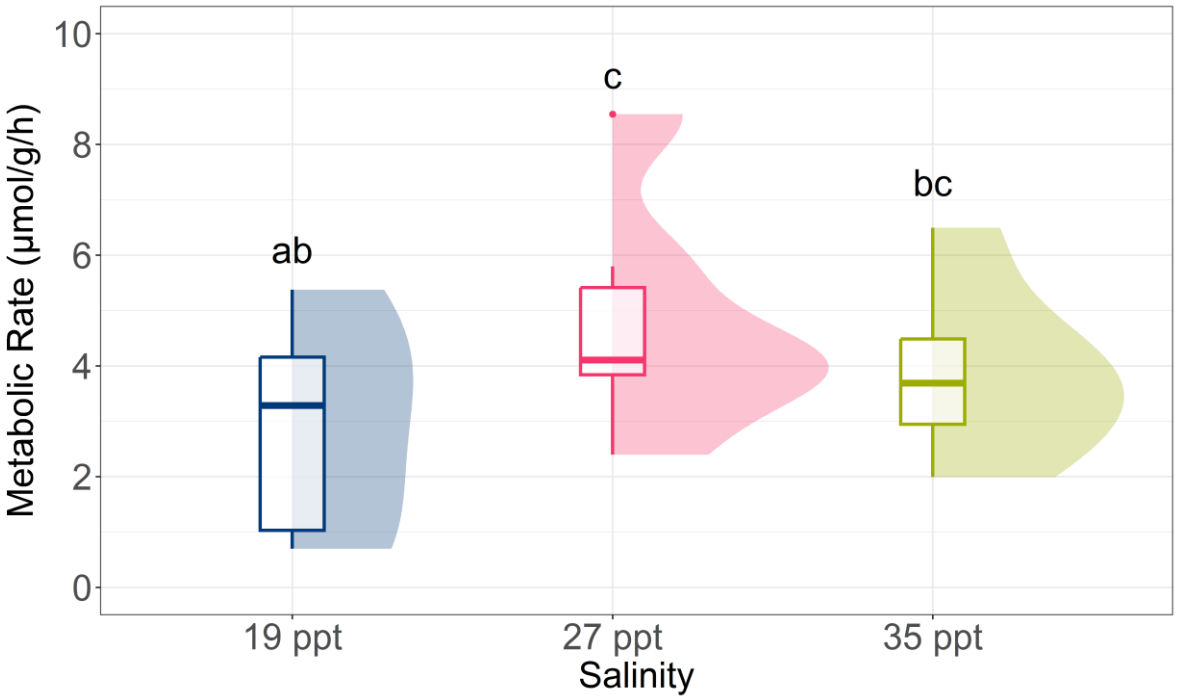


Figure 22: Boxplots and density plots showing the overall mean metabolic rate of mussels exposed to 19 ppt, 27 ppt and 35 ppt for 3 hours. Different letters indicate significant differences between salinities.

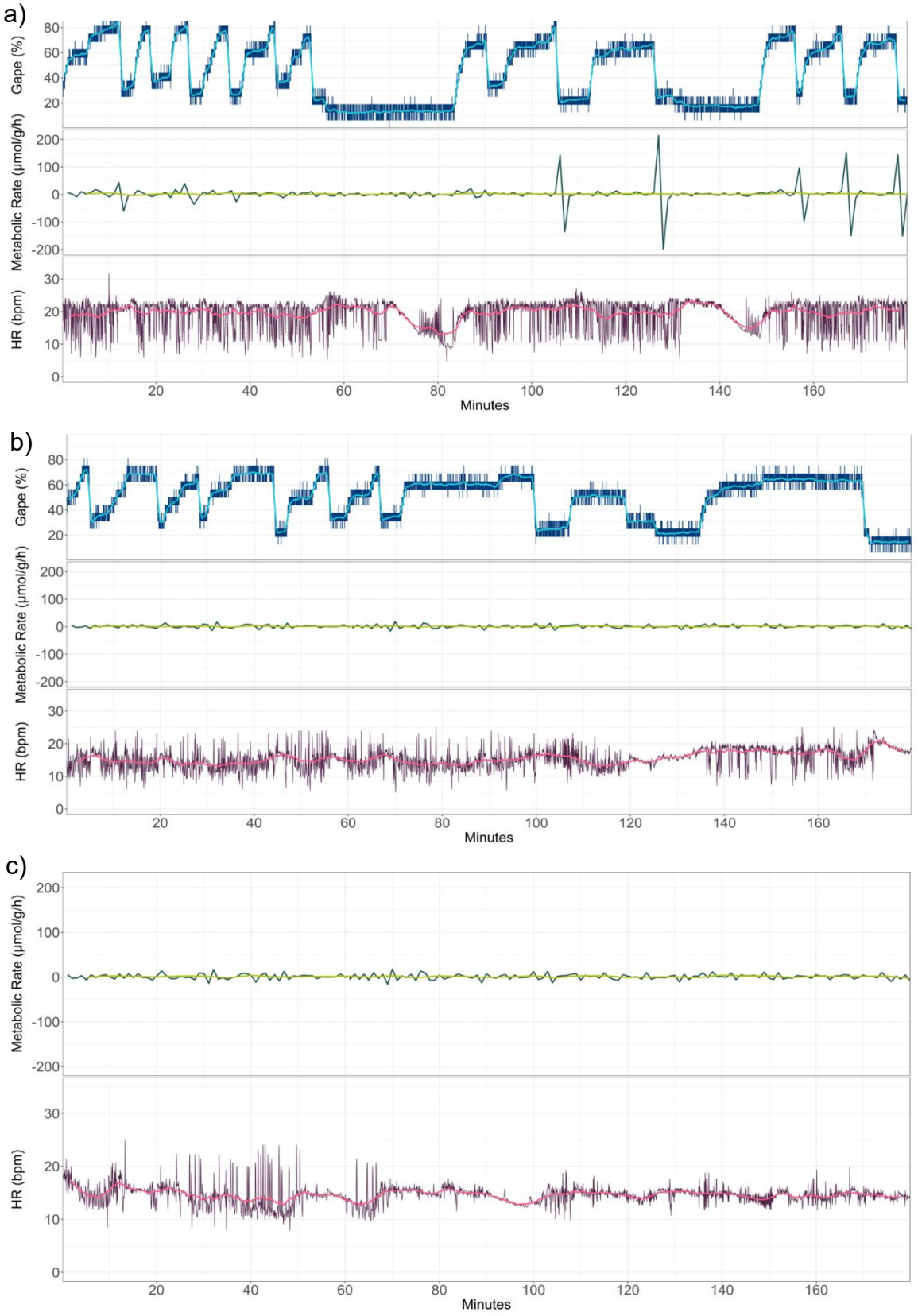
Discussion

a) Mussel gape behaviour

In 95% of trials across all salinity regimes (n=84), the mussels reached a point at which they closed and remained closed in response to falling salinity. This illustrates that mussel gape behaviour is tightly linked to the salinity of its surroundings, an expected response from an osmoconformer.

The mean salinity at shell valve closure of 21 ppt, and retained pallial fluid salinity of 24.51 ppt, were similar to those previously recorded by Davenport (1979), who found that *Mytilus edulis* closed its shell valves at 20.2 (±3.7) ppt and had retained pallial fluid at a salinity of 25.8 (±1.3) ppt when exposed to a 60 minute salinity decline from 33.5 ppt to 0 ppt. In addition, these results also support Davenport's finding that faster rates of salinity change resulted in a higher retained mantle fluid salinity, as the 45 minute salinity decline had the highest pallial fluid. It is also worth noting that Davenport used some much quicker rates of salinity change

Figure 23: Line graphs showing valve gape measured with hall sensors overlayed with the 60 second rolling mean (top), the metabolic rate estimated from oxygen consumption at five minute (light green) and one minute (dark green) intervals (middle), and heart rate overlayed with the 60 second rolling mean (bottom) during a 3 hour exposure to a) 35 ppt, b) 27 ppt, and c) 19 ppt.



(3.75 minutes from 35.5 to 0 ppt), and therefore we may have observed even higher retained pallial fluid salinities if we too had examined equivalent rates of salinity change. However, although Figure 13 shows a slight increase in the

closure salinity as the rate decreased, there were no significant differences between the groups, unlike the Davenport study where a significantly higher closure salinity was demonstrated during the slower rates of salinity change.

Davenport suggested that the differences in the salinity of the retained pallial fluid and at closure are a result of the closure of the exhalant siphon, and thus ceasing of water exchange between pallial fluid and the external medium, which he observed to occur prior to the valve adduction. He also found that the oxygen tension inside mussels was found to be significantly higher in slower rates of salinity change, which suggests that allowing the shell to remain open allows for some diffusion of oxygen through the exposed mantle, whilst closing the exhalant siphon gives the mussel some protection from osmotic stress, and additionally allows for better salinity sensing by osmoreceptors on the inhalant siphon, so any improvement in conditions can be sensed. The salinity of retained pallial fluid recorded in our study was always higher than the minimum salinity of the external seawater during exposure, suggesting that in all cases, the reduced salinity caused the exhalant siphon to close.

Mussels collected in the coastal Teignmouth site had a significantly lower closure salinity than the mussels collected from the Exmouth or estuarine Teignmouth site. As we do not have in-situ measurements of the water conditions at the sites, it is possible that a variation in salinity between the sites is responsible for a differing adaptation in response to osmotic stress. Teignmouth is a partially mixed estuary, which means that during periods of low tide, estuarine mussels are likely to be exposed to reduced salinities as a result of the freshwater river influx. The estuarine mussels had a significantly higher retained pallial fluid salinity than the coastal mussels, which suggests that the earlier closure of the exhalant siphon could be a local adaptation which helps these estuarine mussels deal with lowered salinities. However, the Exmouth mussels had the highest closure salinity of all despite being the furthest site from an estuary, suggesting that this population may actually cope better with salinity changes.

There was a weak but significant correlation between closure salinity and pallial fluid salinity. There are instances where the retained pallial fluid salinity seems to clearly correspond to a small closure prior to the permanent closure of valves, suggesting that although the valves reopened after this point, the exhalant siphon remained shut (see Figure 12e). Additionally, on the three occasions in which the

valves reopened at the lowest salinity, pallial fluid was retained from just before or during their closure prior to reopening. However, this is certainly not the case for all mussels, and there are other occurrences where the pallial fluid has been retained at a point when valve gape is relatively high, and not near a flapping event (see Figure 12b). During each experimental trial (with 8 experimental chambers), only a single experimental chamber contained a salinity probe. Whilst all chambers were fed by a single RO source for dilution, at a standardised rate controlled by the same peristaltic pump, meaning salinity should have been the same in each chamber, a very small level of variation (standard deviation 0.68 ppt) was measured across the final salinities achieved in each chamber. This means that the pallial fluid may have on occasion been retained at a slightly different salinity to that calculated based on the corresponding time of exposure.

The difference between the salinity of the pallial fluid and the salinity at valve closure (pallial-closure interval) was different between most regimes, and showed a general trend of a greater pallial-closure interval at faster rates of salinity change, as shown in Figure 19. This to some extent explains the fact that there was only a weak correlation between pallial fluid and closure salinity, as it illustrates that they were not directly proportional to each other. The fact that pallial-closure interval was greatest in the shortest regime suggests that when the mussel senses a rapid salinity decline, it closes its exhalant siphon early, but the valves continue to remain open until an external salinity of 21 ppt is reached. This mechanism may protect the internal structures of the mussel from osmotic stress, but also allows easy sensing of salinity by the osmoreceptors on the inhalant siphon, so that it can quickly be detected if salinity returns to favourable levels.

The pallial-closure interval was not significantly different between the two locations, which may suggest that environmental salinity does not affect how quickly valve closure occurs after siphon closure.

This study only examined the effects of an acute salinity change on valve closure, and it is likely that if the mussels had remained in 18 ppt water for a prolonged period, the valves would have begun to reopen. In a separate study, where animals were exposed to a continuous 5-day exposure to reduced salinity, mussels exposed to 20 psu were found to remain closed for the first day of exposure, and then reopen for the remaining four days, whereas mussels

maintained at 5 or 10 psu remained closed for the whole 5-day period (Addis et al., 2021).

Many mussels showed flapping behaviours of rapid opening and closing, although this did not seem to be linked to salinity. Flapping has previously been shown to increase in bivalves in response to toxic dinoflagellates (Basti et al., 2009; Comeau et al., 2019; Nagai et al., 2006; Tran et al., 2010), copper (Curtis et al., 2000), oil dispersants (Durier et al., 2021) and diesel (Guterres et al., 2020). Additionally, it is one of the parameters measured by the Mosselmonitor® as an indicator of pollution (Barile et al., 2016). However, salinity does not appear to be a factor which affects flapping, with this behaviour still visible even during the static exposure to 35 ppt. Thus, further investigation is required to determine the interindividual variability of this behaviour, with any difference in the propensity of individuals to display flapping, even under control conditions, having major implications for the use of this parameter as a measure of contaminant exposure, or stress.

In this study, below 20% valve gape was considered to be closed, a threshold which has been used in several other studies measuring gape of bivalves with hall sensors (Ballesta-Artero et al., 2017; Jou et al., 2013; Miller & Dowd, 2017). The differences in sensor range variance meant that some sensors recorded gaping in greater levels of detail than others. Whilst this was standardised somewhat by the use of rolling mean gape to find the point at which valves dropped below 20%, some sensors show variations in valve movement below 20%, suggesting the valves are not always completely closed at this point.

b) Combined gaping, heart rate and oxygen consumption

As a result of the technical issues, sample sizes for this experiment were relatively small. Whilst statistical analyses of this data is included to aid with comparisons, the results should be interpreted with caution given the low sample size. Mean gape was highly variable at both 27 and 35 ppt, which is unsurprising given that mussels naturally alter their valve gape even in optimal conditions. Additionally, there was no significant difference between the average gape in 27 ppt and 35 ppt. Given that the gape behaviour experiments showed that the mean salinity of osmotic stress-induced valve closure is 21 ppt, it is not surprising that valve gape was similar between the much higher salinities of 27 and 35 ppt. Whilst gaping data failed to record for 19 ppt, given that MR was lower in this

salinity, and this is below the mean closure point, it is likely that the mean gape would have also been lower at this salinity.

Salinity had a clear effect on HR, with Figure 21 showing a decrease in HR as salinity decreased. This supports previous studies which have also found that *Mytilus* HR decreases with decreasing salinities (Bakhmet et al., 2005; Braby & Somero, 2006b; Stickle & Sabourin, 1979). When exposing *M. galloprovincialis* to different concentrations of copper, Shen and Nugegoda (2022) also found that there was a significant correlation between gape and HR, with HR beginning to decrease about 4-8 minutes after gape dropped below 40%. This relationship was also found in a previous study into the effect of copper on HR and gape (Curtis et al., 2000). Whilst previously there was no explanation for mussel bradycardia (Bakhmet & Khalaman, 2006), by pairing HR measurements with that of valve movement, the above studies have provided an insight into the strong association between HR and gape behaviour. This is supported by our data, which shows a similar effect of a sharp decrease in HR following a valve closure, after a short delay. There is still a very limited number of studies which measure HR in tandem with other physiological parameters, and future studies should make use of modern technology to measure multiple endpoints concurrently to provide the best understanding of overall animal functioning.

Metabolic rate seemed to be fairly consistent across the three-hour measurement period in all salinities. Figure 22 shows that MR was significantly different between 19 ppt and 27 ppt. Given that both 27 ppt and 35 ppt are above the mean salinity at which pallial fluid is retained and the exhalant siphon is assumed to have shut (24.51 ppt), it would be expected that filtration and therefore consumption of oxygen would have continued at these salinities. It is somewhat surprising that there was no difference in MR between 19 ppt and 35 ppt, although this can likely be explained by the low sample sizes. In Figure 23a, unusual spikes in the metabolic rate can be seen in the 35 ppt exposure, which seem to closely link to the closure of the valves. One possible explanation for this is that the oxygen probe was in very close proximity to the mussel, so that when the mussel shut it forced some of its partially deoxygenated pallial fluid directly over the sensor, causing a sudden drop in oxygen partial pressure, which was subsequently rapidly dispersed by mixing.

Future research

Whilst Davenport provides an interesting insight into the comparisons between valve and exhalant siphon closure, that study was limited by the lack of available technology at the time, with visual observation of siphon closure only offering a crude method of measurement. Few studies have since developed a method for directly recording exhalant siphon activity, with one study attempting to record this with hall sensors (Robson et al., 2009), and others with video cameras showing that exhalant siphon area decreases when water velocity is increased (Newell et al., 2001), and increases when algal concentration increases (Maire et al., 2007). Gape and exhalant siphon area have been compared in the freshwater mussel *Anodonta anatina*, and this suggested that measurement of siphon area gives a quicker response time than with valve movement, relating to the fact that the siphon begins to close before the valves (Escobar-Calderón et al., 2022). This appears to be the only study in recent years which has compared these two measurements, and the technological advancements mean that such investigations are now easier to conduct. This should be utilised with further comparisons of valve gape to exhalant siphon closure under changing salinity in order to determine how these two factors are connected.

Hall sensors give us a valuable understanding of gaping behaviour, but in order for assumptions to be made about the effects of certain valve closures on overall mussel functioning, we must first know how this behaviour is related to other aspects of physiology. This will allow us to gain a greater understanding of the effects of environmental change on the whole organism physiology and functioning of mussels. Additionally, this understanding will allow us to better interpret data from technologies like the Mosselmonitor and MolluSCAN eye which allow us to measure these parameters in situ (Andrade et al., 2016; Barile et al., 2016), creating smart farming solutions to advance aquaculture, and additionally generate better methods for monitoring the impact of climate change.

Conclusion

To summarise, this chapter has found that a declining salinity induces shell valve closure at 21 ppt, and this is not affected by the rate of salinity decline. In contrast, the salinity of the pallial fluid retained by the mussel is higher in faster salinity declines, and this retained pallial fluid has a higher salinity than that at which the shell valves adduct (24.51 ppt). Salinity was found to have a significant effect on

heart rate, which was greatly reduced at lower salinities, and MR was also lower at 19 ppt than at 27 ppt. Additionally, HR was found to be closely linked to gape, with periods of closure being associated with bradycardia. Whilst mussels are a group which have extensively been studied, the fundamental physiological effects of salinity induced closures are still little understood. Gaining this knowledge is of great importance to allow us to better understand how mussels living in already highly changeable environments, like estuaries, will cope with climate change.

Overall Discussion

Current state of the research field, and aims of this thesis

The aim of this thesis was to explore the effects of acute salinity changes on *Mytilus* mussels, both in terms of their physiology, but also their shell valve gaping behaviour. Additionally, it aimed to examine if there were differences in these traits between mussels living in an estuarine site, with variable salinity conditions, and mussels living in a coastal site, with a consistently high salinity. These different sites were also compared in terms of the genetic composition of the mussels, in order to determine if salinity may affect the distribution. Prior to this work, it had been hypothesised that the distribution of hybrid mussels may be to some extent governed by environmental conditions, with *M. edulis* having a greater abundance in sheltered estuarine sites, and *M. galloprovincialis* having a greater abundance at exposed coastal sites (Gardner, 1996). Despite this, few studies have actually examined the physiological and genetic differences simultaneously for mussels from coastal and estuarine sites within a hybrid zone (Gardner & Thompson, 2001), and to our knowledge this comparison has not yet been made in the Southwest UK hybrid zone. Our study therefore sought to make this novel comparison, the results of which would have implications for predicting future distribution shifts as a result of the many changes the ocean is predicted to undergo due to climate change.

The ability for bivalves to adduct their valves in response to unfavourable conditions is one that has been studied to some extent, but there is limited research examining this effect in response to salinity (Addis et al., 2021; Davenport, 1979). However, many physiological traits are largely modified by this closure behaviour, given that it prevents mussels from consuming oxygen, filter feeding, and expelling waste. Recent technological advancement, like the use of magnetic hall sensors to measure gape (Robson et al., 2007), has allowed for a more detailed examination of this behaviour, but only a few studies to date have measured gaping behaviour simultaneously with physiological traits (Lassoued et al., 2019; Shen & Nugegoda, 2022). Additionally, gaping behaviour and physiological endpoints have not yet been measured simultaneously in response to salinity change, despite the fact that we know that low salinity causes valve adduction (Davenport, 1979), which is likely to have a significant effect on

physiology. By making this comparison, we are able to understand the mechanisms behind mussel response to a sudden decline in salinity, the frequency of which is likely to be increasing in the coming years as a result of climate change (IPCC et al., 2021).

General findings

In chapter two, it was found that MR varies significantly with salinity over acute exposures, with a low salinity resulting in a lower MR. However, no difference was found in the MR originating from coastal, lower estuarine or upper estuarine sites. Additionally, genetic analysis using a novel custom designed 60k mussel SNP array found that whilst coastal and lower estuarine mussels were almost entirely *M. edulis*, the upper estuarine population consisted of individuals with higher *M. galloprovincialis* ancestry. This contrasts previous analyses, which found that mussels in the Tamar estuary were almost entirely *M. edulis*, whereas the nearby coastal site was dominated by hybrids (Hilbish et al., 2003).

During experiments for chapter 2, observations showed that in the lowest salinity of 19 ppt, mussels appeared to be closed when the final oxygen reading was taken. As a result of this, it became apparent that valve gape is a key determinant of MR, as short-term stress frequently results in valve closure, subsequently preventing oxygen consumption. As gape was not measured in this experiment, it is difficult to uncouple the physiological response of a reduction in MR from the behavioural response of valve closure. It is possible that all reductions in MR at the lower salinities were the result of partial or complete valve closure, and therefore it is difficult to tell to what degree the response was behavioural or physiological. This helped guide the direction for chapter three to also examine the behavioural response to low salinity.

In the third chapter, a novel hall sensor system was designed and constructed that enabled recording of the valve gape of mussels in response to declining salinity. It was found that *M. edulis* close their shells at 21 ppt in response to an acute decline in salinity over several different rates of change. In addition, it was found that the salinity of the pallial fluid retained inside the shell was higher when the rate of salinity decline was faster. Pallial fluid is assumed to be retained at the point that exhalant siphons stop pumping, preventing the exchange of water with the external environment, and additionally the salinity of this retained fluid has been found to remain stable over time (Davenport, 1979). This means that the

salinity of the pallial fluid can indicate the salinity at which the exhalant siphon closed, and therefore these results suggest that mussels react to a rapid change in salinity by closing their exhalant siphon, thereby allowing their internal environment to remain slightly hyperosmotic to the surroundings. When *Ostrea edulis* were exposed to a salinity decline, it was found that they had closed their valves by 20.5 ppt, suggesting that oysters have a very similar salinity closure response to mussels (Bamber, 2023).

Additionally, this chapter investigated the combined measurements of gape, HR and MR in response to different salinities. It was found that HR is lower in reduced salinities, and MR was significantly lower at 19 ppt than at 27 ppt. MR was slightly higher in chapter 3 than at the same salinities in chapter 2, likely as a result of slightly elevated water temperatures being used in this subsequent experiment. Gape did not vary significantly between 27 ppt and 35 ppt, likely due to being above the salinity determined to induce valve closure (21 ppt). However, HR and gape were found to be closely linked, with a drop in HR occurring during a sustained valve closure. This illustrates the close linkage between physiology and behaviour.

In both data chapters, reduced MRs were shown at lower salinities. Whilst many other studies suggest that MR increases at reduced salinities, this is likely because these studies expose mussels to a reduced salinity over an extended time period (Hamer et al., 2008). In this study, reduced MR was likely a result of valve closure, which protected the internal tissues of the mussel from osmotic stress by isolating it from the external water. This illustrates the differing responses to acute and chronic salinity changes, which are likely to be relevant to different scenarios of future salinity change induced by climate change. Abrupt changes, like increased precipitation and flash flooding, are likely to elicit sudden acute changes in salinity which are likely to induce a behavioural response, causing mussels to close their valves, offering short term protection, but a response that is unsustainable during chronic exposures due to isolation of the mussel from the external environment, resulting in mortality (Addis et al., 2021). On the other hand, chronic changes to salinity caused by ocean freshening, for example in the Baltic Sea, are likely to cause an increase in MR due to the greater energetic costs associated with low salinities (Freitas et al., 2017).

In these studies, several different forms of salinity regime were employed to simulate conditions experienced by mussels in estuaries. In reality, it is incredibly difficult to predict the salinity exposures which mussels face, given the many factors which can affect the fluctuations (see Figure 2). In the Tamar and Teign estuaries from which the mussels for these experiments were collected, partial mixing occurs. This means that in addition to the longitudinal salinity gradient from more saline conditions at the mouth of the estuary to more brackish in the upper estuary, there is also a gradient in which the water becomes more saline with depth. In addition to these factors, salinity varies across the tidal cycle, with greater saltwater input at high tide, and between spring and neap tides, with a larger saltwater intrusion in the estuary at spring tide (Uncles & Stephens, 2011). When combined with weather conditions, such as precipitation and wind force, in addition to the complexities of fluid dynamics, it is almost impossible to predict the salinity exposures mussel populations naturally experience, with such information only obtainable through long term in situ monitoring, which was beyond the scope of this study. However, the salinity regimes used represent a theoretical system within the range mussels are likely to experience in-situ, which allows us to understand the physiological and behavioural responses to salinity changes within a controlled environment. This knowledge can then be applied to in-situ experiments, which help us understand how these effects compare to what mussels actually experience. Studies like that of Domínguez et al. (2020), which use a variety of different salinity regimes which correspond to field measurements, are also an effective way to compare how differences in the salinity exposure regime may influence the response.

In the first experiments of this thesis, mussels were collected from Whitsand Bay in November 2021, a population which has been studied for decades (Bignell et al., 2011; Gilg & Hilbish, 2000; Hilbish et al., 1994; Schneider et al., 2005; Skibinski & Roderick, 1991), alongside samples from the Cremyll and Saltash populations of the Tamar estuary. However, upon return to the Whitsand site in September 2022, it was discovered that a mass mortality of these mussels had occurred (see Figure 24), necessitating a change in direction for the thesis. Mass mortalities have been occurring in increasing frequencies in recent years (Lupo et al., 2021), with repeated exposures to high aerial temperatures shown to increase risk of mortality (Seuront et al., 2019). In summer 2022 the UK experienced heatwaves which resulted in the highest temperatures on record,

with the Southwest reaching temperatures of up to 36 °C (Met Office, 2023). Given that three 6-hour exposures to 35 °C aerial exposure caused 100% mortality in experimental *M. edulis* (Seuront et al., 2019), it is likely that the heatwave temperatures in summer 2022 would have caused a significant amount of mortality. In addition, the bacteria *Francisella halioticida* has recently been identified for the first time in the UK within Tamar mussels (Cano et al., 2022), a species which has previously been associated with mass mortalities of abalone in Japan (Kamaishi et al., 2010), and has additionally been found to correlate with *Mytilus* mortality in France (Charles et al., 2020). It is therefore possible that this infection also contributed to the mussel mortality. Whilst assumptions can be made on the reasons behind the increasing mortality rate, regular large-scale surveying of mussel beds coupled with in-situ logging of environmental conditions with biomimetic loggers will be necessary to confirm a cause.

a) b)

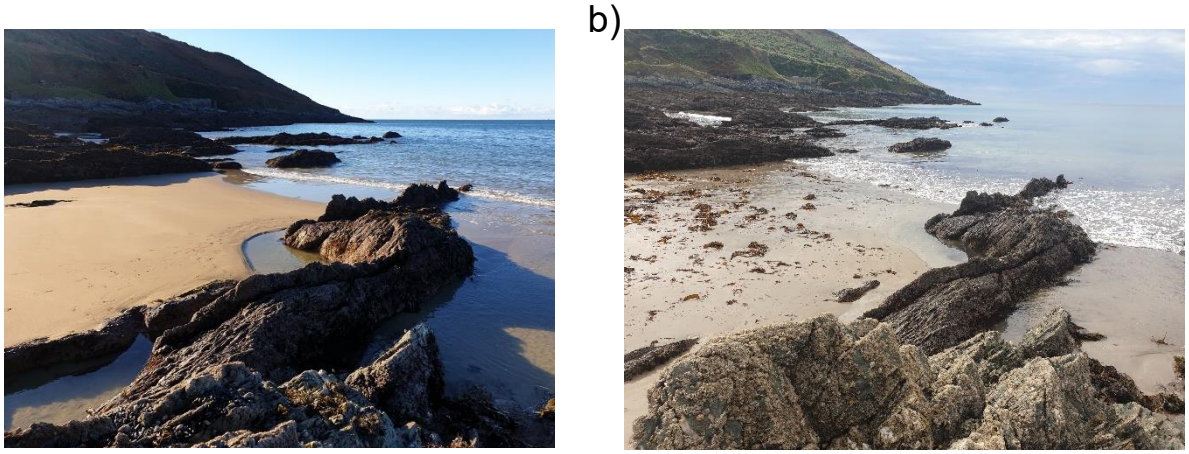


Figure 24: Images of the Whitsand Bay sampling site. a) shows the site on 04/11/21, with mussels visible in the bottom part of the image, and b) shows the site on 12/09/22, with mussels absent.

Future research

In order to be able to accurately predict the fate of marine organisms in a rapidly changing ocean, it is important to conduct experiments which expose them to multiple stressors to create the best reflection of real-life conditions. However, this is often logistically complex, and makes disentangling the variables to understand to what extent each is contributing to the response difficult. In a recent meta-analysis examining the stressors affecting *Mytilus* mortality, it was found that only 25% of studies investigated a combination of factors (Lupo et al., 2021). In the studies which did examine more than one stressor various interaction effects were found, with some studies finding synergistic effects on mortality, and others finding antagonistic effects. This suggests that only examining one stressor may not present the whole picture of how this might affect mussel

functioning when alongside the multitude of other stressors present in the natural environment. This is likely to play a key role in predicting risk factors for mass mortality events and implementing control measures to prevent this. However, there is still a role for single stressor studies, which are the best option for determining cause and effect of specific stressors, which can then be considered when examining these as part of the wider picture.

Additionally, future studies need to examine the impact of these stressors on multiple endpoints in order to gain the best understanding of how overall animal physiology, behaviour and health will be affected. In this thesis, it was shown that in the short-term response to acute salinity change, the valve gape behaviour greatly affected the physiology in terms of both the MR and the HR. This emphasises the importance of recording multiple parameters, as this ensures a comprehensive understanding of the holistic functioning of the organism. Whilst this study has provided a good foundation of knowledge on how gape may impact other physiological parameters, this area is still little understood. In future, this should be expanded to examine these effects over greater periods of time. Additionally, to our knowledge gape and HR have never been measured simultaneously in the field, and therefore a long-term study in which mussels are deployed with gape and HR sensors are attached, alongside a logger to record environmental variables like temperature and salinity, would provide an excellent insight into how these physiological parameters vary with environmental change.

Whilst a few studies have previously observed the connection between valve adduction and reduced heart rate (Curtis et al., 2000; Shen & Nugegoda, 2022), this has previously been recorded as a response to copper, rather than to declining salinities. This unusual response shows the importance of gape as a measurement parameter, as it clearly has a large impact on the physiological response of mussels. As a result of this, if physiological traits which depend on the shell being open like oxygen consumption, HR or feeding rate are measured without also considering gape, it is difficult to determine whether a stressor has directly caused a change in physiological performance, or if it caused valve adduction which then lead to the physiological change. The experiments conducted in this thesis have made it clear that gape is a key measurement parameter, and therefore future research should consider measuring this alongside other endpoints.

As a critical determiner of mussel response to declining salinities, we are in need of improved methodology to study mussel valve movements. Hall sensors have proven to be an excellent way to measure this behaviour, and this can be seen from the increasing number of studies in which they are employed (Addis et al., 2021; Miller & Dowd, 2017; Nagai et al., 2006; Shen & Nugegoda, 2022). However, a conclusive method for analysing this complex and detailed data is yet to be determined. In this study, a threshold of 20% was used as the point of valve closure, based around this being chosen in several other studies (Ballesta-Artero et al., 2017; Jou et al., 2013; Miller & Dowd, 2017), and additionally the fact that valve movements usually appeared stable below this point. By picking out this closure point we were able to simplify and quantitively analyse this complex behaviour. We also converted the raw voltage data output to percentage gape for each mussel, allowing for comparison between individuals. However, the methods of analysis for this data currently vary, and therefore a study which compares previous work and determines several agreed upon methods for quantifying differences in this behaviour would allow for direct comparisons between studies. This is particularly relevant for difficult to interpret data like flapping behaviour, and perhaps the development of an R package designed to analyse bivalve valve movements would make this analysis feasible for many researchers.

When the genetics of mussels in and around the Tamar was assessed previously, in was found that whilst estuarine mussels were entirely *M. edulis*, coastal mussels were hybrids, with the frequency of *M. edulis* and *M. galloprovincialis* greatly depending on size (Bignell et al., 2011; Hilbish et al., 2003). It has previously been hypothesised that *M. edulis* is better able to tolerate salinity fluctuations, and therefore is more likely to dominate estuarine sites. However, our results directly contrasted with this, with the greatest *M. galloprovincialis* allele frequency being found in the upper estuarine site. This has illustrated the temporal variability in the distributions of these species, and suggests that analysis should take place over several years in order to determine the consistency of the species frequencies. Additionally, by regularly collecting data we will be able to see in real time how these distributions may be changing, and create much more accurate predictions of how these changes may continue to occur.

To summarise, this thesis has demonstrated that the relationships between hybrid *Mytilus* distribution, the effect of environmental factors like salinity change, and physiological performance are complex, and need to be studied in greater detail in order to form the full picture necessary to make accurate predictions about the fate of this key group during the changes predicted to occur in the coming years. Additionally, it has highlighted the importance of valve gape as a key measurement parameter which impacts the physiological performance of mussels during acute salinity changes. Future studies need to consider this behaviour, particularly during short-term exposures to stressors which are likely to induce valve adduction.

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