Calcium transport in the Pacific oyster, *Crassostrea gigas*

- in a changing environment

Thesis for the degree of Doctor of Philosophy

By

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DEPARTMENT OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES

GÖTEBORGS UNIVERSITET

CALCIUM TRANSPORT IN THE PACIFIC OYSTER, *CRASSOSTREA GIGAS* - IN A CHANGING ENVIRONMENT

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DISSERTATION ABSTRACT

Pacific oyster, *Crassostrea gigas,* is globally one of the most important farmed bivalve species. A prominent features of the *C. gigas* is the thick $CaCO_3$ shell covering the body of the animal and protecting it from the environment. To be able to produce the shell, the oysters need to take up calcium from the environment and transport it to the shell forming area. The mantle tissue, separating the rest of the body from the shell, is suggested to be of central importance for both uptake of calcium and its transfer to the shell. The final part in this route is the transfer of the ion across the outer mantle epithelium (OME). The Ca has been suggested to be transferred across the OME in one or more of the following forms: as ionic calcium (Ca^{2+}), as calcium bound to proteins or inorganic ligands, as $CaCO_3$ inside vesicles or cells in the hemolymph. The uptake of Ca and other ions for the shell formation, as well as the conditions affecting the calcification process, are dependent on external conditions such as salinity, temperature and pH. As climate change has predicted to change these conditions in the future, also the shell formation of oysters might be affected.

In this thesis, the uptake and transport of calcium from the environment to the shell forming area in *C. gigas* were investigated. Calcium uptake and transport in the hemolymph were analysed by exposing the oysters to water containing radioactive calcium after shell regeneration had been induced through an artificial cut, to accelerate shell formation. The uptake and transport of calcium in the different hemolymph fractions and mantle tissue were then followed. The transfer of calcium ions across the OME was investigated *in vitro* using live OME mounted in specialized Ussing chambers. The kinetics of the Ca^{2+} transport was assessed as were the effects of pharmacological tools inhibiting selected potential Ca^{2+} transporters and channels. Additionally, the mantle genome was searched for these potential ion transporters and channels. The expression of the proteins as well as their cellular localisation in the OME, was confirmed by immunohistochemistry and western blot. Finally, effects of a dilute environmental salinity on the OME ion transfer as well as on the mRNA expression of potential Ca^{2+} transporters and channels were examined

In *C. gigas* calcium was taken up from the environment and transported in the hemolymph mostly as Ca^{2+}. The transfer of Ca^{2+} across the OME consisted of a passive, paracellular component and a transcellular, active transport component. A combination of physiological and functional studies, transcriptome analysis and protein expression analyses through immunological methods made it possible to postulate a model for Ca transfer across the OME of *C. gigas*. The Ca was transferred following two pathway: 1) 60% was transcellularly transported and entered the OME cells through basally located voltage-gated Ca channels (VGCCs) and was then excreted across the apical membrane by Ca^{2+}-ATPases (PMCA) and Na^{+}/Ca^{2+}-exchangers (NCX), the latter using the Na^{+} gradient created by a basal NKAs to function. 2) the remaining 40% was diffusing across the OME through the paracellular pathway. Ionic Ca^{2+} transfer, total active ion transport and paracellular permeability all decreased when *C. gigas* were exposed to diluted seawater (50%). The pattern of changes in mRNA expression of Ca transporters and channels in the OME cells suggest that the cells are

trying to compensate for the decreased Ca levels in the diluted seawater. Expression of intracellular Ca-ATPases (SERCAs), transporting Ca^{2+} into intracellular stores decreases, while membrane bound Ca^{2+} channels and NCX mRNA expression increases. These changes suggest that the cells strive to maintain a high enough intracellular Ca^{2+} concentration to achieve a sufficient Ca^{2+} flow across the OME for shell growth. However, as the Ca^{2+} transfer across the OME decreased when exposed to 50 % seawater, these compensatory mechanisms were not sufficient. Overall, these results indicate that the oyster *C. gigas* may face problems with shell calcification in areas where the salinity of the seawater have been predicted to decreaseas a result of current climate changes.

Keywords: Climate change, Ussing chambers, calcium uptake, sodium/potassium ATPase, calcium ATPase, sodium/calcium exchanger, calcium channel, hemocytes, mantle epithelium

SVENSK SAMMANFATTNING

Stillahavsostronet, Crassostrea gigas, är globalt sett en av de viktigaste odlade arterna av tvåskaliga blötdjur. Utmärkande för *C. gigas* är det tjocka skalet av $CaCO_3$- som skyddar djuret från den yttre miljön. För att kunna producera skalet måste ostron ta upp kalcium från omgivningen och transportera det till skalbildningsområdet. Mantelvävnaden, som separerar resten av kroppen från skalet, föreslås vara av central betydelse för både upptag av kalcium och dess överföring till skalet. Det sista steget i transporten av kalcium till skalbildningsområdet är passagen av kalcium över det yttre mantelepitelet (YME). Kalcium har föreslagits passera över YME i en eller flera av följande former: i jonfrom (Ca^{2+}), bundet till proteiner eller oorganiska ämnen, som $CaCO_3$ inuti vesiklar eller celler i hemolymfan. Upptag av Ca^{2+} och andra joner för skalbildning samt faktorer som påverkar kalcifieringprocessen är beroende av yttre förhållanden såsom salthalt, temperatur och pH. De pågående klimatförändringarna förutspås förändra dessa förhållanden i framtiden vilket gör att även skalbildningen hos ostron kan komma att påverkas.

I denna avhandling undersöktes upptag och transport av kalcium från miljön till det skalbildande området i *C. gigas*. Kalciumupptag och transport i hemolymfan studerades genom att exponera ostron för vatten med radioaktivt märkt kalcium efter det att skalet hade tillfogats en skada för att påskynda skalbildningen. Upptag och transport av kalcium i de olika hemolymfa-fraktionerna och mantelvävnaden studerades. Transport av Ca^{2+} över YME undersöktes *in vitro* genom att levande YME monterades i specialiserade Ussing-kammare. Transportkinetiken för Ca^{2+} studerades, liksom effekter av farmakologiska blockerare av utvalda potentiella Ca^{2+}-transportörer och -kanaler. Mantelgenomet genomsöktes efter potentiella jontransportörer och kanaler. Uttrycket av proteinerna och deras cellulära lokalisering i YME analyserades med hjälp av immunohistokemi och western blot. Slutligen undersöktes effekterna av en utspädd salthalt i omgivande vatten på kalcium transporten och mRNA uttryck av Ca^{2+}-transportörer och -kanaler.

I *C. gigas* togs kalcium upp från miljön och transporterades i hemolymfan huvudsakligen i jonform, som Ca^{2+}. Transport av Ca^{2+} över YME bestod av en passiv, paracellulär komponent och en transcellulär, aktiv komponent. En modell för Ca^{2+}-överföring över YME av *C. gigas* presenteras: 60% av kalciet transporteras transcellulärt där inflödet i YME-cellerna sker genom spänningsregelerade Ca^{2+}-kanaler (VGCC) och utsöndras sedan över det apikala membranet via Ca^{2+}-ATPaser (PMCA) och Na^{+}/Ca^{2+} utbyte (NCX). De återstående 40% av totala Ca2+ flödet diffunderar över YME, paracellulärt, mellan epitelcellerna. Ca^{2+}-transporten, den totala aktiva jontransporten och den paracellulära permeabiliteten minskade när C. gigas utsattes för utspätt havsvatten (50%). Förändringar i mRNA-expression av Ca-transportörer och -kanaler i YME-cellerna tyder på att cellerna försöker kompensera för de minskade Ca-halterna i det utspädda havsvattnet. Expression av intracellulära Ca-ATPaser (SERCA), som transporterar Ca^{2+} till de intracellulära lagren minskar, medan VGCC och NCX mRNA-uttrycket ökar. Dessa förändringar antyder att cellerna strävar efter att upprätthålla en tillräckligt hög intracellulär Ca^{2+}-koncentration för att upprätthålla ett

Ca^{2+}-flöde över YME för skaltillväxt. Eftersom Ca^{2+}-transporten över YME minskade när ostronen exponerades för 50% havsvatten var emellertid dessa kompensationsmekanismer inte tillräckliga. Sammantaget indikerar dett att *C. gigas* kan komma att ha problem med skalbilding i områden där salthalten kan sänkas på grund av klimatförändringar.

Nyckelord: Ussing kammare, klimatförändringar, kalsium upptag, natrium-kalium ATPas, kalsium ATPas, natrium-kalsium utbyte, kalsium kanal, hemocyter, mantel epitel

LIST OF PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. Calcium mobilisation following shell damage in the Pacific oyster, *Crassostrea gigas*. **2016**. Sillanpää, J. K., Ramesh, K., Melzner, F., Sundh, H., and Sundell, K. *Marine Genomics 27: 75-83*.

II. Calcium transfer across the outer mantle epithelium in the Pacific oyster, *Crassostrea gigas*. **2018**. Sillanpää, J. K., Sundh, H., and Sundell, K. S. *Proceedings of the Royal Society B: Biological Sciences 2285:20181676*.

III. Dilution of seawater affects the Ca^{2+} transport in the outer mantle epithelium of *Crassostrea gigas*. **2019.** Sillanpää, J. K., Cardoso, J. C. R., Felix, R. C., Anjos, L., Power, D. M., and Sundell, K. S. *Under revision for publication in Frontiers in Physiology.*

IV. Transcript and protein expression of Ca^{2+} transferring proteins in the mantle of *Crassostrea gigas*. **2019.** Sillanpää, J. K., Anjos, L., Sundh, H., Hasselberg-Frank, L., Power, D. M., and Sundell, K. S. *Manuscript*

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1. INTRODUCTION

In this thesis, I will investigate the underlying mechanisms of bivalve calcium mobilization for shell growth. Anthropogenically driven climate change is an increasing global problem. Marine molluscs have been highlighted as being particularly at risk under future climate change scenarios as it is predicted that their calcified shells will become thinner as seawater becomes more diluted and acidic. This may affect the natural ecological balance and biodiversity as well as the possibility to culture these organisms for production of nutritious and healthy food. However, surprisingly little is known about the mechanisms involved in shell production in bivalves, yet this is fundamental knowledge for our ability to predict the future of these species. Therefore, this thesis has as overall goal to increase the physiological knowledge and understanding of shell growth in one target bivalve species, the Pacific oyster, *Crassostrea gigas*, and how they will fare in a changing climat.

1.1 Bivalves and the society

1.1.1 Sustainable marine aquaculture

The human population is projected to reach 8 billion by the mid-2020s (Figure 1, UN 2019) which has raised questions and uncertainties how the increased demand for food will be met in the future. Aquaculture, the farming of fish, shellfish and seaweed, has been proposed to be one of the solutions to this problem. Aquaculture production worldwide has more than doubled since year 2000 and now almost equals the yield of capture fishing (Figure 1, FAO 2018). Compared to livestock production, aquaculture products have clear advantages such as high nutritional values (FAO 2018), low CO_2 emissions (Nijdam et al., 2012; Cao et al., 2013), decreased land- and water usage as well as potentially increased income for rural communities (Filipski and Belton 2018).

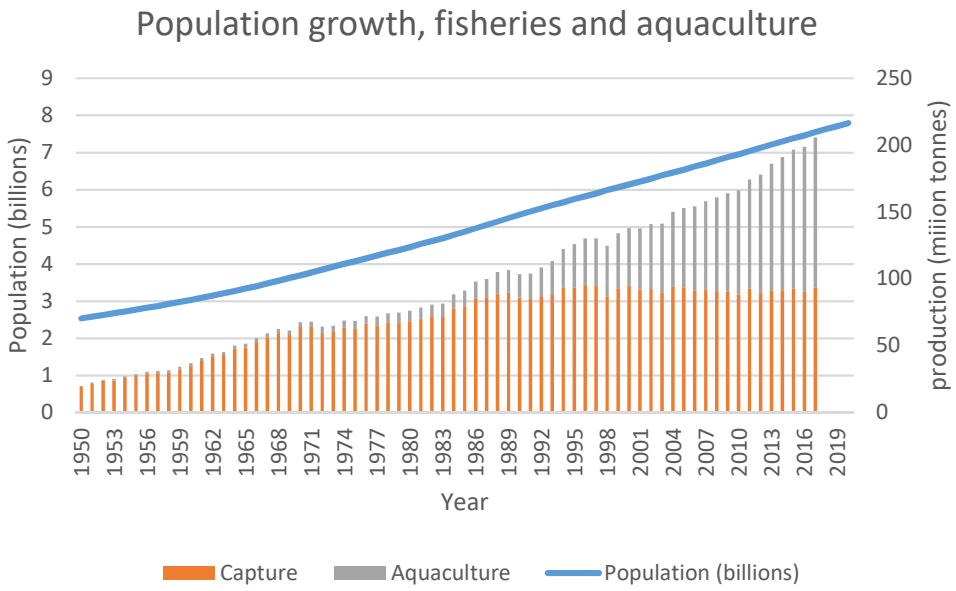

Figure 1. The growth and predicted growth of human population 1950 – 2020 and the changes in the yield of capture fishing and aquaculture 1950 – 2017 (FAO 2018; UN 2019).

However, aquaculture is not without controversies. A portion of the capture fishing is directed for the production of fish meal and fish oil to feed the farmed fish, especially high profit carnivorous fish and shellfish such as salmonid and shrimp species (Naylor et al., 2000; Tacon et al., 2008; Cashion et al., 2017). Although the amount of captured fish used for fish meal and fish oil has decreased the past decades, it still makes up a quarter of the yield of capture fishing, of which 90 % would otherwise be qualified for human consumption (Cashion et al., 2017). The global distribution of the benefits from aquaculture has also been discussed. Some studies suggest that although a large part of the aquaculture production occurs in the developing countries, the products are mainly targeting consumption of a growing middle class in these countries as well as for export to developed countries (Asche et al., 2015; Golden et al., 2016; Cashion et al., 2017). However, other studies suggest that the global increase in aquaculture production has increased the availability of farmed fish for low-income people, since the majority of aquaculture production in developing countries is in fact middle-scale industrial production for domestic markets (Belton et al, 2018).

Overall, an increase in local, medium to small-scale aquaculture production including a shift in consumption towards species at lower trophic levels requiring minimal or no external feed, is a recommended direction. Increased production of bottom grazers such as echinoderms, and filter feeders such as bivalves, along with seaweed, is suggested to be beneficialas both from the perspective of food security and environmental sustainability, compared to farming of carnivorous fish and shellfish (Naylor et al., 2000; Shumway et al., 2003; Nijdam et al., 2012).

1.1.2 Bivalve aquaculture

In 2016, the total global production of molluscs, comprising mostly of bivalves, amounted to approximately 17.1 million tons, which makes up approximatel 21 % of the global aquaculture production excluding seaweed (FAO, 2018). Production had increased by 22 % since 2010 and there are no signs of decline (FAO 2018). Bivalve shellfish aquaculture has been argued to be one of the most sustainable forms of aquaculture (Shumway et al., 2003). Farming bivalves does not require input of nutrients as feed but instead it has the potential to improve water quality as the animals filter seawater and feed on the particulate matter that contains nutrients such as nitrogen and phosphorus (Shumway et al., 2003; Nijdam et al., 2012). The estimates of the emissions from producing 1 kg of edible mussel meat vary between 1 – 2.5 kg CO_2–eq depending on the transport emissions, a value that is one of the lowest among seafood products (Nijdam et al., 2012; Ziegler et al., 2013).

An important role for bivalves exists also within the concept of integrated multitrophic aquaculture (IMTA). The idea of IMTA is to culture fed species such as finfish or crustacea, together with species extracting both dissolved, inorganic (algae) and particulate, organic (filter feedes/bottom grazers such as bivalves) nutrients from the water. IMTA has been suggested to be one of the solution to an expansion of sustainable aquaculture (Troell et al., 2009). Therefore, a global development of IMTA also has the potential to increase the growth of the bivalve production (Granada et al., 2016).

1.1.3 *Crassostrea gigas*: history, aquaculture and ecological impact

Pacific (cupped) oyster (*Crassostrea gigas,* suggested new name *Magallana gigas*) originates from Japan where it has been farmed for centuries, but today it can be found around all continents, excluding the Antarctica (Helm 2005). In 2016 the total aquaculture production of *C. gigas* amounted to 573 617 tons with a value of 1163 million USD (FAO 2018). *C. gigas* is the most farmed oyster species, and the third most farmed bivalve species after Japanese carpet shell

(*Ruditapes philippinarum*) and Chinese razor clam (*Sinonovacula contricta*) (FAO 2018). In the EU, it is the third most farmed bivalve species measured as total mass (after *Mytilus edulis* and *M. galloprovincialis*), and the bivalve species with the highest yield in value (Eurostat 2018) making it an attractive species for aquaculture. Since oyster aquaculture is located mainly in intertidal areas or estuaries, it can create conflicts with other recreational, environmental or economical activities (Kaiser et al., 1998; Forrest et al., 2009). Possible causes of conflict are changes in offshore and benthic environments, a worry for spreading of pests and diseases, and the effects on the ecosystem and other organisms, which can be both positive and negative (Kaiser et al., 1998; Forrest et al., 2009, Callier et al. 2017). Oyster aquaculture usually employs bottom culture, or off-bottom or suspended structures in the form of wooden racks or steel trestles upon which the oysters are laid on long-lines, mesh bags or trays (Helm 2005; Forrest et al., 2009). These structures as well as the boat traffic involved in the harvesting, create a potential disturbance for local communities, as well as compete for space with other offshore activities (Helm 2005).

C. gigas has high tolerance for varying environmental conditions and high growth-rate, which makes it able to survive in fluctuating conditions and has helped it to spread globally (Diedrich 2006; Wrange et al., 2010). In Europe *C. gigas* was first introduced as an aquacultured species in the 1960s. Since it originates from tropical waters, it was assumed it would not be able to reproduce in the colder European climate (Drinkwaard 1999; Strand and Lindegarth, 2014). However, this turned out not be true, and the oysters have been able to form self-sustaining populations and spread throughout the European coastline from Portugal to Norway (Troost et al., 2010; Wrange et al., 2010; Strand and Lindegarth, 2014). One of the problems concerning *C. gigas* aquaculture is its potential spreading to the natural environment (Wrange et al., 2010; Strand and Lindegarth 2014). As *C. gigas* can potentially compete with native species such as flat oyster *Ostrea edulis* and blue mussel *M. edulis* in the northern Europe, as well as modify coastal ecosystems and environments, it is considered to be a potentially harmful invasive species (Ruesink et al., 2005; Troost et al., 2010; Wrange et al., 2010). Once a natural population has been establishes, larval and spat dispersal can spread the oysters even further, a case well illustrated by the spreading of *C. gigas* in Scandinavia. Although *C. gigas* has not been farmed in Sweden or Norway since the 1970s, in 2007 it was reported to be found from multiple locations from the west coast of Sweden and has since been located as north as 60° north in Norway (Wrange et al., 2010). Based on genetic studies and predictions from water currents, the *C. gigas* in Sweden and Norway has most likely spread form Denmark where a wild population has existed since the 1990s (Wrange et al., 2010; Strand and Lindegarth 2014).

However, there are also potential positive effects of oyster aquaculture. Oyster reefs offer habitat and protection to multiple macrofaunal species such as barnacles, anthozoans, hydrozoans, tunicates and ascidians (Markert et al., 2010, Callier et al. 2017). In some cases macrofaunal species diversity and abundance has been reported to be higher in *Crassostrea* reefs compared to *Mytilus* beds (Markert et al., 2010; Hollander et al., 2015, Callier et al. 2017) although this is dependent on the magnitude of the oyster cover (Green et al., 2013). Similar to other filter feeders, oyster aquaculture has the potential to improve water quality by removing nutrients from the water (Beseres Pollack et al., 2013). Additionally oyster farming can offer income opportunities for local farmers with minor investments in infrastructure and equipment, as well as seasonal job opportunities at harvest time since their high tolerance of environmental variance makes them an optimal species for aquaculture (Helm et al., 2005; Strand and Lindegarth 2014).

Temperature and salinity are considered the limiting factors to *C. gigas* spreading, since these factors limit the reproduction and survival (Fabioux et al., 2005; Dutertre et al., 2010; Wrange et al., 2010; Diedrich et al., 2014). The optimal temperature for reproduction in their original habitat in the Pacific range 23 - 27 °C, but they have been able to reproduce even at 15° (Kobayashi et al., 1997; Diederich et al., 2015). Optimal larval growth is reached at 27 °C but similar to the adult oysters, the larvae can survive a wide range of temperatures, from 17 to 32 °C (Rico-Villa et al., 2009). As *C. gigas* has this wide thermal tolerance, a increased spreading of this invasive species further north is anticipated due to increases in surface water temperatures (Dutertre et al., 2010; Diederich et al., 2005). However, for the same reason, the species is also considered a robust farming species, regarding climate induced temperature increases.

1.2. Bivalves – form and function

1.2.1 Functional anatomy

Bivalves are molluscs with two external shells covering the body of the animal. The bivalve shell is made of 95-99 % calcium carbonate ($CaCO_3$), the rest consisting of an organic matrix, which controls the formation of the $CaCO_3$ crystals. An outermost protective organic layer, the periostracum covers the whole shell (Marin et. al., 2008). The internal part of the shell is lined by the mantle tissue, which forms two mantle halves covering the rest of the body (Figure 2). The area remaining between the shell and the mantle is the extrapallial space (EPS) containing the extrapallial fluid (EPF) in which calcification according to traditional theories is thought to happen (Marin et al., 2012).

The mantle halves are connected at the dorsal edge covering the mouth and other organs at the visceral mass. Between the free mantle "leaves" lies the mantle cavity, which is open to the environment and bathes in seawater when the oysters are open during breathing and feeding (Galtsoff 1964). When closed, the shell halves are held together by the posterior adductor muscle located at the anterior ventral end (Galtsoff 1964). The gills are located between the mantle halves (Figure 2). They filter the water and move organic food particles to the labial palps, which then direct them to the mouth, through a short escophagus and further to the gut and intestine covered by a digestive dicerticula. Wastes are excreted at the end of the intestine through the rectum and the anus. Excess water is exctred through the cloaca in the epibranchial chamber between the adductor muscle and the gills. Gonads are located between the digestive diverticula and the epithelium of the visceral mass (Galtsoff 1964). The nervous system of the bivalves is simple and it usually consists of "cranial" node and few major nerves connecting the various parts of the body. A circumpallila nerve crosses the mantle at its edge and radiates smaller radial nerves, which extend from mantle base to the edge.

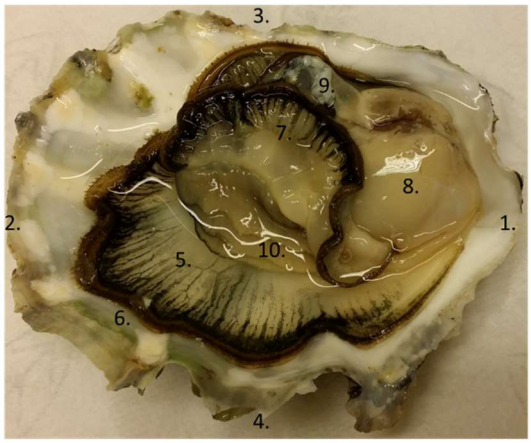

Figure 2. The anatomy of the Pacific oyster, *Crassostrea gigas*, viewed after removal of the right shell. The numbers correspond to: 1, dorsal side of the oyster; 2, ventral side; 3, posterior side; 4, anterior side; 5, pallial left mantle; 6, mantle edge; 7, right pallial mantle; 8, visceral mass; 9, posterior adductor muscle; 10, gills.

The circulatory system

Bivalves have an open circulatory system in which the hemolymph bathes the organs in a solution iso-osmotic to the environment. In *C. virginica*, the main blood vessels are discontinuous and end up in sinuses and the capillaries are irregularly shaped without distinct walls (Galtsoff 1964). The hemolymph

retursn to the heart via two venous sinuses which empty into two auricles. The auricles connect into a single ventricle from which hemolymph is further pumped out through two aortae (Galtsoff 1964). The anterior aorta branches off to smaller arteries, which cross the different parts of the body. The circumpallila arteries follow the mantle edge and branches out small vessels to supply the mantle. The venous system consists of afferent and efferent veins as well as sinuses, which empty through the common efferent vein into the auricles (Galtsoff 1964).

1.2.2 Physiology

Osmoregulatory strategies

Bivalves are considered to be osmoconformers, which means that the hemolymph ion concentration and osmolality follow the environmental conditions, although they do slightly upregulate both osmolality and some ions such as K^+ and Ca^{2+} (Pierce 1982; Thomsen et al., 2010; Alavi et al., 2014). In marine invertebrates ions have been suggested to make up around 70 % of the intracellular osmotic concentration making the intracellular ion concetration lower compared to the extracellular one (Pierce 1982). The difference in osmotic pressure is made up by the organic osmolytes, mostly free amino acids such as taurine and glycine (Shumway et al., 1977; Lin et al., 2016; May et al., 2017).

Since the extracellular fluids fluctuate according to external salinity, the oysters need to regulate cell volume to avoid the cells from shrinking or swelling. This intracellular cell volume regulation is mainly done by adjustment of organic osmolytes. As salinity decreases, free amino acid concentration decreases in *C. gigas* and similar changes have been seen in the expressions of enzymes involved in controlling the amino acid pathways (Zhao et al., 2012; Meng et al., 2012). In *C. gigas*, taurine seems to have a large role in osmoregulation but also glycine, alanine and proline are up regulated during hyperosmotic conditions (Meng et al., 2012). However, also ion transport, especially the excretion of K^+ and Cl^-, has been shown to be involved in the bivalve cell volume regulation (McCarty and O'Neil 1992; Berger and Kharazova 1997). In both hypo- and hyperosmotic conditions *C. gigas* has been noted to downregulate aquaporin expression to decrease the water flow and reduce cell swelling or shrinking due to the osmotic stress (Meng et al., 2012).

The hemolymph in immune response and biomineralisation

Hemolymph protein concentrations are low in bivalves compared to vertebrates and the measured values vary between 0.1 - 0.625 mg/ml (Renwrantz et al.,

1998; Allam et al., 2000; Mello et al., 2010). The hemolymph of many bivalve species is dominated by few major proteins, which can constitute around 80 % the total protein concentration (Xue et al., 2019). Some of the plasma proteins have a function in the immunedefense and their expression is changed in response artificially wounding the animals (Franco-Martínez et al., 2018). Hemolymph proteins have been found in both the extrapallial fluid and the shell matrix (Hattan et al. 2001; Xue et al., 2019). The hemolymph/EPF proteins may also be important for calcium binding and have been noted to change configuration after that.

The circulating cells of the hemolymph, the hemocytes, are immune cells and possibly also involved in shell building (Canesi et al., 2002; Mount et al., 2004; Allam and Raftos., 2015; Lau et al., 2017; Huang et al., 2018). Hemocytes both proliferate in number and change their gene and protein expression when exposed to parasite, bacterial or viral infections (Carballal et al., 1998; Gueguen et al., 2003; Fernández-Boo et al., 2016; Zannella et al., 2017). The main immunological defense mechanism of the hemocytes is phagocytosis but in some bivalve species, also the expression of hydrolytic and oxidative enzymes such as peroxidases and phenyloxidases has been recorded (Bachère et al., 1995; Kuchel et al., 2010; Matozzo and Bailo, 2015). Hemocytes are found in the EPF, to where they most likely have migrated from the secretory cavities located on the outer side of the middle fold of the mantle (Lau et al., 2017; Zhang et al., 2019).

Hemocytes are classified mainly by their shape and size. Traditionally, they are divided into granulocytes (named after many granules visualised inside the cells) and agranular hemocytes or hyalinocytes (only few or no intracellular granules) (Cheng 1975; Lópes et al., 1997; Hine 1999; Matozzo and Bailo, 2015; Zhang et al., 2019). However, there is an ongoing debate about the origin of the different hemocytes with some studies claiming that granulocytes are actually matured hyalinocytes, which would mean that only one type of hemocyte with different stages exists (Ottaviani et al., 1998; Rebelo et al., 2013). While the relative abundances of the different hemocyte types seem to be species dependent, the majority of hemocytes in general has been reported to consist of different type of granulocytes (Hine et al., 1999; Kuchel et al., 2010; Wang et al., 2012; Matozzo and Bailo, 2015). However multiple studies made specifically in the *Crassostrea* species have reported that the majority of the hemocytes are made of agranulocytes (

Lambert et al., 2007; Donaghy et al., 2010; Rebelo et al., 2013; Takahashi et al., 2017). The variation is however large and even in the same species, there has been variations in hemocyte numbers and proportions of subtypes depending

on season, environmental conditions such as pH, and nutriotion leves (Carballal et al., 1998). One of the granulocyte subtypes includes granulocytes containing achromatic, refractive granules suggested to bear $CaCO_3$ crystals and participate in shell biomineralization (Foley and Cheng, 1972; Mount et al., 2004).

1.2.3 Shell biomineralisation

The bivalve shell is made of 95 -99 % $CaCO_3$, the rest consisting of organic matrix such as proteins, chitin and glycoproteins which control the formation of the $CaCO_3$ crystals. Ca^{2+} is suggested to mainly be taken up from the environment while CO_3^{2-} or HCO_3^- can be either taken up or produced metabolically (Schneider and Erez, 2006; Jury et al., 2010; Gazeau et al., 2011; Thomsen et al., 2015; Waldbusser et al., 2015). How shell calcification is controlled is still not understood but it is most likely a co-operation between multiple tissues such as the mantle and the hemocytes.

$CaCO_3$ can naturally form multiple different crystal structures such as calcite, aragonite, vaterite and unstable amorphous $CaCO_3$ (ACC) (Falini et al., 1996; Weiner 2003). In calcifying animals, such as bivalves, the most common forms are calcite and aragonite (Falini et al., 1996). The bivalve shell can be formed completely from either aragonite or calcite, or contain layers from both structures. Two or three layers exist usually, which often differ in morphology, being either prismatic, nacreous, foliated, crossed lamellar, spherulitic or homogenous (Kobayashi and Samata 2006, de Paula and Silveira 2009).Since aragonite can form spontaneously, shells need to have specific control mechanism to produce different crystal structures. This control is mainly thought to be elicited through the organic matric (Zhang and Zhang 2006). The mantle has been suggested to secrete the shell proteins, but also other organs such as hemocytes, gills, digestive gland and labial palps have been shown to express shell proteins (Johnstone et al., 2008; Wang et al., 2013). Furthermore, Wang et al. (2013) suggested that shell proteins not produced by mantle are stored in granules/vesicles possibly alongside $CaCO_3$, in the hemolymph, and later transported through mantle epithelia to the growing shell.

Most of the matrix proteins (though not all) are characteristic to either the nacreous or the prismatic calcite layer, the nacreous layer being the most investigated mineral structure in bivalves due to its high fracture resistance and importance for pearl culturing. Multiple matrix proteins have been identified from the prismatic and nacreous layers functioning as Ca^{2+} chelators, assisting or inhibiting mineral nucleation, regulating crystal shape or determining $CaCO_3$ polymorphism (Miyamoto et al., 1996; Sudo et al., 1997; Samata et al. 1999, Kono et al., 2000; Suzuki et al. 2004; Tsukamoto et al. 2004; Zhang et al., 2006

Suzuki et al. 2009; Song et al., 2019). Known matrix proteins often have an ability to bind calcium due to their posttranslational configuration. They usually contain secondary structures such as β-sheets and random coils that participate in creating the elasticity of the shell (Zhang and Zhang 2012).

Different species and genus exhibit different shell structures, for example *P. fucata* and *M. edulis* have nacro-prismatic shell type while *C. gigas* and *O. edulis* have a mainly foliated, chalk and prismatic calcite shell (Sikes et al., 2000; Esteban-Delgado et al., 2008; Lee et al., 2008; Lee et al., 2011; Checa et al., 2018). The different layers in the bivalve shell are suggested to add a benefit that a single layer would not achieve. While the foliated layer in the *C. gigas* shell has a high fracture resistance, porousness, softness and irregularity of the chalky layer make it fast to deposit and a possible buffer against external impacts (Lee et al., 2008: Lee et al., 2011). Although nacre is the strongest of all the shell strutures, the high organic content makes it costly to produce which might cause some species to opt away from producing it (Marin et al., 2008; Furuhashi et al., 2009).

1.2.4 The mantle tissue – anatomy and function

The mantle is the organ separating the shell from the rest of body. It consists of two lateral mantle folds enclosing mantle cavity and is divided into three separate zones: central zone, pallial zone and marginal zone or mantle edge (Figure 2), though the nomenclature seems to differ slightly between species and studies (Kadar et al. 2009, Bjärnmark et al. 2016). The marginal zone contains three folds: inner-, middle and outer fold, the latter being closest to the shell (Figure 3). The pallial zone and to a lesser degree the central zone, have been shown to synthetize the material for the inner nacreous layer while the edge or outer fold of the marginal zone is related to prismatic layer (Sudo et al., 1997; Takeuchi and Endo 2006). Thus, the edge is mainly responsible for the height and length increment of the shell while the dissolution and recalcification of already existing $CaCO_3$ are mainly controlled by central and pallial zones.

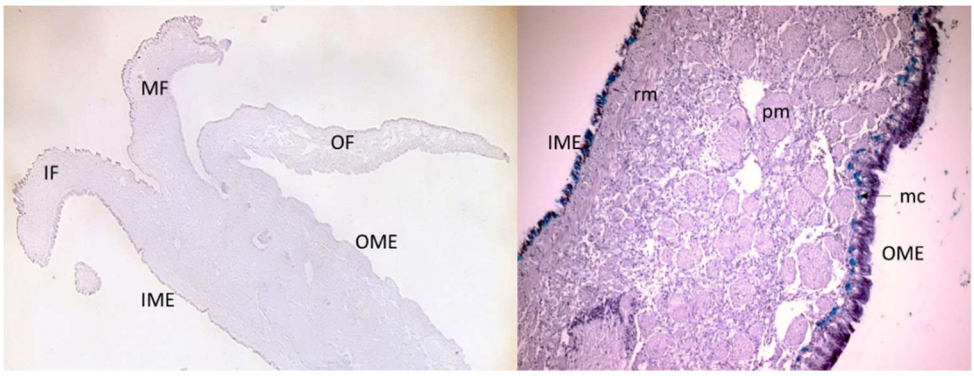

Figure 3. A) The mantle edge consists of inner fold (IF), middle fold (MF) and outer fold (OF). The shell facing epithelium is the outer mantle epithelium (OME) and the epithelium facing the rest of the body and environment is the inner mantle epithelium (IME). B) A cross-section of the mantle showing the OME, IME and the connective tissue between them. Rm;, radial muscle; pm, parallel muscle; mc, mucus cell.

The mantle tissue consists of two epithelial layers, the outer mantle epithelium (OME) in contact with the shell and the inner mantle epithelium (IME) in contact with the environment and rest of the body (Figure 3). The OME cells close to the outer fold of the mantle edge are columnar in shape while those closer to the central zone are almost cubical. Some of the epithelial cells of the OME and IME are responsible for secreting the organic matrix of the shell, as well as mucus. (Marin et al., 2000; Myers et al., 2007; Zhang et al., 2019). On the outer fold of the OME the secretory cells have resemblance to known mucus secretory cells i.e. cells containing acid mucopolysaccharide. The secretory function of the cells seems to decrease moving from edge to central zone (Fang et al., 2008b; Zhang et al, 2019). In *B. azoricus* mantle edge, the extracellular mucus has been shown to have strong calcium-binding abilities (Kadar et al. 2009). However, mucus excretion could also be enhanced reaction used only in shell repair and environmental stress and not involved in the calcium transport of undisturbed individuals (Kadar et al. 2009).

1.3. Calcium – the role in shell building

Although the different shell structures and matrix proteins have been widely studied, the actual process of mineral formation during shell growth is still not elucidated. For the biomineralisation of the shell, both Ca^{2+} and CO_3^{2-} need to be supplied, either taken up or produced metabolically, to the area were the shell growth shall occur. Since *C. gigas* lives in marine environment, there is a surplus of Ca^{2+} in the environment. However, the Ca^{2+} needs to be taken up and transported through the oyster and across the OME to be incorporated into the

shell. Multiple theories on Ca^{2+} up take and transfer to the site of mineralization exists, but the actua mechanisms are still unclear. It is thus, important to investigate potential transport mechanisms and their relative contribution. Once the mechanisms of Ca^{2+} uptake and transfer are known, it opens up for possibilities to study how these mechanisms are regulated and how they will potentially be affected by the ongoing climate changes. In the following chapters, different aspects of Ca uptake and transfer are discussed with focus on the transfer across the OME.

1.3.1 Calcium uptake

Since marine animals live in an environment with direct access to calcium (Ca), the uptake of Ca should not pose a problem. Uptake occurs mainly via the gills, but also through other organs in connection with the surrounding seawater, such as intestine, foot and the outer folds of the mantle (Jodrey 1953; Marin and Luquet, 2004; Fan et al. 2007a; Rousseau et al., 2009). The L-type voltage-gated Ca^{2+} channel (VGCC) subunit β expression has been measured to be highest in the gill tissue and in the hemocytes in *P. fucata* (Fan et al. 2007a). This suggests that the L-type VGCC has a significant role in absorbing ionic calcium (Ca^{2+})from the surrounding seawater across the gills and then transfering the Ca^{2+} to the hemocytes in the hemolymph for storage and transport. VGCCs have also been identified in the gills of *C. virginica* and in the soft tissues of *M. edulis* proposing a function in Ca^{2+} uptake from the seawater since inhibiting the function of these channels partially inhibited the uptake of Cd (taken trough the Ca channels) from the environment (Roesijadi and Unger, 1993; Wang and Fisher, 1999). However the inhibition was only partial, indicating that alternative Ca^{2+} uptake mechanisms exists (Roesijadi and Unger, 1993; Wang and Fisher, 1999).

1.3.2 Calcium transport in the hemolymph

In theory, Ca could be transported in the hemolymph and across the OME in multiple ways: as Ca^{2+}, bound to peptides or proteins, as inorganic complexes, intracellulary or in specialised vesicles (Neff 1972; Nair and Robinson 1998; Mount et al., 2004; Bleher & Machado 2004; Xue et al., 2019). Since bivalves are in constant contact with the environment, and the hemolymph osmolality corresponds the environmental, it would seem logical that the major part of hemolymph Ca would be in the ionic form. This has also been suggested from multiple bivalve species such as *C. gigas* and *M. edulis* (Lannig et al., 2010; Thomsen et al., 2010).

In the quahog *Mercenaria mercenaria,* on the other hand, the concentration of Ca^{2+} in the plasma was assessed to be lower than the environmental Ca^{2+}, and for this species it was suggested that the main part of the Ca was bound to organic ligands (Nair & Robinson 1998). Nair and Robinson (1998) found similar weak ligand binding of calcium to organic substances also in the EPF, which indicates that the Ca transport across the OME could be protein-mediated. In *C. virginica*, the major hemolymph proteins, dominin and segon have been noted to bind Ca^{2+} (Iroh et al. 2011, Xue et al. 2012). These proteins have also been extracted from the shell and the gene expression of the proteins increased both during repair of shell after induced damage and in young oyster which were in a phase of fast calcification (Xue et al., 2019). A similar hemolymph protein in *C. gigas*, cavortin, also binds multiple divalent cations (Scotti et al. 2007 However, neither cavortin nor *C. gigas* segon has been identified in the *C. gigas* shell matrix (Marie et al., 2011a). Similar metal-binding, aggregating hemolymph proteins have been found from other bivalve species too such as *M. edulis*, green-lipped mussel *Perna canaliculus*, flat oyster, *O. edulis* amd Sydney rock oyster, *Saccostrea glomerata* (Hattan et al. 2001; Scotti et al., 2001; Yin et al. 2005; Renwrantz and Werner 2008; Green et al., 2009; Morga et al., 2011). However, the proportions of the total hemolymph Ca that are bound to these proteins has not been reported and their contribution they may have to the Ca transfer to the shell groth area is still unclear. Similarly, if the different hemolymph Ca fractions, ionic, protein-bound or cellular, have different functions in Ca^{2+} transfer or sequestration, has not yet been studied.

1.3.3 Cellular calcium transfer

All eukaryotic cells maintain their intracellular Ca^{2+} concentration low, at approximately 100 nM, compared to the extracellular concentrations. This as Ca^{2+} function as intracellular messenger and the concentrations can momentarily rise by severalfold (Figure 4; Bootman et al., 2001). During such Ca^{2+} bursts the Ca^{2+} is rapidly diffusing from internal or external sources into the cytosol (Berridge et al. 2000). From the extra- and intracellular compartments, Ca^{2+} is diffusing through Ca channels which can be either voltage-gated (VGCC), receptor-operated, mechanically activated or store-operated (Berridge et al., 2000, Bootman et al., 2001). The different types of Ca channels have different functions depending on the cell type and physiological conditions. VGCCs are activated by the depolarization of the cell membrane and thus function in excitable cells such as neuron and muscles but also in general cellular signal transduction (Catterall 2011).

VGCCs can be divided into high-voltage gated (L/P/Q/N/R-types) or low-voltage gated (T-type) channels (Catterall 2011). The different channels are

identified based on the voltage of activation, inhibition by various compounds and the possible diseases caused by mutation in the genes (Catterall 2011). They comprise of five protein subunits (α_1, α_2, β, γ, δ) each of them having multiple possible isoforms. The α_1-subunit forms the channel itself and the other subunits function as assisting proteins (Bootman et al., 2001). This combination of channel types and proteins with multiple isoforms creates a wide array of possible VGCCs. In general, VGCCs perform functions related to Ca inflow in multiple tissues: muscle contraction, hormone secretion, synaptic transmission, enzyme activity and gene expression regulation (Catterall 2011).

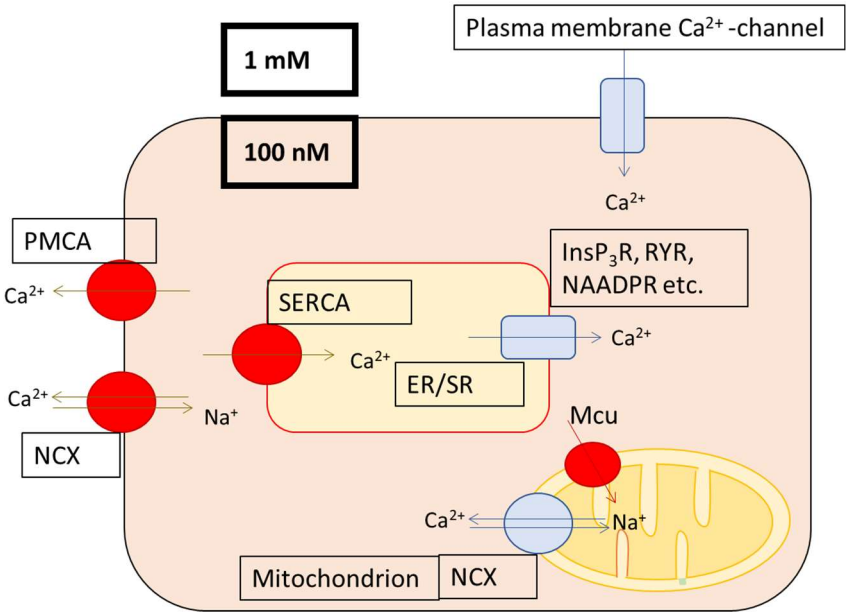

Figure 4. A simplified picture of the cellular Ca^{2+} metabolism. Ca^{2+} is taken into the cells through Ca^{2+} -channels and transported back to the extracellular space via plasma-membrane Ca^{2+} -ATPase (PMCA) or Na^{+}/Ca^{2+} -exchanger (NCX). Alternatively, Ca^{2+} can be stored to the endoplasmic reticulum via the sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) from which it is then released back to the cytoplasm via intracellular Ca^{2+} channels. Ca^{2+} can also be stored in the mitochondria.

The main internal source of Ca is the sarco/endoplasmic reticulum (SR/ER) alongside with mitochondria, Golgi apparatus and small vesicles storing Ca (Figure 4; Berridge et al., 2000). From the ER, Ca is released to the cytosol through intracellular Ca channels. After the Ca^{2+}-transient, the ion is actively transported back into the SR/ER by the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA), a P-type ATPase. Mammals possess three SERCA genes, which can be further spliced to form more variants (Strehler & Treiman 2003).

The isoforms have mostly a tissue specific distribution except for the SERCA2b that has a ubiquitous distribution (Strehler and Treiman 2003). A similar p-type ATPase, signaling pathway Ca^{2+}-ATPase (SPCA) is responsible for transport of Ca^{2+} into the Golgi apparatus (Van Baelen et al., 2004).

The transport of Ca^{2+} from the cytosol to the extracellular space can be achieved by plasma-membrane Ca^{2+}-ATPase (PMCA) or Na^{+}/Ca^{2+}-exchanger (NCX) (Figure 4). PMCA pump Ca out from the cell in exchange for H^{+} ions, though the exact stoichiometry of this transport is still under debate (Thomas 2009; Brini and Carafoli, 2011). It is considered to be a high affinity, low capacity transporter in contrast to the NCX, which is a low affinity, high capacity transporter, having around ten times higher turnover rate for Ca^{2+}than PMCA. While PMCA has a much higher affinity for Ca^{2+}, its lower capacity may lead to saturation in situations where Ca^{2+} needs to be pumped out of the cell quickly. This means that NCX is especially active when pumping out high amounts of Ca^{2+} rapidly, such as after muscle contractions, whereas PMCA is responsible for the fine tuned maintenance of stable intracellular Ca^{2+} levels (Blaustein and Lederer 1999; Brini and Carafoli, 2011). Humans have four PMCA genes, which are further spliced into multiple proteins (Strehler and Treiman 2003). Of these, PMCA1 and thereafter PMCA4 are ubiquitously expressed and found in almost all tissues. PMCA2 and 3 are more tissue specific and mainly expressed in the nervous system with few exceptions. PMCA can be located on both the basal and apical cell membranes, the location depending on the cell type and function (Strehler and Treiman 2003). NCX uses the Na gradient of the cell membrane to exchange one Ca^{2+} ion against three Na^{+} ions. It function as Ca^{2+} extruding or absorbing transporter dependent on the Ca^{2+} and Na^{+} concentration gradients as well as on the plasma membrane potential (DiPolo and Beaugé, 1986; Rasgado-Flores et al., 1989).

PMCA, SERCA and SPCA together with Na^{+}/K^{+}-ATPasea (NKA) all belong to the group of P-type ATPase sharing structural and functional similarities. NKA controls, among other things, the intracellular Na^{+} and K^{+} concentrations and as well as cytosolic Ca^{2+} concentration through the sodium-gradient dependent NCX (Therien and Blostein, 2000). Besides the catalytic subunit common to all P-type ATPases (termed α in NKA), it contains β and γ subunits, and in vertebrates multiple isoforms and splice variant exist for each of the subunit (Therien and Blostein, 2000).

1.3.4 Ca transfer across the OME

The movement of ions across the OME can take two routes: passive diffusion through the paracellular pathway between the OME cells (Neff 1972, Lopes-

Lima et al. 2008) or transcellular active transport, across the cell membranes and through the cells (Coimbra et al. 1988, Beirao & Nascimento 1989, Ramesh et al., 2019). Transcellular Ca^{2+} transport could potentially be carried out by the following transporters: PMCA, NCX and/or different calcium channels. Some of these Ca transporters and channels have been found in the transcriptomes of bivalves such as *C. gigas*, *P. fucata* and *M. edulis* (Fan et al., 2007a; Fan et al., 2007b; Wang et al., 2008; Zhang et al., 2012; Ramesh et al., 2019).

Ca channels have been identified in the mantle tissue of *P. fucata* though the expression was lower than that found in the gills and hemocytes. The expression was mainly focused to the inner fold and the outer side of the middle fold of the mantle edge (Fan et al., 2007a). Using mathematical modelling Carré et al. (2006) suggested that neither paracellular Ca^{2+} diffusion nor active transport of Ca^{2+} by PMCA could alone be sufficient for the Ca^{2+} transfer needed for the shell calcification and suggested Ca channels to have a major role in the Ca^{2+} transfer. This could also explain the incorporation of other elements in the shell since Ca channels are known to be permeable to other divalent cations such as Sr^{2+} or Ba^{2+} (Hess and Tsien 1984; Bourinet et al., 1996). However, Carré et al. (2006) did not discuss the possible participation of NCX on Ca^{2+} transfer. Being a high capacity transporter, NCX could compensate for the low rate of Ca^{2+} transfer by PMCA. In the *C. gigas* larvae ligand-gated Ca channels are proposed to be a part of the regulatory cascade leading to a metamorphosis, but so far no evidence in their participation for shell calcification has been provided (Vogeler et al., 2018; Vogeler et al., 2019).

PMCA-like proteins, with high resemblance to human PMCA3 isoform 3, was identified in the outer and middle folds of the mantle edge of *P. fucata* (Wang et al., 2008). Similarly, a protein binding to a human PMCA1 antibody was found in *C. anadonta* OME cells, though mostly located in the cytosol as opposed to the cell membrane (Lopes-Lima et al., 2008). Cytosolic location of PMCA have also been shown in the calicoblastic cells of coral *Acropora yongei* (Barott et al., 2015). In corals, these cells form the epithelium closest to the calcification site and can be roughly seen to correspond to the OME cells. In the coral epithelial cells also NCX was found to be expressed in intracellular vesicles (Barron et al., 2018). In *A. cygnea*, the appearance of PMCA-like proteins in the OME cells coincided with the period of high shell production further indicating their function in calcium transport (Lopes-Lima et al., 2008). However, the localization of the Ca^{2+} transporters in intracellular vesicles in the calcifying epithelia is not universal. In the *T. squamosa* both PMCA and NCX were located in the apical cell membrane of OME cells indicting a role in the transport of Ca^{2+} to the shell forming area (Ip et al., 2015; Boo et al., 2019). The expression of both these transporters increased after a period of light exposure, a period

that in these clams hosting a symbiotic zooxanthella is connected to calcification. Although NCX, PMCA and VGCCs have also been located in the genome of *M. edulis* and their expression has been shown to increase in periods of active larval calcification (Ramesh et al., 2019), their cellular localisation is not reported any further.

Bivalve mantle cells have also been suggested to produce $CaCO_3$ directly (Addadi and Weiner 2011). Cultured *P. fucata* cells have been seen to create spherical mineral deposits containing ACC, which increased in size and abundancy when Ca^{2+} and Mg^{2+} were added to the culture medium (Xiang et al., 2014; Zhang et al., 2019). Mantle cells also controlled the formation of calcite crystals in the surrounding medium and were seen to attach themselves to the crystal's surfaces indicating that they participate in remodeling the crystals (Xiang et al., 2014; Zhang et al., 2019).

Ca transport has also been suggested to take place in specialised hemocytes (Mount et al. 2004, Xue et al. 2012; Li et al., 2016a). Granulated hemocytes containing $CaCO_3$ crystals could potentially be transferred through the mantle epithelium paracellularly or they could empty their ionic content into the cytosol of the OME cells for subsequent transport to the EPF by the cellular ion transporters (Fleury et al. 2008). $CaCO_3$ crystals have been identified in the hemocytes in both *C. virginica* and *P. fucata* (Mount et al., 2004, Li et al., 2016; Huang et al., 2018). These crystal-bearing hemocytes have been found in both the hemolymph and the extrapallial fluid of *P. fucata* indicating that they at least partly can participate in the calcification (Huang et al. 2018; Zhang et al., 2019). However, most studies so far have investigated crystal-bearing hemocytes in response to artificial shell damage. It is possible that the mechanism during shell repair differs from the regular growth and maintenance of the shell. The picture is further complicated by the fact that damaging the shell creates an immunological response, which also leads to an increase of hemocytes expressing many genes related to cellular and humoral immunity (Allam et al., 2000; Bachère et al., 2015; Huang et al., 2016). The rapid infiltration of hemocytes to the area of the shell damage may be mainly an innate immune response. Summing up, both Ca^{2+} transfer across the OME and the participation of hemocytes have been suggested to be involved in bivalve shell calcification. It seems therefore likely that there are potentially multiple mechanisms involved in shell calcification possibly working togeteher.

1.4. Climate change on shell building

Since the beginning of the industrialization in the 1800s, CO_2 emissions have increased from 280 ppm to approximately 410 ppm and the atmospheric CO_2

is predicted to continue to rise. The predictiction of the atmospheric CO_2 levels at the end of the century range form 490 to over 1000 ppm depending on the scale of implemented mitigating action (IPCC 2014). This will most likely lead to a warming climate, changing weather patterns and increased occurrence of extreme weather events, but also affect the oceans directly by lowering the ocean pH, creating ocean acidification. Oceans are a major sink of atmospheric carbon with one third of the anthropogenic CO_2 emissions to atmosphere being absorbed by the seawater (Orr et al., 2005; IPCC 2014). It is estimated that since the start of the industrial period, the ocean pH has decreased by 0.1 units, which, since the pH is measured on a logarithmic scale, corresponds to an acidification by 26 % measured as the increase of $[H^+]$ (IPCC 2014).

Increasing the partial pressure of seawater CO_2 (pCO_2) has been shown to decrease calcification rate in multiple bivalve species such as *C. gigas*, *M. edulis*, *C. virginica*, *Mya arenaria*, and the scallop *Argopecten irradians* (Gazeau et al. 2007, Ries et al., 2009; Fitzer et al., 2014). However, results have been varying in different studies even for the same species and in some cases high pCO_2 or low pH does not seem to affect calcification or growth rates (Ries et al., 2009; Thomsen et al., 2009; Range et al. 2011). Similarly, the pCO_2 threshold above which calcification and growth are disrupted vary for different species and life-stages (Fitzer et al., 2014; Ventura et al., 2016). Species found in naturally high pCO_2 environments might cope better with decreasing pH. These coping mechanisms are affected by multiple factors, for example, food availability (Melzner et al. 2011). Similarly, increase in temperature has been noted to enhance the effects ocean acidification in *C. gigas* and *M. edulis* (Lannig et al., 2010; Li et al., 2015).

1.4.1 Salinity changes

Besides ocean acidification, global climate change will also affect the salinity of the oceans (IPCC 2014). Since warm air can hold up more moisture, the increase in global temperatures is predicted to intensify both precipitation and evaporation patterns. An increase of 8 % in water cycling has been predicted to follow every degree of increase in temperature (Durack et al., 2012). Precipitation and evaporation patterns are predicted to intensify, making the surface waters more or less saline respectively. Additionally changes in river runoff and glacier and sea ice melting will increase the fresh water inflow which may further lower the salinity in coastal areas. On average, measurements since the 1950's have shown that the Atlantic Ocean has become more saline and the Pacific and Indian Oceans' less saline, and these patterns are suggested to continue in a similar manner in the future (Durack et al., 2012; IPCC 2014). However, local and temporary changes especially in areas having already

fluctuating salinities such as estuarines and river mouths migh experience stronger variations.

Salinity changes will affect marine organism, especially those living in surface waters, estuarines and shallow coastal areas (Tomanek 2012; Zhao et al., 2012; Gonçalves et al., 2017). However, as an euryhaline species with a relatively wide salinity tolerance, *C. gigas* could be expected to survive for prolonged periods in unoptimal salinities (Zhang et al., 2014). Changes in ion metabolism, cell membrane and cytoskeleton proteins, immune response, cell adhesion and communication, signaling pathways, cell cycle as well as Ca-binding proteins caused by prolonged exposure to either low and high salinities have however been reported for *C. gigas* (Zhao et al., 2012; Meng et al., 2012; Zhang et al., 2014).

In salinities lower than full strength seawater, ion concentrations are similarly lowered, which is also reflected in the hemolymph ion concentration as well as in the activity and expression of ion transporters and amino acid metabolism (Meng et al., 2012; Thomsen et al., 2018). Exposure to low salinity (10 ppt) has been shown to downregulate the expression of ion and amino acid transporters as well as voltage-gated ion channels in *C. gigas* (Zhao et al., 2012; Meng et al., 2012). On the other hand, the expression of Ca^{2+}, Cl^{-} and K^{+} channels have in some cases been shown to be upregulated in low salinity, which could be due to intracellular volume regulation and excretion of ions out of the cells to gain an osmotic balance with the extracellular environment (Meng et al., 2012). Aquaporin mRNA expression was downregulated both in hypo- and hyperosmotic conditions in *C. gigas* indicating that the oysters try to adjust the water permeability against cell shrinkage or swelling in changing osmotic conditions (Meng et al., 2012).

There are also indirect effects of new and challenges environmental conditions. When facing un-optimal conditions, the animals also need to allocate more energy to metabolism, defense and repair. Biomineralisation is a costly process requiring not only inorganic minerals but also production of organic substances such as the shell proteins and enzymes involved in calcification. Unoptimal environmental conditions, including low salninity, has been shown to increase standard metabolic rate and calcification costs, decrease mantle and gill ATP levels and oxygen uptake in bivalves such as *C. gigas*, *M. edulis* and *Corbula amurensis* (Navarro 1988; Lannig et al., 2010; Paganini et al., 2010; Wei et al., 2015a; Wei et al.,2015b; Sanders et al., 2018). To save energy for ion and acid-base homeostasis in adverse environmental conditions, non-vital functions such as calcification are reduced (Hüning et al., 2013; Li et al., 2016c).

Since multiple factors affect each other in the seawater chemistry, predicting the effects of climate changes on an ecosystem level is difficult. Similarly, in nature, organisms are affected by multiple stressors such as changes in water pH and salinity, temperature or food availability (Havenhand et al., 2019). To predict the effects of these combined stressor in natural environments we need first to understand how single environmental factors can affect major functions, such as biomineralisation, in marine organisms.

2. AIMS

The overall aim of this thesis is to increase the knowledge on the mechanisms behind bivalve shell biomineralisation and possible effects of a changing environment. The thesis further aims to characterise the physiological mechanisms of calcium uptake and transport in the bivalves, using Pacific oyster, *Crassostrea gigas*, as a model.

To meet this overall aim the following specific aims have been approached:

- to describe the transport of calcium from the environment to the hemolymph, through the mantle tissue and further into the shell forming area
- to characterise the active and passive transport mechanisms for transfer of Ca^{2+} across the outer mantle epithelium (OME) that constitute the final step in the supply of calcium to the shell forming area
- to explore the phylogenetic relationship of calcium transferring mechanisms in bivalves
- to assess if and how these transport mechanisms are physiologically controlled to meet changes in the environment

The following objectives were set up and implemented to meet the aims:

- To analyse pathways of Ca uptake and transport in the hemolymph and mantle tissue of *C. gigas* to assess the mechanisms behind Ca mobilisation from external and internal sources for shell calcification. This was achieved by analysing different calcium fractions of the hemolymph (ionic, bound, cellular), notching the oyster shell to induce accelerated shell regeneration and exposing the oysters to radiolabelled ^{45}Ca in the environment to follow its uptake and distribution in the hemolymph and mantle tissue. (**Paper I**).
- To develop and validate an Ussing chamber methodology to study the ion transport properties of the live and intact OME of *C. gigas,* including sample preparation techniques. This methodology was used to study electrophysiological properties of the mantle epithelium and Ca^{2+} transfer across the OME. From these parameters the contribution of the different ion transfer mechanism i.e. passive and active, was analysed. **(Papers II-III).**

- To identify the presence and expression pattern of Ca^{2+} transporter and channel genes in the mantle tissue of *C. gigas* and their phylogenetic relationships to similar proteins in other bivalves and model organisms (**Papers III-IV**).
- To identify and characterise the cellular localisation of Ca^{2+} transport- and channel proteins in the mantle tissue of *C. gigas* through the development and validation of specific antibodies against these proteins. The specific binding of the antibodies were validated by Western blot and immunohistochemistry (IHC) and the subcellular localisation in the OME cells were investigated using IHC (**Paper IV**).
- To investigate how a dilute environmental salinity affects the Ca transfer mechanisms in the mantle tissue and more specifically in the OME of *C. gigas* using Ussing chamber methodology as well as mRNA expression of chosen ion transporters and channels (**Papers III and IV**).
- To create a model that describes the Ca^{2+} transfer mechanism of the OME of *C. gigas* including potential Ca^{2+} transporters and channels, active and passive pathways as well as other possible routes of Ca^{2+} transfer from the hemolymph to the shell forming area. The model is supported by results from physiological, functional studies, genome analysis and protein expression analysis **(Papers II-IV).**

3. METHODOLOGICAL CONSIDERATIONS

3.1 Uptake and transport of Ca in *C. gigas*

The uptake of Ca from the environment and its transfer in the *C. gigas* hemolymph and mantle tissue was studied in **paper I** to find out which mechanisms are the main contributors in Ca transfer for shell biomineralisation. Studying the calcification process of a new shell in adult oyster can be challenging because it is a dynamic process consisting of both the mineralization of a new shell and the simultaneous dissolution of the old one. Additionally, it is difficult to separate the Ca metabolism required for normal physiological functions from the Ca transport needed specifically for the shell calcification. To accelerate the shell calcification process, an artificial shell regeneration can be induced by damaging or removing a piece of the shell, which leads to a fast re-building of the damaged shell area (Mount et al., 2004; Li et al., 2016a).

To study the uptake of Ca from the environment, as opposed to the mobilization of internal Ca storages, radiolabeled ^{45}Ca can be added to the surrounding medium and followed through its appearance in different compartments within the animal (Wilbur and Jodrey 1952; Jodrey 1953). This separates the uptake of new Ca from the relocation of Ca from the internal storages and allows the turn-over rate of Ca from the environment to the shell to be measured. In **paper I**, both of these approaches were used to study the uptake and mobilization of Ca from the environment after inducing shell regeneration by notching.

3.1.1 Shell notching and induced shell regeneration

Accelerated shell formation can be induced by notching a small fraction of the shell away to create an opening that exposes the mantle to the environment (Mount et al., 2004; Cho and Jeong, 2011; Li et al., 2016a). The subsequent shell repair is then assessed macroscopically by visual examination and/or microscopically, starting from the first appearance of organic deposits followed by mineral formation on the exposed area. In **paper I**, a piece of the shell of adult *C. gigas* was removed by drilling a V-shaped opening in the proximity of the adductor muscle. This was followed by a visual macroscopic examination of the notched area over the experimental period of two weeks to assess the stage of shell repair using the following grading: Stage I: no visible shell repair, Stage II: organic matter formation, Stage III: tanning and surface area increase the organic matter and Stage IV: visible CaCO3 deposit (Cho and Jeong, 2011; Hüning, 2013).

3.1.2 Hemocyte analysis

Previous studies using shell notching have recorded a mobilisation of hemocytes, as well as changes in the relative abundances of different hemocyte subtypes (Mount et al., 2004). After inducing shell regeneration in *C. virginica*, Mount et al. (2004) detected, using scanning electron microscopy (SEM), that some of the granulocytes were carrying crystallised $CaCO_3$ intracellularly. Some of these refractive granulocytes (REF cells) were found at the mineralisation front together with the newly formed prismatic crystals. Additionally, the relative number of the REF cells increased in abundance from 5 % to 15 % of the hemocyte population concomitant with the shell regenerating. This discovery has led to multiple studies in which notching of the shell has been used as a method to study not only the growth process of the new shell but the participation of hemocytes in the calcification process (Kadar 2008; Cho and Jeong, 2011; Li et al., 2016a; Huang et al., 2018). To follow the proliferation of the hemocytes and their participation in the shell building, the total number of hemocytes as well as the proportion of the granulocytes within this population was monitored in relation to the shell regeneration in **paper I**. The hemocyte counting and analysis was done by flow cytometry using gating procedures as presented in Hégaret et al., (2003) and Lambert et al., (2007) at Helmholz Center for Ocean Research in Kiel, Germany.

3.1.3 Calcium uptake and transfer in the hemolymph and mantle tissue

Ca in the hemolymph can potentially be transported by multiple pathways: as free Ca^{2+}, bound to Ca-binding proteins or small organic molecules, chelated with inorganic ligands, or in vesicles or specialised hemocytes (Coimbra et al., 1993; Nair and Robinson, 1998; Mount et al., 2004; Marin et al., 2012; Li et al., 2016a). In most studies where hemolymph Ca concentration has been analysed, only one form, usually the total Ca or Ca^{2+} has been measured. So far, fractionation of the hemolymph to different compartments has been only reported in a quahog *Mercenaria mercenaria* in which both total Ca and Ca^{2+} were measured directly, and additionally the hemolymph was separated by equilibrium dialysis (Nair and Robinson, 1998). In **paper I** the Ca concentration in the hemolymph was assessed by fractionating the hemolymph to different compartments: total plasma Ca (Ca_T), ionic (Ca_I), Ca bound to proteins larger than > 30 kDa (Ca_P), Ca bound to small organic and inorganic ligands (Ca_F; free or filtrate Ca, including Ca^{2+}) and intracellular Ca in the hemocytes (Ca_H) (Figure 5). Since the total Ca concentration in the hemocytes is very small compared to the other hemolymph fractions, the results considering the cellular Ca were not reliable. Due to this, and the fact that the cells were not examined by advanced

microscopy techniques, the participation of hemocytes in Ca transfer could not be completely verified.

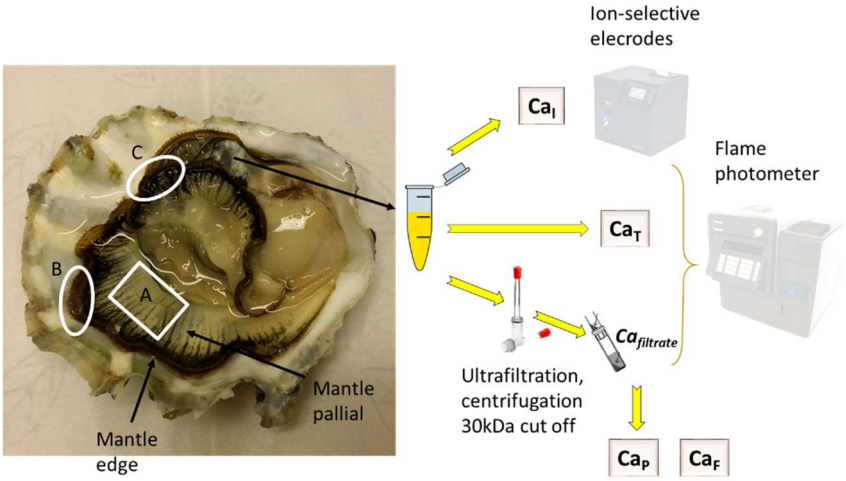

Figure 5. A simplified description of the sampling for **papers I-IV**. The mantel samples for Ussing methodology were taken from area A (**papers II-III**). The samples for mRNA extraction as well as for immunohistochemistry were taken for areas B-C (papers **III-IV**). To study the Ca^{2+} speciation in the hemolymph, a sample was drawn from the adductor muscle and fractionated to different hemolymph compartments. Adapted from **papers I** and **III**.

When the oysters open their shells for foraging and respiration, the surrounding water will come in direct contact with the mantle and the rest of the body, and both ions and other small molecules can be relatively freely exchanged with the surroundings. Therefore adding radiolabeled ^{45}Ca to the surrounding medium has been used as a method to study the uptake of Ca from the environment, as well as its accumulation in the different compartments in the oyster such as the shell, different soft tissues and the hemolymph (Wilbur and Jodrey 1952; Jodrey 1953). The use of ^{45}Ca allows even very small changes in Ca^{2+} uptake to be recorded. In the experiments for **paper I**, half of the oysters were kept in a water containing ^{45}Ca to study the uptake of Ca from the environment as well as the turn-over rate in the mantle and different hemolymph fractions. The other half was kept in similar conditions but without the addition of ^{45}Ca. Half of the oysters in both groups had their shells notched to induce shell regeneration. Following the exposure, the hemolymph and mantle tissue from both groups were sampled. The hemolymph was fractionated as described above and both the Ca concentrations from the non-radiolabelled samples and the ^{45}Ca activity from the radiolabeled samples were measured from the

hemolymph fractions. The hemolymph protein concentration was measured in the non-exposed oysters to assure the seawater contamination was not distorting the results.

3.2 Ca transfer in the mantle of *C. gigas*

Once taken up from the environment to the hemolymph and mantle tissue, Ca then needs to be transported in the mantle tissue, and finally across the OME to the shell forming area. The mechanisms involved in the Ca tranfer can be studied using multiple approaches including transcriptomics, proteomics and localization of Ca^{2+} transporting proteins using immunohistochemistry or in situ hybridization. However, looking only at the gene or protein expression may only provide an indication of the type of potential transporters present in the tissue, but does not reveal anything about their actual function or if there are multiple mechanisms working alongside each other. Since the function of the proteins is what makes them relevant from a physiological perspective, a method studying the Ca^{2+} transfer in tissue *in vivo* was chosen. To study the Ca^{2+} transfer as well as the electrophysiological properties of the *C. gigas* OME, an Ussing chamber methodology was developed, validated and employed in **papers II** and **III**. Since the potential transfer forms in the hemolymph (ionic, bound, cellular) can be applied on the transfer of Ca across the OME as well, the contribution and kinetics of Ca^{2+} transfer were further studied in **paper II** by manipulating the "hemolymph" Ringer concentration.

3.2.1 Ussing chamber methodology for the C. gigas OME mantle

The Ussing chamber methodology is an *in vivo* method developed to study the transport of ions and molecules across an epithelium (Ussing and Zerahn, 1951). The epithelium can be separated from the surrounding tissues and kept viable by bathing it in a physiological salt solution. By separating the apical and basal cell membranes of the epithelium, the transport of molecules both through the paracellular pathway between the epithelial cells and the transcellular transfer across the cell membranes can be measured. Radiolabeled ions and small molecules can be added on one side of the epithelium and sampled at time intervals from the opposite site to determine the accumulation over time to calculate the transfer rate. In **papers II** and **III**, the Ussing chamber technique together with radiolabeled ^{45}Ca and ^{3}H-mannitol was used to assess Ca transfer across the OME as well as to measure the paracellular permeability.

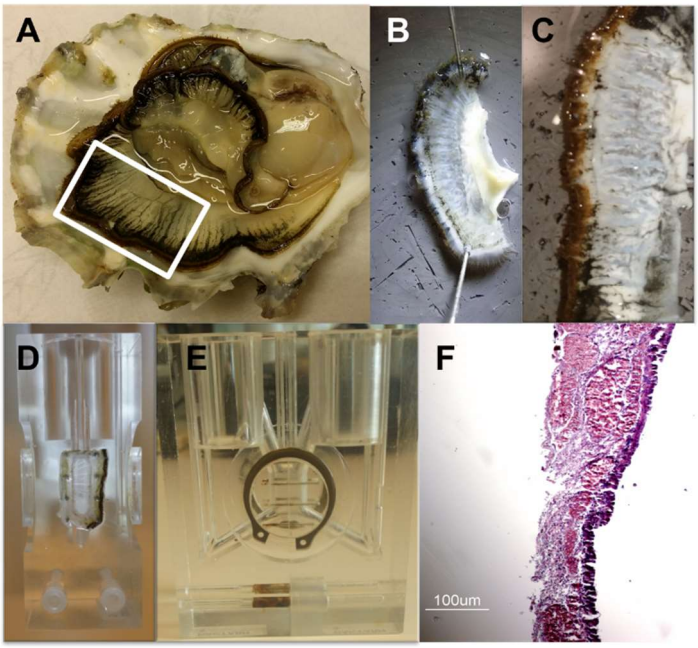

Figure 6. The outer mantle epithelium (OME) preparation for the Ussing chamber methodology. The mantle is sampled from the left ventral pallial mantle as close to the edge as possible. The inner mantle epithelium and connective tissue are peeled off (B-C) and the OME is mounted on an Ussing chamber (D-E). F shows the final preparation with the OME and pallial muscles remaining attached to it (adapted from **Paper II**).

Active transport of charged molecules in leaky epithelia creates a potential difference across the apical and basal cell membranes of the epithelium. This can be measured by placing two voltage (U) electrodes on the opposite sides of the tissue, close the epitheium. Further, by applying an additional pair of current (I) electrodes to the chambers alternating adptive voltages can be added to the epithelium, that generate corresponding currents (I). The voltages are randomly applied to generate μI that alternate between positive and negative values, within the range of a fixed min and max value, resulting in zero net charge. When the U/I pairs assessed are fitted to a straight line, the slope of the line represents the transepithelial resistance (TER). The TER is composed of resistance across both basal and apical cell membranes (in series) in parallel to the resistance across the tight-junction proteins in vertebrates or septate junctions in invertebrates between the epithelial cells (Tambutté et al., 2011). Due to the very high TER across the lipid cellular membrane, the measdured TER mainly reflects the paracellular shunt resistance created across the junction proteins in leaky epithelia (Sundell and Sundh 2012). The short circuit current (SCC) is

calculated as SCC = −TEP/TER. SCC is a measure of the net flow of ions across the epithelium while TEP describes the net ion distribution of ions between the apical and basal compartmets. TER, TEP and SCC were monitored in **papers II** and **III** to describe the electrical properties of the OME of *C. gigas*, and the effects that different treatments had on the transport mechanisms.

Ussing chamber methodology has been used to study the electrical parameters as well as transport of ions across the OME of bivalve species such as *A. cygnea* and *Unio complanatus* (Coimbra et al., 1988; Hudson 1992). However, the methods to prepare the OME samples have not been decribed besides the fact that the OME is separated from the rest of the mantle. In **papers II** and **III**, an approximately 1 x 2 cm piece of the left, ventral mantel was removed from the oyster. The IME and as much of the connective tissue as possible were removed by blunt dissection while bathing the mantle tissue in cold Ringer solution, and the OME was mounted into an Ussing chamber with the hemolymph side as the electrical ground (Figure 6). While the IME can be removed smoothly, the connective tissue can at times be difficult to remove completely (Figure 6). Depending on the season, the glycogen storages in the connective tissue of the mantle vary, which makes acquiring standardised samples difficult. The integrity of the preparation was controlled by looking at the electrical parameters and ^{3}H-Mannitol transport across the OME of the tissue to reveal potential holes in the tissue. Unfortunately, these could only be assessed reliably after the measurement was finished, leading to some wasted samples especially for **paper II** when the technique was still being optimised. The degree of which OME could be separated from the OME and connective tissue was later confirmed by histological analysis (Figure 6).

3.2.2 Kinetics of calcium transport

The overall mechanisms of ion transport across an epithelium can be described by using kinetic modelling depending on the type of transfer, its constituents and the rate of transport (Korla and Mitra, 2014). In general, active ion transport can be divided into primary transport and secondary transport. Primary transport mainly uses energy in the form of ATP to drive the ion transport across a membrane while secondary transport uses the concentration gradient of one ion across the cell membrane (often created by primary transport) to drive the transport of another ion or substance to the same (symport) or different (antiport) direction (Korla and Mitra, 2014).

In order to characterise the active and passive transport mechanisms for transfer of Ca^{2+} across the OME, the Ussing chamber methodology was used to measure the kinetics of Ca transfer across the OME by manipulating the Ca^{2+}

concentration of the Ringer solution bathing the hemolymph side of the OME (**paper II**). The transfer rate of Ca^{2+} in different hemolymph Ca^{2+} concentrations was calculated and both Michaelis-Menten modelling and allosteric sigmoidal kinetics were used to describe the Ca^{2+} transfer across the OME.

Michaelis-Menten kinetics was developed and published in 1913 originally to describe the enzymatic reaction of sucrose inversion catalysed by invertase. Besides enzymatic reactions, is can also be used to describe the uptake and transfer of ions (Craemer and Dueck, 1962; Feher et al., 1992; Cornish-Bowden 2013). It is described by the equation

$$Y = \frac{V_{max} * X}{K_m + X}$$

in which V_{max} describes the limiting rate of conversion in the enzyme reaction, or maximum transport rate in the case of ion transport; and K_m is the Michaelis constant which corresponds the concentration when the transport rate is half of the maximal (Cornish-Bowden 2013).

Allosteric sigmoidal model describes the transfer as

$$Y = \frac{V_{max} * X_h}{K_{half}^h + X_h}$$

in which V_{max} and K_{half} equate those from the Michaelis-Menten kinetics and h is the Hill constant describing the degree of co-operative binding in the case on enzyme activity, and the participation of multiple transport components in the case of ion transport (Ahearn 1978).

3.2.3 Pharmacological methods to study the Ca^{2+} transfer across the OME

In theory, multiple Ca^{2+} transporters and channels could participate in the Ca^{2+} transfer across the OME of *C. gigas*. To study the function of these transporters in the Ca^{2+} transfer across the OME, multiple specific inhibitors were chosen: vanadate (inhibits ATPases in general), ouabain (NKA), caloxins 1A1 and 1c2 (PMCA), verapamil (VGCCs) and ORM-10103 (NCX) (**Paper II**). Since these inhibitors have been mostly developed against the human/mammalian isoforms of the aforementioned proteins, only limited information exists about their function in marine invertebrates in general, and even less on the function specifically in bivalves. The effects of ouabain and vanadate on the short-circuit

current of *A. cygnea* have been studied before (Coimbra et al., 1988; Machado et al., 1990) though the latter had no effect on the SCC. Verapamil has been shown inhibit Cd uptake in the gills of *M. edulis* (Roedijadi and Ungern 1993) and Ca^{2+} uptake in the coral *Stylophora pistillata* (Tambutté et al., 1996). Caloxins were ordered based on the sequences reported in Pande et al. (2005) and Pande et al., (2008), and they have not been used so far to study the existence of PMCAs in invertebrates. Similarly, ORM-10103 is a newly developed NCX inhibitor still lacking information about its effects and usage in invertebrates (Jost et al., 2013).

3.3 Ca^{2+} transporters and channels in the mantle tissue of *C. gigas*

Since the model constructed using the pharmacological tools in **paper II** suggested the involvement of plasma-membrane Ca^{2+} -ATPase, VGCCs and potentially Na^+/Ca^{2+} -exchanges controlled by the Na^+-gradient created by Na^+/K^+ -ATPase, the next step to validate the model was to investigate if these proteins were truly expressed in the mantle tissue of *C. gigas*. First, the published genome of the *C. gigas* was searched for the potential transporters and channels. Based on the genome search and phylogenetic analysis, candidate genes were then selected to be analysed for mRNA expression. Additionally, to validate the protein expression on the OME as well as determine the subcellular localization, *i.e* if the membrane proteins are located on the basal or apical cell membrane or possibly in intracellular membrane enclosed vesicles, was investigated by designing specific antibodies and studying their binding by immunohistochemistry.

3.3.1 Genomic approach

Phylogeny is the study of evolutionary relationships between different organisms usually using different evolutionary or phylogenetic trees to describe them (Gregory 2008). Evolutionary trees can describe the relatedness of genes, species or whole taxa but also the historical pattern of ancestry and descent (Gregory 2008). Two different methods to calculate phylogenetic relatedness were used in **paper III**, the maximum likelihood (ML) and the Bayesian inference (BI) methods.

The name maximum likelihood is derived from the fact that the tree with the highest probability to explain the observed sequence similarities is chosen. The method also calculates the probability that the presented tree is correct. ML method takes into account that multiple mutations can happen at the same site (Holder and Lewis 2003). Since all possible mutation pathways are considered, it makes the tree relatively powerful if slow to calculate (Holder and Lewis

2003). BI method is closely related to the ML method but it also calculates in the posterior possibility, a set of parameters which are proportional to the likelihood of the model being correct (ML method) multiplied by the prior probability of the events leading to the result. Therefore, the BI method allows prior information taken into consideration while constructing the trees. However, in most cases these prior probabilities are artificially chosen and represent cases that have either neglible meaning or the same probability. Bootstrapping is a method to verify the reliability of the produced tree by re-sampling the original to produce a "pseudo-replicate". Low value of bootstrap indicates that the original results could not be repeated and have a high chance of being unreliable (Holder and Lewis 2003).

In **paper III** the genome of *C. gigas* published by Zhang et al., (2012) was searched for potential ion transporters and channels involved in the mantle Ca^{2+} metabolism. The retrieved sequences were used to search the assembled *C. gigas* genome to confirm their identity and to identify additional family members. Sequence homologues for the genes were searched from NCBI and Ensembl Metazoa Genomes databases as well as from the available mantle transcriptome data for *Mytilus galloprovincialis* (Bjärnmark et al., 2016). Homologue sequences for the transporter and channel genes were retrieved from bivalve species (*C. virginica, P. fucata, T. squamosa, Mizohopecten yessoensis, Hyriopsis cumingii*), other molluscs (*Lottia gigantea, Octopus bimaculoides*), annelids (*Helobdella robusta, Capitella teleta*) and a branchiopod (*Lingula anatina*) and for comparative purposes from protostomian and vertebrate model organisms (*Drosophila melanogaster, Caenorhabditis elegans, Homo sapiens, Danio rerio*). Multiple sequence alignments for each gene family were used to construct phylogenetic trees using the ML and BI methods.

The sequences retrieved for the phylogenetic analysis were additionally used to design primers to study the mRNA expression of the Ca^{2+} transporters and channels in the mantle tissue. Since only one isoform was found for the proteins excluding NCX, choosing and designing primers was made relatively easy. For NCX, the isoform most resembling the human isoforms was chosen since it was assumed the function of this protein corresponds most that of a real NCX (On et al., 2009). The mRNA expression was measured from the whole mantle instead of the OME to increase sample size and to keep the integrity of the sample as good as possible. However, this might have affected the interpretation of he results since the separation of Ca^{2+} transfer for shell growth from general Ca^{2+} metabolism used for other function was impossible.

3.3.2 Ca^{2+} transporting membrane proteins

Once a preliminary model of Ca^{2+} transfer was achieved by pharmacological studies in **paper II**, a follow up visualisation and localisation of the transporter and channel proteins was done to confirm the model and to further investigate the subcellular location of the transporters and channels (**paper IV;** Figure 5). Based on the gene sequenced retrieved for **paper III**, antibodies were designed against NKA, PCMA, NCX and L-type VGCC. These proteins were chosen based on the results from the inhibition experiment in **paper II** which suggested that all of these membrane proteins are located on the OME cells and possibly participate in Ca^{2+} transfer to the shell forming area. However, while the antibodies were being designed, a simultaneous study measuring changes in mRNA expression of the Ca^{2+} transporters and channels after exposure to salinity stress (described below) revealed that T-type VGCC and SERCA were the transporters responding most strongly. However, it is likely that these responses were mostly due to their function in osmoregulation, instead of changes in the Ca^{2+} transfer to the shell forming area. Therefore, the original reasoning to choose the aforementioned proteins for immunohistological analysis remains valid.

Antibodies were designed based on the modelling of the structure of the proteins, so that they would bind on the extracellular or intracellular domains and not on the transmembrane sections. The validation of the specific binding of the antibodies was assessed with Western blot methodology.

3.4 Effects of salinity on Ca^{2+} transfer

Salinity of the oceans is predicted to change in the future, and the occurrence of extreme events to increase, especially on estuarines, coastal areas and areas receiving increased precipitation. The changes in seawater chemistry are not uniform and in fact the predicted trend shows areas with already a higher than average becoming more saline and the opposite. *C. gigas* is an euryhaline estuarine species which has a relative high tolerance against environmental variations in salinity (Helm 2005). However, prolonged periods in unoptimal salinity have shown to cause physiological changes, including in ion regulation (Zhao et al., 2012; Meng et al., 2012). Therefore, it is important to know how mechanisms related to shell calcification are affected by a changing salinity. Knowing these physiological changes will also help to understand the competitive benefit the oysters have against species with a lesser toleration against salinity changes.

Multiple experiments involving exposure of marine bivalves to either low or high salinity have been made to study the different strategies these animals have in coping with the salinity change. The aim of these studies is often to predict how future changes in ocean salinity will affect bivalve survival and production (Shumway 1977; Zhao et al., 2012; Meng et al., 2013; Velez et al., 2016; Peng et al., 2019). Salinity has been previously shown to affect many physiological functions in bivalves including but not limited to, osmoregulation and ion transport, energy reserves and metabolism as well as protein and mRNA expression (Berger and Kharazove, 1997; Zhao et al., 2012; Meng et al., 2013). Effects on ion transport and osmoregulation are especially an interesting subject since these functions directly affect the hemolymph and EPF conditions and thus the calcification process (Sanders et al., 2018). In **papers III** and **IV**, the *C. gigas* were exposed to two different salinities, full seawater or 32 ppt (SW), which corresponded the salinity where the oysters had been acclimatised prior to the start of the experiment; and 50 % of the full seawater or 16 ppt (50% SW). In both experiments, the oysters were exposed for a period of two weeks and following parameters where assessed:

Mantle electrophysiology

In **paper III**, the Ussing chamber methodology was used to assess the effects of reduced salinity on the mantle electrophysiology and ion transport, measuring TER, TEP and SCC, the OME permeability using ^{3}H-mannitol, as well as the total Ca^{2+} transfer using radiolabeled ^{45}Ca.

Mantle physiology

Histological analysis was used to study the tissue anatomy and the changes brought by the decreased salinity in **paper IV**. Haematoxylin-eosin-alcian blue stain was used to visualize the individual cells in the mantle tissue as well as the mucous-producing cells and the other secretory cells on the OME and IME.

mRNA expression

Low environmental salinity has been noted to affect the gene expression of multiple ion channels such as voltage-gated Na^{+} and Ca^{2+} channels as well as voltage-dependent K^{+} and Ca-activated K^{+} channels in *C. gigas* (Meng et al. 2013). Additionally, some ion and amino acid transporters have noted to be down-regulated in low salinity in *C. gigas* (Zhao et al., 2012). These results can be interpreted as an adaptation to the new salinity, but the changes can potentially also affect Ca^{2+} transfer to the EPF and thus the calcification process. In the salinity experiments for **papers III** and **IV**, mRNA expression

of NKA, PMCA, SERCA, NCX and L,- and T-type VGCCs were measured to analyse how exposure to dilute seawater affected the expression of these genes (Figure 5).

Since the mRNA expression of same genes was measured after a two-week long experimental period to the same salinities, it was also used as a control to assess the repeatability of the experimental design between the two experiments for **papers III** and **IV**. However, since the experiments were performed in different seasons, one in November 2017 and another one in March 2018, these parameters can only be held as a rough estimate. Seasonal variation has been measured to affect ion transport in *M. edulis*, *A. cygnea* and ark-shell *Scapharca inaequivalvis* though in natural conditions these changes can also be related to changes in the water temperature (Borgatti et al., 2003; Lopes-Lima et al., 2008) which in the experiments was held in constant 10° C.

4. RESULTS AND DISCUSSION

4.1 Mechanistics of shell repair in *C. gigas*

Bivalves, such as oysters, need to take up Ca in order to build and maintain their shells. However, following the calcification process of an adult oyster can be difficult. Therefore, to induce a temporary peak in shell calcification, shells can be artificially damaged to accelerate the transfer of Ca to the shell building area. This allows following the shell building from the first organic deposits to the fully recovered shell. Additionally, changes in the normal Ca uptake and transfer followed by the shell damage can be recorded to reveal which transfer mechanisms are active in the shell regeneration.

4.1.1 Shell notching and induced shell regeneration

At the start of the experiment for paper I, half of the oysters were nothced, i.e. a triangular piece was cut away from the shell thus creating a direct opening to the mantle from the environment. The deposition of a new shell was visually followed during the two-week long experiment. First organic deposits appeared on 20 % of the oysters two days after induced shell damage by notching in **p.aper I**. First tanning of the organic layer was seen on day 4, and by day 7, 50 % of the oyster were already depositing $CaCO_3$. All of the oysters were depositing $CaCO_3$ by day 11. Mount et al., (2004) discovered visible $CaCO_3$ crystals 48 hours after shell notching in the *C. virginica* oysters, which was notably earlier than observed in **paper I**. In notched *P. fucata* new prisms were found from the regenerating shell area 96 h after the injury was caused (Huang et al., 2018). Temperature has been shown to affect calcification rate in *C. virginica* and *P. fucata* (Waldbusser et al., 2011; Li et al., 2016c). This could be one factor resulting in the slower rate of calcification in *C. gigas* in comparison to that seen in *C. virginica* as the experiment in **paper I** was performed at 10 °C compared to 18 °C in Mount et al., (2005). However, also species differences are probably important as the results form **paper I** match the rate of shell repair in *P. fucata* even though the water temperature in that study was as high as 25 °C (Huang et al., 2018).

4.1.2 Hemocytes after induced shell damage

During the experimental period after notching the oyster shells, the number of hemocytes as well as specifically the number of granulocytes was followed to determine their participation in shell calcification. The number of granulocytes

increased in the notched animals during the experimental period of two weeks (**paper I)**. This result is similar to those reported for *C. virginica* and *P. fucata,* in which shell damage also increased the proportion of granulocytes (Mount et al., 2004; Zhang et al., 2019). Refractive granulocytes have been suggested to participate in shell building, either through delivery of $CaCO_3$ crystals to the shell forming area (Mount et al., 2004; Li et al., 2016a) and/or by transporting of matrix protein, necessary for the calcification process, to the same area (Johnstone et al., 2008). Multiple Ca-binding proteins have been shown to be expressed in hemocytes sampled from the EPF of *P. fucata* and the expression of some of the proteins has been shown to increase after the shell was notched (Huang et al., 2018). Hemocytes have also been found suspended between the $CaCO_3$ sheets in regenerated shell of *P. fucata* (Li et al., 2016a) suggesting they might be involved in the actual calcification process and not only in delivering components for the shell. Similar to the hemocyte, the mantle cells have also been seen to precipitate $CaCO_3$ (Xiang et al., 2014). The proteome of the hemocytes differs notably from that of the mantle which indicates that the functions of the two compartments are not completely overlapping and that the tissues most likely have different functions in cell calcification (Huang et al., 2018). The mantle most likely has the main role in calcification, transporting both mineral constituents for CaCO3 as well proteins for the shell matrix (). However, it is not yet completely clear, if the contribution of the hemocytes is additional to the mantle controlled calcification even in situations of normal shell growth or if it is a form of acute delivery of $CaCO_3$ crystal when the shell is damaged.

Although the number of granulocytes increased due to the notching of the shell (**paper I)**, agranular hemocytes formed the clear majority in both notched and un-notched animals. This result corresponds to those reported previously for *C. gigas* (Lambert et al., 2007; Donaghy et al., 2010; Takahashi et al., 2017), whereas in other bivalve species granulocytes have been shown to form the major hemocyte population (Hine et al., 1999; Kuchel et al., 2010; Wang et al., 2012; Matozzo and Bailo, 2015). In fact, the proportion of granulocytes in **paper I** was uncharacteristically small, 1 - 5 %, while in other studies the *C. gigas* granulocytes have been reported to make up around 8 – 17 % of the total hemocyte population (Delaporte et al., 2003; Donaghy et al., 2010; Takahashi et al., 2017). The total hemocyte count showed a tendency to increase after notching, but the change was not significant due to the high variation between individuals ($p = 0.059$; **paper I**). The total count of hemocytes in the un-notched animals was $2.089 * 10^6$ ml^{-1} ($\pm$ 0.460) which is slightly higher but still in range with the values previously reported for *Crassostrea* species, 0.876 – $1.207* 10^6$ ml^{-1} ($\pm$ 0.460) (Cho et al., 2011).

It is difficult to assess for certain whether the changes in cell populations were due to an immune reaction caused by the notching and exposing the mantle to the environment that contains possible antigens, or due to the participation of the cells in the Ca transfer for shell regeneration. Cellular Ca did not change during the notching experiment in **paper I.** However, due to the very low Ca concentration in the hemocytes in combination with a high sample variation these results should not be viewed as definitive. As granulocytes have been shown to be the main cell type in immune defense, (Hine et al., 1999), the increase in granulocyte count in **paper I** could be due to an immune reaction against the notching. The expression of immune defense -related genes in hemocytes have also been shown to increase after shell notching, further supporting that their role in the immune defense may be the main factor causing the hemocyte and more specifically the granulocyte proliferation (Wei et al., 2017; Huang et al., 2018). Delivery of CaCO3 crystal could theoretically be related to immune defense, as peal forming oyster are known to isolate an invading particle in a CaCO3 layers in the form of a pearl (Allam et al., 2015). However, as *Crassostrea* species rarely produce pearls, this mechanism is not perhaps likely for these oysters.

4.2. Uptake and transport of calcium in *C. gigas*

Once taken up into by the animal, Ca needs to be transported in the hemolymph through the mantle tissue to the extrapallial space.

4.2.1. Calcium uptake and fractions in the hemolymph

Induced shell regeneration by notching was used to follow the paths of Ca uptake and transfer to the shell forming area. Calcium concentration in the different hemolymph fractions was measured to find out in which form Ca is transported in the hemolymph. The hemolymph Ca was divided into following fractions: the total hemolymph Ca (Ca_T), Ca^{2+} (Ca_I), Ca bound to ligands < 30 kDa, including also the Ca^{2+} (Ca_F), Ca bound to proteins > 30 kDa (Ca_P) and Ca in the hemocytes (Ca_H). To follow specifically the uptake of Ca from the environment as opposed to the mobilization of internal Ca storages, half of the oyster were kept in water containing radiolabeled ^{45}Ca while half were kept in normal seawater. Total Ca concentration was measured in the different hemolymph fractions from the animals kept in normal seawater, while the amount of ^{45}Ca from the different fractions was measured from those kept in the water containing ^{45}Ca.

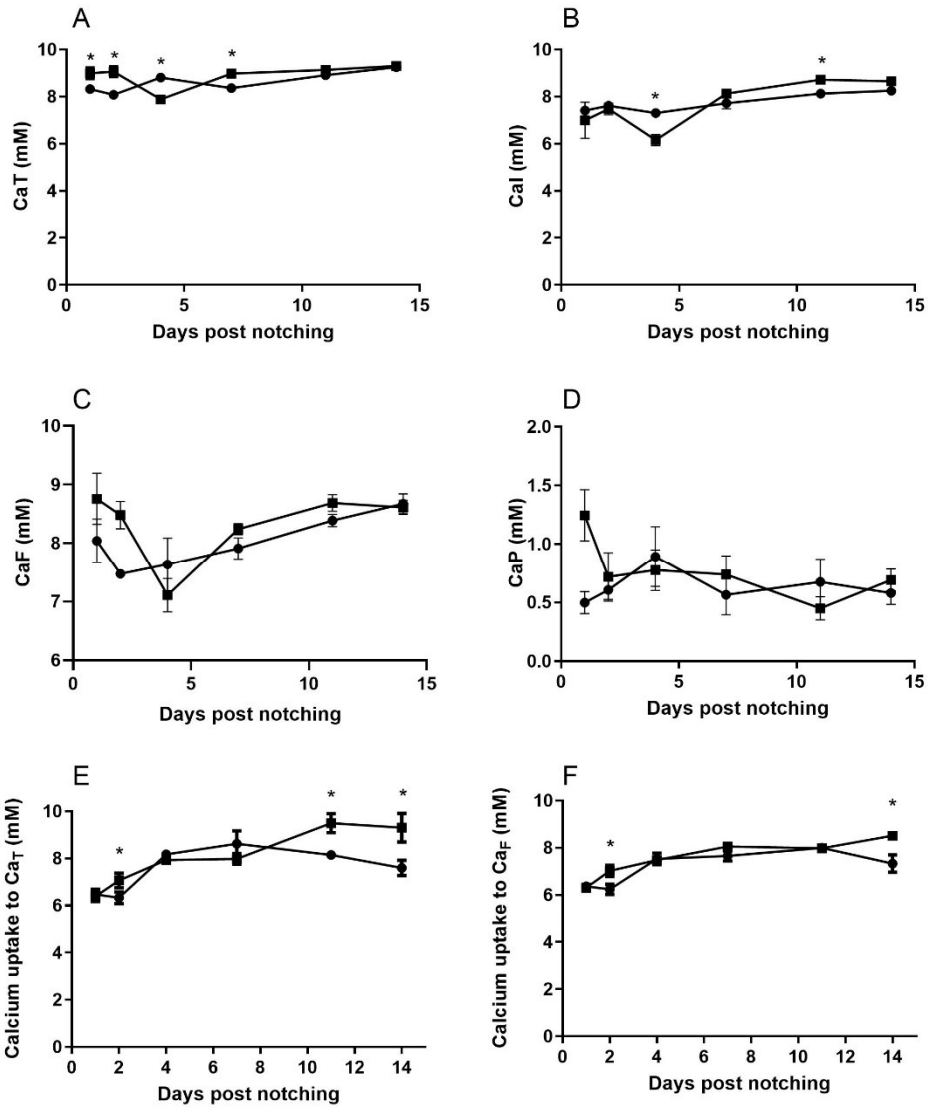

Figure 7. The hemolymph results from the notching experiment. A – D depicts the Ca concentrations in the different hemolymph fractions in the oyster exposed to non-radiolabelled water: A = total Ca (Ca_T); B = Ca^{2+} (Ca_I); C = Ca bound to ligands < 30 kDa (Ca_F); D = Ca bound to proteins > 30 kDa (Ca_P). E – F depicts the uptake of Ca in the oysters exposed to water containing ^{45}Ca: E = ^{45}Ca uptake to Ca_T; F = ^{45}CA uptake to Ca_F. The stars denote significant differences between treatments (■ notched; ● control). Values are described as average ± standard error of mean. Adapted from **paper I**.

In the notched animals, a direct opening is created to the environment as a piece of the shell is cut away. To return to the normal stage, the oysters will rebuild the shell quickly. As seen from visually examining the notched area, the first $CaCO_3$ deposits appear within days from the notching (**Paper I**). To accomplish this fast calcification, shell components need to be transported to the notched area. For Ca this means mobilization from the environment or internal storages which can be seen as increased uptake and possible changes in the proportions of different Ca fractions (Figure 7). Following the notching, on day 2, an increase can be seen in the uptake of ^{45}Ca from the environment in the notched animal indicating an increased turn-over rate of Ca to the shell from the hemolymph (Figure 7). The uptake of Ca from the seawater to the shell has been noted to take only hours in previous experiments with *C. virginica* (Wilbur and Jodrey 1952; Jodrey 1953). Therefore its possible that some changes in the uptake of Ca were not visualised in this experiment. Possibly there existed a continual transfer of Ca from the environment to the shell forming area, but the proportion of Ca being actively mobilised to the shell is small and being constantly renewed from the environment. Ca can be additionally mobilised from the internal storages, such as from the mantle tissue, possibly to enable the Ca to be more efficiently transported to the damaged area via the hemolymph. This mobilization leads a temporary increase in the hemolymph Ca_T and Ca_F concentration (Figure 7). Similar change was not seen in the Ca_I fraction which implies that the increase in Ca_T was due to a change in the complex-bound fraction. In the temporal perspective, a comparison between notched and control animals reveals that also the protein bound Ca_P was higher in notched oysters compared to un-notched on day 1, although this difference disappeared on day 2. The difference in Ca_F between notched and un-notched animals was on the other hand larger on day 2 compared to day 1 (Figure 7).

After the initial mobilization from the internal storages, Ca is then being transported to the shell forming area which temporarily depletes the hemolymph Ca storages. In contrast to the Ca mobilised from the internal storages, the Ca transported to the shell forming area is mainly in the form of Ca_I as a drop was seen in Ca_I and Ca_T, but not in the other forms on day 4 in the non-radiolabelled oysters (Figure 7**, paper I**). Following the mobilization concomitant with the drop in the hemolymph Ca_T, the oysters also start to visibly deposit $CaCO_3$. To replenish the hemolymph Ca, uptake from the environment, also mainly in the form of Ca_I, is increased after the visible shell calcification has started. This was visualised in **paper I** both in the increased amount of ^{45}Ca in Ca_F and Ca_T in the hemolymph of the oysters exposed to ^{45}Ca, as well as in the return of Ca_I and Ca_T to normal level in the non-radiolabelled oysters (Figure 7). Ca^{2+} was shown to be both passive and actively transported across the OME to the extrapallial space in **paper II**, further supporting the

notion that Ca^{2+} is the hemolymph fraction involved in shell calcification. This would indicate that in oysters, ionic hemolymph Ca is not tightly controlled but acts as fluctuating source of free Ca easily transported in the body to be used for cellular function and possibly also in shell building. On the other hand, protein-bound Ca functions as more controlled reservoir or has a specific function.

Calcium in the *C. gigas* hemolymph was found as ionic, ligand-bound (< 30 kDa), protein-bound and intracellular calcium. Main part (77 – 95 %) was found in the ionic form with minor parts bound in the proteins. These results were consisted with those reported by Thomsen et al. (2010) for *M. edulis* and Lannig et al., (2010) for *C. gigas*. However, in a study on the quahog *Mercenaria mercenaria*, Nair and Robinson (1998) measured Ca^{2+} concentration of only 0.4 mM (of the total [Ca] of 10.9 mM) and the authors postulated that most of the hemolymph Ca instead is bound to Ca-binding proteins. This was however not the case for *C. gigas* in **paper I** where CaP formed only 3- 13 % of the total hemolymph Ca. Similarly, Lannig et al., (2010) measured Ca^{2+} concentration of 7.2 mM using ion chromatography.

The major hemolymph proteins in *Crassostrea* species have been reported to bind Ca^{2+} along with other divalent ions, but the amount of the hemolymph Ca^{2+} bound to proteins has not been further studied (Scotti et al., 2007; Xue et al., 2012). However, Ca^{2+} binding proteins in *C. virginica* have been found in between the $CaCO_3$ crystals in the EPS, indicating some role in the shell building (Xue et al., 2012). In their study, Nair and Robinson (1998) used multiple Ca analysis techniques including ion-specific electrodes which is why their differing results from other marine bivalves are interesting. Even if marine bivalves can upregulate their ions to some extent compared to the environment as shown in **paper I, III** and **IV**, they are still in close contact with the seawater and should by default exchange some ions with it. Nair and Robinson (1998) sampled the hemolymph by draining the tissues, while in papers **I-III,** hemolymph was sampled from the adductor muscles which increases the risk of contamination from the seawater. However, since the measured hemolymph protein concentrations (unpublished) are in line with those published for *Crassostrea* species, major contamination seems unlikely. In general, bivalve hemolymph has a low protein concentration compared to vertebrates (Renwrantz et al., 1998).

4.2.2. Calcium uptake into the mantle tissue

Mantle is the organ separating the rest of the oysters' body from the shell which means that all transport of shell components must happen in the mantle tissue.

The uptake of ^{45}Ca into the different mantle areas was followed after notching the oyster shells to investigate the participation of the mantle tissue in Ca^{2+} uptake and transport. Induced shell damage increased the Ca content of the upper mantle, in the area closest to the notch (mantle 2, figure 8) as well as in the lower mantle near the hinge (mantle 3, figure 8) (**paper I)**. In similar studies using radiolabeled ^{45}Ca it has been noted that the turnover rate of Ca from the seawater to the shell is rapid taking only hours (Wilbur and Jodrey 1952; Jodrey 1953). In *C. virginica*, the mantle Ca concentration has been shown to be 2.5 higher compared to the seawater Ca concentration. However, only small amount is being actively renewed and thus participating to the shell calcification while the bulk Ca in the mantle remains as an intact storage (Jodrey 1953). If there is an equally rapid turnover of Ca inside the mantle in *C. gigas*, this may explain why only a few of the examined mantle areas showed an increase in Ca content (**paper I)**. In support for this, the time series for mantle areas 2 and 3, in the notched oyster, show that the ^{45}Ca uptake was higher compared to the control both at the beginning (days 1-2) and at the end (day 14). It seems that once the notch creates an opening to the environment, there might immediately exist some exchange with the environment, which increased the proportion of radiolabeled Ca in the mantle tissues. As apparent from the hemolymph Ca_T, the Ca concentration in the hemolymph increased immediately following the notching seemingly without a parallel increase in the ^{45}Ca uptake from the environment. This may suggest that the initial increase in hemolymph CaT is due to mobilisation of internal stores of Ca i.e. in the mantle. Once the mantle tissue has released this Ca to the hemolymph, the Ca storages need to be replenished by increased uptake of environmental Ca directly into the mantle tissue. The intermediate uptake in the hemolymph is not apparent as there is an overall increased turn-over rate of Ca.

As described in **paper I**, there was a statistical difference between the notched and control animals in mantle ^{45}Ca uptake only in mantle areas 2 and 3. However, looking more closely to temporal changes in the different mantle areas revealed an increase in ^{45}Ca uptake on day 4 also in mantle 1 which is the area located closest to the notch (Figure 8). This increase, which leveled out after day 7, coincides with the drop in the hemolymph Ca_T and Ca_Lon day 4. This seems to repeat the effect seen in the beginning of the experiment where the uptake of Ca is first seen in the mantle tissue after which it is moved to the hemolymph as seen in the increase of radiolabeled Ca_T on days 11 -14. This further supports the notion that the mantle either participates in the uptake of Ca directly from the seawater or at least functions as temporary storage for Ca taken up for example the gills.

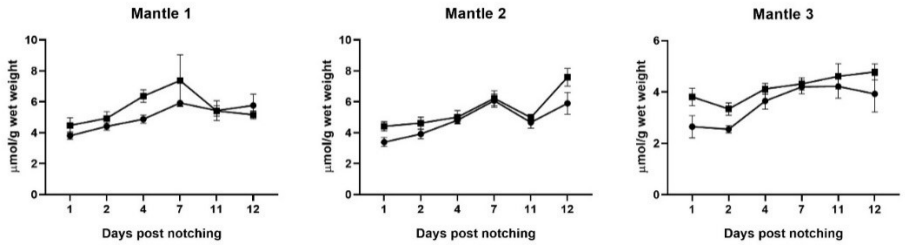

Figure 8. The mantle ^{45}Ca uptake during the two week long experimental period On general, the was a difference between the two treatments in mantles 2 and 3 (■ notched; ● control) as well as an overall effect of time. Values are described as average ± standard error of mean. Adapted from paper I.

New shell has been shown to be deposited twice as fast on the posterior shell compared to center and anterior (figures x and y; Wilbur and Jodrey 1952). This fast turn-over rate on the posterior mantle which is where the notch was located, could explain the lack of apparent increase in the mantle 1 Ca concentrations in the beginning of the experiment. Similarly there was no change in the gill Ca uptake even though gills are suggested to be one of the main organs in responsible for Ca uptake (Fan et al., 2007a).

4.3. Ca transfer across the outer mantle epithelium of C. gigas

Calcium taken up into from the seawater and transported into the mantle tissue and in the hemolymph to the shell forming area. The final layer of transport is the outer mantle epithelium (OME), which separates the mantle from the extrapallial space. The OME has been suggested to function actively in the shell calcification, both in controlling the mineralization of the $CaCO_3$ (Huang et al., 2018) and in producing the mantle matric proteins (Zhang and Zhang 2012).

4.3.1 Functional morphology of the mantle

Based on the histological analysis, the mantle consists of two epithelial layers, which connect at the mantle edge forming three distictive folds (figure 1; **paper IV**). Similar structure has been recorded for multiple bivalve species such as *C. virginica, M. galloprovincialis* and *P. fucata,* and seems to be almost universal among bivalves (Galtsoff 1964; Fang et al., 2008; Kadar et al., 2009; Bjärnmark et al., 2016). Between the two epithelial layers there are also muscle tissues, a longitudal radial muscle close to the inner mantle epithelium (IME) and multiple parallel muscles radiating from and perpendicular to the radial muscle located in the middle of the mantle and close to the OME. The mantle also contains nerves as well as connective tissue. The OME cells close to the outer fold of the

edge are long and columnar in shape; while closer to the pallial mantle they become more cubical and shorter in length. Similar change can be seen in the IME cells. In the pallial mantle, OME cells are shorter and stockier, while the IME layers seems more spacious and the cells are farther apart from each other.

The three mantle folds are distinctive from each other regarding the morphology. The inner and middle fold and closely connected to each other, and morphologically also share a larger similarity with each other as compared to the outer fold. The inner fold is large and thick, and the radial muscle continues into the inner fold. Between the inner and middle fold is sometimes located a small extra fold which could be an artifact caused by the handling of the samples. The outer fold of the mantle is often curved inward, longish in shape and gradually transform into the OME at its outermost side.

Analysis of the differential expression of the Ca^{2+} transporters and channels using immune histochemistry in the mantle edge also revealed differences between the folds (**paper IV**). The outer fold expressed the Ca^{2+} transporters and channels consistently showing especially strong staining for all the antibodies on the inner side close to the periostracal groove. Inner side of the middle fold as well as the inner fold stained for NKA and NCX, but only minimally or not at all for PMCA and L-type VGCC (**paper IV**).

4.3.2 Electrophysiology of the mantle

The Ussing chamber methodology was used in **papers II** and **III** to study the electrophysiological properties of as well as the ion transport across the OME especially focusing on the Ca^{2+} transfer across the OME. In general, all of the electrophysiological parameters showed a large variation between individuals, which made the interpretation of the data challenging. However, this might be excepted since even though the oysters used in the experiments for **paper II-IV** were cultivated, they are cultivated in open cages in fluctuating environments and thus subjected to natural variation in environmental conditions leading to most likely high natural variation between individuals. The faming site where the oysters for **papers II-IV** were ordered (Huîtres-Baudit, Charente-Maritime, France) uses near caught wild caught spat to produce the oysters. The oysters used in **paper I** were collected from a natural population from Sylt, Germany.

TER describes mainly the paracellular shunt resistance across the tight junction proteins between the epithelial cells and therefore also the permeability of the tissue through these junction proteins (Sundh and Sundell, 2012). In invertebrates, epithelial cells are connected by zona adherens, gap junctions and septate junctions (Gilula and Satir, 1971; Hudspeth and Revel 1971;

Aschenbrenner and Walz, 1998). Of these, the first two have been shown to be completely permeable to ions, which has led to a suggestion that it is the septate junctions that correspond the vertebrate tight junction proteins and thus regulate the passage of ions across an epithelium (Aschenbrenner and Walz, 1998; Banerjee et al., 2006). However, the exact permeability of septate junctions seems to be dependent on the species and the tissue in which they are expressed (Aschenbrenner and Walz, 1998). In *A. cygnea*, septate junctions together with zona adherens and gap junction were seen to connect the OME cells (Bleher and Machado, 2004). These junctions were shown to be somewhat permeable to ions such as calcium and lanthanum, though the movement of these ions was still impaired at the inter-septate areas inside the septate junctions. Similar junction proteins have been visualised in the mantle of *R. philippinarum* and *M. mercenaria*, though their permeability has not been further studied (Neff 1972; Paillar and LePennec 1993).

The TER in **paper II** was on average 31.4 ± 3.07 Ohm * cm^2 while that in **paper III** was 20.1 ± 3.87 Ω*cm^2 for animals exposed to full seawater (for those in 50% SW the mean TER was 28.1 ± 4.80 Ω*cm^2). The differences can possibly be, at least partly, explained by seasonal differences as stated above, and/or to the large individual variations. Based on the TER values, the OME can be characterised as a leaky epithelium, (Frömter and Diamond 1973; Anderson and VanItallie,; 2007; Sundh and Sundell, 2012) which would imply that part of the transport of ions and other small molecules would be via a passive, diffusional paracellular pathway between the epithelial cells. The high permeability of the OME is reflected in the transfer of Ca^{2+} across it, of which approximately 40 % is executed by passive transfer, including the transfer through the paracellular pathway (**paper II)**. Paracellular transfer of ions across the OME has previously been studied mainly in the *A. cygnea* where also a part of the Ca transfer from the hemolymph to the EPF was proposed to be executed through the paracellular pathway (Coimbra et al., 1988; Bleher and Machado, 2004).

TEP, the potential difference across the epithelial layer, describes the net ion transfer including both paracellular and transcellular pathways and was in the *C. gigas* OME 0.19 ± 0.10 mV in **paper II** and around -0.5 ± 0.36 mV in **paper III** (with the hemolymph side as electrical ground) irrespective of the salinity treatment. The TEP was very close to 0 in all preparations, however, the slightly positive potential in **paper II** may reflect a net transfer of positives charges to the EPF i.e possible positively charged ions such as Ca^{2+}. The negative potential in **paper III**, on the other hand implies that there is a net transfer of negative charges across the OME, possibly in the form of HCO_3^-, Cl^- or amino acids.

The short circuit current, SCC, is the third electrical parameter continuously measured in the Ussing chamber set-up. It reflects the I in the Ohms law: $U = R * I$ and describes the active transport taking place in the live OME mounted in the chambers. The SCC was positive in both studies, 21.4 ± 6.4 uA * cm^{-2} in **Paper II** and 41.7 ± 22.2 μA*cm in full SW in **paper III** (SW 50% 10.6 ± 10.9 μA*cm^{-2}). As seen from the results of **paper III**, the SCC decreases as salinity goes down. However, the salinity in **paper I**I corresponded that used in the FW in **paper III** so salinity alone cannot explain the changes in the SCC. Individual and seasonal variation can also account for the changes, especially for **paper II** which was run during a whole year. Looking only at the SCC from the control samples run while testing the inhibition with ORM-10153 (run in July - September), the values show 36.1 ± 1.29 μA*cm, further confirming that the values are either depended on random variation or seasonal patterns. These results together imply that although there is a net negative total transfer across the OME in **paper III**, the part that constitutes from the active transport is net positive. The major cation which could be transported is the Ca^{2+}. However, changing the Ringer Ca^{2+} concentration did not affect any of the electrochemical parameters (**paper II)** suggesting manipulating the Ca^{2+} flow across the OME has no major consequence to the total ion current. In *A. cygnea*, Ca^{2+} transfer has been shown to contribute a major part to the SCC of the OME, but the transfer has been shown to vary greatly between seasons (Coimbra et al., 1988; Lopes-Lima et al., 2008; Lopes-Lima et al., 2009). In *C. gigas* Ca^{2+} transfer is lowest in the winter perhaps causing the negative TEP in **paper III** (Lejart et al., 2012).

A seasonal variation has been noted to exist in the TEP and SCC in *A. cygnea* (Coimbra et al., 1988; Lopes-Lima et al., 2008; Lopes-Lima et al., 2009), and a similar seasonal differences most likely affected the results in **paper II** in which the measurements were run continuously during one year. Net calcification in *C. gigas* has been shown to be greatest in June and minimal in December (Lejart et al., 2012) which would suggest that also Ca^{2+} transfer is decreased in winter time. Additionally, periods of acidosis can lead to the dissolution of the shell and the movement of Ca^{2+} from the EPF to the hemolymph which could affect the net charge of ions moving across the OME. The oyster reproduce around mid July to August which leads to the loss of carbohydrate and fat storages and an overall lower fitness (Soletchnik et al., 2002). The experiment in **paper II** were conducted during the whole year which probably evens out any effects due to seasonal variations in net calcification and reproductive cycle, but also adds to the overall individual variation seen in the electrical parameters. The experiment in **paper III** was on the other hand run during a very short period, two consecutive days in November. This is a period the oysters are suggested to have a period of low net calcification which can perhaps been seen in the

negative TEP indicating that the transfer of positive ions to the EPF is lower compared to the experiments for **paper II** . Similarly, SCC was notably lower in the experiment for **paper III** compared to **paper II** indicating a lower level of active ion transport as well. The ion transporters in **paper IV** were consistently expressed higher in the left mantle compared to the right one, a difference that was not seen in **paper III**.

4.3.3 Kinetics of calcium transport

To investigate the kinetics of Ca^{2+} transfer across the OME and the methods of transport potentially used, a gradient of Ringer Ca^{2+} concentrations (2 – 20 mM) was used on the hemolymph side of the OME in the Ussing chambers. In high environmental Ca^{2+} concentrations (≥ 10 mM), the Ca^{2+} transfer across the OME followed approximately a linear relationship described by the equation $y = 0.1678x + 2.2722$ ($R^2 = 0.98$) (**paper II)**. This linear component describes the passive, diffusional paracellular component of the Ca^{2+} transfer, is directly dependent on the hemolymph (or in the situation in the Ussing chambers, the Ringer solution on the hemolymph side of the epithelium) Ca^{2+} concentration. At hemolymph Ca^{2+} concentration of 10 mM, the paracellular diffusion amounted to approximately 40 % of the total Ca^{2+} transfer across the OME. Hemolymph Ca^{2+} concentration in the control oysters in **paper I** was 7.74 ± 0.18 mM; in the oyster from the kinetics measurement in **paper II** 5.99 ± 0.10 mM and the oyster from the full SW in **paper III** 5.87 ± 0.19 mM which, when extrapolated suggests a passive component amounting 38 – 43 % in the different studies.

When the linear, passive component was subtracted from the total Ca transfer, the remaining portion revealed a saturable transport component which was described using both Michaelis-Menten ($R^2 = 0.89$) and allosteric sigmoidal kinetics ($R^2 = 0.95$) modelling (Ahearn 1978; Cornish-Bowden 2013). Although the R-value for the sigmoidal model was higher, when the two models were compared using an extra-sum-of squares F-test, Michaelis-Menten kinetics proved to be the model best describing the data. The Michaelis- Menten constant, K_m, of 6.2 clearly suggest that, at least parts of the transcellular Ca^{2+} transport of the OME can be attributed to high affinity, low capacity transporters. In the case of active Ca^{2+} transport across the oyster OME, this would most probably be credited plasma membrane Ca^{2+}-ATPases (PMCA; Blaustein et al., 2002). This is confirmed by the results of the inhibition studies in **paper II** and well as antibody-binding in **paper IV**. The specific PMCA inhibitor caloxin 1a1 showed 32 % inhibition of the total Ca^{2+} transfer (**paper II**) and the antibodies, specifically developed for PMCA show specific binding to the OME cells (**paper IV**). Thus, the present thesis provide three

independent evidence for functional and active PMCA involved in the Ca^{2+} transport across the OME of *C. gigas*. Interestingly, kinetic analyses of the saturable component reveales a Hill coefficient of 1.99 which can roughly be used as an indicator of the presence of more than one type of binding sites present (Lolkeema and Slotboom, 2015). As the PMCAs only have one type of binding site for Ca this suggests the presence of an additional active transporter of Ca^{2+} in the OME, e.g. the sodium coupled Ca-exchanger (NCX). Although the presence of NCX could not be directly confirmed by the inhibition study in **paper II**, inhibition of Ca transfer across the OME by a specific NKA inhibitor ouabain indicated the existence of a Na^{+} -coupled Ca^{2+} transporter i.e. NCX (**Paper II**). Additionally, the antibody-binding in **paper IV** revealed it to be expressed in the OME mostly located on the apical cell membrane. In the calcified hard-coral *Stylophora pistillata* similar studies modelling the Ca^{2+} uptake into the coelenteric compartment have revealed that the Ca^{2+} uptake follows both apparent diffusion and Michaelis-Menten kinetics indicating that similar to *C. gigas*, the transfer consists of a passive and an active component (Tambutté et al., 1996).

4.3.4. Phylogeny of Ca^{2+} transporters and channels in the mantle of bivalves

The functional studies for **paper II** suggested the involvement of VGCCs, PMCA, and most likely NCX on the Ca^{2+} transfer across the *C. gigas* OME. Since functional studies strongly suggested the involvement the aforementioned proteins in Ca^{2+} transfer across the OME, the next was to confirm their presence in the oyster genome as well as determine the number of potential isoforms expressed in the mantle tissue. Therefore, the genome of *C. gigas* (Zhang et al., 2012) was scanned for potential Ca^{2+} transporters and channels as well as related proteins (**paper III)**. Three genes were annotated as plasma membrane Ca^{2+}-ATPase, four as sarco/endoplasmic reticulum Ca-ATPase isoform A and seven as voltage-dependent L-type calcium channel alpha-1 subunit isoform c were found from the original publication. Comparing the accession numbers of the identified genes using EnsemblMetazoa and NCBI databases further identified these genes as α-NKA, PMCA, SERCA, secretory pathway Ca^{2+}-ATPase (SPCA), and type L-, T-, D- and A- VGCCs as well as three sodium channel genes which were not considered in the further analysis. The D-type channel on further examination was annotated as a L-type VGCC subunit α-1C and A-type shared some similarities (30 – 40 %) to both L-type and R-type VGCCs. However, while running the phylogenetic trees, the D-type channel grouped together with the vertebrate P-type channels and when comparing sequence similarities does in fact share a high similarity with the human P-type compared to the similarity with the L-type (57% vs 45%) (**paper**

III). NCX was not found from the genome published by Zhang et al., (2012) but it was located from the mantle proteome from **paper IV** so similar phylogenetic trees compared to the other transporters.

All the ATPase proteins and VGCC, apart from the confusions about the L/D/P-type transporters, possessed only one single isoform in the *C. gigas* genome based on the genome search. These results seem to match with other mollusc species in which single isoforms were also located (**paper III**). In invertebrates in general, fewer isoforms seem to exist when compared to the situation in vertebrates. This is the case also for the well-studied model-organisms, such *Caenorhabditis elegans* and *Drosophila melanogaster* that possess only a single isoform of the studied ATPases and Ca channels. The expection to this is the NCX, which according to the phylogenetic analysis seemed to possess multiple isoforms of NCX. However, only one of them resembled the vertebrae isoform and seemed to possess a true NCX- function. In invertebrates in general there seems to be only one NCX isoform expressed however some species like the *C. gigas* can have multiple pseudo-isoforms which share similar motifs to NCX but lack the transport function of the real NCX (On et al., 2008).This was confirmed by the phylogenetic analysis where the invertebrate NCXs grouped into a three different subgroups only one of them resembling the vertebrate isoforms. The two other groups consisted of a clade containing most of the mollusk NCX genes while the last one contained one CgiNCX and the branchiopod sequences.

In general, marine invertebrates commonly only have one or two NKA subunit-α isoforms, which has been merited to their relatively stable marine environment (Sáez et al., 2009; Thabet et al., 2016). All of the studied bivalve and mollusc species only expressed a single α-NKA isoform (**paper III**). However, multiple α- subunits have been identified in invertebrates living in environments with fluctuating salinity, further supporting the function of this NKA isoform in adaptation and/or acclimation to varying environmental osmotic and ionic conditions (Sáez et al., 2009; Lind et al., 2013). In general only one SERCA gene has been found from invertebrates with the exception of some parasitic blood worms (Altschuler et al., 2012). In *P. fucata*, three SERCA isoforms have been reported (Fan et al., 2007b) but based on the phylogenetic analysis in **paper III** these differ only at the C-terminal and are most likely splice variants. In vertebrates, SERCA 2b is considered to be the ubiquitously expressed housekeeping variant of the ATPase and the invertebrate variants are suggested to have the largest resemblance with this particular isoform (Altschuler et al., 2012). However, as shown in **paper III**, the vertebrate SERCA multiplication appears to has happened at the base of the vertebrate division suugesting that any resemblance between specific vertebrate isoforms and invertebrate

SERCAs is probably functional. Additionally, Fan et al., (2007b) reported the highest sequence similarity of the *P. fucata* SERCA to the human isoform 3 (Fan et al., 2007b). There is not much information about the SPCA transporters in general, and even less for marine invertebrates, besides its likely function in transporting Ca^{2+} to the Golgi apparatus. The gene expression of SPCA was measured in sea urchin *Strongylocentrotus purpuratus* in relations to egg development but no change in SPCA expression was detected (in contrast to PMCA and SERCA which increased their expression during the 30 h measurement) (Jayantha Gunaratre and Vacquier 2007).

4.3.5 Cellular localisation of the Ca^{2+} transporters and channels

The functional studies using pharmacological inhibitors in the Ussing chambers suggest that the transfer of Ca^{2+} across the OME is performed by PMCA, VGCCs and possibly a NCX co-transporter (**paper II**). The suggested presence of these aforementioned transporters and channels was further confirmed by the visualization of them through immunohistochemical analyses by binding of specific antibodies (**paper IV)**. To achieve this, custom made antibodies were developed against PMCA, NKA, NCX and L-type VGCC. The specific binding of the antibodies was analysed by Western blot. Through visualising the binding of the antibodies, the subcellular localization of the membrane-proteins suggested by the inhibition study could also be confirmed. The information from the IHC together with that from the functional studies was used to construct a model of Ca^{2+} transfer across the OME from the hemolymph to the extrapallial space for shell building.

Verapamil inhibited the Ca^{2+} transfer across the OME when added to the hemolymph side, but not when added to the shell side in the Ussing chambers. This indicates the presence of either a L-type or T-type VGCC on the basal OME cell membrane (**paper II**), as even though verapamil traditionally is viewed upon as a L-type VGCC inhibitor, it has also been shown to inhibit T-type VGCC and Na channels in high concentrations (Norris and Bradfort, 1985; Bergson et al., 2011). The immunohistochemistry staining confirmed the existence of a L-type VGCC in the OME cells. However, its expression was not strictly located to the basal or basolateral cell membrane, as may have been expected for a function in the transfer of Ca^{2+} from the hemolymph to the shell building area in the EPF (Figure 9A-B). The location suggests a double function for L-type VGCC, both in transferring Ca^{2+} into the OME cells from the hemolymph for shell calcification, but also in the potential transfer of Ca^{2+} to the opposite direction.

If the conditions of the EPF are unfavorable to calcification, the shell might also be dissolved. This can happen if EPF pH decreases as by dissolving the shell, the CO_3^{2-} ion is released to be used as a buffer against the pH change (). The inner shell containing the newly calcified material has been shown to function as a buffer for Ca ions in low salinity (Akberali 1980). When the clam *Scrobicularia plana* was bathed in a solution containing radiolabeled ^{45}Ca to allow the follow up of new shell calcification and then exposed to 50% salinity, the newly deposited ^{45}Ca disappeared quickly from the inner shell indicating that the shell can be used as a labile buffer in case the environmental conditions change (Akberali 1980). Perhaps the apical L-type VGCC participates in the Ca^{2+} transfer from the EPS across the OME back to the hemolymph and mantle tissue.

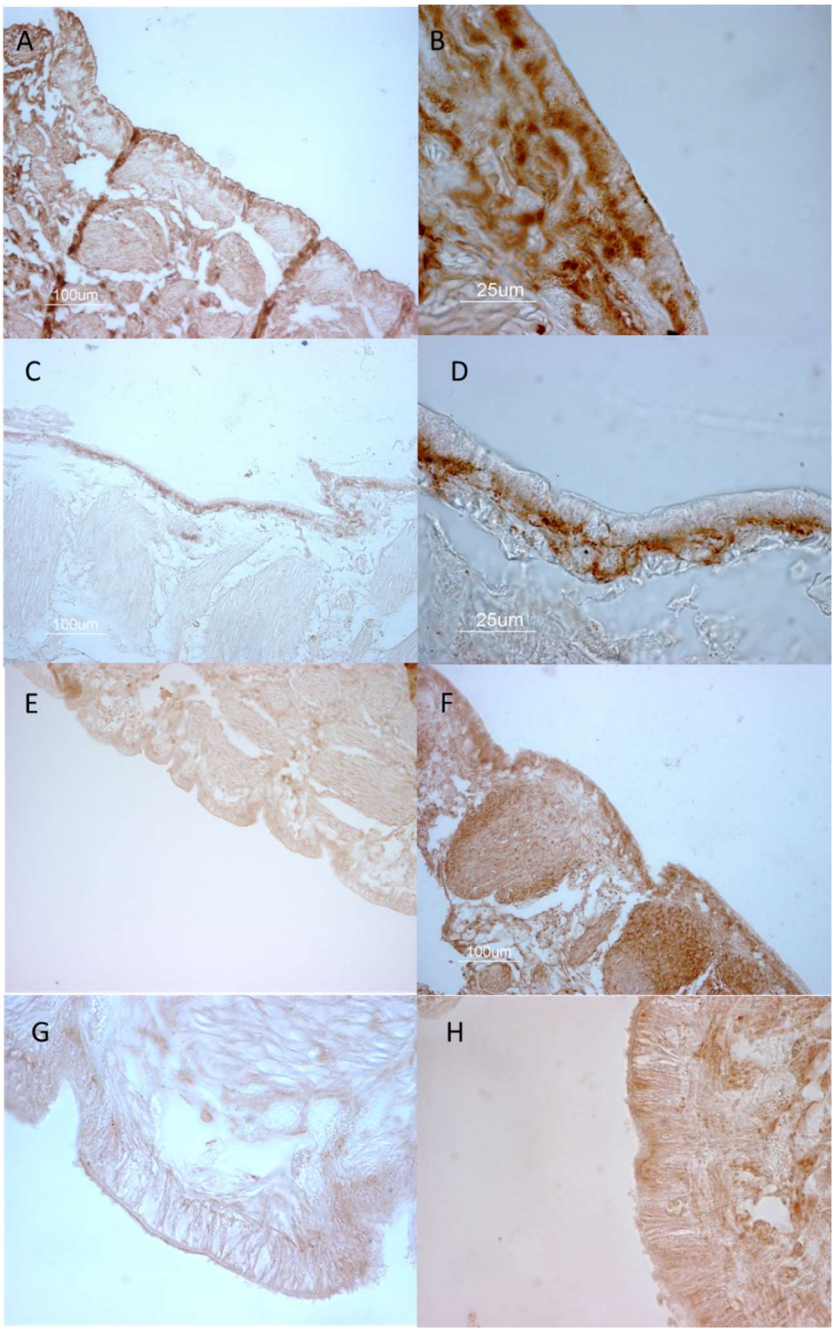

Figure 9. The expression of L-type VGCC (A-B), NKA(C-D), PMCA (E-F) and NCX (G-H) in the OME of *C. gigas*.

The non-specific ATPase inhibitor, vanadate, inhibited Ca^{2+} transfer when added form the apical, shell side of the OME in the Ussing chambers study (**paper II**). However, since vanadate is a general P-ATPase inhibitor binding to the phosphorylation site, the assessed inhibition could have either been due to the inhibition of PMCAs or NKAs, especially since a decrease in the Ca^{2+} transfer was also detected after inhibition by the specific NKA-inhibitor ouabain (**paper II**). Ouabain, on the other hand, only inhibited the Ca^{2+} transfer when added from the hemolymph side, clearly suggesting a basal location for the NKA (Figure 9C-D). An ubiquitously and basolaterally expressed NKA has also been demonstrated in the OME of *T. squamosa* and *A. cygnea* (da Costa et al., 1999; Boo et al., 2017). In the *C. gigas* the basal/basolateral localization of the NKA in the OME was confirmed by IHC (**paper IV**).

To investigate further whether the decrease in Ca^{2+} transfer caused by vanadate was due to an inhibition of NKA or PMCA, specific PMCA inhibitors, caloxins, were tested (paper II). Ca^{2+} transfer was inhibited by the caloxin 1a1 by 32 % when added from the shell side indicating the participation of apical PMCA on Ca^{2+} transfer. The results suggesting an apically located PMCA, as predicted by the inhibition, were confirmed by the antibody binding on the apical OME membrane although a weak binding was also located inside the mantle cells or between them (Figure 9E-F, **paper IV**). The apical PMCA therefore most likely contributes to the Ca^{2+} transfer for shell calcification. The basal PMCA might have a similar function to the apical L-type VGCC in transporting Ca^{2+} from the OME cells back to the hemolymph.

Another caloxin, caloxin 1c2, which according to the literature is a more pontent PMCA inhibitor (Pande et al., 2008), did not have any significant effect (**paper II**). Both caloxin 1a1 (Ki = 86 μM) and 1c2 (Ki = 2.3 μM) have highest affinity to PMCA4 even though they do inhibit other isoforms also, but then in higher concentrations (Pande et al., 2008). According to the sequence analysis in **paper III**, CgiPMCA shares the highest sequence similarity with human PMCA3 and PMCA4 isoforms (59 %). However, focusing the comparison specifically at the reported caloxin binding site reveals larger differences between the *C. gigas* and human isoforms. Both caloxins bind at the first extracellular loop of the PMCA, but at slightly different regions, caloxin 1a1 at residue 133-147 and caloxin 1c2 at residues 115-131 (Pande et al., 2005; Pande et al., 2008). Interestingly, the *C. gigas* extracellular domain has been predicted to span residues 119-137 (Liliana Anjos, personal communication), which makes it smaller and slightly differently positioned compared to the human PMCA4. This would indicate that the caloxin 1a1 binding site is completely missing from the *C. gigas* even though there was a clear inhibition of Ca^{2+} transfer caused by it. Therefore the effect of caloxin 1a1 on CgiPMCA raises questions. It could be that the inhibition by the

caloxin 1a1 is less dependent on the actual amino acid sequence and more on the configuration of the extracellular domains but since a model of the CgiPMCA is still lacking, the configurational similarities between it and the human PMCAs cannot be confirmed. Caloxin 1a1 has also noted to inhibit SERCA by around 60 % at a concentration of 200 μM by creating a disulfide bridge between the cysteine residues between SERCA and caloxin 1a1 (Pande et al., 2008). Because caloxin 1c2 does not contain any cysteine and can thus not form any disulfide-bridges, this would explain the apparent lack of inhibition even though the similarity between the binding sites in human PMCA4 and CgiPMCA was higher (50 %). However, the CgiPMCA extracellular domains do not contain cysteine residues according to the current model, although in few places they are flanked by cysteine. The inhibition due to a disulfide bridge cannot however be completely excluded, since a slight shift in the location of the extracellular domains in the current CgiPMCA model would open the cysteine residues for binding. The inhibition by caloxin 1a1 could also be due to an unknown toxic effect. However, if an overall damage to the OME tissue had been present, an increase in Ca^{2+} transfer would be expected due to a loss of tissue integrity.

There was no direct evidence on the participation of NCX in transport of $Ca^{2+}$to the shell building area, as the specific NCX inhibitor ORM-10103 did not have any effect on the Ca^{2+} transfer (**paper II)**. Not much data have been published on the ORM-10103, regarding neither its specificity nor its exact physiological function. Therefore, it is difficult to determine if the lack of inhibition is due to the lack of a critical sequence required for its binding from the *C. gigas* NCX protein. However, the inhibition of NKA by oubain decreased Ca^{2+} transfer across the OME by 25 %, which indicated that a Na^{+}-coupled Ca^{2+} transfer mechanism exists in the OME cells. The existence of NCX was further confirmed by measuring mRNA expression in the mantle in **papers III** and **IV**, and by IHC in **paper IV**. As demonstrated with IHC, an antibody staining was detected in the OME, mostly located at the apical cell membrane, but also with some unclear binding between the OME cells (Figure 9G-H). The presence of specific NCX binding in the apical membrane clearly supports a role of NCX in the bulk transport of Ca^{2+} across the OME. This is further supported by studies in other bivalve species, e.g *M. edulis* where the NCX gene expression increased in relation to larval calcification *(*Ramesh et al., 2019). Apical NCX has also been located in *T. squamosa* (Boo et al., 2019). In *T. squamosa*, PMCA and NCX gene expression increased during light exposure, which in this species, containing symbiotic zooxanthella that provide energy to the clams, is related to calcification (Ip et al., 2017; Boo et al., 2019). NCX has also been located in the calcifying epithelia of the hard coral *Acropora yongei* (Barron et al., 2018).

PMCA and L-type VGCC staining was visualised also in the hemocytes. Although participation of hemocytes ion shell calcification could not be confirmed in **paper I**, they have been previously suggested to carry $CaCO_3$ crystals to the shell forming area (Mount et al., 2004; Huang et al., 2018). The expression of PMCA and L-type VGCC in the hemocytes could be related to the concentrating of Ca^{2+} ions intracellularly for $CaCO_3$ crystallisation. However, intracellular Ca^{2+} is also involved in other function such as immune response (Kotturi et al., 2006) which means that based on the evidence from this thesis, the participation of hemocytes on calcification this cannot be stated conclusively.

Occasionally, the NCX, PMCA and L-type VGCC antibodies showed staining inside the OME cells, not in clear contact with either apical or basal cell membrane. Similar intracellular binding of membrane proteins has been previously reported in *A. cygnea* (L-type VGCC), *P. fucata* (PMCA) and coral *Acropora yongei* (NCX) (Lopes-Lima et al., 2008; Wang et al., 2008; Barron et al., 2018). The OME cells of *P. fucata* have also been noted to deposit $CaCO_3$ directly (Xiang et al., 2014). Possibly, intracellular vesicles containing the abovementioned transporters and channels are concentrating Ca^{2+} ions inside them to be transferred across the apical membrane to the EPS by exocytosis of either Ca^{2+} or $CaCO_3$. Another possible explanation for the intracellular staining is a storage of the membrane proteins in vesicles for then to be quickly incorporated into the OME, upon stimulation i.e. "trafficking". A similar mechanism of temporary internalization and recycling of Ca^{2+} transporters has been located in developing *Xenopus* oocytes (El-Jouni et al., 2008). Since hemocytes were seen in some samples to be located on or between the OME cells, the occasionally seen, seemingly intracellular binding could be actually due to the hemocytes.

4.4. Proposed model of Ca^{2+} transfer across the OME of *C. gigas*

Combining the results in the current thesis lead to the proposition of the model presented in figure 10, for transfer of Ca^{2+} across the OME of *C. gigas*. Calcium is transported in the hemolymph mainly in the ionic from, as Ca^{2+}. Based on the kinetic study Ca^{2+} also crosses the OME cells both through a passive, paracellular pathway and through passive and active components of a transcellular pathway. The transcellular pathway consists of an initial uptake into the OME cells across the basolateral membrane, a transport through the cell and finally an extrusion into the extrapallial space (EPS), across the apical cell membrane.

The flow across the basolateral side of the OME is passive and occur through voltage-gated Ca channels (VGCCs; Figure 10). VGCCs are located in both the

basal and the apical cell membrane where they can allow a regulated inward flow of Ca2+, along its concentration gradient, into the OME cells. The functional studies demonstrated that Ca^{2+} transfer decreased only when the VGCC inhibitor, verapamil, was added from the hemolymph side, indicating a basal location for the channels. However, the antibody staining showed that both the basal and apical cell membrane were expressing L-type VGCC. This suggests double functions for the VGCCs in the OME cells. The L-type VGCC, located in the basal membrane, is involved in the bulk of Ca^{2+} transfer from the hemolymph to the extrapallial space for calcification, by enabling inflow of Ca^{2+} into the OME cells. This can possibly also be accompanied by other types of Ca channels, such as the T-type VGCC. As calcification is a dynamic process depending also on the carbonate chemistry, pH and hemolymph ion concentration, there may be situations when the transport of Ca^{2+} away from the EPS is required. Thus, for the apically located VGCC's, a possible role can be during situations of decreased environmental salinity, as discussed in 4.5, or low pH in the EPS. A diffusional flow of Ca^{2+} back into the cells from the EPS would liberate CO_3^{2-} ions for buffering capacity, i.e. at decreased pH.

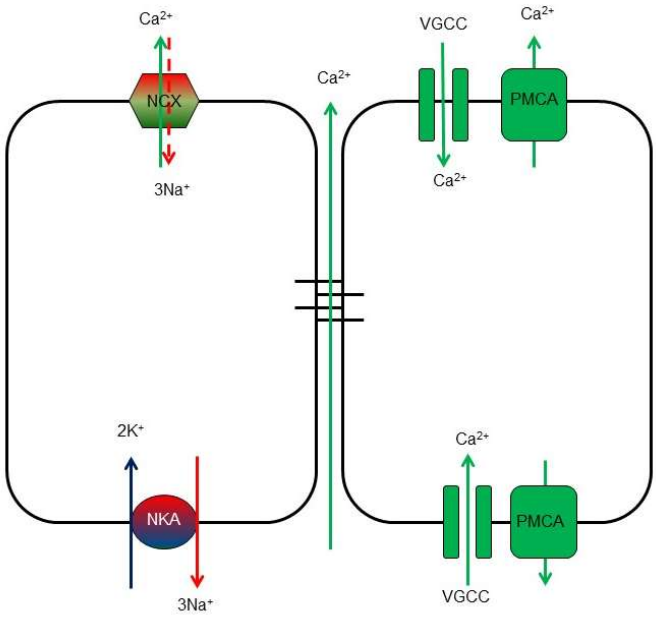

Figure 10. A proposed model of Ca^{2+} transfer across the OME of *C. gigas*. Ca^{2+} is taken into the OME cells through basolateral VGCCs and extruded further to the EPS by apical PMCAs and NCXs. Apical L-type VGCC and basal PMCA participate in the transfer of Ca^{2+} to the opposite direction.

The final step in the transfer of Ca^{2+} across the OME is taken care of by apically located PMCA and NCX that actively transport Ca^{2+} from the OME cells to the EPS for shell calcification (Figure 10). The existence and location of PMCA was confirmed by both functional studies and immunohistochemistry staining. The Ca^{2+} transfer was inhibited when the PMCA inhibitor, caloxin, was added from the shell side indicating an apical location for the transporter. Visualising the OME cells using IHC showed both apical and basal staining for the PMCA. The apical PMCA most likely participates in the bulk transport of Ca^{2+} from the OME cells into the shell growth area, while the basal PMCA might have a similar function as the apical L-type VGCC, transporting Ca^{2+} away from the EPS back to the OME and possibly hemolymph in situations when a retrograde transport is needed.

The addition of the NCX inhibitor, ORM-10103, did not result in any decrease Ca^{2+} transfer. However, this inhibitor is specific for mammalian NCX and not validated in oysters and thus the inhibitory effect in oyster may be low. Instead, the functional studies showed a decrease in the Ca^{2+} transfer by the specific NKA-inhibitor oubain when it was added to the hemolymph side of the OME, but not when added to the apical side. This clearly indicates a basal location for the NKA in the OME cells, as well as the existence of a Na^{+}-coupled Ca^{2+} transport mechanism, probably the NCX. Although the functional studies did not give any indication on the location of the NCX, it was speculated that it would be located on the apical cell membrane since according to the kinetics study, a complementary active transport mechanism, to the PMCA, were suggested for the active transport of Ca^{2+} to the EPS for shell growth. Both the presence and apical location of the NCX was confirmed by the antibody staining using IHC. Also the NKA antibody showed a clear basolateral staining in the OME cells confirming the localization suggested by the functional studies.

4.5. Effects of salinity on Ca^{2+} transfer

4.5.1 Physiological responses to a dilute environment

Ca^{2+} transfer across the OME of *C. gigas* decreased when the oysters where exposed to 50% diluted seawater. This could be potentially caused by either a decrease in the paracellular Ca^{2+} transfer or in the active transfer of Ca^{2+} transcellularly. The total active transport of ions, measured as SCC also decreased in dilute seawater by 75 % compared to full seawater (SW: 41.7 ± 22.2 $\mu A*cm^{-2}$; 50% SW 10.6 ± 10.9 $\mu A*cm^{-2}$) although due to the high variation between individuals, the change was not statistically significant. The decrease in SCC could be caused by a decrease in the activity of the Ca^{2+} transporters, PMCA and NCX. Although PMCA mRNA expression did not change in **paper**

III, the mRNA expression was measured only once at the end of the experiment so earlier changes would not be detected. Additionally, changes in mRNA expression do not always reflect the changes in protein activity, as the latter is also affected by posttranslational modifications as well as protein synthesis and degradation rate (Greenbaum et al., 2003). However, it is likely that the decrease in NKA expression also affected the SCC in **paper III**. NKA makes up a major part of bivalve energy consumption (Lannig et al., 2010; Frieder et al., 2017) and is the major osmoregulatory transporter in the mantle tissue. Compared to the other transporters, that are highly expressed in the connective tissue of the mantle, NKA is almost exclusive expressed in the OME as seen in the immunohistochemistry results (**paper IV)**, which would indicate that changes in its mRNA expression are also reflected in the ion transport of the OME.

The TER (SW 20.1 ± 3.87 $\Omega*cm^2$; 50% SW 28.1 ± 4.80 $\Omega*cm^2$) showed a tendency to increase when the oyster were exposed to dilute seawater in **paper III**. Although oysters, as well as other bivalves, are considered osmoconformers mostly following the environmental osmolality and ion concentrations, bivalves have been noted to regulate their water content as well as ion concentrations to some degree when facing suboptimal salinities (Shumway 1977b; Fuhrmann et al., 2018). This might be the case for *C. gigas* in **paper III** as well, since the increased resistance leads to a decreased paracellular permeability. The decrease in permeability was also shown in the decreased 3H-Mannitol transfer in the 50 % SW in **paper III**.

4.5.2 Effects of dilute environment on mRNA expression

In both studies the mRNA expression of NKA, PMCA, SERCA, NCX as well as T-type and L-type VGCCs were measured. However, the results on the mRNA expression of all the transporters differed between the two studies. This may be due to seasonal variations as the calcification rate of bivalves are known differ depending on the season (Lejart et al., 2012) and paper III was conducted over in November 2017 whereas paper IV in the March 2018. Additonally, in **paper IV** only few individuals from the full SW (4-5 individuals per treatment) could be measured for mRNA expression due to difficulties in RNA extraction and a large individual variation in the qPCR results. NKA, SERCA and both L-type and T-type VGCC mRNA expression in **paper IV** were higher in the left compared to the right mantle, a difference that was not seen in **paper III**. This regional difference, despite randomization of both sampling, mRNA extraction and qPCR analysis, may suggest an actual difference in the two mantle regions as it was apparent also with a low sample size. The difference between the two mantle halves has not been much studied in comparison to the differences between the different mantle zones. The calcification rate of the left shell has

been shown to be faster and the produced shell thicker in *C. virginica* (Galtsoff 1964). There are also clear differences in the *C. gigas* shell, revealed by a visual examination. The left shell is curved, thicker and rougher, while the right shell is thinner and smoother. These differences indicates that there probably exist difference in the calcification rate as well as the function of the Ca^{2+} transporters and channels in *C. gigas* as well (Galtsoff 1964).

Exposure to 50% SW salinity decreased NKA expression in **paper III** but not in **paper IV**. In marine bivalves, NKA is suggested to participate in osmoregulation, though results from low-salinity experiments are contradicting, showing that low salinity can either increase, decrease or have no effect on NKA activity or gene expression (Willmer 1978; Borgatti et al., 2003; Paganini et al., 2010 Lin et al., 2016; Peng et al., 2019). In some studies where NKA activity or mRNA expression have been measured over time after an exposure of molluscs to low salinity, an initial increase in NKA activity followed by a decrease back to or even below, basal level have been shown (Sommer & Mantel, 1991; Jia and Liu, 2018; Peng et al. 2019). When the environmental salinity suddenly changes, there might be a need to quickly adjust the ion uptake before other long-term adaptation mechanisms, including adjustment of organic osmolytes, are effective. This is followed by an adjustment in the expression of ion transporters to meet the new ion concentration, which aims to maintain the ion gradient over cell membranes stable as is seen in **paper III**. It could be that the oysters in **paper III** and **IV** were in a different stagse of acclimation to the new salinity. Perhaps in the oysters from **paper IV** NKA mRNA expression had first increased, but was already back to the basal value by the end of the experiment. In the barnacle *Balanus improvisus*, the different NKAα splice variant responded differently to salinity change (Lind et al., 2013). Since only one NKA isoform was found from the genome of *C. gigas* (**paper III**), no variability regarding isoforms or splice variants could not be detected. As the *C. gigas* genome considering ion transport has not been thoroughly investigated, the existence of different NKA variants with tissue/condition specific expression patterns cannot be ruled out.

SERCA mRNA expression decreased in 50% SW in **paper III** but not in **paper IV.** When the environmental as well as hemolymph Ca^{2+} concentration decreases, maintaining the uptake and intracellular concentration in the OME cells becomes more challenging and less sequestration into ER/SR is needed. Similarly to **paper III**, the mRNA expression of SERCA has been noted to decrease in the shrimp *Litepenaeus vannamei* in hyposaline (3 ppt) conditions compared to hypersaline (40 ppt) in the digestive gland, hepatopancreas, muscle and epithelia but not in the gills in which the effect was the opposite (Wang et al., 2013c). This further supports the notion that the SERCA expression is

adjusted in concordance with the changes in the environmental Ca^{2+} concentration.

T-type VGCC mRNA expression increased in the 50% SW in **paper III** but the expression did not change in **paper IV**. Especially the T-type VGCC has previously been shown to activate Ca-gated K^+ channels, which have been suggested to have a role in the cell volume regulation in marine molluscs facing low salinities (Berger et al., 1977: McCarty & O'Neil, 1992; Snutch et al., 2003). Similar increase in VGCC expression has been reported before in *C. gigas* exposed to low salinities, and in the same study a simultaneous increase in the expression of Ca-gated K^+ channels was also recorded (Meng et al., 2013). The expression of Ca-gated K^+-channels were not investigated in this thesis but the increased expression of T-type VGCC mRNA could indicate a similar role in intracellular volume regulation as described previsously for *C. gigas*.

NCX mRNA expressions was higher in the 50% SW in the right mantle in **paper III** but again there was no difference in **paper IV**. The increase in NCX mRNA expression can also be a result of the decreased Na^+ concentrations of the hemolymph. Perhaps the mRNA expression is increased to compensate for an inherent decrease in the transport rate caused by the decreased Na^+ concentration in the hemolymph. To maintain the normal Na^+/Ca^{2+} balance, more NCX would then be required to keep the same exchange level. NCX expression may also be coupled to the function of the other Ca^{2+} transporters and channels. When Ca^{2+} first enters the cells through the VGCCs, it can be either immediately transported back out of the cell by PMCA and/or NCX or bound by intracellular Ca-binding proteins and/or sequestered into Ca-storing compartments such as the ER. Since the expression of T-type VGCC increases and the expression of SERCA decreased in the 50 % SW, together with the increased NCX expression this indicates that the oysters aim to keep the Ca^{2+} flow to the EPS for shell growth stable. However, since Ca^{2+} transfer across the OME did decrease in the 50 % SW, these compensatory mechanisms were not sufficient (**paper III**).

5. CONCLUSIONS AND FUTURE PERSPECTIVES

This PhD thesis concludes that the calcium needed for biomineralisation in the marine bivalve Pacific oyster, *Crassostrea gigas,* is externally derived ionic Ca^{2+}. The Ca^{2+} is transported in the hemolymph and within the mantle tissue to the outer mantle epithelium (OME), where it is transferred across to supply the extrapallial space (EPS) with Ca^{2+} for shell calcification and growth. This thesis presents a model for the active and passive transfer mechanisms present in the OME as well as their proportional contributions. Both transport pathways are

affected by changes in the environmental salinity with a decreased supply of Ca^{2+} in dilute seawater. These results suggest that the current global climate changes will most likely affect calcification in this species.

All bivalves have a constant need of calcium for basal growth of the shell. During periods of fast growth or repair of damaged shells the calcium supply to the EPS needs to be increased. In *C. gigas* this thesis work concludes that this supply is provided by absorption of environmental Ca^{2+}mainly through the mantle tissue. The absorbed calcium is transported in the hemolymph mainly (77 – 95 %) as dissolved calcium ions. However, both protein- and complex bound Ca are also present in the hemolymph. These fractions function as calcium buffers and stores that rapidly can be mobilised when the calcium demand is increased. For example, at the initiation of a period of shell repair complex- and protein bound calcium are first mobilised to the EPS. If the increased demand of calcium is prolonged, the oysters increase absorption of environmental calcium, followed by an increased transfer of calcium across the OME resulting in an overall increased turnover of calcium from the environment to the shell forming area.

For *C. gigas,* it was further concluded that the calcium transferred across the OME was also mainly in the ionic form, both regarding paracellular and transcellular pathways. The participation of hemocytes in supply of Ca to the EPS cannot be excluded, but the proportion of Ca inside the hemotocytes was negligible compared to the other hemolymph Ca fractions two weeks after initiation of shell repair mechanisms through notching of the shell. However, the relative importance of the different Ca fractions, transporting mechanisms and their regulation during basal shell growth and in a longer time perspective require further investigation, as these mechanisms may differ from those involved in the immediate repair of a damaged shell.

The transfer of Ca^{2+} across the OME of *C. gigas* consisted of transcellular component (60 % of total Ca transfer) actively transporting Ca^{2+} across the apical membrane. This is executed by plasma membrane Ca^{2+}-ATPases (PMCA, 32 %) and Na^{+}/Ca^{2+}-exchangers (NCX; 25 %) in combination with passive diffusion of Ca^{2+} across the basal OME membrane executed by voltage-gated Ca channels (VGCC). It further consists of one passive, paracellular component amounting to approximately 40 % of the total Ca transfer. The present thesis presents a model for the transfer of Ca^{2+} across the OME of *C. gigas*. This model concludes that Ca^{2+} enters the OME cells via basolaterally located VGCCs. Ca^{2+} is then transported to the EPS via apically localised PMCA and NCX. Additionally, apically situated L-type VGCC and basal PMCA provide a regulated possibility for transfer of Ca^{2+} in the opposite direction, which can be necessary e.g. during oversaturation of Ca^{2+} or decreased pH in the EPS. To

complement this model, the participation of the other potential membrane bound calcium channels and transporters should be investigated in the future. As this thesis work also revealed the importance of the endoplasmic reticulum in the Ca^{2+} metabolism of the mantle cells, the full elucidation of the intracellular calcium storing mechanisms and their participation on the shell building should be researched further.

It could be concluded from the phylogenetic analyses that *C. gigas* and other bivalves have closely related Ca^{2+} transporters and channel which suggests a common ancestor. Only single genes seem to be present for most of the transporter and channels analysed, the sole exception being NCX from *C. gigas*. Further analyses of the NCX genes revealed that only one of the identified *C. gigas* isoforms resembled the vertebrate NCX isoforms. This indicates that the other NCX genes present in the phylogenetic tree are the result of an early duplication, before the protostome-deuterostome division, and that in the vertebrates the other genes of this subfamily do not exist anymore.

Ca^{2+} transfer across the OME decreased when the oysters where exposed to seawater diluted to 50%. A similar decrease was detected in the total active ion transport and the paracellular ion transfer of the epithelium. The Ca^{2+} transporters and channels in the C. *gigas* OME are regulated at the level of the transcriptome in order to compensate for the decreased Ca^{2+} concentration in the environment. The Ca^{2+} transporters and channels in the C. *gigas* OME are regulated at the level of the transcriptome in order to compensate for the decreased Ca^{2+} concentration in the environment. The membrane bound VCCG channels and NCX showed increased mRNA expression while NKA and SERCA were downregulated, indicating a decreased sequestration of Ca^{2+} into the endoplasmic reticulum thus adding Ca^{2+} to the intracellular pool available for transport to the EPS. However, these compensatory mechanisms were not sufficient to compensate fully for the decrease in environmental calcium as the Ca^{2+} transfer to the shell forming area decreased. It can be concluded that possible future dilution of coastal seawater, as a result of climate change, will probably result in disturbed shell biomineralisation in *C. gigas,* at least in a short term perspective. This, in turn, may lead to physiological and immunological stress on the oysters, as an intact shell is the main mechanism of defence and protection. The capability of oysters to acclimate to lowered salininty during prolonged exposure is not known and as climate change is predicted to change the oceans' salinity alongside several other factors, such as temperature and pH, further studies using multiple stressors are needed to predict how bivalves will fare in the future.

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