THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Ecophysiologie Adaptative

École doctorale GAIA
Biodiversité, Agriculture, Alimentation, Environnement, Terre, Eau

Unité de recherche MARBEC
Marine Biodiversity, Exploitation and Conservation

COPING WITH SALINITY AND TEMPERATURE CHANGES: A FOCUS ON THE GILL RESPONSE IN EUROPEAN SEA BASS *Dicentrarchus labrax*

Présentée par Waliullah MASROOR
Le 26 Avril 2019

Sous la direction Mme Catherine LORIN-NEBEL
et Mme Emilie FARCY

Devant le jury composé de

Mme Delphine DESTOUMIEUX-GARZON, Directrice de Recherche, CNRS, Université Montpellier	Présidente
M. Gert FLIK, Professeur, Radboud University, Pays Bas	Rapporteur
Mme Anne-Sophie MARTINEZ, Maître de Conférences, Université de Caen Normandie	Rapporteur
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Dedication

I dedicate this thesis to my beloved younger brother Abdul Muqeet Umair (late) (2007-2018) not to see him in my family any more. I never thought about this day that upon completion of my thesis I won't be able to see him celebrating my PhD.

Acknowledgement

Many people have inspired and supported me for completion of this thesis, but to the greatest extent, I wish to express my heartfelt gratitude to my supervisors Dr. Catherine Lorin-Nebel and Dr. Emilie Farcy. I will be indebted to them that they reshaped my academic career by accepting me as a PhD student and this means a lot to me. It is merely possible because of their encouragement and guidance this thesis never would have eventuated. Thank you for giving me this opportunity, never letting me give up, and always finding time for me. Also special thanks to them they teach me many more things that could have been learnt before starting my PhD. I remember when I started my PhD, it was difficult for me to find myself and I was always lost then it were my supervisors their feedback and troubleshooting made my way to move forward. They deserved more than words and I truly appreciate for their corrections and presentation skills they taught me.

I wish to thank the jury members of my thesis: Gert Flik, Anne-Sophie Martinez, Jack Falcon and Delphine Destoumieux for generously offer their time and review of this document. I admire magnificent suggestions of thesis committee members: Helena D'Cotta, Delphine Destoumieux, Benjamin Geffroy and Julien Issartel that improved my research work and they endorsed my efforts of each year.

I submit my heartiest gratitude to Prof. Guy Charmantier for his guidance in my experiments. I am deeply indebted to my other lab members: Jehan-Herve Lignot, Patricia Cucchi, Eva Blondeau-Bidet, Sophie Hermet, Romain Gros and colleagues: Thibaut, Quanquan, Khalid, Elsa, Laura, Thomas, Dimitri for their invaluable help in the lab and sometime outside the lab when it comes to speak French. My country fellows were nice and supportive at all stages I humbly extend my thanks to all.

To my loving parents, sisters and brothers, I offer my sincerest thanks for their unswerving support for my stay in France and of my work and for the patience and understanding that they have shown despite me having far too much time away from them. With the thesis now finished, it's finally time for us to meet in home country. I look forward to all the exciting times that lie ahead. My joy knows no bounds in expressing my cordial gratitude to my loving wife and daughter. They were always by my side and served my inspiration to complete my studies. I would like to express my gratitude for being with me in France and my wife's interest in baking bakery items were the great part I enjoyed most.

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List of abbreviation

AE	Anion exchanger $Cl^-/HCO3^-$
CFTR	Chloride channel “Cystic fibrosis transmembrane conductance regulator”
ClC-3	Chloride channel 3
CIRBP	Cold-inducible RNA-binding protein
CO_2	Carbon dioxide
CTM	Critical thermal methodolgy
FW	Fresh water
HSPs	Heat shock proteins
IPCC	Intergovernmental Panel on Climate Change
MRCs	Mitochondrion rich cells/ionocytes
NBC	Na^+/HCO_3^- cotransporter
NHE	Na^+/H^+ exchanger
NKA	Na^+/K^+-ATPase pump
NKCC	$Na^+/K^+/2Cl^-$ co-transporters
OCLTT	Oxygen- and capacity-limited thermal tolerance
pO_2	Partial pressure of oxygen
PVCs	Pavement cells
Rh	Rhesus
SW	Seawater
UNEP	United Nations Environmental Programme
VHA	V-type H^+-ATPase
WMO	Worldwide Metrological Organization

Chapter I

Introduction

Chapter I - Introduction

1.1 Environmental variability in Mediterranean coastal areas: present and future

1.1.1 Characterization of West Mediterranean lagoons and estuaries

The average sea surface temperature in Western Mediterranean Sea roughly varies from 14 °C to 26 °C (Shaltout and Omstedt 2015). Habitats like lagoons and estuaries are characterized by more highly variable environmental parameters as shown in Table 1. Lagoons and estuaries are transition zones between land and ocean. An estuary is commonly considered as the mouth of the coastal river while a coastal lagoon is an embayment separated from the coastal ocean by barrier islands (Yáñez-Arancibia et al. 2011). The temperature and salinity of water in estuaries vary with the water flow of the rivers and the tides of sea as the estuaries are connected to both land and ocean or sea. The temperature and salinity in lagoons are generally less influenced by the tides because the body of water is more enclosed. However, the influence of other factors such as sunshine, evaporation and precipitations can be significant (Dalrymple et al. 1992). These strong environmental fluctuations are particularly challenging for organisms living in these areas to maintain stable physical, chemical and biological parameters (Feyrer et al. 2015). Coastal rivers are characterized by a strong temporal variability of water flow which influences physical parameters like temperature. For example in the Têt river in southwestern France, the difference between average summer and winter temperature is about 14.4 °C (Ludwig et al. 2004). Lagoons are shallow costal habitats where organisms are commonly exposed to environmental fluctuations. The salinity of Mediterranean lagoons for example can vary from 0 ppt to 50 ppt according to the considered lagoon (Newton and Mudge 2003) (Table 1).

In the life cycle of certain fish species, such as sea bass, lagoons and estuarine habitats are essential and critical because of the use of such habitats for feeding and nursery areas. Sea bass undertake seasonal migration towards lagoons and estuaries in the spring. At these periods, heavy precipitation can occurr exposing fish to rapid salinity decrease.

Table 1 Minimum and maximum temperature and salinity of the lagoons in the vicinity of Montpellier and Perpignan coast, France[1]

Mediterranean lagoons	**Temperature**		**Salinity**	
	Min	**Max**	**Min**	**Max**
Etangs de la Camargue gardoise	8.9 °C	32.5 °C	15 ‰	30 ‰
Etang de l'Or	6.5 °C	29.5 °C	7 ‰	31 ‰
Etangs palavasiens	4 °C	28 °C	28 ‰	45 ‰
Etang de Vendres	5 °C	25 °C	2 ‰	30 ‰
Etang de Bages Sigean	5 °C	25 °C	30 ‰	35 ‰
Etang de La Palme	3 °C	31 °C	8 ‰	55 ‰
Etang de Canet Saint-Nazaire	2 °C	26.5 °C	3.8 ‰	39.9 ‰

1.2 Future: what modifications are expected in the context of climate change?

1.2.1 Climate change – global context

The Intergovernmental Panel on Climate Change (IPCC) was formed jointly in 1998 by the United Nations Environmental Programme (UNEP) and the worldwide Metrological Organization (WMO). The IPPC, UNEP and WMO main objective is to provide a clear scientific view on the ongoing status of climate change and its potential environmental and socio-economic impacts. The projected increases of temperature documented by the synthesis report of the IPPC (IPCC 2015) are most reliable and their recommendations serve as the key reference for mitigation of global warming. The increase of global average air and ocean temperatures are evidence for warming climate systems (IPCC 2015).

[1] Réseaux de suivi lagunaire du Languedoc Roussillon: Bilan des résultats 2013. Ifremer Rapport RST/LER/LR/13.03, Sète, France. http://archimer.ifremer.fr/doc/00148/25940/24004.pdf

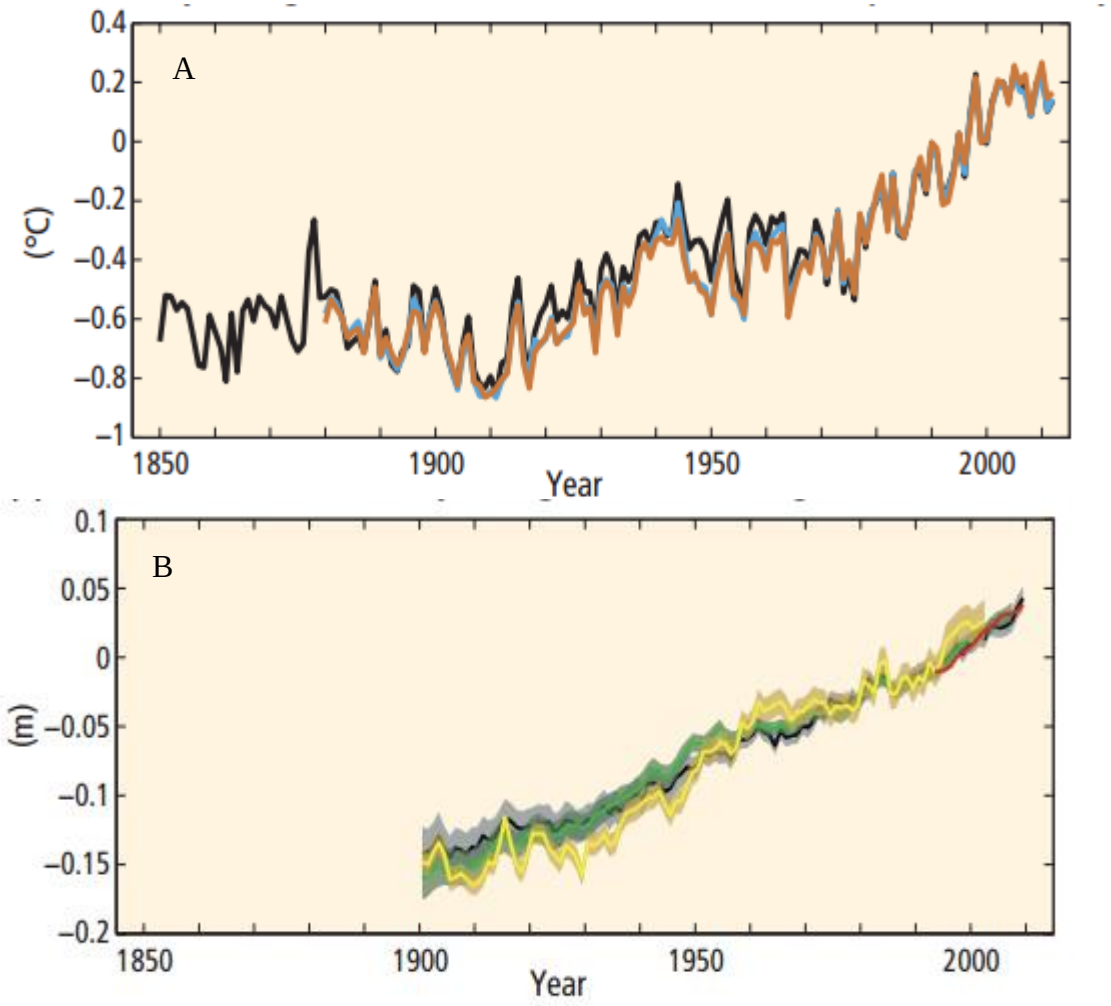

Fig. 1: (A) Annually and globally averaged combined land and ocean surface temperature anomalies relative to the average temperature over the period 1986 to 2005. Colors indicate different data sets. (B) Annually and globally averaged sea level change relative to the average temperature over the period 1986 to 2005 in the longest-running data sets. Colors indicate different data sets. All datasets are aligned to have the same value in 1993, the first year of satellite altimetry data (red). Where assessed, uncertainties are indicated by coloured shading (IPCC, 2015)

The Earth's average temperature has warmed up by approximately 0.65 °C to 1.06 °C during the period from 1850 to 2012 (Fig. 1). According to the scenario (low emission or high emission), models predict increases in mean air temperature of 1.1 to 6.4 °C by the year 2100 (IPCC 2015). The magnitude of the regional temperature increase appears to be correlated with latitude: higher latitudes are predicted to experience a larger temperature change than tropical and subtropical latitudes. Climate change may occur due to natural variability or as a result of anthropogenic activity. According to IPCC, increase in global average surface temperature from 1951 to 2010 is mainly explained by increase in greenhouse gases concentration and other anthropogenic forcing together. The most influential factors of

anthropogenic activities are the increase of carbon dioxide (CO_2) level arising from fossil fuel combustion, release of aerosols or particular matter, extensive land use and deforestation.

Climate change will influence different sectors and region's economy by mean of its intensity to those which are sensitivity to climate change. Many continental or oceanic natural resources are vulnerable. In different part of the regions, hydrological systems have been altered by the changing precipitation or melting snow or ice. This affects water resources in terms of quality and quantity.

1.2.2 Climate change impacts on Mediterranean Sea

There are strong natural variations in the Mediterranean sea but overall, there is a discernible trend of increased salinity and warmer temperature in key water masses over the last 50 years (Cacho et al. 2001; Mariotti et al. 2008; Mariotti 2010). The Western Mediterranean deep water warming is in agreement with recent atmospheric temperature changes over the Mediterranean Sea (Luterbacher et al. 2004). The salt content of the Western Mediterranean deep water has also been steadily increasing during the last 50 years, mainly attributed to decreasing precipitation over the region since the 1940s (Krahmann and Schott 1998; Mariotti et al. 2002), to increasing of evaporation (Mariotti et al. 2008; Mariotti 2010), and to anthropogenic reduction in the freshwater inflow (Rohling and Bryden 1992).

1.2.3 Climate change impacts on coastal estuaries and lagoons habitats

Habitats like coastal lagoons and estuaries are potentially more vulnerable to climate change by several factors such as sea level, open ocean temperature, precipitation and storms (Anthony et al. 2009). Indeed, climate change influences a multitude of physical and chemical processes in coastal areas (e.g. increase of temperature, salinity changes, acidification, decreased oxygen solubility, sea level rise), that may deeply affect biological functioning of species living in these habitats. Coastal areas are threatened to urbanization such as land-use changes. Therefore, the coastal areas are particularly threatened by climate change especially the deltas, low-lying coasts, wetlands, lagoons and estuaries.

Climate change will produce a series of direct and indirect impacts on coastal zones, but the magnitude of these impacts is not well known in most of cases. The existing literature aiming to evaluate the environmental impacts of climate change on Mediterranean coastal wetlands is scarce, and most of the few existing studies are quite speculative. In relation to the deltas,

most of the research has been carried out in the Ebro, Po and Rhône, and focuses primarily on the effects of rising sea level, which is a direct consequence of climate change. Regarding the coastal lagoons, the existing studies focus on the impacts on hydrology and salinity. The impacts of factors such as higher temperature or changes in rainfall were poorly studied. Much more research is needed to quantify and predict the effects of climate change on Mediterranean coastal wetlands, where the expected impacts are certainly not negligible.

If we focus on projection data for the local area (*i.e.* Northwestern Mediterranean), the tendencies are generally the same than what is projected for global trends in the Mediterranean Sea. According to Gualdi et al. (2013), studies based on global climate models or uncoupled regional climate model simulations give a general picture of more warm/hot extremes temperature and fewer cold extremes, which is consistent with the changes observed in the Mediterranean Sea over the last few decades (Goubanova and Li 2007; Diffenbaugh et al. 2007; Giannakopoulos et al. 2009; Fischer and Schär 2010). However, in Northwestern Mediterranean, a local increase of extreme precipitation events may be expected. In Figs 2 and 3, in most parts of the Mediterranean region the scenario considering all climatic changes (indicated as "SCE" in red, Fig. 2) shows an increase in the intensity of heavy precipitation events (expressed as the 95^{th} percentile of daily precipitation on wet days) through the course of the year. **These projections highlight that species living in enclosed body of water such as lagoons may be exposed to an increased frequency of extreme events such as heavy rains and warmer temperatures.**

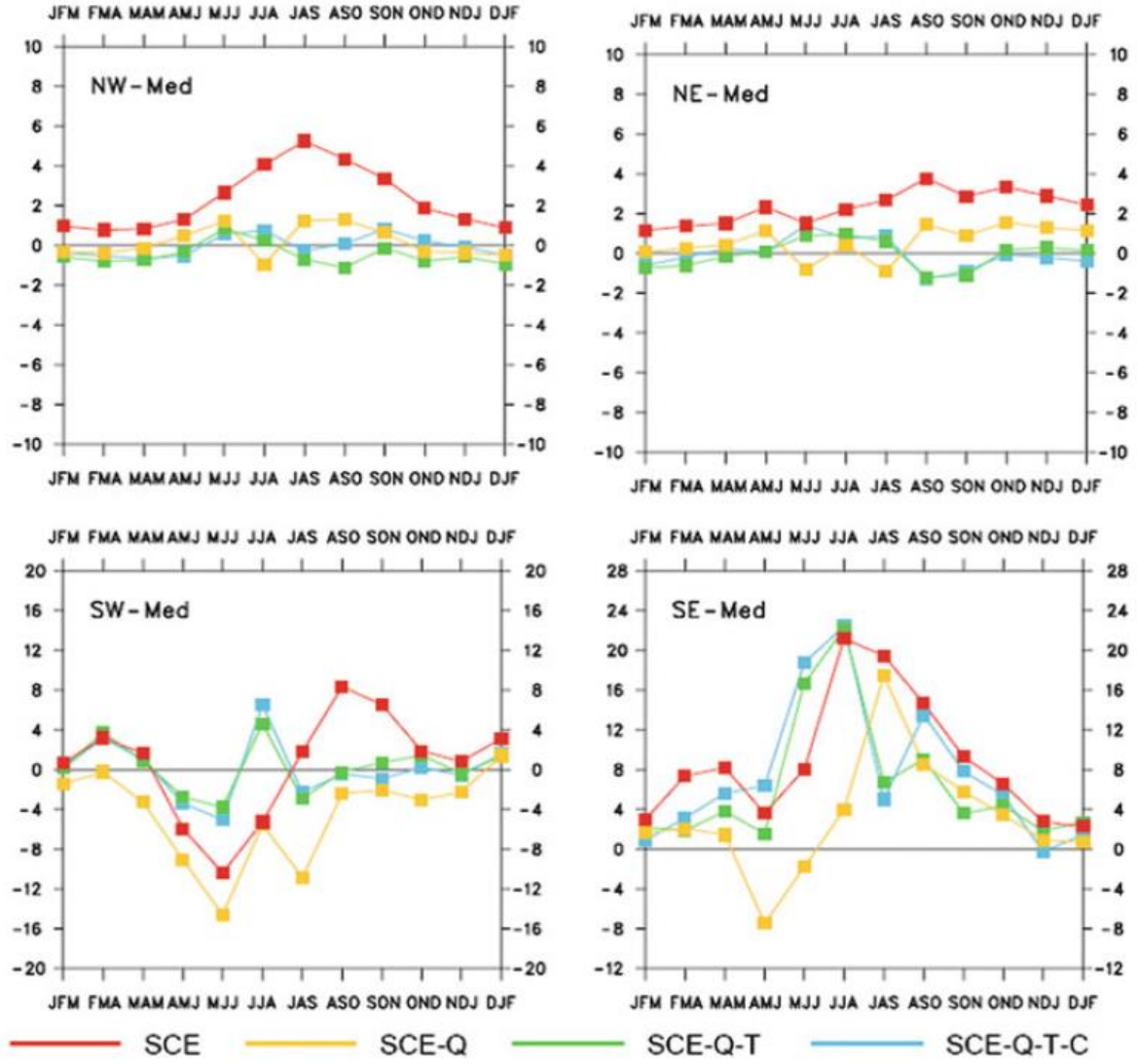

Fig. 2: Greenhouse gases changes in the 95th percentile of daily precipitation for four different scenarios for the period 2071-2100 with respect to present-day climate conditions for the period 1971-2000 simulated with the HIRHAM regional climate model. The values represented are averages (land points only) for four parts for different seasons, defined as overlapping 3-month periods. Units are [mm]. SCE: scenario considering all climatic changes; SCE-Q: scenario that excludes the general increase in specific humidity throughout the atmosphere; SCE-Q-T: scenario that also excludes the increase in temperatures at the sea surface as well as throughout the atmosphere; SCE-Q-T-C: scenario that also excludes the large-scale circulation changes (from Gualdi et al., 2013)

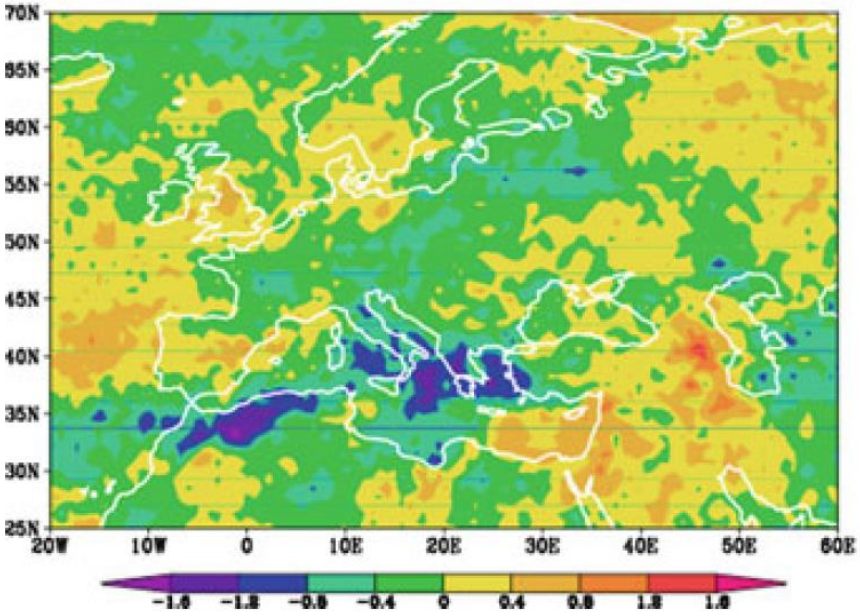

Fig. 3: Climate change trend (2021–2050 minus 1961–1990) in frequency of days with extreme precipitation in January obtained from INGV (from Gualdi et al., 2013)

1.3 Climate change impacts on aquatic animals

1.3.1 Biodiversity and species extinction

Coastal species are often subjected to a mixture of stressors (Przeslawski et al. 2015) which makes coastal ecosystems vulnerable to the effects of global warming (Pörtner et al. 2001; Pörtner 2002; Daufresne et al. 2004) . The above mentioned stressors induce changes in species abundance, habitat loss and distribution shifts, leading to extinctions of species (Perry et al. 2005) and an overall decrease or change in biodiversity (review in Pereira et al. 2010). For example, Daufresne et al. (2004) analyzed fish abundance over the last 20 years in the upper Rhone River (France) and showed an increasing dominance of southern and eurythermal species in the fish community. Therefore, there is a need of research on species that tolerate and survive in these unstable habitats. Improving our knowledge of physiology and ecology of marine and estuarine fishes is thus relevant in the context of climate change (review in Roessig et al., 2004).

1.3.2 Habitat distribution

Increasing temperature is known to be one of the most influential factor that determines species distribution (Pörtner et al. 2001; Pörtner 2002). Habitat fragmentation leads to small,

isolated populations that have an increased risk of extinction because of stochastic environmental fluctuations and inbreeding depression (Mills and Smouse 1994). Perry et al. (2005) observed important changes in abundance and extension of distributional ranges in marine species during a period of 25 years. Hence, Chevaldonne and Lejeusne (2003) showed replacement of endemic species, reduction in abundance and changes in distribution during warming with an overall increased temperature tolerance. Prediction of such distributional changes demands to determine heat tolerance that correlates with organism performance in natural habitats (Pörtner 2002).

1.3.3 Thermal performance

Organisms have different capacities in coping, acclimating and adapting to environmental temperature and its variation (Beitinger and Lutterschmidt 2011). In particular, the physiological processes for thermal performance are different between temperate and tropical regions. Temperate organisms are often exposed to a wider range of temperature compared to tropical organisms. The temperate climate is cooler and more variable requiring a broader thermal tolerance and higher acclimation plasticity (Pörtner 2001). Tropical organisms live near to their upper thermal limits as they experience narrow seasonal variation in temperature (Storch et al. 2014). Measures of thermal tolerance may be important to consider in the context of global climate change (Beitinger and Lutterschmidt 2011). For example, lower and upper thermal tolerance of aquatic animals is being compared using critical thermal methodology (CTM). It is defined as the "maximum temperature that can be tolerated by an organism. In fish, this variable is most often measured by acutely increasing the temperature (e.g., by 2 °C h^{-1}) until the fish loss equilibrium" (Currie and Schulte 2014). The more integrative concept of oxygen- and capacity-limited thermal tolerance (OCLTT) defines thermal constraints on the capacity for oxygen supply to the organism in relation to oxygen demand (Pörtner et al. 2017). These and other approaches give a mechanistic explanation about the borders of the niche of functional capacity of animals with regard to temperature and species interactions (Pörtner and Peck 2011).

1.3.4 Acidification and hypoxia

Projected levels of ocean warming and acidification can alter oxygen supply (Widdicombe and Spicer 2008; Heuer and Grosell 2014; Hu et al. 2016). Higher temperature and CO_2 can cause hypoxia in fish through increased oxygen demands and decreased capacity for oxygen

supply to the tissues (Pörtner and Knust 2007; IPCC 2015; Przeslawski et al. 2015). For example, occurrence of elevated temperature and CO_2 increase the sensitivity of fish because of the functional link and it reduces tissues functional capacities including tissues involved in oxygen supply (Pörtner and Knust 2007). Temperatures changes can thus affect acid-base status, including shifts in extracellular and cellular pH. As a result, partial pressure of oxygen (pO_2) levels decrease in the body fluid and higher temperatures reach critical levels earlier than at normoxia. Such effects would favor that animal are limited in pH regulation capacity and cause a setpoint to acid-base regulation, notably when temperature extremes are already causing hypoxemia (hypoxia in body fluids) (Pörtner and Peck 2011). Thermal sensitivities differ among species and which will cause changes in species interactions and thereby functional shifts observed at the ecosystem level (Pörtner and Knust 2007).

1.4 Environmental stress and fish acclimation

1.4.1 Temperature acclimation

Temperature is a limiting factor in the distribution of organisms and reproductive processes. Thermal acclimation in animals involves several behavioral and physiological features including avoidance, tolerance and thermoregulation. Two types of responses exist in animals regarding changing temperatures, 'poikilothermy' *vs* 'homeothermy'. In poikilotherm animals, the body temperature varies according to the environment notably due to a low capacity to produce heat and to control body temperature. In contrast, homeothermic animals maintain a constant body temperature regardless the external temperature notably through an active thermoregulation and an increased capacity of thermal isolation. Fishes belong mostly to the poikilothermic group. The range of a temperature tolerance for fishes is from a few degrees below 0 °C up to about 45 °C (Schulte 2011a). Eurythermal species like viviparous *Poeciliopsis*, annual killifish *Austrofundulus limnaeus*, goby *Gillichthys mirabilis* and common killifish *Fundulus heteroclitus* live at large temperature ranges whereas other species live at a much narrower range of temperatures, and are known as stenotherms e.g. sharp-spined notothenia *Trematomus pennellii* and emerald notothen *Trematomus bernacchii* (Schulte 2011a; Currie and Schulte 2014). In fish, the rapid loss of internally generated heat to the aquatic environment is notably due to branchial respiration. The gills are vascularized and represent a large surface for potential heat loss. Temperature greatly affects physiological

performance and biochemical reactions in ectothermic animals (Schulte 2011a, 2015; Moyes and Schulte 2014).

Physiological performance is highly dependent on oxygen availability that also depends on environmental parameters (Sollid et al. 2005). Generally, aquatic animals extract oxygen from water, and water has a very low content of dissolved oxygen compared to air (Kramer 1987). A dissolved oxygen concentration[2] of 5 mg/L or more is suitable for aquatic life. A dissolved oxygen level that is out of this range can have adverse effects on aquatic life and modifies water quality[3]. Hence, increasing temperatures result in a lower solubility of oxygen in the water (Pörtner et al. 2001; Pörtner 2002, 2008). Phenotypic changes in teleost species exposed to oxygen stress linked to temperature increases have been reported in several studies, notably regarding the gill level (Sollid and Nilsson 2006; Tzaneva et al. 2011, 2014).

Thermal tolerance is an essential physiological phenomenon for ectotherms and is strongly dependent on the ambient temperature (Somero 2005; Moyes and Schulte 2014). The phenotype plasticity in response to a change in body temperature depends on the capacity of modulating gene expression in an adaptive manner (Somero 2005). As explained by Horowitz (2007), organisms use diverse adaptive strategies to ensure their existence in a thermally heterogeneous environment. Effects of global warming are species dependent and can be determined by the acclimatory response. Organisms in their natural environment have to be acclimatized to a complex of factors, whereas in laboratory conditions, only one or two factors are generally considered. Many parameters could be analyzed in ectotherms notably thermal acclimation referred to as eccritic temperature, thermal optimum, thermal preferendum, selected temperature or preferred temperature (Hutchison and Maness 1979). In this thesis, we will refer to temperature acclimation, in contrast to evolutionary adaptation, as "within lifetime" phenotypic adjustments involving a suite of modifications that allow an organism to shift its thermal optimum for numerous physiological activities to new temperature ranges to efficiently reach acclimation homeostasis (Horowitz 2007).

Temperature effects have been found to affect different levels of organization from genes to organism (Willmer et al. 2005; Schulte 2011a). Several studies have shown that temperature affects lipid composition of membranes (Crockett 2008; Grim et al. 2010; Rodríguez et al. 2017), membrane fluidity (Zehmer 2005; Kammer et al. 2011; Gonzalez et al. 2013) and

[2] http://academic.keystone.edu/jskinner/Limnology/Water_Chemistry_LectureNotes.htm
[3] https://www.fondriest.com/environmental-measurements/parameters/water-quality/dissolved-oxygen/

protein structure and function (Kultz and Somero 1996; Buckley et al. 2006). Enzyme activities such as gill Na^+/K^+-ATPase (NKA) activity can depend on acclimation temperature (Fiess et al. 2007; Sardella et al. 2008; Mitrovic and Perry 2009; Zydlewski and Wilkie 2012). For example, in the common carp *Cyprinus carpio,* NKA activity was decreased at low acclimation temperatures (15 and 22 °C) and was significantly higher at 29 °C acclimated fish (Metz et al. 2003). The effects of temperature on biological processes can be determined by the Arrhenius plot (*e.g* Metz et al. 2003; Michael et al. 2016a) in order to determine the activation energy at each temperature. It seems important to assay the enzyme activity (*e.g*. NKA) at the acclimation temperature of the fish in order to have better interpretations of the physiological condition. The NKA activity can be assayed at two or a series of temperatures and is then plotted in relation to temperature (Metz et al. 2003; Michael et al. 2016a). A change in membrane fluidity according to temperature typically results in a breakpoint of the Arrhenius plot indicating a change in membrane protein function (Metz et al. 2003; Moyes and Schulte 2014; Fields et al. 2015).

Enzyme adaptation to temperature over evolutionary time is based on the amino acid changes in which stabilization (for higher temperature adaptation) and destabilization (adaptation to colder temperatures) occur in areas of the molecule whose motions are necessary for catalysis (Fields et al. 2015). At high temperatures, the maintenance of protein stability is particularly important (Deller et al. 2016). In most extreme conditions, like in the hyperthermophilic bacterium *Thermotoga maritima*, proteins, thermal stability is inferred by subtle changes in the amino-acid composition (Zhou et al. 2008). Some fish species inhabiting environments with steep temperature fluctuations have modulated amino acid composition (through substitutions of 1-2 amino acids) in order to optimize protein function at low temperatures (*e.g*. maintain Michaelis constant (Km) of pyruvate in a narrow 0.15-0.35 mM range) (Fields and Houseman 2004; Fields et al. 2015; Yang et al. 2015). Hence, functional and structural properties of proteins can be of importance in the balance between rigidity (that arise from cold stabilization) and flexibility (from warm stabilization) of structure that will greatly affect protein activity (Hochachka and Somero 2002; Fields et al. 2015).

Molecular studies have been conducted to analyze fish responses to increased temperatures (Podrabsky and Somero 2004; Buckley et al. 2006; Chou et al. 2008; Logan and Somero 2010, 2011; Evans et al. 2011). Eurythermal fish can tolerate wide ranges of temperature and maintain physiological and biochemical processes through the modulation of the expression of numerous genes. In Medaka *Oryzias latipes* using a microarray analysis on skeletal muscle

tissues exposed to 10 and 30 °C for 5 weeks, 11 genes involved in energy metabolism and muscle atrophy were significantly upregulated at 10 °C. On the other hand, at the higher temperature, 20 genes encoding myofibrillar proteins and heat shock proteins were highly expressed. This is highlighting the importance of heat shock proteins (HSPs) at high temperatures to avoid denaturation of other proteins (Ikeda et al. 2017). Following temperature stress, it is common to observe increased expression of HSPs. HSPs and other molecular chaperones interact with stress-denatured proteins in order to maintain their native structures and prevent aggregation and degradation (Hartl 1996). Podrabsky and Somero, (2004) have used cDNA microarray analysis to examine changes in gene expression in liver of killifish *Austrofundulus limnaeus* subjected to constant temperatures of 20, 26 and 37 °C for up to two weeks and then to environmentally realistic daily fluctuations in temperature between 20 °C and 37 °C. The authors have shown that small heat shock proteins appeared to play an important role in response to fluctuating temperatures, while larger molecular mass chaperones such as HSP70 and HSP90 responded more strongly to a chronic high temperature stress.

Gills have been in the center of interest in several studies due to their key role in multiple physiological processes (Gracey et al. 2004; Evans et al. 2005; Buckley et al. 2006; Nilsson 2007; Chou et al. 2008; Buckley and Somero 2009; Healy et al. 2010; Logan and Somero 2010, 2011; Quinn et al. 2011; Evans et al. 2011; Jeffries et al. 2012; Tan et al. 2012; Huth and Place 2013; Liu et al. 2013; Jeffries et al. 2014). Temperature has been reported to affect gill morphology in order to optimize oxygen uptake (Sollid et al. 2005; Mitrovic and Perry 2009). Temperature can in fact alter the gill surface area which can thus favor or not the mechanisms of respiration, ion transport, acid base regulation and nitrogen excretion. Temperature is also known to affect gene expression at the gill level, such as in *Danio rerio*, where low temperatures caused an upregulation of numerous genes that are involved in ionoregulation and acid-base balance (Chou et al. 2008). In longjaw mudsucker *Gillichthys mirabilis*, gene expression patterns of different biological processes were compared between 9 °C, 19 °C and 28 °C. At the higher temperatures (19 °C and 28 °C were significantly different to 9 °C) genes encoding proteins involved in proteolysis, ion transport and suppression of cell division were upregulated. A decreased cell division suggests that sublethal thermal stress may result in reestablishing the energy flow in cells toward repair processes (Logan and Somero 2010). The effect of temperature on ion transport will be discussed in section 1.4.4 on the combined effects of temperature and salinity in fish.

As reported above, global warming is thought to have strong impacts on fish physiology in the future (Pörtner, 2008, 2002; Pörtner et al., 2001; Coppes and Somero, 1990; Ficke et al., 2007; Poloczanska et al., 2013). Finally, "who is looser and winner in the era of climate change" has been discussed by Somero, (2010) in exactly these words in his review. "Depending on generation time, population size, the level of genetic variation in a population and other factors, adaptive evolution of proteins potentially may occur rapidly enough to "keep pace with" climate change. Species possessing this potential for rapid evolution could emerge as (survivors) in a warming climate" (Somero, 2010). Further, a more challenging problem in adapting to climate change arises for species that have lost genetic information required for life at increasing temperatures as a consequence of long evolutionary periods under highly stable conditions of low temperature like Antarctic marine stenotherms. Such losses of protein-coding genes and gene regulatory mechanisms may in many cases be essentially irreversible, and may place cold-adapted stenothermal species in extreme vulnerability from the predicted rise in air and sea temperatures (review in Somero 2010). Moreover, while species are historically acclimated or adapted to changes in climate, the rapid rate of current climate change coupled with increasingly fragmented and impaired habitats present unprecedented challenges for modern species. Currently, the consequences of such environmental changes on fish population dynamics are poorly understood and long-term rearing experiments related to the plasticity and adaptability of life-history traits are now needed.

1.4.2 Salinity acclimation

According to salinity tolerance, fishes are considered either stenohaline or euryhaline (Kultz 2015). A larger number of fishes are stenohaline, which means that they are highly sensitive to salinity changes as shown in channel catfish *Ictalurus punctatus* and goldfish *Carassius auratus* (Altinok 2001). Stenohaline fish inhabit osmotically stable environments as oceans, freshwater lakes and streams (Kultz 2015). On the other hand, some fish species tolerate high salinity ranges and are thus euryhaline, for example rainbow trout *Oncorhynchus mykiss*, Gulf sturgeon *Acipenser oxyrinchus desotoi* and sea bass *Dicentrarchus labrax*. Physiology of fishes changes according to the ionic composition of the environment. All vertebrates have to regulate intracellular and extracellular ionic compositions in order to maintain normal operation of cellular and biochemical reactions. Some aquatic environments are challenging because of fluctuating ionic compositions that directly affect the homeostasis of body fluids.

Table 2 indicates the concentration of major solutes of different aquatic environments. In seawater, Cl^- and Na^+ are major osmolytes whereas in hard fresh water, Ca^{2+} can also be a key osmolyte.

Table 2 Major solutes (mmol.L^{-1}) in different aquatic habitats[1].

Ions (mmol L^{-1})	Sea water	River water	Hard river water	Soft lake water
Na^+	439	0.39	6.13	0.17
Cl^-	513	0.23	13.44	0.03
K^+	9.3	0.04	0.11	-
Ca^{2+}	9.6	0.52	5.01	0.22
Mg^{2+}	50	0.21	0.66	0.15

[1]Table adapted from the book fish physiology, chapter 6. Gill ionic transport, acid-base and nitrogen excretion) (Hwang and Lee, 2007)

Fish have evolved different strategies to maintain body fluids and ionic homeostasis. There are two main osmoregulatory strategies, called hypo- and hyper-osmoregulation (review in Kultz 2015). In the process of hypo-osmoregulation, fish maintain body ion composition below that of seawater (SW). In SW, there is a passive loss of water across the gills and fish must drink to maintain fluid balance. The intestine actively absorbs salts and secondarily water in order to avoid dehydration. The excess salts are secreted actively across the gills (and skin epithelia in larvae). At low salinities, teleost fish hyperosmoregulate and maintain blood osmolality higher than the surrounding water. In a dilute environment, there is a diffusive ion loss and an osmotic water gain across the large surface area of the gill epithelium. Fish gain ions by active ion uptake that occurs through highly specialized cells, called ionocytes or mitochondrion-rich cells (MRCs) (Marshall and Grosell 2006). MRCs are also involved in other functions like acid-base regulation, nitrogen excretion and Ca^{2+} uptake. Therefore, MRCs are playing an important role in acclimation to various environments (review in Kaneko et al. 2008).

Euryhaline species are more effective in their capacity to tolerate salinity fluctuations and salinity extremes (Hwang and Lee 2007; Hiroi and McCormick 2012). Amphihaline fishes like salmonids are capable of making large scale migration from inland freshwater systems to rich and fertile ocean habitats for feeding. They then migrate back to their natal stream to reproduce (McCormick et al. 1998; Cooke et al. 2011). Salmons adjust themselves with an

appropriate level of osmoregulatory preparedness. This occurs at specific life stages, for example at the metamorphosis called smoltification when smolts become able to enter seawater thanks to a change in hormonal control and osmoregulatory mechanisms activated by environmental changes (temperature and photoperiod essentially). Freshwater entry of adults is also prepared through a downregulation of gill NKA despite being in full strength seawater (Cooke et al. 2011). Contrary to amphihaline species, euryhaline species undertake seasonal migrations towards lagoons and estuaries and are subject to large changes of abiotic environmental variables including salinity. Teleost fishes of intertidal habitats like mudskippers and killifish are capable to tolerate high salinity fluctuations and are able of rapidly modify their osmoregulatory mechanisms in response to salinity changes (Schulte 2011b). This is also the case of the European sea bass, a marine teleost fish able to migrate between the sea and lagoons (Potts 1995; Pawson et al. 2007). Sea bass are able to maintain their blood osmolality relatively constant independently of environmental salinity (Varsamos et al. 2001).

Irrespective to environmental salinity (FW, brackish water, SW or hypersaline), teleost fishes maintain their blood osmolality at around 300-380 $mOsm.kg^{-1}$. Some fish species are osmoconformers, like hagfish for example. Elasmobranchs are slightly hyperosmotic to SW due to the accumulation of urea and trimethylamine-N-oxide (TMAO) in the blood (944 to 1,095 $mOsm.kg^{-1}$) as an adaptive strategy to avoid dehydration (Marshall and Grosell 2006).

Several studies have investigated teleost fishes to understand the osmoregulatory process following salinity changes (Lin et al. 2003; Sardella et al. 2004b; Lorin-Nebel et al. 2006; Hiroi and McCormick 2007; Bossus et al. 2013; Blondeau-Bidet et al. 2016). In fish, the major osmoregulatory organs are the gills, kidney, gastrointestinal tract, rectal gland (elsmobranches), urinary bladder, skin (in larvae) and opercular membrane (Marshall and Grosell 2006). Gill plasticity can be considered as an adaptive and reversible change in teleost fish depending on environmental conditions (Sollid and Nilsson 2006; Gilmour and Perry 2018). Salinity change can lead to morphological modifications at the gill level in order to optimize ion uptake (review in Marshall 2002). MRCs size, number and distribution varies according to salinity (review in Marshall 2002; Evans et al. 2005).

Teleost gills have been at the center of interest to investigate MRCs subtypes (FW subtypes notably) to investigate ionoregulatory pathways in fish. The ultrastructure and localization of ion transporters in MRCs required convincing cellular and physiological evidence to support

the proposed ionoregulatory pathways (Hwang and Lee 2007). The gill is a multifunctional organ and is a major site for gas exchange, ion transport, acid-base regulation and nitrogenous waste excretion (Wilson and Laurent 2002; Evans et al. 2005; Marshall and Grosell 2006). The specific ionocytes/mitochondrion-rich cells (MRCs) present in the gills present numerous ion transporters in apical and basal membranes involved in transepithelial ion uptake and excretion as well as intracellular regulation (Marshall and Grosell 2006). In sea bass gills, NKA is transcriptionally regulated following salinity change (Blondeau-Bidet et al. 2016) and its activity is also modulated (Jensen et al. 1998; Nebel et al. 2005). Changes in branchial NKA activities in euryhaline teleost are thought to be a classical response for salinity change (Jensen et al. 1998; Lin et al. 2003; Tang et al. 2010b). It consists of two subunits, α and β. Two isoforms of subunit α1 were identified in sea bass (Blondeau-Bidet et al. 2016) and in other teleosts (Seidelin et al. 2000; McCormick et al. 2009; Armesto et al. 2014), and seem to be transcriptionally regulated following salinity change.

In SW, Na^+ and Cl^- secretion is affected by three critical transporters, the Na^+/K^+-ATPase (NKA), the $Na^+/K^+/2Cl^-$-1 cotransporter (NKCC1) located in basolateral membranes of ionocytes, and the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel, located in apical membranes (Hwang and Lee 2007; Hwang et al. 2011; in review Hiroi and McCormick 2012). The NKA provides an electrochemical gradient to regulate the process of Na^+ and Cl^- secretion. Other ions transporters have been identified in SW gills involved in acid-base regulation (for example the apical Na^+/H^+ exchanger NHE2 and 3) and nitrogen excretion (involving apical Rhcg and basal Rhbg) (Nakada et al. 2007a; Braun et al. 2009).

In FW gills, ion uptake is a critical process and different ionocyte models have been established to better understand ion uptake in fish. The pioneering work done on FW fish gills by Krogh (1937, 1938) suggested that Na^+ and Cl^- uptake are respectively linked to NH_4^+ and HCO_3^- excretion which intimately links osmoregulation and acid-base regulation processes. Later studies have investigated different pathways for apical Na^+ uptake. Na^+ uptake has been shown to be linked to acid secretion via V-type H^+-ATPase or NHE2 or 3 and nitrogen excretion via Rh glycoproteins (Nakada et al. 2007a; Yan et al. 2007). The electroneutral NHE2 or 3 mediates Na^+ uptake and H^+ secretion in FW teleost gills (Evans 2011; Hwang et al. 2011) including sea bass (Blondeau-Bidet et al. 2019). Another pathway has been investigated that involves an apical Na^+ - Cl^- contransport via a Na^+ - Cl^- contransporter (NCC2). NCC2 has been suggested to be localized in apical membranes of ionocytes in several teleosts following FW transfer (Hiroi et al. 2008; Inokuchi et al. 2008, 2017). Various

basolateral transporters have been identified in several fish species for example Cl^- channels (ClC-2 or 3), the Na^+/HCO_3^- cotransporter (NBC1) or HCO_3^-/Cl^- exchangers (for example anion exchangers, AE), that facilitate basal Na^+ or Cl^- uptake and will be analyzed in this work (in review Evans 2011; Hwang et al. 2011).

1.4.3 Acid base regulation

The preservation of constant blood pH is essential to maintain cellular functions. Intertidal environmental conditions are fluctuating regarding salinity, temperature and oxygen levels. All these parameters greatly affect acid-base status in fish (Reeves 1977; Evans et al. 2005). In temperate areas, water temperature often increases during the day and decreases at the night whereas salinity status is highly influenced by temperature (evaporation) and climate conditions (precipitations, tights, freshwater input). Water ionic composition is known to affect acid-base balance in fish. Fish gills are major sites for acid-base regulation and contribute by 90% to the organism's total acid-base regulation (Evans et al. 2005; Marshall and Grosell 2006). FW species maintain ionic balance by an uptake of Na^+ in exchange of H^+/NH^+_4 (acidic residues) whereas Cl^- is taken up in exchange of HCO^-_3 (basic residue) (Krogh 1938). Subsequently, a change in Na^+ or Cl^- in the water body may lead to metabolic acid-base disturbance in fish (alkalosis or acidosis). Depletion of Na^+ in the water can lead to acidosis and on the other hand Cl^- depletion caused blood alkalosis (review in Goss et al. 1998) and has to be restored by respiratory or metabolic compensations. Ectotherms generally decrease their blood pH as temperature increases to secure protein conformation (Johnston and Dunn 1987; Somero 1995). In fish, this is mostly achieved by accumulating HCO^-_3 in extracelluar spaces (Truchot 1988). Hyperventilation is a general respiratory response to hypoxia and contributes to restore O_2 levels and to avoid hypercapnia by excreting excess CO_2 to the water. Net transport of acid-base relevant molecules between the animal and the environment using transporters expressed mostly in the gill epithelium is another mechanism to compensate acid-base disturbances. The effects of salinity and/or temperature on acid-base status have been studied by several authors (Reeves 1977; Goss et al. 1998; Claiborne et al. 2002; Tresguerres et al. 2005; Shaughnessy et al. 2015; Fehsenfeld and Wood 2018). Proteins involved in acid secretion include apical V-type H^+-ATPase (VHA) and Na^+/H^+ exchangers (NHEs) as well as cytoplasmic carbon anhydrase 2 (CA2) (Georgalis et al. 2006; Hsu et al. 2014). Apical $Cl^-/HCO3^-$ anion exchangers (AE), basal Na^+/HCO_3^- cotransporter 1 (NBC1) are involved in base absorption or secretion. (Truchot, 1988).

1.4.4 Combined effects of temperature and salinity

The simultaneous changes of several abiotic parameters can become stressfull for fish. In aquatic ecosystems, fish are routinely subjected to a combination of different stressors. The combination of two or several stress will require a physiological response that optimizes acclimation to all of these stressors. Following a temperature stress, for example, water dissolved oxygen is low and fish need more surface area to ensure gas exchange. Gill remodeling can be costly for fish metabolism and also affects other gill functions. Salinity and temperature changes represent an excellent example of two different stress conditions that occur in coastal ecosystems simultaneously. High or low temperatures are known to affect osmoregulatory organ morphology (e.g. gill) and thus affect osmoregulatory capacity (e.g. blood osmolality and NKA activity). The effects of temperature and salinity on hydromineral balance are highly variable among species (Imsland et al. 2003; Metz et al. 2003; Sardella et al. 2004a; Fiess et al. 2007; Sardella et al. 2008; Vargas-Chacoff et al. 2009b). Several studies have reported changes in blood osmolality following thermal stress as in turbot *Scophthalmus maximus,* Mozambique tilapia *Oreochromis mossambicus* and Mozambique tilapia hybrids *Oreochromis mossambicus x O. urolepis hornorum* (Imsland et al., 2003; Sardella et al., 2008, 2004a). Gill structure (grouper *Epinephelus coioides*, threespinse stickleback *Gasterosteus aculeatus,* zebrafish *Danio rerio* and gambusia *Gambusia affinis*) (Caberoy and Quinitio 2000; Uliano et al. 2010; Gibbons et al. 2018), and Na^+/K^+-ATPase activities (Stuenkel and Hillyard 1980; Kültz and Somero 1995; Sardella et al. 2008; Vargas-Chacoff et al. 2009a; Arjona et al. 2010; Michael et al. 2016a) are clearly modified following heat stress.

Few studies have shown the interactive effects of elevated temperature and salinity decrease (< 8‰) on fish species (Kultz and Somero 1996; Caberoy and Quinitio 2000; Metz et al. 2003; Nichols and Playle 2004; Uliano et al. 2010; Gibbons et al. 2018). Vargas-Chacoff et al. (2009) have stated that extreme temperatures alter the metabolic response at different salinities, as it is metabolically expensive for an animal to acclimate to both environmental parameters. In another study conducted on Mozambique tilapia, *Oreochromis mossambicus,* exposed to a combined challenge, gill NKA activity, glucose metabolism and osmoregulatory hormones were altered following salinity and temperature acclimation (Fiess et al. 2007). This shows that alteration of physiological processes is part of the adaptive response in order to survive under adverse conditions.

Several studies have assessed microscopic examination of teleost gills under combined stress (Metz et al. 2003; Mitrovic and Perry 2009; Gibbons et al. 2018). Gill morphology can be affected by temperatue and salinity. Temperature is also a potent modifier of ionocytes density, as reported in common carp *Cyprinus carpio* (Metz et al. 2003). Gill remodeling may affect ion transport flux and gas exchange (Kreiss et al., 2015b), by altering branchial respiratory surface area and water-blood diffusion distance (in review Nilsson 2007). It has been shown in European eel, *Anguilla anguilla*, that temperature acclimation induced morphometric changes in fish gills that increased branchial water-blood barrier by thickening the gill strucutre (Tuurala et al. 1998). The change in gill ionocyte number is a response that reflects fish capacity to regulate ion transport to cope with environmental challenges (review in Hwang et al. 2011).

Molecular techniques have been applied in the study of combined stress in gills of several teleost species (Metz et al. 2003; Brauer et al. 2005; Mitrovic and Perry 2009; Mladineo and Block 2009; Michael et al. 2016a; Gibbons et al. 2018). In long-jawed mudsucker *Gillichthys mirabilis*, a euryhaline and eurothermic fish, the effects of acute thermal stress on gene expression were examined at 9 °C, 19 °C and 28 °C (Logan and Somero 2011). Acute heat stress induced mRNA expression in genes encoding different ion transporters involved in osmoregulation and ammonia transport (for example the ammonia transporters *rhbg* and *rhag*) (Logan and Somero 2010, 2011). In zebrafish *Danio rerio* acclimated to 12 °C and compared to 28 °C (control), proteins involved in ionoregulation were highly upregulated in cold (12 °C) acclimated fishes. In the tropical milk fish, *Chanos chanos*, Kang et al. (2015) have shown that the protein abundance of HSP 70 at a low temperature increased in the gills at both salinities (FW and SW) to protect the cell and maintain native functions. Table 3 summarizes the effect of combined stress and the reported responses, allowing a better insight into the mechanisms of combined stress.

Marine species are exposed to multiple stressors including natural environmental stressors but also increase effects of anthropogenic stressors, particularly increasing global temperatures, reduced pH, increased salinities or pulses of decreased salinity (Adams 2005; Przeslawski et al. 2015; Chatzinikolaou et al. 2016; Lange and Marshall 2017). Environmental salinity influences ion transporters regulation and acid-base regulation (Evans and Somero 2008). However, the interactive effects of salinity and temperature on gill morphology, enzyme activity and ion transport gene expression have not previously been examined in sea bass, which justifies this study.

Table 3 Different effects of temperature on osmoregulatory processes analyzed in several fish species. Studies are listed in alphabetical order.

Species	Acclimation		Results	References
Mummichog *Fundulus heteroclitus*	Control 20 °C	5 °C for 24 days at 32 ‰	Gill remodeling occurred at low temperature SW acclimated fish, to limit surface area for diffusive NaCl gain while maintaining the function of gill ionocytes (NaCl secretion) as oxygen solubility is high in cold water and oxygen demand is low.	(Barnes et al. 2014)
Killifish *Fundulus heteroclitus*	Control 15 °C	20 °C, 23 °C and 30 °C for three weeks	Gill surface area was modified at all temperatures tested showing an important thermal plasticity in killifish gills.	(McBryan et al. 2016)
Rock cod *Eleginops maclovinus*	Control 10 °C	14 and 18 °C for two weeks	Blood osmolality did not change significantly but gill NKA activity was affected by temperature, at each temperature fish modulated enzymatic NKA activity.	(Oyarzún et al. 2018)
Goldfish *Carassius auratus*	Control 18 °C two weeks	7 and 25 °C for two weeks	Gill modification reported for each acclimated temperature.	(Tzaneva and Perry 2010)
Largemouth bass *Micropterus salmoides*	Control 20 °C for two weeks	8 °C, 15 °C, 25 °C 32 °C for 1h and 6h	Physiological parameters (Na^+, Cl^-, K^+) were measured under temperature stress. The rates of ion exchange are not positively related to temperature but are upregulated via cortisol to compensate decreased ion levels at low temperature.	(Vanlandeghem et al. 2010)
Ray-finned *Schizothorax prenanti*	Control 11 °C	16 °C, 21 °C, 26 °C and 31 °C for two weeks	Branchial NKA activity was significantly increased when temperature was high or low compared to the optimum temperature.	(Yang et al. 2018)
Shortnose sturgeon *Acipenser brevirostrum*	Control 15 °C for 4 weeks	Critical thermal tolerance measured	Na^+ and Cl^- levels were not affected by temperature stress but plasma K^+ concentration is significantly higher. Thermal stress did not change the expression of heat shock protein 70 and 90, but glucose level and oxygen consumption rate increased.	(Zhang et al. 2017)

1.4.5 Objectives

The general objective of the present study was to investigate the combined effects of temperature increase and salinity decrease in the European sea bass in order to analyze its physiological capacity to tolerate abrupt salinity change in either temperate condition (18 °C) or warm condition (24 °C).

For this, we examinated:

- whole organism hydromineral balance through blood osmolality measurements (chapter III)
- ion balance through plasma Na^+ and Cl^- assays (chapter III)
- gill morphology and plasticity using light microscopy and scanning electron microscopy (chapter III)
- gill ionocyte and mucocyte density using histology and immunocytochemistry (chapter III and V)
- branchial gene expression analysis using qRT-PCR (chapter IV and V)

These physiological traits were compared between fish in temperate (18 °C) and warm (24 °C) seawater conditions and following salinity transfer at the two respective temperatures.

Chapter II

Materials and Methods

Chapter II - Materials and Methods

2.1 European sea bass *Dicentrarchus labrax*

The sea bass *Dicentrarchus labrax* (Linnaeus 1758) (Table 4) is a euryhaline marine teleost fish. The sea bass is commonly found along the coasts of the north-east Atlantic Ocean and the Mediterranean Sea as well as in the Black Sea (Pawson et al. 1987; Pickett and Pawson 1994; Potts 1995; FAO 2005). The sea bass is a marine teleost species that tolerates a wide range of salinities and migrates to estuaries, lagoons, coastal waters and rivers (Pawson et al. 1987; Dufour et al. 2009). Sea bass possess strong osmoregulatory capacities and tolerate salinity ranges from fresh water (FW) up to 90 ‰ (Pawson et al. 1987; Barnabé 1989; Potts 1995). Sea bass are eurythermic fish and the range of temperature tolerance is 5-28 °C (Barnabé 1989).

2.2 Life cycle of sea bass

The spawning occurs offshore during the late spring and summer months (Kelley, 1988) and after metamorphosis from larvae to early juveniles (mean length ~15mm), sea bass migrate to suitable nursery habitats that are mostly brackish waters of lagoons and estuaries (Kelley 1988; Jennings and Pawson 1992). Juveniles and adults seasonally migrate to river mouths and to coastal lagoons, and few adults can enter freshwater areas like rivers (Pawson et al. 1987; Barnabé 1989).

Table 4 Taxonomy of sea bass

Kingdom	Animalia
Phylum	Chordata
Class	Actinopterygii
Order	Perciformes
Family	Moronidae
Genera	*Dicentrarchus* (Gill, 1860)
Specie	*Labrax* (Linnaeus 1758)

2.3 Sea bass as an experimental fish

Salinity acclimation occurs mainly because fish living with a wide range of salinity have developed hypo- and hyper-osmoregulatory strategies. The reason of selecting sea bass as an experimental fish, is their diverse distribution in various lagoons and estuaries (Dufour et al. 2009) that are characterized by salinity and temperature variation (Table 1). However, tolerance to salinity change may vary with other environmental parameters that can affect osmoregulatory processes. Other than temperature, pH and dissolved oxygen level potentially vary in shallow waters. For instance, few data are available on temperature effects on the osmoregulatory processes in sea bass. It underscores the need to investigate the sea bass physiology when fish are challenged to a suit of environmental stress to study osmoregulatory strategies. We can take the benefit of rearing sea bass in the laboratory for conducting controlled environmental variables. The wide-ranging laboratory experiments cover sampling that enables to analyze multiple levels from cell to organism.

2.4 Experimental conditions

All experimental protocols were conducted according to the guidelines of the European Union (directive 86/609) and of the French law (decree 87/848) regulating animal experimentation. European sea bass *Dicentrarchus labrax* were obtained from the Ifremer Station at Palavas-les-Flots (Hérault, France). Water was aerated and mechanically/biologically filtered (Eheim System, Lens, Pas-de-Calais, France). Fish were maintained in 200 L tanks (8-13 fish/tank). All fish were acclimated to sea water (SW) at 18 °C (temperate SW), SW at 24 °C (warm SW), then transferred to FW at 18 °C (temperate FW) and FW at 24°C (warm FW) respectively (Fig. 4). The experiment lasted four weeks. Fish were pre-acclimated for two weeks to either temperate (18 °C) or warm (24 °C) conditions (with a temperature increase of 0.2 °C/h), then each batch was divided into 2 groups that either remained in SW (at 18 or 24 °C) or were challenged to FW (at 18 or 24 °C). Experimental design for this study is presented more in details in Figure 4. Ionic composition (in $mEq.L^{-1}$) of the FW Na^+ (0.12), K^+ (0.04), Ca^{2+} (5.70), Mg^{2+} (0.29), Cl^- (0.98), NO_3^- (0.06) and SO_4^{2-} (0.61).

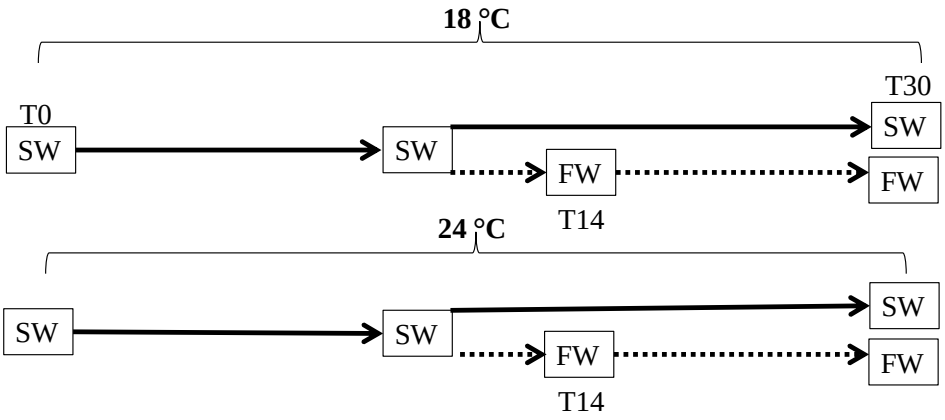

Fig. 4: Experimental design in sea bass. *D. labrax* were challenged for 14 days (T14) to 18 °C or 24 °C in seawater (SW) then challenged to fresh water (FW)

Temperature, salinity, oxygen and nitrogen levels were checked daily. Fish were fed twice a week with fish granules (Aphymar feed, Meze, Herault, France) until 2 days before sampling. Fishes length and body weight were recorded of each sampled fish. Fish weight and length were 86.87±20.23 g (mean ± SD) and 20.77±1.32 cm. Fish were sacrificed with an overdose of anaesthetic (2-phenoxy-ethanol, 500 mg L^{-1}) and blood sampling was done immediately. Later fish was dissected in order to collect gill tissues. Gill tissue for microscopy was fixed in Bouin (for immunohistochemistry) or gluteraldaldehyde 2% (for SEM). For biochemistry (NKA activity), tissue samples were immersed in tubes containing SEI buffer (300 mM sucrose, 20 mM Na_2EDTA, 100 mM imidazole, pH 7.4) and stored at -80 °C until further investigation. For qPCR analysis, gill tissues were flash frozen in liquid nitrogen in Trizol and stored at -80 °C until further analysis. On overview of subsequent analyses is given in Table 5.

Table 5 Different observations were used at different levels of animal

Observation	**Aim**	**Biological scale**	**Chapter**
Osmotic pressure	To analyze teleost osmoregulation capacity	Organism	III
Microscopy analysis	Gill histology	Tissue	III, V
Immunofluorescence	Localization of proteins in cellular compartments using antibodies	Tissue, protein and cell	III
Scanning electron microscopy	Structure of the gill epithelium	Tissue	III and V
Real time PCR	Quantifying the expression of messenger RNA	Gene	IV and V

2.5 Blood osmolality

Blood was sampled from the caudal vessel using a 1-mL syringe coated with heparin (Li-heparin, Sigma-Aldrich, France). Blood osmolality was measured in 8-13 fish per condition. The osmolality of 20 µL of blood was measured on an Advanced 3300 micro-osmometer using internal standards of 100, 300 and 1000 $mOsm.kg^{-1}$.

2.6 Sodium ion levels

Plasma was obtained by centrifugation of 8 min at 10000 g at 4 °C, then was stored at -20 °C. Flame photometry (Model 410[4], Sherwood, Cambridge, UK) was used for the determination of Na^+ ion level in the plasma. Plasma Na^+ concentration was determined by using a standard curve of Na^+, K^+ from 0 to 400 $mEq.L^{-1}$ and plasma at a 1/1000 dilution in MilliQ water. Before the determination of sodium concentration of a sample, all standard solutions emission intensities were measured. Three replicates were measured for each sample.

2.7 Chloride ion levels

The titration method was used for the determination of the Cl^- concentration (Schales and Schales 1941). Chloride was determined by a chloride titrator (AMINCO, Maryland, USA) coulometric titration with silver ions. The current is constantly passed between a pair of silver electrodes, which release silver ions into the titration solution at a constant rate. Once the chloride ions have combined with the silver ions, free silver ions will appear in the solution and change conductivity that is detected by a pair of electrodes. A switch is then activated to terminate the measurement and to stop a timer. The amount of chloride precipitated is proportional to the elapsed time. Therefore, the elapsed time of blank, standard and samples was determined for the calibration factor then the concentration of Cl^- was reported in mmol/l.

2.8 Light microscopy and immunofluorescence analysis

2.8.1 Tissue sectioning

Histology is regularly used for the investigation of tissue structure (Anderson 2012). The first left gill arch of the animal from each condition was fixed for 48 hours by immersion in Bouin's fixative. Rinsing was carried out in 70% ethanol to remove Bouin's completely from

[4] http://www.sherwood-scientific.com/flame/sh410.html

the tissue. Samples were dehydrated in ascending grades of ethanol prior to impregnation and embedding in Paraplast® (Sigma-Aldrich, USA). Transverse sections (4 µm) were cut on a Leitz Wetzlar microtome (Leica, Rueil-Malmaison, Ile-de-France, France). Tissues sections were collected on poly-L-lysine-coated slides and dewaxed with clearing agent HistoChoice MB®.

2.8.2 Gill staining

For the observation of gills, slides were stained with Masson's trichrome (hematoxylin, acid fuchsin and anilin blue) or sections were stained with Alcian Blue pH 2.5 (DIAPATH) Periodic Acid (CARLO ERBA) and Schiff (SIGMA). Hematoxylin and eosin stains are basic dyes that stain nuclei (giving bluish color) and nucleus (giving pinkish color) respectively.

2.8.3 Morphometric measurement

Masson's trichrome staining sections were observed under Leica Diaplan microscope and gill lamellae were photographed at a magnification of x40. The pictures were used to determine filament thickness, lamellar length, lamellar width at top and base. Twenty-seven measurements per animal (total N=3) were determined for lamellar parameters and for filament thickness 18 measurements per animal used for morphometric analyses. The mucous cells density were counted on 200 µm of filament length (n= 15 measurements per conditions, N=3 animal per conditions).

2.8.4 Immunofluorescence observation

For determining sub-cellular localization of proteins (for example Na^+/K^+-ATPase (NKA)) using immunohistochemical analysis, three fish were used from each condition. Slides for immunolocalization were dewaxed by clearing agent HistoChoice® MB, hydrated through a descending series of ethanol baths (from 100% to 50%). Slides were rinsed in phosphate buffer saline (PBS) and immersed for 10 min into 0.02% Tween 20, 150mM NaCl in PBS, pH 7.3. Tween with PBS used to saturate all non-specific potential binding sites in the membrane. After blocking in 5% skimmed milk (SM) in PBS at 37 °C for 20 min, the slides were rinsed twice with PBS. Primary labeling was performed for 2 h or overnight at room temperature or at 4 °C, respectively, in a humidity chamber. Mouse monoclonal anti-NKAα5 was used as a first antibody at a concentration of 8µg/ml in SM/PBS at 0.5 %. After three washes in PBS to

remove unbound antibody, the sections were incubated for 1 h with secondary antibody. We used Alexa 488 donkey anti-mouse at 4µg/ml. Following washes, sections were mounted in an anti- bleaching mounting medium (Immunohistomount, Santa Cruz Biotechnology). Slides were observed with a Leica Diaplan microscope equipped with a special filter for fluorescence set and coupled to a Leica DC 300F digital camera and FW4000 software. Gill sections were photographed using objectives with a magnification of x25 (ionocytes density) and x40 (ionocytes area).

2.8.5 Counting

The images were obtained by a Leica Diaplan microscope coupled to a Leica DC 300F digital camera and the FW4000 Software. Positively stained cells for NKA were counted over 300 µm of selected filaments of three fish from each condition. The numbers of measurements were 10 on each filament from three different fish of each condition. On the other hand for the area of mucous in each mucous cell was measured and indicated as mucous cell size on 200 µm located within the interlamellar area.

2.8.6 Ionocytes area measurements

The images photographed on the Leica Diaplan microscope were used to determine ionocytes area with the help of image J software. These vales of areas were taken on those ionocytes where the nucleus was visible. The 25 measurements on three fish were done on the ionocytes stained for NKA.

2.9 Scanning electron microscopy

In order to investigate the branchial external morphology, Scanning Electron Microscopy **(**SEM) was used in this study. The gills of four fish from each condition fixed in 2% glutaraldehyde solution adjusted to 380 $mOsm.kg^{-1}$, buffered at pH 7.4 with 0.1 $mol.L^{-1}$ in cacodylate buffer. The gill tissues were post fixed in 2% osmium tetroxide for 1h, then rinsed in water, and dehydrated in an ethanol series (50-96%). The samples were thoroughly air-dried and mounted to specimen stubs with adhesive carbon tabs, coated with gold, using sputter coater and examined with a FEI Quanta 200 ESEM using the conventional mode and the Thornley-Everhart secondary electron detector. The magnification >2000x were used for each sample to examine the apical opening of ionocytes and subsequent examination of the

superficial structure (for example microridges of pavement cells) of filament epithelium. Images were taken and saved as digital images, which were later used for determination of apical opining of ionocytes, mucous density and pavement cells microridges examination.

2.10 Gill Na^+/K^+-ATPase activity measurement

The gill tissue was analyzed for NKA activity via a 96-well microplate spectrophotometric assay, that determines the rate at which functioning, sample-derived ATPase can hydrolyze ATP and previously confirmed this assay conditions by Nebel et al. (2005). Sea bass gill samples stored in the SEI buffer were thawed on ice, homogenized in 300 µl of buffer containing 250mM sucrose, 5mM $MgCl_2$, pH 7.4 and using a Retsch Mixer mill MM400 (Haan, Germany), (frequency:30Hz, 2 times for the 30s) and centrifuged. After centrifugation at 1800 g (5 min at 4 °C), the supernatant was used for protein quantification by the Bradford method (Bradford;Bio-Rad, France) (Bradford 1976) and NKA activity determination. The reaction was initiated by adding an assay mixture containing 100 µl assay buffer (buffer was contained salts NaCl, KCl, $MgCl_2$), ATP (adenotriphosphate) and reagents that enzymatically couple ADP (adenine dinucleotide phosphate) production with NADH (nicotinamide adenine dinucleotide + hydrogen) oxidation. The enzyme reaction was measured by repeated 630 nm absorbance reading for 10 min on a plate reader at room temperature to quantify liberated P_i using I-control 2.0 software (Infinite M200 plate reader, TECAN trading AG, Switzerland). NKA specific activity was calculated from the difference ATP hydrolysis at 37 °C (V_{max}) and at 24 °C and 18 °C ($V_{apparent}$) in triplicate wells in the presence or absence of 13.4 mM KCl or 1.4 mM ouabain. The ouabain is used as a potent NKA inhibitor. Values were normalized to the sample's total protein concentration and reported in µmol Pi mg $protein^{-1}$ h^{-1}.

2.11 RNA extraction purification and reverse transcription

Gill tissues were collected from SW and FW exposed to the temperate and warm condition. The epithelium of the gills was scraped with a sterile scalpel. Total RNA was extracted using Trizol® reagent according to the manufacturer's instructions. RNA quantity and purity were assessed by measuring the A260/A280 ratio using the NanoDrop® ND-1000 V3300 Spectrometer (Nanodrop Technology Inc., Wilmington, Delaware, USA). RNA quality was checked using Agilent bioanalyzer using electrophoretic trace method in order to get the RNA integrity number (RIN) and it was satisfactory (RIN score >6). One microgram of the total RNA was treated with 1U of DNase I amplification grade (Invitrogen™, Life Technologies).

Reverse transcription was performed using 200 U of M-MLV reverse transcriptase (Invitrogen™) and the first strand of complementary DNA (cDNA) was generated using 250 ng of random primers (Invitrogen™), dNTPs (10 mM) and 40 U of RNAse out recombinant (Invitrogen™), following manufacturer's instruction.

2.12 Quantification at the transcript level

The specific primers (forward and reverse) for different transporters are listed in Table 6. Quantitative PCR analyses (qPCR) were performed with LightCycler® 480 Real-Time PCR System (Roche, Mannheim, Baden-Wrüttemberg, Germany), using 2X LightCycler-FastStart DNA Master SYBER-Green I™ Mix (Roche), forward and reverse primers (at a final concentration of 0.5 µM) and cDNA (either equivalent to 1.6 ng RNA using manual pipetting (2 ul of cDNA diluted at 1/64 in a final volume of 5 ul) or equivalent to 0.4 ng RNA using robot pipetting (0.5 ul of cDNA diluted at 1/64 in a final volume of 1.5 ul). The qPCR condition were: denaturation at 95 °C for 10 min, followed by 45 cycles of repeat amplification (95 °C, 10s) hybridization (60 °C, 10 s) and elongation (72 °C, 10 s), and final step at 40 °C for 30 s. A melting curve program was performed to control the amplification specificity, and the amplification products were sequenced. Three reference ARN were selected according to the recommendation of Mitter et al. 2009: EF1α (encoding elongation factor 1 alpha), FAU (40S ribosomal protein S30) and 18S rRNA. Ultra-pure water was used as no-template control in the qPCR. Efficiencies were determined and given in Table 6. Since, the 18S rRNA was found were unstable, it was discarded as a potentially good reference gene. The relative expression ratio of a target gene was calculated using the ΔCt method with the formula: Efficiency $(E)^{-\Delta Ct}$ (Pfaffl 2001), using the efficiency of each primer pair calculated in gill tissue. The geometric mean of the two reference genes was also used as recommended by Vandesompele et al. (2002). Henceforth, the normalized expression of each analysed gene is referred to as 'gene expression'. Finally, the choice was made to present expression data normalized with reference gene (EF1α) in this manuscript.

Table 6 Primer sequences used for qPCR in this study

ID genome sequences	Target gene	Primer name	Sequence (from 5′ to 3′)	Amplicon size	Efficiency	Reference
DQ501276	*cftr*	CFTR F CFTR R	GACTGATGCGTTCGGTAG CCTCAATGACATCTCCTTC	215	1.917	(Bodinier et al., 2009)
DLAgn_00080120	*slc12a2*	NKCC1 F NKCC1 R	TCAGCTCACAGTTCAAGGCC GCCGCTATGGACTCCACAA	102	2.08	(Lorin-Nebel et al., 2006)
AJ866727	*ef1α*	EF1-F EF1-R	GGCTGGTATCTCTAAGAACG CCTCCAGCATGTTGTCTCC	239	2.024	
KP400258	*nka α1a*	NKAα1a F NKAα1a R	CCTCAGATGGCAAGGAGAAG CCCTGCTGAGATCGGTTCC	146	1.89	(Blondeau-Bidet et al. 2016)
KP400259	*nka α1b*	NKA α1b F NKA α1b R	AGCAGGGCATGAAGAACAAG CCTGGGCTGCGTCTGAGG	204	1.99	
FM004681	*fau*	FAU F FAU R	GACACCCAAGGTTGACAAGCAG GGCATTGAAGCACTTAGGAGTTG	150	2.029	(Mitter et al. 2009)
LAgn_00038210	*Slc12a3-like*	NCC-like F NCC-like R	ATGATGAGCCTCTTCGAGCC GCTGCTCTCATCACCTTCTGT	278	1.94	(Blondeau-Bidet et al. 2019)
DLAgn_00204050	*slc9a3*	NHE3 F NHE3 R	GGATACCTCGCCTACCTGAC AAGAGGAGGGTGAGGAGGAT	251	1.98	
DLAgn_00076370	*atp6v1a*	VHA-A F VHA-A R	GGCAGTCACATCACAGGAGG CCAGCTCCATCACCACATCG	154	1.98	
DLAgn_00018050	*atp6v1b2*	VHA-B F VHA-B R	TTGCCATAGTCTTCGCAGCC CTTCTCGCACTGGTAGGC	194	1.90	
DLAgn_00222650	*rhbg*	RHBG F1 RHBG R1	CCTCATGGTGACCCGAATCC GCCTGCACTCTGTCCACATA	218	1.97	
DLAgn_00166370	*rhcg1*	RHCG1 F RHCG1 R	TCAGGGAATTGTGTGACCGC AGAATCAAGTCCACGCTGGG	118	2.01	
JN998891	*ClC3*	ClC-3 F ClC-3 R	CAAGTACAGCAAGAACGAGGC ACAGCGTCTTGAGAGGGAAG	146	2.069	(Bossus et al. 2013)
DLAgn_00169960	*hsp90b1*	HSP90B1 F HSP90B1 R	CTACCAGCTGGCTGACACAA CCGCTTTATCCTCAGAGTCG	161	1.456	This study
DLAgn_00070720	*hsp90ba*	HSP90BA F HSP90BA R	GTGGAGAAGGAGCGTGACAA CTGAGCCCACATCCTCGATC	115	1.442	

Chapter III

Coping with environmental changes: from the cell to the whole organism

Chapter III - Coping with environmental changes: from the cell to the whole organism

3.1 Preamble

Several studies have analyzed the effects of temperature on osmoregulatory mechanisms in teleost fish species (Stuenkel and Hillyard, 1980; Kültz and Somero, 1995; Metz et al., 2003; Fiess et al., 2007). Changes in hydromineral balance as a result of temperature acclimation has been reported in many fish species (Metz et al. 2003; Fiess et al. 2007; Sardella et al. 2008; Mitrovic and Perry 2009; Vargas-Chacoff et al. 2009a). In the study by Fiess et al. (2007), the authors have shown that the interactive effects of environmental salinity and temperature have modulated plasma osmolality in the Mozambique tilapia *Oreochromis mossambicus.* According to Metz et al. (2003) this change is explained by altered gill Na^+/K^+-ATPase (NKA) activity and by a change in ionocytes recruitment. Energized NKA pumps in the gills are temperature-sensitive as generally admitted in enzymes. Temperature-dependent changes in NKA activity depend on the considered salinity, as shown in several species such as pupfish and tilapia (Fiess et al., 2007; Stuenkel and Hillyard, 1980).

Acclimation to environmental changes also requires cellular and tissue modifications that contribute to physiological compensation. For example, gill ionocytes proliferate on the lamellae which cause a thickening of the blood to water diffusion barrier and thus impedes respiratory gas transfer. Gill remodeling in response to environmental parameters affects functional surface area that will either favor osmoregulation or respiration, a phenomenon called as osmorespiratory compromise (Sollid et al., 2005; Sollid and Nilsson, 2006).

Previous studies on juvenile sea bass transferred to FW at temperate condition (18-20 °C) showed low and variable blood osmolalities (ranging from 214 to 316 $mOsm.kg^{-1}$) several weeks after salinity challenge (Nebel et al., 2005). In seawater, blood osmolality levels are more stable and ranged from 360 to 380 $mOsm.kg^{-1}$. Freshwater transfer induced in this species the appearance of lamellar ionocytes and an increase in branchial NKA activity (Jensen et al., 1998; Nebel et al., 2005). In the sea bass, however, no investigation based on physiological and morphological parameters has been carried out to analyze the interactive effects of temperature increase (24 °C) and salinity decrease (transfer from SW to FW).

3.2 Main findings

This chapter is evaluating the effects of temperature increase on osmoregulatory process in European sea bass *Dicentrarchus labrax*. Sea bass juveniles have been pre-acclimated to seawater (SW) at 18 °C (temperate) and 24 °C (warm) for two weeks and were then transferred to SW or freshwater (FW) at the respective temperatures. Blood osmolality and ion levels (Na^+ and Cl^-) were measured at different temperature in order to determine osmoregulatory ability in sea bass at organismal level. The fish gill is a multifunctional organ involved in osmotic and ionic regulation. It is directly exposed to salinity and temperature changes. It was thus used to provide a comprehensive understanding of morphological and molecular parameters that allowed fish to cope with combined effects (salinity transfer and temperature increase). We have analyzed NKA protein localization and semi-quantitative intensity level using immunolabeling. Ionocyte number was quantified and apical opening of these cells were observed. At the molecular level, the NKA activity was assayed at the acclimation temperatures (24 °C and 18 °C, termed $V_{apparent}$) and at the maximal temperature at 37 °C (V_{max}).

Hydromineral balance

After four weeks in SW (T30), blood osmolality and Cl^- level did not change. FW transfer induced changes in ion levels and we noticed that blood osmolality was highly correlated to Cl^- levels ($r=0.9738$, $p<0.0001$) but not to Na^+ levels ($r=0.09715$, $p>0.05$). Interestingly, Na^+ level was significantly decreased in SW at 24 °C (147.9±18.96 $mOsm.kg^{-1}$, SD±SEM) *vs* SW at 18 °C (174.5±36.56 $mOsm.kg^{-1}$). Temperature acclimation resulted in a change in ion level as reported in many fish species (Metz et al. 2003; Fiess et al. 2007; Sardella et al. 2008; Mitrovic and Perry 2009; Vargas-Chacoff et al. 2009a). The decreased Na^+ level may also indicate a change in the Na^+ transport mechanisms at the gill or kidney levels or an altered acid-base regulation (Tresguerres et al., 2005). Na^+ uptake at the gill level is generally linked to acid secretion, a topic that will be discussed in Chapter IV. Direct transfer to FW for two weeks resulted in decreased blood osmolalities as previously reported in sea bass (Bossus et al., 2011; Jensen et al., 1998). In the FW warm condition however, increased temperature seems to affect differently the ion composition of the blood. Cl^- levels were higher in FW warm compared to FW temperate condition. This explained the maintained blood osmolality in FW warm condition compared to the FW temperate condition. Increased temperatures seem somehow to favor the maintenance of Cl^- blood levels maybe as a compensation of decreased

Na^+ levels. Plasma Na^+/Cl^- ratio is therefore higher in FW compared to SW in temperate condition. In warm condition Na^+/Cl^- ratio is lower in SW (0.93±0.03) and FW (1.07±0.03) that can be mild and transient metabolic acidosis. Metabolic acidosis and alkalosis will be discussed in Chapter IV. The compensation of ion level may be a strategy of coastal fish species to enter lagoon-like habitats characterized by temperature and salinity fluctuations (Newton and Mudge 2003) and sometimes contrasted environmental conditions such as those tested in this study.

Na^+/K^+-ATPase (NKA) activity

The NKA is the main osmoregulatory pump responsible for maintaining the electrochemical gradient across the membrane. Assaying apparent NKA activity seems essential for proper physiological interpretation and to our knowledge, this is the first time that sea bass NKA has been assayed at acclimation temperature. Our study clearly show that using the physiological more relevant assay conditions, FW transfer results in a slight though not significant increased NKA activity ($V_{apparent}$) in temperate condition, contrary to warm conditions where NKA activity ($V_{apparent}$) does not change following salinity transfer. On the other hand, maximal NKA activity (V_{max}) (an estimate of the maximized NKA activity reflecting total amount of enzyme, as stated by Metz et al. (2003)) was higher in FW-acclimated sea bass compared to SW-acclimated fish as it was anticipated from previous studies (Jensen et al., 1998; Nebel et al., 2005). Gill NKA activity (V_{max}) was overall lower in warm compared to temperate conditions which suggests decreased net branchial active ion transport in sea bass acclimated to warm temperatures and may contribute to ion imbalance in warm acclimated fish. Expression of branchial NKAα1 paralogs will be presented in Chapter IV (page -59).

Gill structure and morphometry

Gill morphological parameters were analyzed at the different temperatures. Ionocytes density increased following transfer to FW which is certainly linked to the higher NKA activity (V_{max}) at this salinity, as ionocytes are rich in NKA at the basolateral membrane. Overall, ionocytes number and area decreased in warm compared to temperate conditions which probably explains the lower NKA activity in FW warm condition. An increased ionocyte density following FW transfer has been reported in numerous teleost species (Lin et al., 2003; Riou et al., 2012; Seo et al., 2009) and has been previously shown in sea bass (Bossus et al., 2013; Nebel et al., 2005; Varsamos et al., 2002). Increased number of ionocytes on filament and lamella in FW temperate condition induce higher lamella width and filament thickness. The

morphological adjustment of the gill is linked to the recruitment of ionocytes required for effective ion uptake at temperate condition. Higher salinity and temperature may lead to low dissolved oxygen level in the water. Fish may need larger lamellar surface area for effective diffusive gas exchange. The high lamella length in warm FW and SW temperate suggests a strong need for O_2 uptake in this media. However, it was expected higher lamellar surface area in SW warm condition but fish did not remodel gill surface area.

Salinity clearly affects apical opening of ionocytes. Small apical openings (or pits) are recorded in SW to avoid excess ion diffusion and net ion uptake. Larger ionocyte openings are observed in FW to increase surface available for ion uptake (Choi et al., 2011; Seo et al., 2009; Varsamos et al., 2002). In our study, measurements of apical opening area could not be done due to numerous mucous cells on the gill boundary. This high amount of mucous will be further discussed in Chapter V. Main changes in the apical surface of the gill were observed in pavement cell microridges. Fish epithelia comprises many different cell types of which about 90 % are pavement cells (Wilson and Laurent, 2002). In our study, changes in microridges surface presented less concentric rings in warm temperature-challenged fish in SW only. This modification of the apical surface of the gill boundary as a response to increased temperatures might be linked to a change in membrane fluidity, but this is a hypothesis and requires further investigations.

3.3 Conclusion

In conclusion, our data suggest that fish were effective osmoregulator, as they are able to maintain relatively high blood osmolality in SW when exposed to temperature increase. Sea bass efficiently hyper-osmoregulates in FW warm condition. At the gill level however, gill remodeling occurs notably following FW transfer at both temperatures. NKA activity, ionocytes size and density seem to be lower in warm FW which also is an indication of an altered branchial ion uptake capacity in warm FW. An imbalance between Na^+ and Cl^- uptake mechanisms is suggested and will be discussed in Chapter IV. In summary, salinity and temperature stress seems to affect sea bass gill epithelium. Thus it could be expected to encounter a more severe alteration of hydromineral balance following long-term FW transfer (> 2 weeks) at this warm temperature due to osmorespiratory compromise.

Research article I

Effect of combined stress (salinity and temperature) in European sea bass *Dicentrarchus labrax* osmoregulatory processes

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Published in

Journal of Comparative Biochemistry and Physiology, Part A 2015 (2018) 45-54

http://dx.doi.org/10.1016/j.cbpa.2017.10.019

Comparative Biochemistry and Physiology, Part A 215 (2018) 45–54

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Effect of combined stress (salinity and temperature) in European sea bass *Dicentrarchus labrax* osmoregulatory processes

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ARTICLE INFO

Keywords:
Gill plasticity
Hydromineral balance
Ionocytes
NKA activity
Osmoregulation
Teleost
Thermal acclimation

ABSTRACT

European sea bass *Dicentrarchus labrax* undertake seasonal migrations to estuaries and lagoons that are characterized by fluctuations in environmental conditions. Their ability to cope with these unstable habitats is undeniable, but it is still not clear how and to what extent salinity acclimation mechanisms are affected at temperatures higher than in the sea. In this study, juvenile sea bass were pre-acclimated to seawater (SW) at 18 °C (temperate) or 24 °C (warm) for 2 weeks and then transferred to fresh water (FW) or SW at the respective temperature. Transfer to FW for two weeks resulted in decreased blood osmolalities and plasma Cl^- at both temperatures. In FW warm conditions, plasma Na^+ was ~15% lower and Cl^- was ~32% higher than in the temperate-water group. Branchial Na^+/K^+-ATPase (NKA) activity measured at the acclimation temperature ($V_{apparent}$) did not change according to the conditions. Branchial Na^+/K^+-ATPase activity measured at 37 °C (V_{max}) was lower in warm conditions and increased in FW compared to SW conditions whatever the considered temperature. Mitochondrion-rich cell (MRC) density increased in FW, notably due to the appearance of lamellar MRCs, but this increase was less pronounced in warm conditions where MRC's size was lower. In SW warm conditions, pavement cell apical microridges are less developed than in other conditions. Overall gill morphometrical parameters (filament thickness, lamellar length and width) differ between fish that have been pre-acclimated to different temperatures. This study shows that a thermal change affects gill plasticity affecting whole-organism ion balance two weeks after salinity transfer.

1. Introduction

The European sea bass *Dicentrarchus labrax* (Linnaeus 1758) is an important aquaculture species. It is considered as highly euryhaline (tolerating salinities from fresh water (FW) up to 90‰) and eurythermic (tolerates temperatures from 5 to 28 °C) (Barnabé, 1989; Kousoulaki et al., 2015). *D. labrax* is commonly found on the coasts of the north-east Atlantic Ocean, the Mediterranean Sea and the Black Sea. In the Mediterranean region, *D. labrax* juveniles and adults undergo seasonal migrations (in spring) from the sea to lagoons occasionally travelling upstream into rivers (Dufour et al., 2009). Salinities of Mediterranean lagoon habitats range from close to fresh water up to hypersaline waters (> 45‰) (Newton and Mudge, 2003). In these habitats, it is not rare that temperatures reach mean temperatures close to 25 °C from late spring to end of August. In autumn, as temperatures of lagoons decrease to 5–6 °C, sea bass migrate back to the sea, where spawning occurs in winter (Dufour et al., 2009). In order to cope with the salinity drop caused by high precipitations that are common in autumn and spring season in Mediterranean lagoon habitats, sea bass have to be able to efficiently osmoregulate at different temperatures encountered in the wild. Gills are considered as the major organ involved in active ion absorption (review in Evans et al., 2005). In FW, mitochondrion-rich cells (MRCs) also called ionocytes are involved in transepithelial ion uptake, acid-base regulation and ammonia excretion (review in Hwang et al., 2011). The basolateral Na^+/K^+-ATPase (NKA) is considered as a key pump enabling active transport of ions within these cells (review in Marshall, 2002).

Previous studies on juvenile sea bass transferred to FW at temperate conditions (18–20 °C) showed low and variable blood osmolalities (ranging from 214 to 316 $mOsm{\cdot}kg^{-1}$) several weeks after salinity challenge (Nebel et al., 2005). In seawater, blood osmolality levels are more stable and ranged from 360 to 380 $mOsm{\cdot}kg^{-1}$. FW transfer induced in this species the appearance of lamellar ionocytes, and an increase in branchial NKA activity (Jensen et al., 1998; Nebel et al., 2005). No data are available on osmoregulatory mechanisms in sea bass acclimated to increased temperatures.

In other teleost species, several studies are available on the effect of temperature on osmoregulatory mechanisms (Stuenkel and Hillyard,

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http://dx.doi.org/10.1016/j.cbpa.2017.10.019
Received 19 July 2017; Received in revised form 17 October 2017; Accepted 17 October 2017
Available online 19 October 2017
1095-6433/

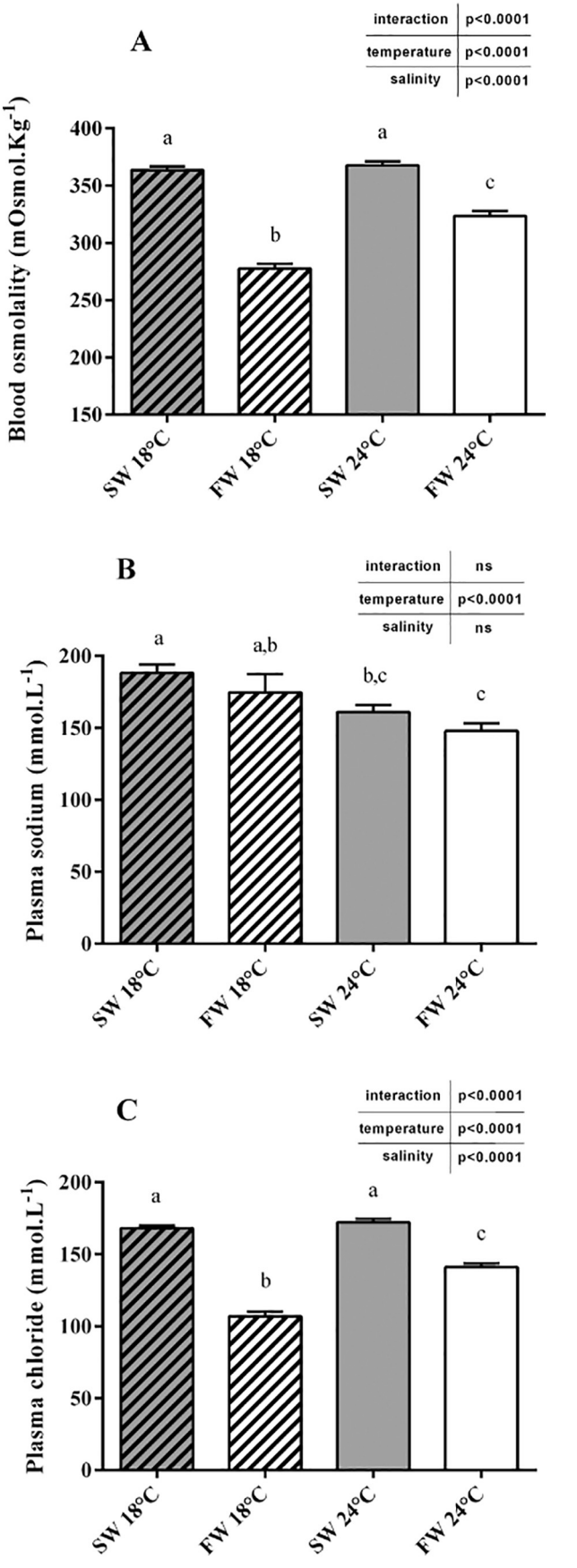
1980; Kültz and Somero, 1995; Metz et al., 2003; Fiess et al., 2007; Sardella et al., 2008; Vargas-Chacoff et al., 2009; Radaelli et al., 2010; Uliano et al., 2010). Na^+/K^+-ATPase activity can depend on acclimation temperature, but the effect of temperature depends on the range of temperatures that have been tested and the species considered (McCarty and Houston, 1977; Stuenkel and Hillyard, 1980; Imsland et al., 2003; Metz et al., 2003; Morrison et al., 2006; Fiess et al., 2007; Sardella et al., 2008; Mitrovic and Perry, 2009; Zydlewski and Zydlewski, 2012).

Several investigations reported differences in gill NKA activity according to the temperature used in the assay (acclimation ($V_{apparent}$) vs maximal temperature (V_{max})) (McCarty and Houston, 1977; Metz et al., 2003; Sardella et al., 2004a; Sardella et al., 2008; Michael et al., 2016). In FW carp, where three temperatures were tested (15, 22 and 29 °C), higher branchial NKA expression and activity (V_{max}) were reported at 15 °C (Metz et al., 2003). NKA activity assayed at acclimation temperature ($V_{apparent}$) however was not increased at this temperature and maximal NKA ($V_{apparent}$) levels were recorded at 29 °C. The authors state that cold temperatures induce MRC number and NKA expression in order to compensate for the lower NKA enzyme activity. In Mozambique tilapia acclimated to FW or SW and then transferred from 26 °C to 15, 25 and 35 °C for 2 weeks, osmotic disturbances have been recorded in the low-temperature group in FW with decreased plasma osmolalities and NKA activities (assayed at the acclimation temperature, $V_{apparent}$). Osmotic alterations were also recorded in SW in this low-temperature group with increased plasma osmolalities and decreased NKA activities ($V_{apparent}$) (Sardella et al., 2008). Interestingly, maximal NKA activity (V_{max}) peaked at 15 °C in SW as well as the size of MRCs, which could be interpreted as a compensatory response following cold stress.

Temperature-dependent change in NKA activity depends on the considered salinity, as shown in several species such as pupfish and tilapia (Stuenkel and Hillyard, 1980; Fiess et al., 2007). In tilapia, a significant increase in NKA activity was recorded only in 200% SW but not in FW and SW at temperatures ranging from 20 to 35 °C (Fiess et al., 2007). In juvenile turbot exposed to different salinities (15, 25 and 33‰) and temperatures (10, 14, 18, and 22 °C) for 3 months, lowest gill NKA activities, plasma chloride and osmolality were recorded at 15‰, that corresponds to a salinity close to the iso-osmotic point (Imsland et al., 2003). Low gill NKA activity is an interesting parameter to consider for aquaculture studies as it leads to reduced energy expenditure for osmoregulation and possibly increased growth rate (Imsland et al., 2003; Árnason et al., 2014; Michael et al., 2016). In turbot, the optimal temperature-salinity combination for aquaculture was estimated at around 15.8 ± 4.1‰ at 17.6 ± 2.2 °C (Imsland et al., 2003).

In salmonids, it is well known that temperature is a key factor affecting the smoltification-related increase in branchial NKA activity (Handeland et al., 2013). In Atlantic salmon *Salmo salar*, where branchial NKA activity is supposed to increase during the smoltification process as a pre-acclimation to the seawater salinity, a different increase in NKA activity has been reported according to the temperature, with higher enzyme activities at high temperatures (19 °C vs 5 °C group), which can be linked to better hypo-osmoregulatory capacities in the 19 °C group (Handeland et al., 2000). In the same species, gill NKA activity, plasma Cl^- and growth-hormone have been shown to be affected by temperature (8.3 vs 12.7 °C) and photoperiod (Handeland et al., 2013).

In several species, gill morphology has been altered under temperature stress alone (Sollid et al., 2005) or combined with other stress (Metz et al., 2003; Tzaneva et al., 2011). Gill remodeling in response to environmental parameters (like salinity, temperature and oxygen) affects functional surface area that will either favor osmoregulation or respiration, a phenomenon termed osmorespiratory compromise (Sollid et al., 2005; Sollid and Nilsson, 2006; Perry et al., 2010; Tzaneva et al., 2011). In goldfish *C. auratus* and carp *C. carpio*, important gill



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remodeling has been shown following temperature acclimation with the formation of an interlamellar cell mass (ILCM) in cold water (7 °C) to reduce respiratory surface area (Sollid et al., 2005; Mitrovic and Perry, 2009). Mitochondrion-rich cells (MRCs) of *C. auratus* have been shown to be larger in size, more abundant and differently located (confined in the outer part of the ILCM, that is devoid of a blood supply) after acclimation to cold temperatures (7 °C compared to 25 °C). Despite the higher number of MRCs, NKA activity (V_{max}) was lower in cold conditions, probably linked to the decreased blood and oxygen supply at the basal part of MRCs (Mitrovic and Perry, 2009).

The aim of this study is to investigate the effects of temperature acclimation (18 °C or 24 °C) on the establishment of hyperosmoregulation in sea bass following SW to FW transfer. This study will analyze branchial NKA activity (V_{max}, $V_{apparent}$), MRC density and size, as well as the morphometry of gills. Furthermore, blood osmolalities and plasma ion levels will be measured to assess osmoregulatory status of fish in order to get a comprehensive view on the effects of combined temperature and salinity stress in this species.

2. Materials and methods

2.1. Experimental conditions

European sea bass *Dicentrarchus labrax* were obtained from the Ifremer Station at Palavas-les-Flots (Hérault, France). Fish were brought to the Montpellier University and maintained for one week in 3500 L tanks containing natural seawater (SW) from the Mediterranean Sea at 38‰ and 18 °C, under a 12 h light/12 h dark photoperiod. Fish were then transferred to 200 L tanks (14 fish/tank, density of 6–7 kg/m^3, two replicates) to be acclimated either at 18 °C or 24 °C (with a temperature increase of 0.2 °C/h). After two weeks of temperature acclimation, fish were transferred directly either to dechlorinated tap water (fresh water, FW) or to SW (7 fish/tank, two replicates for each condition) and maintained in this salinity two weeks before sampling. Ionic composition (in mEq·L^{-1}) of the FW was Na^+ (0.12), K^+ (0.04), Ca^{2+} (5.70), Mg^{2+} (0.29), Cl^- (0.98), NO_3^- (0.06) and SO_4^{2-} (0.61). Water was aerated and mechanically/biologically filtered (Eheim System, Lens, Pas-de-Calais, France). Temperature, salinity, oxygen and nitrogen levels were checked daily. Fish were fed twice a week with fish granules (Aphymar feed, Mèze, Hérault, France) until 2 days before sampling. At the end of the experiment, fish were anesthetized in a solution of phenoxy-2-ethanol (0.24 mL·L^{-1}) prior to any manipulation. The sampled fish average fork length was 20.77 ± 1.32 cm (mean ± SD) and average weight was 86.87 ± 20.23 g (mean ± SD). Four groups were compared: SW at 18 °C (temperate SW), SW at 24 °C (warm SW), FW at 18 °C (temperate FW) and FW at 24 °C (warm FW). These experiments were conducted according to the guidelines of the European Union (directive 86/609) and of the French law (decree 87/848) regulating animal experimentation.

2.2. Blood osmolality and plasma ion levels

Blood osmolality and plasma ions were measured in 8–12 fish per condition. Blood was sampled from the caudal vessel using a 1-mL syringe coated with heparin (Li-heparin, Sigma-Aldrich, France). The osmolality of 20 µL of blood was measured on an Advanced 3300 micro-osmometer using internal standards of 100, 300 and 1000 mOsm·kg^{-1}. Plasma was obtained following centrifugation of 8 min at 10,000*g* at 4 °C. 15 µl of plasma was used to determine chloride concentration using a chloride titrator (AMINCO, Maryland, USA). Plasma sodium levels were determined by flame photometry (Sherwood, Cambridge, UK) using a standard curve of Na^+, K^+ from 0 to 400 mEq·L^{-1} and plasma at a 1/1000 dilution in MilliQ water. Mean Na^+/Cl^- ratio was determined for each condition.

Fig. 1. Blood osmolality (A), plasma sodium (B) and chloride concentration (C) of sea bass exposed to SW and FW at 18 °C (temperate) and 24 °C (warm). Different letters indicate significant differences between conditions (two-way ANOVA followed by a Fisher LSD post hoc test, $p < 0.05$, $N = 8–12$). The table above each graph gives the results of two-way ANOVA with temperature and salinity as the main factors. Data are expressed as means ± SEM. FW: fresh water; SW: seawater.

2.3. Branchial histology, morphometric analyses and immunolocalization of Na^+/K^+-ATPase

The first left gill arch of three animals per condition was fixed for 48 h by immersion in Bouin's fixative. After rinsing in 70% ethanol, samples were dehydrated in a graded ethanol series and embedded in Paraplast (Sigma). Transverse sections (4 µm) were cut on a Leitz Wetzlar microtome, collected on poly-L-lysine-coated glass slides and were stained using the Masson's Trichrome Staining Protocol. Slides were observed under Leica Diaplan microscope, and gill lamellae sections were photographed at a magnification of × 400. For morphometric analyses, several parameters were measured using the software Image J (ImageJ 1.51f) (http://rsbweb.nih.gov/ij/): filament thickness (n = 18 measurements per animal, N = 3 animals per condition), lamellar length, lamellar width at top and base (n = 27 measurements per animal, N = 3 animals per condition) (Fig. 6).

For immunolabeling of the Na^+/K^+-ATPase, sections were dewaxed (LMR), hydrated through a descending series of ethanol baths (from 100% to 50%) and rinsed in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.4, Sigma). Slides were then immersed for 10 min into 0.02% Tween 20, 150 mM NaCl in PBS, pH 7.3. After blocking in 5% skimmed milk (SM) in PBS at 37 °C for 20 min, the slides were rinsed twice with PBS. Primary labelling was performed for 2 h at room temperature in a humidity chamber with the primary monoclonal antibody (α5) anti-Na^+/K^+-ATPase (Hybridoma Bank, University of Iowa) diluted in PBS at 10 µg·mL^{-1} in 0.5% SM-PBS. After three washes in PBS to remove unbound antibody, the sections were incubated for 1 h with a secondary antibody (donkey anti-mouse Alexa Fluor® 488 (Invitrogen, Life Technologies) at 10 µg·mL^{-1}). Following washes, sections were mounted in an anti-bleaching mounting medium (Immunohistomount, Santa Cruz Biotechnology) and observed with a Leica Diaplan microscope equipped with a special filter for fluorescence set (450–490 nm) and coupled to a Leica DC 300F digital camera and FW4000 software. Gill sections were photographed using objectives with a magnification of × 25 (MRC density) and × 40 (MRC area) and images were analyzed with Image J software (ImageJ 1.51f). The number of MRCs located on the filaments and lamellae were counted on 300 µm of filament length in each picture on one side of the filament (n = 10 measurements per animal, N = 3 animals per condition). MRC area was measured using cells where the nucleus was visible (n = 25 measurements per animal, N = 3 animals per condition).

2.4. Scanning electron microscopy

A small section of the 1st left gill arch of four fish from each condition was fixed for 24 h at 4 °C by immersion in 2% glutaraldehyde solution adjusted to 380 mOsm·kg^{-1}, buffered at pH 7.4 with 0.1 mol·L^{-1} cacodylate buffer. Samples were then kept in 0.5% glutaraldehyde with diluted SW adjusted at 380 mOsm·kg^{-1} until proceeding. Then, samples were rinsed 3 times for 30 min in cacodylate buffer and post-fixed for 4 h at room temperature in buffered 1% OsO_4. After extensive washes in buffer, the samples were thoroughly rinsed with water, dehydrated through an ethanol series (50%–96%), and 1,1,1, 3, 3, 3-hexamethyldisilazane (EMS, Hatfield, USA) (2 baths of 1 min), and were subsequently air-dried and attached to specimen stubs with adhesive carbon tabs. Samples were coated with gold for 160 s (560 mÅ), using the BAL-TEC SCD 050 sputter coater and examined with a FEI Quanta 200 ESEM using the conventional mode (low vacuum) and the Thornley-Everhart secondary electron detector.

Table 1
Plasma Na^+/Cl^- ratio of sea bass exposed to SW and FW at 18 °C (temperate) and 24 °C (warm). Data are expressed as means ± SEM (*N*). Different letters indicate significant differences between conditions (Dunn's test, $p < 0.05$). FW: fresh water; SW: seawater.

SW 18 °C	FW 18 °C	SW 24 °C	FW 24 °C
1.12 ± 0.04[a] (9)	1.65 ± 0.12[a] (8)	0.93 ± 0.02[b] (13)	1.07 ± 0.03[a,b] (13)

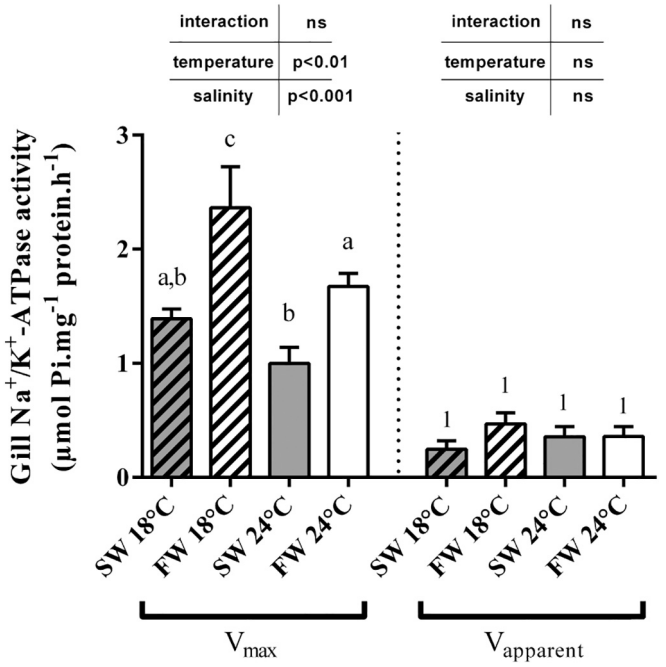

Fig. 2. Gill Na^+/K^+-ATPase (NKA) activity of sea bass exposed to SW and FW at 18 °C (temperate) and 24 °C (warm). NKA activity was assayed at optimal temperature (V_{max}) and at the acclimation temperature of the fish ($V_{apparent}$). Different letters (V_{max}) or numbers ($V_{apparent}$) indicate significant differences between conditions (two-way ANOVA followed by a Fisher LSD post hoc test, $p < 0.05$, $N = 8–12$). The table gives the results of two-way ANOVA with temperature and salinity as the main factors. Data are expressed as means ± SEM. FW: fresh water; SW: seawater.

2.5. Na^+/K^+-ATPase activity measurements

NKA activity was measured on the first and second right gill arch from 8–12 fish per condition. After sampling, gills of each fish were stored at −80 °C in SEI buffer (300 mM sucrose, 20 mM Na_2EDTA, 100 mM imidazole, pH 7.4). Homogenization was performed in 300 µl of buffer containing 250 mM sucrose, 5 mM $MgCl_2$, pH 7.4 and using a Retsch Mixer mill MM400 (Haan, Germany) (frequency: 30 Hz, 2 times for 30 s). After centrifugation at 1800*g* (5 min at 4 °C), the supernatant was used for protein quantification (Bradford; Bio-Rad, France) and NKA activity determination. NKA activity, expressed in µmol Pi mg protein^{-1} h^{-1}, was determined using a microplate method adapted from Flik et al. (1983) and previously validated in sea bass by Nebel et al. (2005). NKA specific activity was calculated from the difference in ATP hydrolysis at 37 °C (V_{max}), 24 °C (V_{app} for 24 °C acclimated fish) or 18 °C (V_{app} for 18 °C acclimated fish) for 20 min after the addition of 100 µL assay buffer (3 mM Na-ATP, 100 mM NaCl, 30 mM imidazole/Hepes, 0.1 mM EDTA, 5 mM $MgCl_2$, pH 7.4) amended with either 13.4 mM KCl or 1.4 mM ouabain. The enzyme reaction was stopped by the simultaneous addition of ice-cold trichloroacetic acid at 10% and color reagent (660 mM H_2SO_4, 9.2 mM $(NH_4)_6$ $Mo_7O_{24}{\cdot}4H_2O$, 330 µmol·L^{-1} $FeSO_4{\cdot}7H_2O$), followed by a 10-min reaction at room temperature to quantify liberated P_i (630 nm absorbance, Infinite M200 plate reader, TECAN trading AG, Switzerland).

2.6. Statistics

Statistical analyses were performed using Graphpad Prism (version 6, GraphPad Software Incorporated, La Jolla, CA 268, USA). Normality and homogeneity of variance were respectively checked using D'Agostino-Pearson test and Bartlett test. For data fitting homogeneity of variance requirement, a two-way factorial analysis of variance with temperature and salinity as the main factors was performed; critical differences between groups were appraised using the Fisher least-square difference test. For data not fitting homogeneity of variance and data issued from histology sections, a non-parametric Kruskal-Wallis test followed by a multiple comparison Dunn's test was used. Linear correlations were determined using the Spearman correlation. Data are presented as means ± SEM, and the level of statistical significance was set at $p < 0.05$.

3. Results

3.1. Blood osmolality and plasma ion levels

For sea bass maintained in SW, blood osmolality was not significantly different between temperatures (Fig. 1A). After transfer to FW, blood osmolalities were significantly decreased by ~23% and ~12% in temperate and warm conditions, respectively. Plasma chloride levels were significantly decreased by ~35% and ~17% in temperate and warm FW conditions (Fig. 1C). Salinity, temperature and interaction between both exerted a significant effect on blood osmolality and plasma chloride (Fig. 1A, C, two-way ANOVA, $p < 0.0001$). Overall, blood osmolality was highly correlated to plasma chloride levels ($r = 0.9738$, $p < 0.0001$), but not to plasma sodium levels ($r = 0.09715$, $p > 0.05$). Plasma sodium levels were not significantly modified following FW transfer at temperate and warm temperature, but Na^+ levels were significantly lower in warm FW compared to temperate FW conditions (Fig. 1B). The temperature is the only factor exerting an effect on plasma Na^+ levels (Fig. 1B, two-way ANOVA, $p < 0.0001$). Na^+/Cl^- ratio in SW at 24 °C was significantly lower than in temperate conditions in SW and FW but was not significantly different from Na^+/Cl^- ratio in FW at 24 °C. Na^+/Cl^- ratio was slightly but not significantly increased in FW 18 °C compared to SW 18 °C (Table 1).

3.2. Branchial Na^+/K^+-ATPase activity

Branchial NKA activity at acclimation temperatures (18 °C or 24 °C, $V_{apparent}$) did not change between different salinity and temperature conditions and was significantly lower than NKA activities assayed at 37 °C (V_{max}) (Fig. 2). When NKA activity was assayed at 37 °C, NKA activity was higher in FW compared to SW conditions by 67% and 80% at the temperate and warm temperature, respectively. Overall, lower NKA activities (V_{max}) were measured in warm conditions. There was a significant salinity effect ($p < 0.001$) and temperature effect ($p < 0.01$) but the interaction between temperature and salinity did not exert an effect on NKA activities (V_{max}) (Fig. 2, two-way ANOVA).

3.3. Branchial histology, morphometric analyses and immunolocalization of Na^+/K^+-ATPase

MRCs were detected by NKA immunolabeling in gill filaments (in

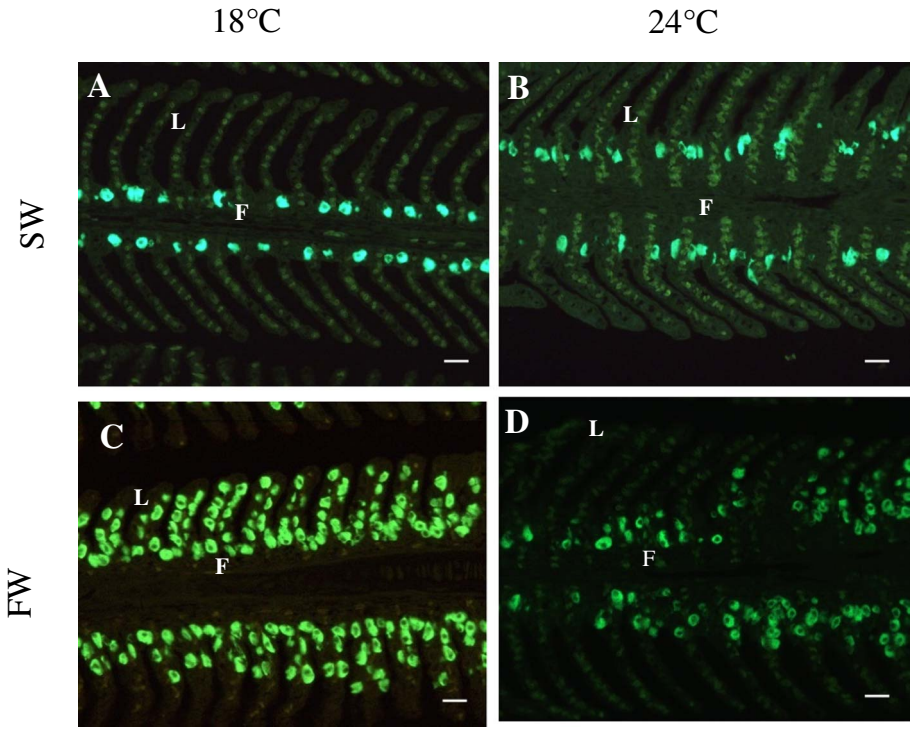

Fig. 3. Immunolocalization of gill Na^+/K^+-ATPase of sea bass exposed to SW (A, B) and FW (C, D) at 18 °C (temperate, A, C) and 24 °C (warm, B, D). F: filament, L: lamellae, FW: fresh water, SW: seawater, Scale bar: 20 μm.

SW and FW conditions) and in lamellae (almost exclusively in FW conditions) (Figs. 3, 4). Contrary to SW conditions, where temperature does not affect MRC density, fish acclimated to FW temperate conditions had significantly more MRCs on lamellae and filaments than fish acclimated to FW warm conditions (Figs. 3, 4A). Moreover, the MRC area was significantly decreased in FW warm condition (Fig. 4B).

Lamellar width at the base (L.wd.b., ranging from 17–25 μm) and at the top (L.wd.t., ranging from 9–12 μm) was significantly higher in SW warm compared to the SW temperate condition whereas no effect was recorded in FW conditions (Figs. 5, 6A, B). Lamellar length (L.l.) however was significantly higher in FW warm compared to FW temperate conditions and no temperature effect was recorded in SW conditions (Figs. 5, 6C). Decreased filament thickness (F.th.) was measured in FW warm compared to FW temperate conditions whereas no effect was recorded between temperature regimes in SW (Figs. 5, 6D).

3.4. Scanning electron microscopy

The apical features of MRCs revealed different types of apical surfaces in sea bass exposed to different salinity regimes. Sea bass acclimated to FW showed some MRCs with wide and large apical surfaces with a few but well apparent microvilli (Fig. 7C, D). MRCs of sea bass acclimated to SW showed narrow and small, apical surfaces forming an apical pit (Fig. 7A, B). As a lot of mucous was detected on the gill surface (not shown), MRC apical surfaces could not be quantified among salinities and temperature regimes. Pavement cells (PC) showed well-developed microridges on apical surfaces at both salinities (Fig. 7A, C, D). In SW warm conditions, microridges were less developed, and nearly absent in the centre of the pavement cells (Fig. 7B).

4. Discussion

This study provides information on osmoregulatory mechanisms at two temperatures (18 and 24 °C, temperate and warm, respectively) in response to a salinity challenge from SW to FW.

4.1. Warm temperatures affect hydromineral status in fresh water

Direct transfer to fresh water resulted in decreased blood osmolalities as previously reported in sea bass (Bossus et al., 2011; Jensen et al., 1998). Blood osmolality is notably correlated to Cl^- levels ($r = 0.9738$, $p < 0{,}0001$) and not to Na^+ levels ($r = 0.09715$, $p > 0{,}05$). Changes in plasma ion levels as a result of temperature acclimation have been reported in many fish species (Metz et al., 2003; Fiess et al., 2007; Sardella et al., 2008; Mitrovic and Perry, 2009; Vargas-Chacoff et al., 2009) and could thus be expected in sea bass. This study has shown maintenance of overall hydromineral balance (blood osmolality and main plasma ion levels) in SW, 2 weeks after salinity transfer. In FW warm conditions however, there was an effect of temperature that seems to differ among ions (Na^+ levels were lower whereas Cl^- levels were higher in FW warm compared to FW temperate conditions). Na^+/Cl^- ratio is slightly higher in FW (1.65 ± 0.12) compared to SW (1.12 ± 0.04) in temperate conditions unlike warm conditions, were Na^+/Cl^- ratio is similar in SW (0.93 ± 0.02) and FW (1.07 ± 0.03). A Na^+/Cl^- ratio of 1.50 ± 0.01 was measured in FW-acclimated sea bass by Jensen et al. (1998), that is similar to our results in temperate FW. The authors concluded in this study that a high Na^+/Cl^- ratio in FW compared to SW could indicate a metabolic alkalosis. In sea bass maintained in warm conditions, the absence of a high Na^+/Cl^- ratio following FW transfer might indicate a different handling of acid-base status. This could be linked to an imbalance in branchial ion transporters/channels and uncoupled Na^+ vs Cl^- uptake. If branchial Na^+ uptake is more affected by warm temperatures, it is likely that the gill MRCs mainly involved in Na^+ uptake (comparable to HR cells in zebrafish (Lin et al., 2006) or PNA-MRCs in rainbow trout (Reid et al., 2003)) are more affected by warm temperatures than other MRC types involved in Cl^- uptake. Na^+ uptake is generally linked to H^+ excretion but has not been elucidated in sea bass. Transporters/channels like the Na^+/H^+ exchangers (NHE2 or 3), the acid sensing ion channel (ASIC) as well as the Na^+/HCO_3^- cotransporter 1 (NBC1) and the V-H^+-ATPase will be investigated in future studies (Hwang and Lee, 2007; Evans, 2011; Hwang, 2011; Hwang et al., 2011; Dymowska et al., 2014). Despite lower Na^+ levels, blood osmolality was higher in FW warm conditions than FW temperate conditions which indicate a strong

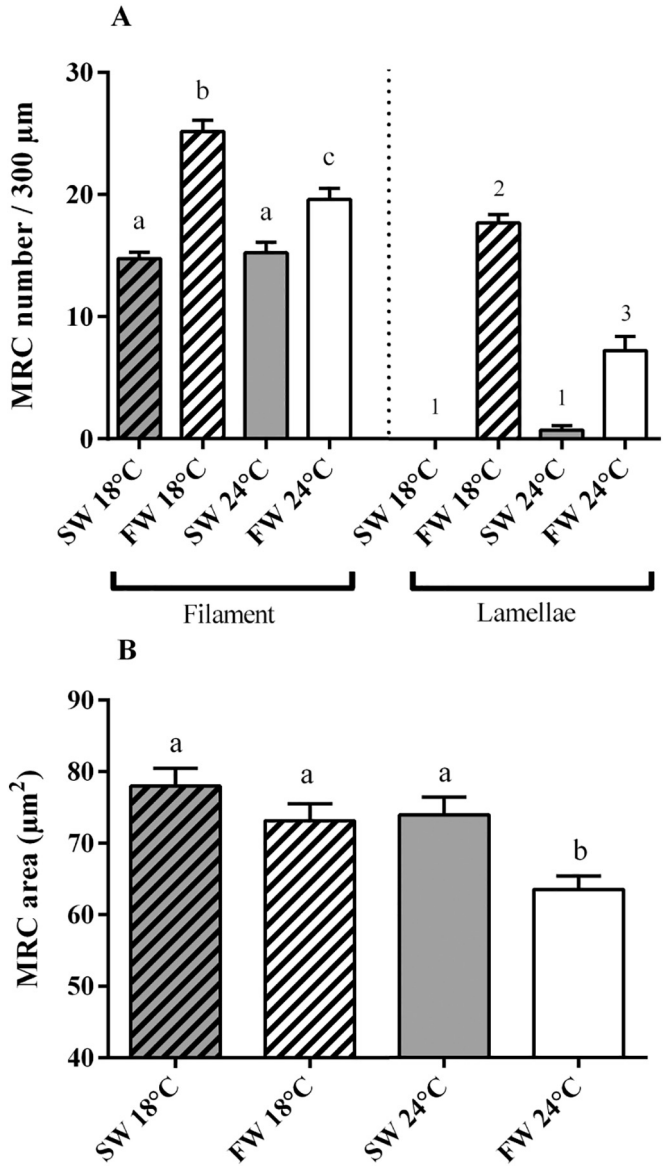

Fig. 4. Mitochondrion-rich cell number on filaments and lamellae per 300 µm of gill filament and MRC area (B) in sea bass exposed to SW and FW at 18 °C (temperate) and 24 °C (warm). Different letters (filaments) and numbers (lamellae) indicate significant differences between conditions (Kruskal-Wallis test followed by a Dunn's multiple comparison test, $p < 0.05$, $N = 3$, 10–25 measurements per individual). Data are expressed as means ± SEM. FW: fresh water; SW: seawater.

capacity to osmoregulate and to compensate low Na^+ levels through increased Cl^- uptake at low salinity and high temperatures. This allows fish to stay in lagoon-like habitats characterized by instable (Newton and Mudge, 2003) and sometimes contrasted environmental conditions such as those tested in this study.

4.2. Branchial Na^+/K^+-ATPase activity and mitochondrion-rich cell area and density

Gill parameters were affected by different temperature regimes. Maximal NKA activity (V_{max}) (an estimate of the maximized NKA activity reflecting the total amount of enzyme, as stated by Metz et al., 2003) was higher in FW-acclimated sea bass compared to SW-acclimated fish as was expected from previous investigations (Jensen et al., 1998; Nebel et al., 2005). Increased NKA activity (V_{max}) in FW is certainly linked to the higher number of MRCs recorded in filaments and lamellae in FW-acclimated fish suggesting increased active transport for ion uptake. Overall, maximal NKA activity (V_{max}) was lower in warm compared to temperate conditions which suggests decreased net branchial active ion transport in sea bass acclimated to warm temperatures. NKA activities ($V_{apparent}$) measured at the acclimation temperatures (24 °C and 18 °C) were significantly lower (by around 5-fold) than NKA activities assayed at 37 °C (V_{max}) and there were no significant differences between salinity and temperature conditions. In temperate conditions, however, there is a tendency of increased apparent NKA activity ($V_{apparent}$) in FW but without significant difference, due to the high variability of data. This inconsistency between NKA activities assayed at different temperatures (V_{max} and $V_{apparent}$) has already been reported in previous studies (McCarty and Houston, 1977; Doneen, 1981; Metz et al., 2003; Sardella et al., 2004a; Sardella et al., 2008). Assaying apparent NKA activity seems essential for proper physiological interpretation and has, to our knowledge, been done for the first time in sea bass gills. Our results clearly show that using physiological assay conditions, FW transfer results in a slight though not significant increased NKA activity ($V_{apparent}$) in temperate conditions, contrary to warm conditions where NKA activity ($V_{apparent}$) does not change following salinity transfer. The difference between assay temperatures could be linked to the differential activation of different isoenzymes (like NKA alpha isoforms, see Blondeau-Bidet et al., 2016) in warm compared to temperate waters, which remains to be investigated in future studies.

As stated above, FW-acclimated sea bass have a higher density of MRCs on lamella and gill filaments, which is consistent with other studies dealing with FW transfer in euryhaline teleosts (Lin et al., 2003; Seo et al., 2009; Riou et al., 2012) and has been previously shown in sea bass (Varsamos et al., 2002; Nebel et al., 2005; Bossus et al., 2013). What is new in this investigation is that high temperatures negatively affect MRC number and area in FW. We hypothesize that this decrease could lower branchial ion uptake capacities and might also affect hydromineral balance after long term FW acclimation (> 2 weeks). The lower size and number of MRCs may also partly explain that NKA activity ($V_{apparent}$) does not increase in FW warm compared to FW temperate conditions. In common carp, temperature seems also to affected MRC size with larger MRCs in 15 vs 29 °C acclimated fish (Metz et al., 2003). In sea bass, contrary to FW conditions, MRC size was not affected in SW conditions, which partially explains maintenance of hydromineral balance in warm SW-acclimated fish.

4.3. Gill remodeling, an essential response to environmental changes

Gill remodeling occurred in sea bass exposed to different salinity and temperature regimes. Several studies have addressed gill remodeling in fish related to temperature and/or salinity changes that are likely to affect the osmorespiratory compromise (Sollid et al., 2005; Sardella and Brauner, 2007; Mitrovic and Perry, 2009; Perry et al., 2010; Barnes et al., 2014). Negative interaction between gas exchange and ion regulation has been postulated in several studies (Randall et al., 1972; Mitrovic and Perry, 2009; Turko et al., 2012). In SW at 24 °C, lamellar width was increased compared to SW at 18 °C. This is not linked to the MRCs, as these cells are located on the filament only. Increased lamellar width is probably linked to a thickening of respiratory cells and might increase blood to water distance and affect gas exchange in warm SW. Sea bass transferred to FW in control temperate conditions show higher lamella width and filament thickness, and lower lamellar length, all parameters probably linked to the recruitment of numerous MRCs required for effective ion uptake. At the temperature ranges that we have used in our study, low-salinity waters have generally higher dissolved O_2 levels than SW (5.2 mL/L in distilled water compared to 6.4 mL/L at 35‰ at a temperature of 20 °C, according to Ivanoff, 1972). We can thus suggest that sea bass maintained in temperate FW have higher dissolved O_2 levels than those maintained in temperate SW and seem to have a sufficient lamellar surface area to maximize diffusive gas transfer. The observed gill remodeling

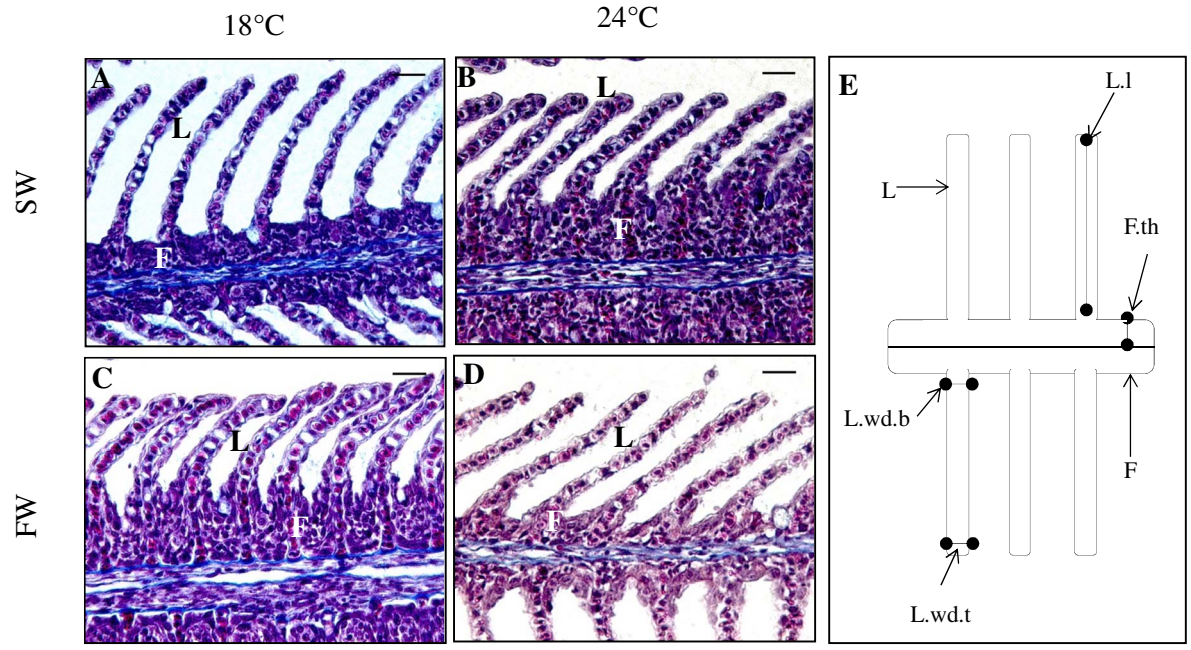

Fig. 5. Masson's Trichrome staining of gills in sea bass exposed to SW (A, B) and FW (C, D) at 18 °C (temperate, A, C) and 24 °C (warm, B, D) and schematic diagram (E). F: filament, L: lamellae, F.th.: filament thickness, L.l.: lamellar length, L.wd.b.: lamellar width at base, L.wd.t.: lamellar width at top, FW: fresh water, SW: seawater, Scale bar: 20 μm.

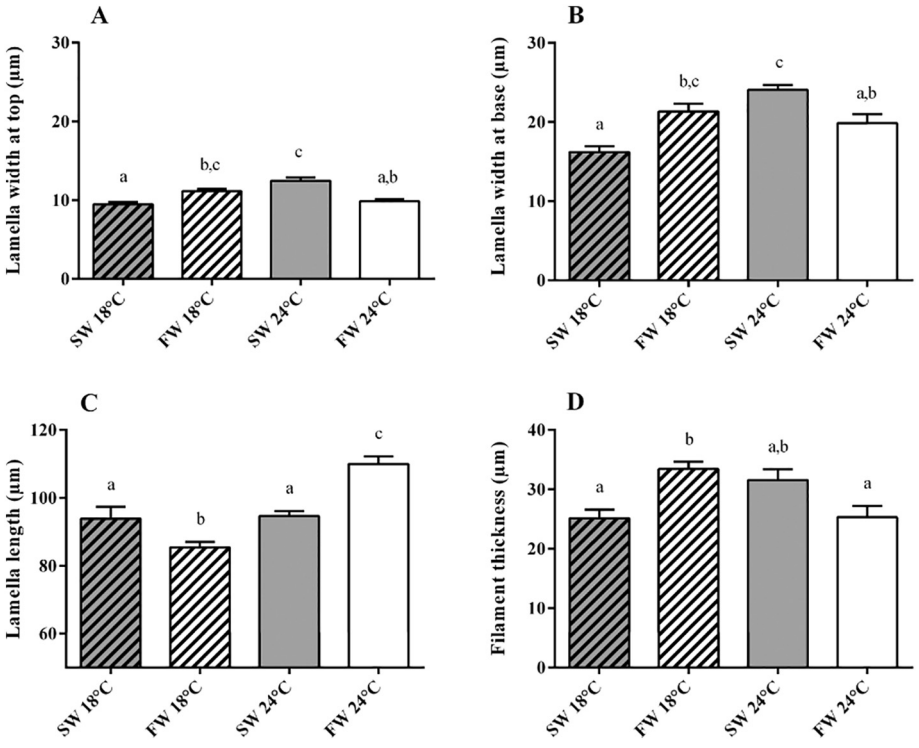

Fig. 6. Lamella width at the top (A), lamella width at the base (B), lamella length (C) and filament thickness (D) of sea bass exposed SW and FW at 18 °C (temperate) and 24 °C (warm). Different letters indicate significant difference between conditions (Kruskal-Wallis test followed by a Dunn's multiple comparison test, $p < 0.05$, $N = 3$, 18–27 measurements per individual). Data are expressed as means ± SEM. FW: fresh water; SW: seawater.

(following FW transfer in temperate conditions) seems to be in favor to maximize ion uptake. Chatelier et al. (2005) addressed oxygen uptake rate in sea bass maintained at 14 °C across salinities ranging from FW to SW and did not show any difference between salinities, however further studies should address oxygen uptake in this species, as only three fish were analyzed in FW. SW to FW transfer in warm conditions affected all measured parameters in an opposite way than what was observed in temperate conditions (decreased lamella width, increased lamella length and slightly decreased filament thickness in FW warm). This can be explained partially by a lower increase of MRC density in FW warm (compared to FW temperate) and the recruitment of smaller MRCs in FW warm conditions. The high lamella length in warm FW suggests a stronger need for O_2 uptake in this media, as O_2 is less available in warm waters. These data suppose that gill remodeling following FW transfer occurs to maximize gas exchange rather than osmoregulation in warm waters. Our results are inconsistent with the study on five coral reef fishes investigated at temperatures ranging from 29 °C to 34 °C, where the base of lamellae and length of the lamellae did not change with increasing temperatures (Bowden et al., 2014). It is likely that high temperatures are experienced differently between temperate and tropical reef fishes. Absence of gill remodeling could be disadvantageous in tropical fishes that have to maintain O_2 uptake mechanisms at high

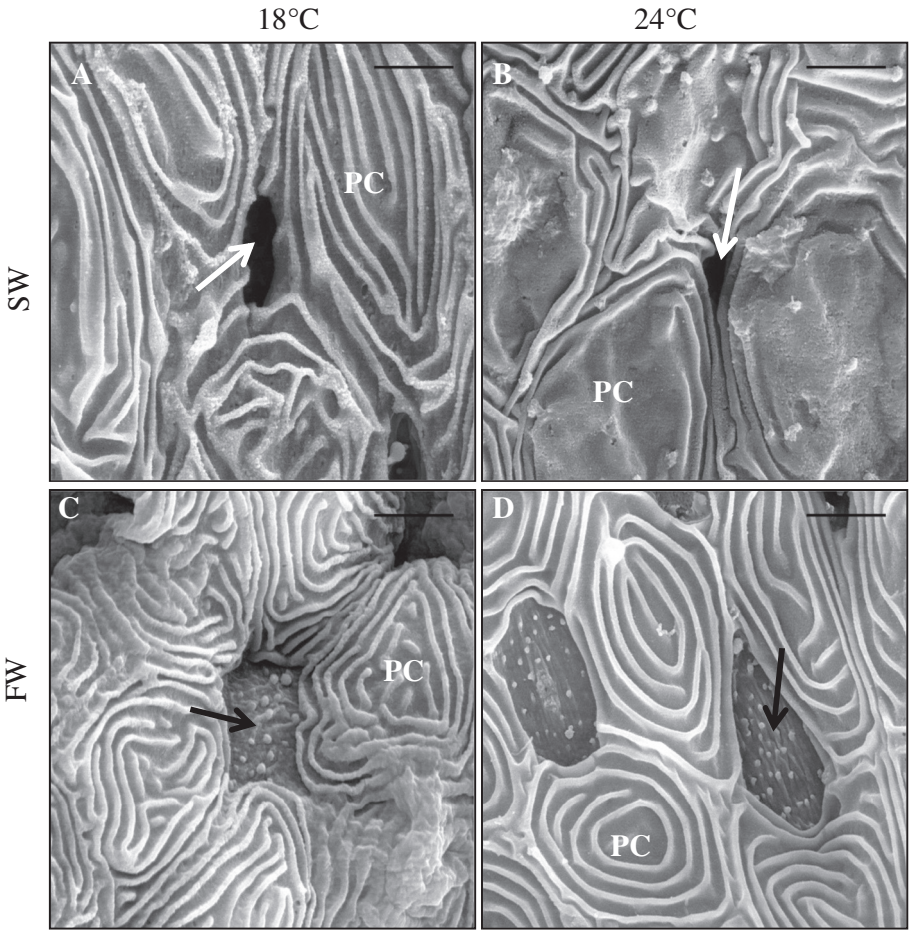

Fig. 7. Scanning electron micrographs of sea bass gills exposed to SW (A, B) and FW (C, D) at 18 °C (temperate, A, C) and 24 °C (warm, B, D). Arrows indicate apical pits of mitochondrion-rich cells (MRCs) in SW (A, B) and large apical MRC openings in FW (C, D). Note that apical microridges of pavement cells (PC) are less developed in warm SW (B). F: filament, L: lamellae, FW: fresh water, SW: seawater. Scale bar: 2 μm.

temperatures, except if other mechanisms compensate for low environmental O_2 conditions. In response to air exposure or hypoxia, gill morphology of *Kryptolebias marmoratus* and *Gymnocypris przewalskii* also showed high plasticity and remodeling capability (Ong et al., 2007; Matey et al., 2008).

Apical structure of the branchial epithelium of sea bass has already been shown by Varsamos et al. (2002) at different salinities. As shown in numerous species, salinity clearly affects apical opening with small apical pits in SW to favor ion secretion and large MRC openings in FW to optimize ion uptake. No change in apical openings of MRCs could be observed in our study among temperatures, but measurements of apical areas could not be done due to numerous mucous cells masking apical cell surfaces. There was a clear difference regarding apical microridges of pavement cells (PVCs) between sea bass acclimated to different temperatures in SW. As > 90% of the gill surface correspond to PVCs, the convoluted microridges present on the PVCs have been defined as important interaction areas between the branchial epithelium and ambient water (Wilson and Laurent, 2002). Changes in apical microridges of pavement cells have been reported in other species during transfer to SW and/or FW (Daborn et al., 2001; Carmona et al., 2004; Sardella et al., 2004b; Seo et al., 2009; Choi et al., 2011; Chasiotis et al., 2012). In our study, changes in microridges surfaces were observed in warm temperature-challenged fish in SW only, probably as a response to increased temperatures and possibly linked to a change in membrane fluidity, but further investigations are required to prove that.

5. Conclusions

In summary, we have focused on the effect of salinity (SW vs FW) and temperature (18 temperate vs 24 °C warm) that are representative of environmental conditions encountered by migrating sea bass in the wild, with a focus on the onset of osmoregulatory mechanisms following FW transfer. Sea bass are euryhaline and eurythermic, as they are able to maintain relative high osmolalities in fresh water at both temperatures tested. At the gill level however, a high plasticity has been observed and gill remodeling seems to occur notably following FW transfer at both temperatures. It seems that gill remodeling occurs to maximize FW ion uptake in temperate water which is not the case in warm FW, where diffusive gas exchange seems to be maximized. NKA activity, MRC size and density seem to be lower in warm FW which also points to an altered branchial ion uptake capacity in warm FW, probably through an imbalance between Na^+ and Cl^- uptake mechanisms. We conclude that salinity and temperature stress seems to affect sea bass gill epithelium and it could be expected to encounter a more severe alteration of hydromineral balance following long-term FW transfer (> 2 weeks) at this warm temperature due to osmorespiratory compromise.

Acknowledgements

The authors would like to thank Alain Vergnet and Béatrice Chatain from Ifremer Palavas for providing fish and Evelyse Grousset for gill sectioning. The authors would like to thank Prof. Tsung-Han Lee as well as Prof. Guy Charmantier for their advices. Part of this project was funded by the OSMOVILL project (PRC n°231789, CNRS-MoST).

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Chapter IV

Coping with environmental changes: gene expression

Chapter IV - Coping with environmental changes: gene expression

4.1 Preamble

Gills are the most important site for active ion transport with highly specialized ion-transporting cells called ionocytes (discussed in Chapter I and III). In eurytherm and euryhaline species, the density and distribution of gill ionocytes are supposed to change as a response to temperature and salinity changes (Metz et al., 2003; Mitrovic and Perry, 2009). As shown in Chapter III, warm temperature (24 °C) affected ionocyte density in the freshwater condition which suggests altered ion transport mechanisms (Masroor et al., 2018). Ion transport alteration at the gills may result in plasma osmolyte imbalance (review in Burton, 1986) as we have shown in European sea bass for plasma Na^+ and Cl^- (Chapter III, Masroor et al. 2018). Fish ionoregulation is highly dependent on temperature as shown in several species (Fiess et al., 2007; Imsland et al., 2003; Metz et al., 2003). Few studies have addressed the combined effects of both salinity and temperature at the gene expression level (Morrison et al. 2006; Chou et al. 2008; Michael et al. 2016a; Vargas-Chacoff et al. 2018; Gibbons et al. 2018). Gill membrane transporters and channels contribute directly (e.g. Na^+/H^+ exchanger or Na^+/Cl^- cotransporter) or indirectly (e.g. ammonia transporters) to osmoregulation and acid-base regulation processes. Changes in expression of these transporters have been reported in numerous species regarding salinity transfers, but the effect of increased temperature on the expression of these genes is much less known, notably following salinity change. It has been demonstrated that acclimation to elevated temperature affects the expression of genes in different organs of fishes (Morrison et al. 2006; Kyprianou et al. 2010; Logan and Somero 2011; Logan and Buckley 2015; Podrabsky and Hand 2015; Michael et al. 2016a; Hu et al. 2016).

Previous studies on juvenile sea bass have analyzed gene expression level of different ion transporters notably upon freshwater challenge in temperate conditions. An increased expression level of *nka α1a* (*atp1a1.a)*, *nhe3* (*slc9a3)*, *ncc2a* (*slc12a3-like)* and *rhcg1* was measured following a two-week FW challenge (Blondeau-Bidet et al. 2019). On the contrary, *nkcc1* (*slc12a2)*, *cftr* and *clc3* (*clcn3)* were downregulated in FW compared to SW conditions (Lorin-Nebel et al. 2006; Bodinier et al. 2009; Bossus et al. 2013; Blondeau-Bidet et al. 2019). So far, the effects of increased temperatures on branchial mRNA expression of ion transporters have not been documented in sea bass, notably comparing different salinities.

4.2 Main findings

This Chapter IV is evaluating the effects of warm temperature preacclimation on the mRNA expression level of different ion transporters in European sea bass *Dicentrarchus labrax* in seawater and following freshwater transfer. The experimental conditions were the same than discussed previously (Chapter II and Chapter III). The results showed that a temperature increase affected the expression of several genes involved in osmoregulation, acid-base balance and ammonia excretion in gills, at both tested salinities. In Chapter III, we reported that plasma Na^+ levels were decreased in warm (24 °C) compared to temperate conditions (18 °C) at both salinities. On the contrary, plasma chloride levels were increased in FW warm conditions (24 °C) compared to FW temperate conditions (18 °C) and no effect of temperature was reported in SW (Masroor et al., 2018). These results may point to enhanced Cl^- uptake and decreased Na^+ uptake (or enhanced Na^+ loss) at the gill level but also other osmoregulatory tissues could be involved (for example the posterior kidney). Additionally, it suggests the activation of diverses transport pathways between warm and temperate conditions. Our results brought new knowledge in integrated physiological and molecular research of sea bass by analyzing the effect of warm temperatures on ion transport processes. In summary, several branchial genes that we have analyzed exhibited a significant increase in expression at 24 °C in SW and to a lesser extent in FW.

Some key results of the present study demonstrated a response of sea bass to altered plasma sodium, chloride level and sodium/chloride ratio measured in Chapter III. For example, *cftr* levels plotted against blood chloride levels (Fig. 2, P-87) showed that the high *cftr* levels in warm-acclimated SW fish were not specifically linked to higher plasma chloride levels when we compare to temperate conditions. The high expression of *cftr* in SW warm conditions certainly contributes to the high capacity of sea bass to maintain plasma chloride levels in SW after a long-term warm acclimation (4 weeks). Similarly, sea bass responded to a possible blood acidosis (measured by a low plasma Na^+/Cl^- ratio) (Jensen et al., 1998; Masroor et al., 2018) by an overexpression of *nhe3* and as well as *vha-a* and *vha-b* in warm conditions. Additionally, *vha-a* and *vha-b* are negatively correlated to the Na^+/Cl^- ratio (Table 3, P-85). This reinforces the role of branchial VHA in proton excretion. For future studies, subcellular localization of VHA has to be investigated in order to better understand the role of VHA in sea bass.

To evaluate the mechanisms involved in the switching from hypo to hyper-osmoregulation under temperature stress, we have analyzed branchial mRNA expression of transporters involved in ion uptake. Fish transferred from SW to FW showed higher branchial NKA activity at both temperatures. In FW, NKA maximal activity was lower in warm compared to temperate conditions. Temperature strongly affected *nka α1a* expression in SW with a significantly higher expression of *nka α1a* in SW at 24 °C compared to 18 °C. This indicates a high active ion transport capacity and certainly contributes to the maintenance of NKA activity in warm SW. No increase of *nka α1a* expression was recorded following FW transfer in warm conditions, unlike temperate conditions, probably contributing to the lower NKA activity in FW warm *vs* FW temperate conditions and suggesting altered ion uptake in warm FW. *Ncc2a* and *nhe3* have then been analyzed, both of these transporters are involved in ion uptake (Na^+ and Cl^-) and are expected to be highly expressed in temperate FW compared to SW conditions. The expression level of *ncc2a* and *nhe3* are both negatively correlated to plasma Na^+ and Cl^- levels. These transporters are apically localized in sea bass FW-type ionocytes and are thus essential for transepithelial Na^+ and Cl^- uptake (Inokuchi et al. 2017; Blondeau-Bidet et al. 2019). *Ncc2a* is upregulated upon warm temperature acclimation, in SW and following FW transfer. Contrary to *nhe3* and *nka α1a*, there is a significant difference in *ncc2a* expression between SW warm and FW warm, suggesting an induction of NCC2-type cells to take up Cl^- and Na^+ in warm FW. In Chapter III we have observed a decreased density of lamellar ionocytes in warm-acclimated fish challenged to FW (Masroor et al., 2018). In sea bass, different ionocyte cell types have been distinguished and it seems that NHE3-type cells are localized on lamellae whereas NCC2-type cells are detected on gill filaments (Blondeau-Bidet et al. 2019). This decrease in lamellar ionocytes might be linked to the absence of NHE3 induction in warm FW conditions *vs* SW conditions.

In this study, we also showed that *rhcg1* was highly upregulated in warm temperature-challenged fish at both salinities. In FW, RHCG1 might operates in concert with NHE3 and VHA to take up Na^+ and excrete NH_3 forming a metabolon as shown in other species, but this remains to be analyzed further in sea bass (Catches et al. 2006; Chang et al. 2009; Nawata et al. 2010; Heuer and Grosell 2014). At higher temperature, the induction of ammonia excreting channels might also indicate an enhanced metabolism that could be measured in future investigations. The underlying mechanisms of ammonia excretion are still to be determined in sea bass.

4.3 Conclusion

In present study, temperature affected the expression of several branchial transporters. This clearly shows an effect of warm temperature on the ion transport machinery of sea bass at the gill level with the most striking response in SW, indicating a compensating response to temperature stress in this condition. This response contributes to the maintained whole-organism ion homeostasis in warm-acclimated sea bass to SW conditions, notably regarding plasma chloride levels. In FW, the observed plasma ion (Na^+ and Cl^-) imbalance measured is probably linked to a less effective response at the gill level, suggesting an imbalanced induction of genes encoding for Na^+- *vs* Cl^- uptake. Other osmoregulatory tissues as well as other genes (notably Cl^-/HCO_3^- exchangers that are supposed to contribute to Cl^- uptake) should be analyzed to have a broader view on the effect of temperature on osmoregulatory processes.

Research article II

Effect of salinity and temperature on the expression of genes involved in branchial ion transport processes in European sea bass *Dicentrarchus labrax*

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Submitted in

Journal of Thermal Biology

Acknowledgements

Sea bass were generously donated by the Ifremer Palavas. We would like to thank Philippe Clair from the qRT-PCR CEMEB platform for his technical guidance.

Abstract

The responses of European sea bass to temperature increase and salinity decrease were investigated measuring mRNA expression levels of main genes involved in ion transport. Juvenile fish were pre-acclimated to seawater (SW) at 18 °C (temperate) or 24 °C (warm) for two weeks and then transferred to either fresh water (FW) or SW at the respective temperature. Unlike temperate conditions, there is no induction of Na^+/K^+-ATPase α1a (*nka α1a*) and Na^+/H^+ exchanger 3 (*nhe3*) following FW transfer in warm conditions. This is linked to the high expression of these genes in warm SW. Na^+/Cl^--cotransporter (*ncc2a*) expression however is induced following FW transfer in warm conditions. Main transporters involved in ion excretion ($Na^+/K^+/2Cl^-$-1, *nkcc1* and cystic fibrosis transmembrane conductance regulator, *cftr*) as well as nitrogen excretion (Rh-glycoproteins, *rhcg1* and *rhbg*) and acid-base regulation (V-type H^+-ATPase, *vha-a and b*) are highly expressed in SW warm conditions *vs* FW warm. Overall, our results suggest a higher induction of ion transport processes in warm conditions and more strikingly in SW. This is linked to a strong interplay between diverse ion transporters in order to coordinate physiological responses at the gill level.

Key words: *Dicentrarchus labrax,* ion transporter, gill, mRNA expression, thermal acclimation, salinity

Introduction

Temperature is considered a main factor affecting organism distribution, life-history traits and biological processes (Crockett and Londraville, 2006; Hutchison and Maness, 1979; Schulte, 2011). The effects of temperature on ion-regulatory mechanisms and other physiological processes have been shown in several fish species (Burton, 1986; Chou et al., 2008; Gibbons et al., 2018; Kreiss et al., 2015; Metz et al., 2003; Michael et al., 2016; Morrison et al., 2006) including recently in the European sea bass *Dicentrarchus labrax* (Masroor et al., 2018). Ion homeostasis is mainly relying on ion pumps such as the branchial Na^+/K^+-ATPase (NKA) and V-type H^+-ATPase (VHA), both creating an electrochemical gradient for active ion transport across gill basolateral and apical membranes (Evans, 2008; Evans et al., 2005, 1999; Hwang and Lin, 2014; Vasić et al., 2008). It has been reported that active ion transporters represent a higher thermal sensitivity than carrier-mediated diffusive transporters (Moyes and Ballantyne, 2011) which could lead to imbalances between active and passive ion transport. Ion transport alteration at the gills due to high temperatures can also occur due to changes in membrane integrity and fluidity that could affect the proper insertion and function of ion transporters (Moyes and Ballantyne, 2011). As Na^+ and Cl^- are thought to be taken up by different uptake pathways, plasma ion imbalance likely affect Na^+/Cl^- ratio which could lead to acid-base imbalance (Goss et al., 1998; Jensen et al., 1998). The fish gill is a plastic organ involved in gas exchange, ion regulation, acid-base balance and nitrogen excretion and significantly contributes to physiological homeostasis in changing environments (Evans et al., 2005). In response to temperature and salinity changes, fish gills are subject to significant morphological remodeling (Metz et al., 2003; Tzaneva and Perry, 2010) and changes in ionocyte density and distribution (Metz et al., 2003; Mitrovic and Perry, 2009). We have previously shown that gill morphological parameters differed in sea bass that have been pre-acclimated at two different temperatures, 24 °C and 18 °C in seawater (SW) and fresh water (FW) with particularly a less ionocyte density in warm FW (Masroor et al., 2018). Plasma Na^+ levels were decreased in warm (24 °C) compared to temperate conditions (18 °C) at both salinities. On the contrary, plasma Cl^- levels were higher in FW warm compared to temperate conditions and no effect of temperature was reported in SW. These results may point out an effect of increased temperatures on enhanced FW Cl^- uptake and decreased Na^+ uptake (or enhanced Na^+ loss) at the gill and/or kidney levels. Membrane transporters and channels, including Na^+/H^+ exchangers (NHE2/3), VHA, ammonia transporters (Rhesus (Rh) glycoproteins, mainly RHBG and RHCG1), NKA and Na^+/Cl^- cotransporters (NCC2)

contribute directly or indirectly to ion homeostasis. Basolateral $Na^+/K^+/2Cl^-$ (NKCC1) and apical cystic fibrosis transmembrane conductance regulator (CFTR) are involved in ion excretion in SW (Hwang and Lin, 2014). The mechanisms for ion uptake in FW have been reviewed in several reports and involve a multitude of transporters working together to take up Na^+ and/or Cl^- ions in exchange of different counterions, mainly H^+ and HCO_3^- (Dymowska et al., 2012; Hsu et al., 2014; Hwang, 2009). In sea bass maintained in FW for two weeks, gill NHE3 and NCC2 have been detected in different ionocyte subtypes at the apical cell part (NHE3-type cell and NCC2 cell), coupled to basolateral NKA in order to absorb Na^+ and Cl^- (Hwang et al., 2011; Kumai and Perry, 2012; Tang et al., 2011). ClC-3 is a volume-activated chloride channel involved in transepithelial Cl^- transport (Bossus et al., 2013; Tang and Lee, 2011) and cell volume regulation (Duan et al., 1999, 1997; Hermoso et al., 2002; Wang et al., 2000). Changes in expression of these and other transporters have been reported in numerous species following salinity transfers, but the effect of increased or decreased temperature on the expression of these genes is less known (Chou et al., 2008; Gibbons et al., 2018; Hu et al., 2016; Kyprianou et al., 2010; Logan and Buckley, 2015; Logan and Somero, 2011; Michael et al., 2016; Morrison et al., 2006).

Several paralogs exist in fish for *nka α1* with potentially different functions and expression patterns depending on the considered species (Hwang and Lee, 2007). Two paralogs have been identified in sea bass, *nka α1a* and *nka α1b*, with *nka α1a* being the most expressed paralog in sea bass gills whatever the considered salinity (Blondeau-Bidet et al., 2016). Temperature generally increases NKA transcript expression and protein activity but the response highly depends on the species and even the population (Michael et al., 2016; Morrison et al., 2006). Michael et al. (2016) have reported population-specific differences regarding mRNA expression levels of *nka* in cod *Gadus morhua* populations maintained at the same temperature but originating from a different thermal niche. The European sea bass *Dicentrarchus labrax* (Linnaeus 1758) is an important aquaculture species along the Mediterranean and Atlantic coasts. It is considered as a highly eurythermal (tolerates temperatures from 4 to 35 °C) and euryhaline species (tolerating from FW up to 90 ppt) (Barnabé, 1989; Dülger et al., 2012; Madeira et al., 2013). In the wild, sea bass adults and juveniles are frequently exposed to fluctuations of environmental parameters, notably during their stay in lagoons and estuaries (Dufour et al., 2009; Newton and Mudge, 2003). Temperature acclimation and preference have been studied in sea bass by investigating oxygen consumption (Dalla Via et al., 1998), fish distribution (Trancart et al., 2016), food intake (Dülger et al., 2012; Person-Le Ruyet et al., 2004) and swimming speed (Claireaux et

al., 2006). *D. labrax* optimal growth rate was reported at 25 °C (Person-Le Ruyet et al., 2004) which is close to the warm temperature analyzed in our study.

In this study, we analyzed branchial ion regulatory mechanisms at the transcript level with a particular focus on genes involved in osmoregulation, acid-base regulation and ammonia excretion. We compared mRNA levels of *nka α1a* (*atp1a1a*), *nka α1b* (*atp1a1b*), *cftr*, *nkcc1* (*slc12a2*), *nhe3* (*slc9a3*), *ncc2a* (*slc12a3-like*), *clcn3* (*clc-3*), *vha-a* (*atp6v1a*) and *vha-b* (*atp6v1b2*), *rhbg* and *rhcg1* in gills from fish acclimated at two different temperatures (18 °C and 24 °C) and transferred from SW to FW.

Material and Methods

Experimental conditions

Experimental conditions have been previously described in Masroor et al. (2018). Briefly, juvenile sea bass from a Western Mediterranean population were obtained from the Ifremer Station at Palavas-les-Flots (Herault, France). Fish were brought to the Montpellier University and maintained for one week in 3,500 L tanks containing natural seawater (SW) form the Mediterranean Sea at 38 ppt and 18 °C, under a 12 h light/12 h dark photoperiod. Fish were transferred to 200 L tanks (14 fish/tank, density of 6-7 kg/m3, two replicates per conditions) to be acclimated either at 18 °C or 24 °C (with a temperature increase of 0.2 °C/h). After two weeks of temperature acclimation, fish were transferred directly either to dechlorinated tap water (fresh water, FW), or to SW (7 fish/tank, two replicates for each conditions) and maintained in this salinity two weeks before sampling. Ionic composition (in $mEq.L^{-1}$) of the FW was Na^+ (0.12), K^+ (0.04), Ca^{2+} (5.70), Mg^{2+} (0.29), Cl^- (0.98), NO_3^- (0.06) SO_4^{2-} (0.61). Water was aerated and mechanically/biologically filtered (Eheim System, Lens, Pas-de-Calais, France). Temperature, salinity, oxygen and nitrogen levels were checked daily. A quarter of the water volume was changed every two days. Fish were fed twice a week with fish granules (Aphymar feed, Meze, Herault, France) until 2 days before sampling. At the end of the experiment, fish were anesthetized in a solution of phenoxy-2-ethanol (240 ppm) prior to tissue collection. The fish used for the experiment had a length of 20.77±1.32 cm (mean ± SD) and average weight was 86.87±20.23 g. Four groups were compared: SW at 18 °C (temperate SW), SW at 24 °C (warm SW), FW at 18 °C (temperate FW) and FW at 24 °C

(warm FW). These experiments respected the guidelines of the European Union (directive 86/609) and of the French law (decree 87/848) regulating animal experimentation.

RNA extraction purification and reverse transcription

Gill tissues were collected from gills of SW- and FW-exposed sea bass in temperate and warm conditions. The epithelium of the first gill arch was scraped with a sterile scalpel, immerged in Trizol® reagent and flash frozen in liquid nitrogen. Tissues were then stored at -80 °C until analysis. Total RNA was extracted using Trizol® reagent according to the manufacturer's instructions. RNA quantity and purity were assessed by measuring the A260/A280 ratio using the NanoDrop® ND-1000 V3300 spectrometer (Nanodrop Technology Inc., Wilmington, Delaware, USA). RNA quality was checked using Agilent bioanalyzer (Agilent) using electrophoretic trace method. One microgram of the total RNA was treated with DNase I amplification grade (Invitrogen™, Life Technologies). Reverse transcription was performed using 200 U M-MLV reverse transcriptase (Invitrogen™) and first strand of complementary DNA (cDNA) was generated using 250 ng of random primers (Invitrogen™), dNTPs (10 mM) and 40 U of RNase OUT (Invitrogen™), following manufacturer's instruction.

Quantification at the transcript level

Specific primers (forward and reverse) for different transporters are listed in Table 1. Quantitative real-time PCR analyses (qRT-PCR) were performed using the LightCycler® 480 Real-Time PCR System (Roche, Mannheim, Baden-Württemberg, Germany) with 2X LightCycler-FastStart DNA Master SYBER-Green I™ Mix (Roche), forward and reverse primers (at a final concentration of 0.5 µM) and cDNA. The qRT-PCR conditions were: denaturation at 95 °C for 10 min, followed by 45 cycles of repeated amplification (95 °C, 10s), hybridization (60 °C, 10 s) and elongation (72 °C, 10 s), and a final step at 40 °C for 30 s. A melting curve program was performed to control the amplification specificity, and the amplification products were sequenced. *ef1α* (encoding elongation factor 1α) was used as reference gene as in previous studies performed on salinity challenged sea bass (Blondeau-Bidet et al., 2016; Lorin-Nebel et al., 2006) and as recommended by Mitter et al. (2009). Ultra-pure water was used as a no-template control in the qRT-PCR. Efficiencies were determined and given in Table 1. The relative expression ratio of each target gene was

calculated using the ΔCt method with the formula: Efficiency $(E)^{-\Delta Ct}$ (Pfaffl, 2001) and the efficiency of each primer pair.

Statistical analysis

Statistical analyses were performed using Graphpad Prism (version 6, GraphPad Software Incorporated, La Jolla, CA, 268 USA). Normality and homogeneity of variance were respectively checked using D'Agostino-Pearson test and Bartlett test. When necessary, data were log-transformed to fit homogeneity of the variance assumption. Two-way factorial analysis of variance with temperature and salinity as the main factors was performed. Critical differences between groups were appraised using the Fisher's least-square difference test. Linear correlations were determined using the Spearman correlation. Data are presented as box and whisker plots showing median, minimum and maximum values. Level of statistical significance was set at $p<0.05$. Linear correlation analysis was carried out with data from mRNA levels from this study and physiological parameters (plasma Na^+ and Cl^- levels, plasma Na^+/Cl^- ratio) recently reported in Masroor et al. (2018) obtained in the same fish (Table 3).

Results

Quantitative gene expression

For *nka α1a* (*atp1a1a)* and *nka α1b* (*atp1a1b),* there was a significant salinity effect (Table 2, two-way ANOVA, $p<0.01$). Temperature and interaction between both parameters (salinity and temperature) exerted a significant effect only on *nka α1a* expression (Table 2, two-way ANOVA, $p<0.01$). In temperate conditions (18 °C), fish challenged to FW exhibited a significantly higher expression of *nka α1a* compared to SW controls (Fig. 1A). In warm conditions (24 °C), *nka α1a* expression was not significantly different between salinities (Fig. 1A). Moreover, a significant higher *nka α1a* expression was measured in SW warm compared to SW temperate.

On the other hand, *nka α1b* expression did not change in temperate conditions in SW and FW (Fig. 1B). Conversely, a significant higher *nka α1b* expression was recorded in warm conditions in SW *vs* FW- exposed fish (Fig. 1B).

For *cftr* and *nkcc1* (*slc12a2)*, there was a significant salinity (*cftr*, $p<0.0001$; *nkcc1*, $p<0.001$) and temperature effect (*cftr*, $p<0.0001$; *nkcc1*, $p<0.05$) but the interaction between temperature and salinity did not exert an effect on both of these genes (Table 2, two-way ANOVA). *Cftr* expression was significantly lower in FW than in SW at both temperatures (Fig. 1C). In both salinities, fish acclimated to 24 °C had a significantly higher expression of *cftr* than at 18 °C. Regarding *nkcc1*, fish challenged to FW had a significantly lower expression than SW fish, at both tested temperatures. *Nkcc1* expression did not change significantly between both tested temperatures (Fig. 1D) in the FW- and SW-exposed groups.

For *ncc2a* (*slc12a3-like)* and *nhe3* (*slc9a3)*, there was a significant salinity (*ncc2a*, $p<0.0001$; *nhe3*, $p<0.01$) and temperature effect (*ncc2a*, $p<0.001$; *nhe3*, $p<0.01$). Interaction between both parameters did not exert a significant effect (Table 2, two-way ANOVA). In temperate conditions, fish exhibited a significantly higher expression of *nhe3* in FW compared to SW. In warm conditions however, no differences were observed in the expression level of *nhe3* between both salinities. In SW warm conditions, fish showed a significantly higher expression of *nhe3* compared to fish acclimated to SW temperate conditions (Fig. 1E). In temperate and warm conditions, *ncc2a* expression was significantly higher in FW compared to SW (Fig. 1F). In SW, *ncc2a* expression was higher in warm compared to temperate conditions (Fig. 1F).

For *clc-3* (*clcn3)*, the temperature ($p<0.0001$) was the only factor exerting a significant effect (Table 2, two-way ANOVA). *Clc-3* expression was 2 and 1.6-fold higher at 24 °C compared to 18 °C in SW and FW, respectively (Fig. 1G).

Regarding expression of *vha-a* (*atp6v1a*), there was a significant salinity ($p<0.01$) and temperature effect ($p<0.0001$) but the interaction between temperature and salinity was not significant (Table 2, two-way ANOVA). In the case of *vha-b* (*atp6v1b2*), the temperature ($p<0.05$) is the only factor exerting a significant effect (Table 2, two-way ANOVA). In temperate conditions, no differences were observed between salinities. In warm conditions however, significantly lower *vha-a* expression was detected in FW compared to SW. *Vha-a* expression was 2 and 1.6-fold higher at 24 °C compared to 18 °C in SW and FW respectively (Fig. 1H). *Vha-b* expression was significantly higher in fish challenged to 24 °C in SW, compared to all other conditions (Fig. 1I).

For *rhbg*, there is no effect of neither salinity nor temperature (Table 2, two-way ANOVA, Fig. 1J). No difference in *rhbg* expression was observed between all analyzed conditions. In case of *rhcg1*, there was a significant salinity ($p<0.05$) and temperature effect ($p<0.0001$) but

the interaction between temperature and salinity did not exert an effect on *rhcg1* expression (Table 2, two-way ANOVA). No difference in *rhcg1* expression was observed between salinities in temperate and warm conditions. *Rhcg1* expression was significantly increased by 4 and 3.3-fold at 24 °C compared to 18 °C in SW and FW, respectively (Fig. 1K).

Correlations

mRNA levels of transporters that are involved in ion secretion (*cftr* and *nkcc1*) and of key pumps, *nka α1b* and *vha-a*, showed a positive correlation with plasma Cl^- level and negative correlation with Na^+/Cl^- ratio, as shown in Table 3. mRNA levels of transporters involved in ion uptake, *nhe3* and *ncc2a*, showed a negative correlation to plasma Cl^- and Na^+ level. mRNA levels of *clc-3* and *vha-b* are negatively correlated to Na^+/Cl^- ratio. *Rhcg1* was negatively correlated to plasma Na^+ levels. *Nka α1a* and *rhbg* were not correlated to the analyzed blood parameters.

Discussion

European sea bass raised in SW were acclimated to two environmental relevant temperatures, 18 °C and 24 °C, in order to investigate the molecular mechanisms underlying acclimation to warm temperature followed by a salinity decrease. The results showed that increased temperature affected the expression of several genes involved in osmoregulation, acid-base balance and ammonia excretion in gills, at both tested salinities.

In this study, we confirm previous results with higher branchial *nka α1a* mRNA levels in temperate FW conditions compared to SW (Masroor et al., 2018; Blondeau-Bidet et al., 2016; Jensen et al., 1998). In warm conditions however, *nka α1a* expression was similar between both salinities, which partially explain changes observed previously at the protein activity level (Masroor et al. 2018). NKA activity was increased in FW warm *vs* SW warm but to a lesser extent than in temperate conditions, probably due to a lack of transcription induction in FW warm compared to SW warm. Despite *nka α1a* has been identified as a main *nka* paralog in sea bass osmoregulatory tissues (Blondeau-Bidet et al., 2016), other paralogous genes encoding for NKA (*nka α1b, α2, β, …*) may be of importance in warm conditions and may help to better understand protein activities. It is known in the literature that *nka* expression is modulated by temperature change (Michael et al., 2016; Mitrovic and Perry, 2009; Nilsen et

al., 2007) as it seems to be the case in our study in SW notably. Posttranscriptional processes as differential NKA phosphorylation might also be worth investigating in warm *vs* temperate conditions (Féraille et al., 1999).

Ion excretion mechanisms

In SW, NKA generates the driving force for ion excretion involving basolateral NKCC1 and apical CFTR (Evans et al., 2005). *Nkcc1* and *cftr* mRNA levels are higher in SW compared to FW, as previously shown in sea bass (Bodinier et al., 2009; Lorin-Nebel et al., 2006) and numerous other teleost species (Hiroi et al., 2005; Inokuchi et al., 2017; McCormick et al., 2003; Nilsen et al., 2007). Contrary to *nkcc1*, whose expression is strongly affected by salinity only and to a much lesser extent by temperature, there seems to be an additive effect of high salinity and increased temperature on *cftr* expression. Thus, fish exposed to both, high salinity and high temperature (SW, 24° C) have greater *cftr* expression levels than fish exposed to SW without temperature increase (Fig. 2). Other than plasma chloride levels, there are probably other factors triggering *cftr* expression in warm SW, contributing to maintain constant blood chloride levels in warm SW (shown previously by Masroor et al. (2018). Branchial *nka α1b* mRNA expression seems to be affected only by salinity with slightly higher expression in SW than in FW. This tendency has already been shown in long-term (2.5 years) acclimated sea bass to FW *vs* SW controls (Blondeau-Bidet et al., 2016). We did not observe a switch from *nka α1a* to *nka α1b,* a phenomenon previously reported in salmons (McCormick et al., 2009). However, it seems that significant differences in *nka α1b* mRNA expression between SW and FW are observed only under specific circumstances as long-term acclimation to extreme salinities (Blondeau-Bidet et al., 2016) or increased temperatures (this study).

Ion uptake mechanisms

Sea bass gills display remarkable plasticity when it comes to adjusting ion transport in response to salinity changes (Masroor et al., 2018; Nilsson, 2007). The switch from hypo- to hyper-osmoregulation is achieved by the activation of ion transporters that are involved in ion uptake (Blondeau-Bidet et al., 2019; Hwang et al., 2011). Other than NKA, key transporters involved in branchial Na^+ and Cl^- uptake, such as *ncc2a* and *nhe3,* are negatively correlated to plasma Na^+ and Cl^- levels, as expected, and higher expressed in temperate FW *vs* SW conditions. These transporters are apically localized in sea bass FW-type ionocytes and are

thus essential for transepithelial Cl^- and/or Na^+ uptake (Blondeau-Bidet et al., 2019; Inokuchi et al., 2017). Few studies have investigated the interactive effects of temperature and salinity on these transporters. In a recent study on stickleback *Gasterosteus aculeatus* from a marine ecotype, the combination of low salinity and low temperature (4 °C and 0.3 ppt) had no interactive effect on the expression of *nhe3*, contrary to *nka*, where an additive effect was observed (Gibbons et al., 2018). In European sea bass, similar patterns of expression were observed for *nhe3* and *nka α1a* (Fig. 1A, E), showing a clear induction of these two transporters in warm SW conditions *vs* temperate SW but no change in expression between salinities in warm conditions. No apical NHE3 expression has been observed so far in temperate SW conditions (Blondeau-Bidet et al., 2019) and protein localization should be investigated further, notably in SW warm conditions. *Ncc2a* is upregulated upon warm temperature acclimation, in SW notably. In FW conditions, a slight but not significant increase is observed. Contrary to *nhe3* and *nka α1a*, there is a significant difference in *ncc2a* expression between salinities in warm conditions, suggesting an induction of *ncc2a* expressing cells to take up Cl^- and Na^+. In previous studies we have observed a decreased density of lamellar ionocytes in warm-acclimated fish challenged to FW (Masroor et al., 2018). Lamellar ionocytes are essentially NHE3-type cells whereas NCC2-type cells have been essentially detected on gill filaments (Blondeau-Bidet et al., 2019). This decrease in lamellar ionocytes (NHE3-type) might partially be linked to the lack of *nhe3* induction in warm FW conditions. It is likely that the proportion of NCC- *vs* NHE3-type cells is different in warm FW conditions, but this needs to be investigated further using immunocytochemistry.

In pufferfish *Tetraodon nigroviridis* gills, *clc-3* mRNA expression did not change between FW and SW groups as we have shown in this study in sea bass maintained at 18 °C. This differs from previous results in sea bass, where *clc-3* mRNA expression was lower in FW than in SW whereas protein levels seemed to be higher in FW (Bossus et al., 2013; Tang et al., 2010). In tilapia, *clc-3* mRNA expression was higher in deionized water in comparison to FW and SW (Tang et al., 2010) and several studies suggest an involvement of ClC-3 in basolateral Cl^- uptake in FW (Bossus et al., 2013; Tang and Lee, 2011, 2007). In this study, we showed that *clc-3* expression is strongly affected by temperature but not by salinity. The high *clc-3* expression in warm SW is surprising and might be linked to an overall increased ion transport and increased need to regulate ionocyte cell volume.

Effect of temperature on acid-base regulation

It is well known in marine fish that acid-base homeostasis is mainly regulated at the gill and kidney level (Heuer and Grosell, 2014). The link between ionic regulation and acid-base balance with regard to low pH has been investigated in several species (Dymowska et al., 2012; Kwong et al., 2014). At the gill level, acid secretion is thought to be coupled to Na^+ uptake either through a Na^+/H^+ exchanger (NHE3 or NHE2) or VHA. In SW, the model with NHEs is more likely given the favorable Na^+ gradient for Na^+/H^+ exchangers. The increased mRNA expression of *nhe3* as well as *vha-a* and *vha-b* in SW warm conditions could be a response to the lower blood Na^+/Cl^- ratio (Masroor et al., 2018), that could indicate blood acidosis triggering acid excretion mechanisms. An overexpression of *nhe3* together with carbonic anhydrase 2 (*ca2*) and Na^+/H^+ exchanger (*nbc1*) has been reported in Osorezan dace *Tribolodon hakonensis* gills when fish were challenged to acidic waters compared to fish maintained in neutral waters (Hirata et al., 2003). In this latter species, apical NHE3 clearly participates to acid excretion as well as in other species studied (Hiroi et al., 2008; Inokuchi et al., 2008; Ivanis et al., 2008). In zebrafish maintained in acidic FW, *vha-a* mRNA expression as well as the density of VHA-enriched ionocyte subtype (called HR cells) were increased following 7 days of acid exposure (Chang et al., 2009). *Vha-a* is negatively correlated to the Na^+/Cl^- ratio ($r=-0.6471$, $p<0.0001$) and may be involved in proton excretion in sea bass gills, however the subcellular localization of VHA is not yet clear in this species. Apical (Sullivan et al., 1995; Yan et al., 2007) as well as basolateral (Catches et al., 2006; Malakpour Kolbadinezhad et al., 2018; Uchiyama et al., 2012) localization of VHA has been reported in fish. Basolateral VHA, by pumping protons out of the cell to the blood, would generate a favorable electrochemical gradient for apical Cl^-/HCO_3^- exchange and could thus participate to HCO_3^- excretion and chloride uptake (Piermarini and Evans, 2001) whereas apical localization would rather be involved in acid secretion. Interestingly, a high correlation is also observed between *vha-a* and blood chloride levels ($r=0.903$, $p<0.001$).

Effect of temperature on nitrogen excretion

In a previous study on sea bass challenged to FW temperate conditions, *rhcg1* was significantly upregulated compared to SW temperate but *rhbg* did not change between SW and FW conditions (Blondeau-Bidet et al., 2019). This suggests a different handling for nitrogen excretion when comparing different salinity regimes (Frick and Wright, 2002). In our study, we can observe a slightly but not significantly increased *rhcg1* expression in FW

compared to SW. More strikingly, a significant upregulation of *rhcg1* was observed in warm temperature-challenged fish at both salinities. In gill ionocytes, RHCG1 might operate in concert with NHE3 and VHA at the apical cell part as shown in other species, but this remains to be analyzed further in sea bass (Heuer and Grosell, 2014; Nawata et al., 2010). In this study, temperature did not affect *rhbg* expression. In longjaw mudsucker *Gillichthys mirabilis* maintained in SW, warm temperatures (28 °C) seemed to enhance the expression of different transport related genes including branchial *rhbg* and *vha* compared to lower temperature groups (9 °C and 19 °C) and suggested an increased ammonia excretion in warm conditions (Logan and Somero, 2010). Nawata et al. (2010) showed that in seawater-maintained pufferfish (*Takifugu rubripes*) exposed to ammonia, gill *rhcg1*, *vha, nkcc1, nka* and *nhe3* were upregulated, which suggests a tight cooperation between different ion transporters expressed in ionocytes under high ammonia. The involvement of Rhesus proteins (Rh) in ammonia transport processes remains to be clarified in sea bass gills as well as the functional link with other ion transporters, as VHA and NHE3 (Nawata et al., 2010, 2007). However, our data strongly point to a role of *rhcg1* in ammonia excretion in warm temperatures, probably linked to increased metabolism, as shown previously in the same sea bass lineage challenged to increased temperatures (Claireaux and Lagardère, 1999). In another study, Person-le-Ruyet et al. (2004) have shown in the same species a 3-fold increased mean daily ammonia excretion rate at 25 °C than at 13 °C which is in accordance with our data.

Conclusion

In this study focusing on the mechanisms involved in freshwater acclimation at different temperatures, we showed that branchial transcript levels of most analyzed transporters were significantly affected by warm temperatures. We showed a more striking effect of temperature on gene expression patterns in SW warm conditions compared to FW warm. In FW warm conditions, a differential induction of transporters involved in Na^+ *vs* Cl^- uptake might explain previous data obtained on blood parameters (Masroor et al. 2018). Increased *rhcg1* mRNA expression points to a potential up-regulation of ammonia excretory pathways as a response to enhanced metabolism in warm conditions.

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Table 1 Sequences and efficiencies of the primers used for qRT-PCR in this study. F: forward primer; R: reverse primer; Sequence ID: identification number from sea bass genome or GenBank identification number.

Sequence ID	Target gene	Primer name	Sequence (from 5′ to 3′)	Amplicon size	Efficiency	Reference
KP400258	*atp1a1a*	NKAα1a F NKAα1a R	CCTCAGATGGCAAGGAGAAG CCCTGCTGAGATCGGTTCC	146	1.89	(Blondeau-Bidet et al. 2016)
KP400259	*atp1a1b*	NKA α1b F NKA α1b R	AGCAGGGCATGAAGAACAAG CCTGGGCTGCGTCTGAGG	204	1.99	(Blondeau-Bidet et al. 2016)
DQ501276	*cftr*	CFTR F CFTR R	GACTGATGCGTTCGGTAG CCTCAATGACATCTCCTTC	215	1.917	(Bodinier et al. 2009)
DLAgn_00080120	*slc12a2*	NKCC1 F NKCC1 R	TCAGCTCACAGTTCAAGGCC GCCGCTATGGACTCCACAA	102	2.08	(Lorin-Nebel et al. 2006)
JN998891	*clcn3*	ClC-3 F ClC-3 R	CAAGTACAGCAAGAACGAGGC ACAGCGTCTTGAGAGGGAAG	146	2.069	(Bossus et al. 2013)
DLAgn_00204050	*slc9a3*	NHE3 F NHE3 R	GGATACCTCGCCTACCTGAC AAGAGGAGGGTGAGGAGGAT	251	1.98	(Blondeau-Bidet et al. 2019)
DLAgn_00038210	*slc12a3-like*	NCC-like F NCC-like R	ATGATGAGCCTCTTCGAGCC GCTGCTCTCATCACCTTCTGT	278	1.94	
DLAgn_00076370	*atp6v1a*	VHA-A F VHA-A R	GGCAGTCACATCACAGGAGG CCAGCTCCATCACCACATCG	154	1.98	
DLAgn_00018050	*atp6v1b2*	VHA-B F VHA-B R	TTGCCATAGTCTTCGCAGCC CTTCTCGCACTGGTAGGC	194	1.90	
DLAgn_00222650	*rhbg*	RHBG F RHBG R	CCTCATGGTGACCCGAATCC GCCTGCACTCTGTCCACATA	218	1.97	
DLAgn_00166370	*rhcg1*	RHCG1 F RHCG1 R	TCAGGGAATTGTGTGACCGC AGAATCAAGTCCACGCTGGG	118	2.01	
AJ866727	*ef1α*	EF1-F EF1-R	GGCTGGTATCTCTAAGAACG CCTCCAGCATGTTGTCTCC	239	2.024	(Lorin-Nebel et al. 2006)

Table 2 Two-way ANOVA results of gill gene expression with salinity and temperature as the main factors. ns: not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. N= 8-13 per condition.

Genes	Interaction	Salinity	Temperature
nka α1a (*atp1a1a*)	**	**	**
nka α1b (*atp1a1b*)	ns	**	ns
cftr	ns	****	****
nkcc1 (*slc12a2*)	ns	***	*
nhe3 (*slc9a3*)	ns	**	**
ncc2a (*slc12a3-like*)	ns	****	**
clc-3 (*clcn3*)	ns	ns	****
vha-a (*atp6v1a*)	ns	**	****
vha-b (*atp6v1b2*)	ns	ns	*
rhbg	ns	ns	ns
rhcg1	ns	*	****

Table 3 Spearman correlation for gill gene expression *vs* plasma Cl^-, Na^+ and Na^+/Cl^- ratio. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. N= 8-13 per condition.

Genes	**Cl^- level**	**Na^+ level**	**Na^+/Cl^- ratio**
nka α1a (*atp1a1.a*)	-0.2798	-0.2858	-0.02371
nka α1b (*atp1a1.b*)	0.3303*	-0.01329	-0.3031*
cftr	0.5442***	0.07930	-0.3796*
nkcc1 (*slc12a2*)	0.4615**	-0.05339	-0.3365*
nhe3 (*slc9a3*)	-0.3059*	-0.3437*	0.005587
ncc2a (*slc12a3-like*)	-0.3913**	-0.4037**	-0.02552
clc-3 (*clcn3*)	0.1989	-0.2980	-0.4097**
vha-a (*atp6v1a*)	0.4903***	-0.2927	-0.6471****
vha-b (*atp6v1b2*)	0.2822	-0.2027	-0.3201*
rhbg	0.1040	-0.09353	-0.07771
rhcg1	-0.1198	-0.3655*	-0.1780

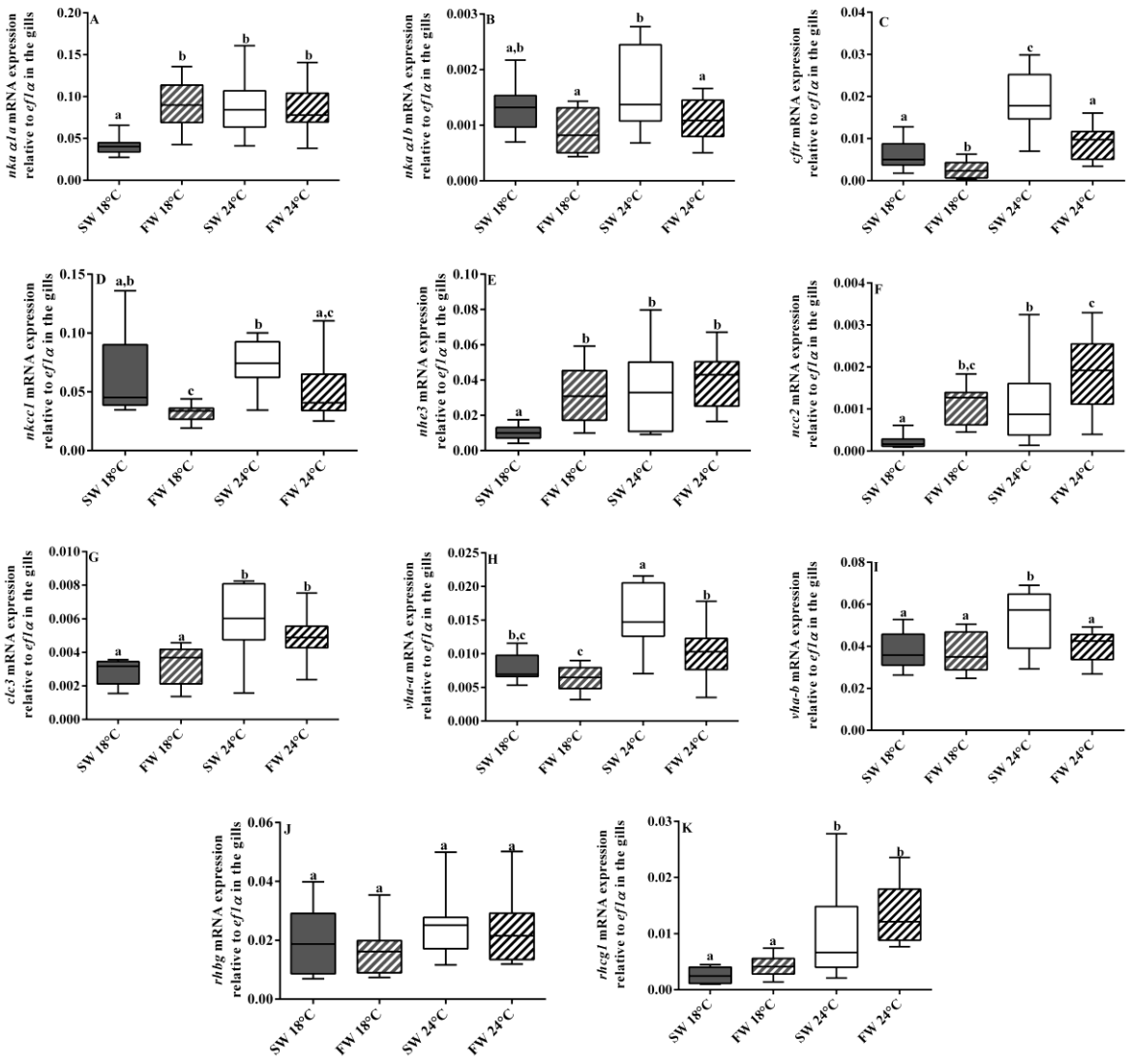

Fig. 1: Box and whisker plot showing the median, minimum and maximum mRNA expression level of *nka α1a* (*atp1a1a*) (A), *nka α1b* (*atp1a1b*) (B), *cftr* (C), *nkcc1* (*slc12a2*) (D), *nhe3* (*slc9a3*) (E), *ncc2a* (*slc12a3-like*) (F), *clc-3 (clcn3)* (G), *vha-a* (*atp6v1a*) (H), *vha-b* (*atp6v1a*) (I), *rhbg* (J) and *rhcg1*(K) in gills of sea bass exposed to FW and SW at 18 °C (temperate) and 24 °C (warm). mRNA levels were normalized to *ef1α*. Different letters indicate significant differences between conditions (two-way ANOVA followed by a Fisher Least Significant Difference (LSD) post hoc test $p<0.05$, N= 8-13). SW: seawater; FW: fresh water.

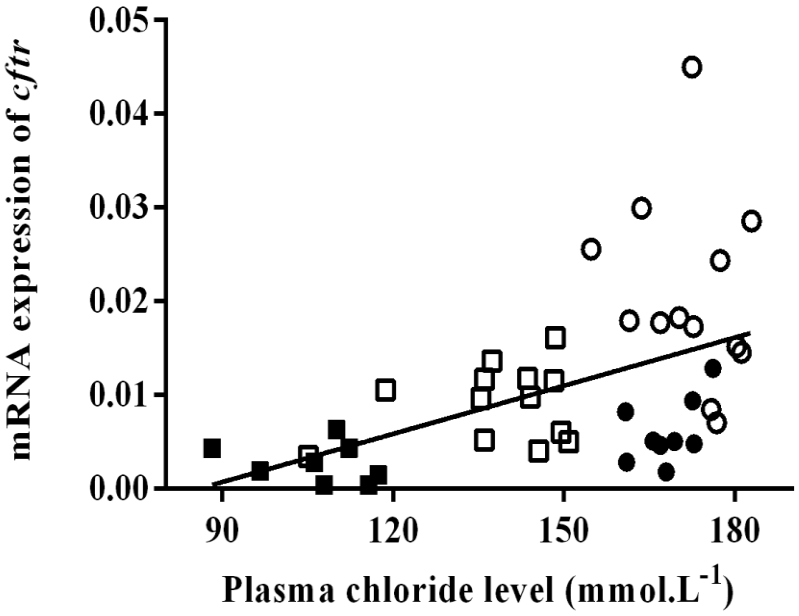

Fig. 2: Gill mRNA expression level of *cftr* plotted against plasma chloride level. mRNA levels were normalized to *ef1α*. Circles and squares represent SW and FW values. Filled and open symbols represent temperate (18 °C) and warm (24 °C) conditions. Spearman correlation was r=0.54. Linear correlation between *cftr* and chloride level for all groups was significant ($p<0.001$, N= 43)

Chapter V

Temperature and salinity stress affect gill mucus and heat stress-related proteins

Chapter V - Temperature and salinity stress affect gill mucus and heat stress-related proteins

5.1 Preamble

In this study, while analyzing apical openings of ionocytes, we observed that gill surface was covered by mucus as visible under scanning electron microscopy (SEM) (Fig. 5). Thus, this observation prompted us to analyze mucus production in fish gills.

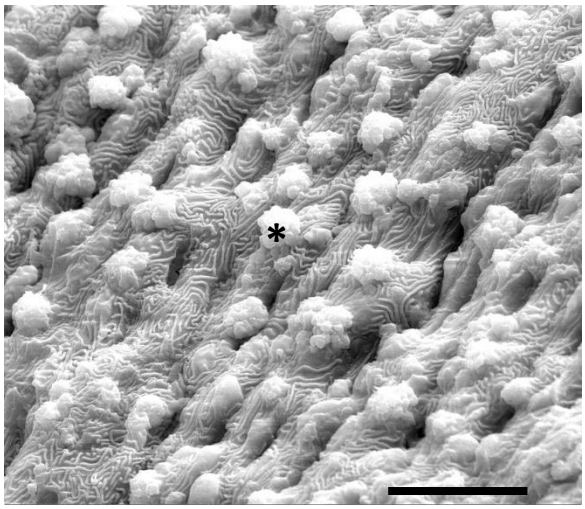

Fig. 5: Scanning electron micrographs of sea bass gills exposed to fresh water (FW) at 18 °C (temperate). The asterisks indicate mucus. Scale bar: 10 µm.

It has been reported that mucous layers serve as important boundary between internal and external environment of fish and are therefore important for several biological functions (Shephard 1994; Dash et al. 2018; Reverter et al. 2018). A decrease in salinity or an increase in temperature can have detrimental effects on biological systems due to the direct involvement of the gill epithelium to ionoregulation, oxygen uptake and nitrogen excretion. Many studies have shown that skin mucus is a major determinant in fish health (Matey et al. 2011; Parra et al. 2015; Blair et al. 2017; Fernández-Alacid et al. 2019). Increased mucus production is thought to be an indicator of increased stress response in fish gills during thermal and salinity stress as well as in the presence of pollutants or pathogens. Expression of skin mucus-related proteins involved in the immune reponse have been shown abundantly in *D. labrax*, which indicates that local signaling networks are present in the mucosal surface of this species (Cordero et al. 2015). However, rapid induction of innate mucus immunity in turbot *Scophthalmus maximus* (Huang et al. 2011) and Atlantic salmon *Salmo salar* (Jensen et

al. 2015) following temperature exposure has been regulated by multiple mechanisms and is not determined solely by fish mucus. Hartl (1996) proposed that heat shock proteins (HSPs) are inducible by thermal stress. These proteins also play important roles in maintening cellular.

In this study, we will focus on the influence of a salinity and/or temperature stress on the mucus production in sea bass gills. For that purpose, the abundance of branchial mucus and mucous cell number was also investigated using histology and PAS staining. mRNA expression of mucins (mucin 5 notably) will be measured in future investigations. The mRNA expression of *hsp90b1, hsp90ba* was measured at the branchial level in sea bass as a proxy of thermal stress.

5.2 Major highlights

- As expected, fish upregulated both analyzed HSP90 encoding genes (*hsp90b1*, *hsp90ba*) in warm conditions of both considered salinities. Thermal increase from 18 °C to 24 °C required the induction of chaperone proteins.

- The number of interlamellar mucus cells was increased after FW transfer at 18 °C. This highlights the role played by in fresh water acclimation and ion regulation, by trapping ions.

- Following warm-acclimation, the area of mucus stained within the cell is increased.

Overall, production of mucus, overexpression of HSPs likely require for sea bass when they migrate to estuaries and lagoons, where temperature and salinity are frequently fluctuate.

Research article III (in prep)

Temperature and salinity stress affect gill mucus and heat stress-related proteins

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Introduction

Mucus

Fish mucus layers represent the main surface of exchange at the interface between fish and the environment. They possess important biological and ecological functions (review in Reverter et al., 2018). Mucosal surfaces are known as the first line of defense against different external abiotic and biotic stressors such as thermal and salinity stress as well as pollutants or pathogen infections (Powell et al., 2008; Blair et al., 2017; Matey et al., 2011; Parra et al., 2015).

Skin mucus was the most extensively studied mucosal surface in teleosts since it represents a major determinant in fish health and a main defense mechanism against pathogens (Dash et al., 2018). Fish epidermal mucus constitutes a gel-like structure that contains different humoral immune factors (e.g. antimicrobial peptides, lysozymes, lectins, immunoglobulin, complement, C-reactive protein and proteolytic enzymes). These immune components are secreted by goblet cells and provide the primary defense against different pathogenic microbes, acting as a barrier between fish and their immediate niche (Shephard, 1994; Koppang et al., 2015; Peterson, 2015; Cabillon and Lazado, 2019; Cordero et al., 2015). The major function of mucus includes entrapment and sloughing of microbes. Immune-related proteins have been shown to be expressed abundantly in the mucosal surface of *D. labrax* skin mucus (Cordero et al., 2015). To our knowledge, the role of gill mucus in immune protection has been less investigated.

In fish, there are many other proposed roles for mucus, including respiration, ionic and osmotic regulation, reproduction, etc. (Shephard, 1994). Increased mucus production is thought to be a major determinant to protect epithelium from various environmental stressors (Matey et al., 2011, 2008). Among fluctuating environmental parameters, temperature is considered as a major parameter affecting fish physiology (Hutchison and Maness, 1979; Schulte, 2011). The mucous cells coverage was increased in warm temperature-challenged Atlantic salmon *Salmo salar* pre-acclimated to 10 °C (Jensen et al., 2015). Several studies highlighted that mucus plays a protective role in case of thermal stress, especially regarding the susceptibility to diseases (Quiniou et al., 1998; Wentworth et al., 2018).

Several studies have shown a change in mucus secretion and number/or size of mucous cells following salinity change. The involvement of mucus in ionic regulation in fishes remains still

unclear but according to Shepard (1994), the mucus recycles ions by trapping them in the mucous layer covering the gill epithelium and might change the accessibility for the ions to transport proteins. A study on Arctic grayling *Thymallus arcticus* reported changes in whole filament thickness and increased mucous cells number following high salinity exposure (Blair et al., 2017). An increase in the mucous cell density and a change in the proportion of mucosubstances (*i.e*. neutral, acidic and sulphated glycoproteins stained in gill mucous cells) has been shown in traira *Hoplias malabaricus* and jeju *Hoplerythrinus unitaeniatus* exposed to acute salinity decrease (Moron et al., 2009).

Regarding response to hypoxia, increased mucus layers reduce diffusional transport of O_2 in different teleost species (Ultsch and Gros, 1979). Indeed, Vatsos et al. (2010) have shown in sea bass that chronic environmental hypoxia and increased level of nitrate in the water increased the skin mucous cell coverage and the size of superficial mucous cells. Hypoxia caused discharge of mucus from enlarged mucous cells as shown in the gills of rainbow trout *Oncorhynchus mykiss* and Amazonian oscar *Astronotus ocellatus* (Matey et al., 2011).

In Salmo salar *Salmo salar* smolts exposed to different aluminum concentrations at low pH for 3 days, an increased secretion of mucus was observed in skin and gills (Berntssen et al., 1997), thus suggesting a protective role for mucus against aluminum toxicity at low pH, by trapping aluminium. Altogether, these studies highlight that an adjustment of gill surface mucous cells/mucus properties is required to cope with environmental changes in the aquatic environment (e.g. temperature, salinity, pathogens, hypoxia, pH, pollutant) and that mucous production is plastic.

Heat Shock Proteins

Heat-shock proteins (HSPs) are part of a superfamily of stress proteins, highly conserved across species. As protein chaperones, HSPs bind other proteins by stabilizing them in case of a cellular stress. These proteins also play key roles in maintaining protein structure and folding. Thus, HSPs have a crucial role in intracellular protein homeostasis. Following temperature stress, it is common to observe increased expression of HSPs in fish (Dietz, 1994; Dietz and Somero, 1992; Ikeda et al., 2017). HSPs and other molecular chaperones interact with denatured proteins in order to maintain their native structures and prevent aggregation and degradation (review in Hartl, 1996). Podrabsky and Somero (2004) have used cDNA microarray analysis to examine changes in gene expression in liver of killifish *Austrofundulus limnaeus* subjected to either constant temperatures of 20, 26 and 37 °C for up to two weeks or

to environmentally realistic daily fluctuations in temperature between 20 °C and 37 °C. These authors have shown that small heat shock proteins appeared to play an important role in response to fluctuating temperatures, while larger molecular mass chaperones such as HSP70 and HSP90 responded more strongly to a chronic high temperature stress. Several authors showed that HSPs might contribute to mucosal defense mechanisms, especially in the gastrointestinal tracts (Tsukimi and Okabe, 2001). In fish, HSP70 has been found in skin mucus of European sea bass *D. labrax* (Cordero et al., 2015), gilthead sea bream *Sparus aurata* (Jurado et al., 2015) and lumpsucker *Cyclopterus lumpus* (Patel and Brinchmann, 2017). The role of this protein has still to be clarified but HSPs may act through protecting key enzymes and other proteins related to cytoprotection or immunity.

Objectives

In teleost fish, gill play important roles in oxygen uptake, osmotic and ionic regulation, acid-base regulation, and excretion of nitrogenous wastes (Chou et al., 2008; Evans et al., 2005; Hwang, 2009; Mitrovic and Perry, 2009; Nilsson, 2007; Perry and Gilmour, 2006). Gill mucus can potentially interfere with these different functions since it may trap ions or may change the gas diffusion rate. To date, most research on fish mucus has focused on immune-related molecules, but few studies have analyzed other physiological roles in response to changing environmental parameters. The mucus matrix is produced by goblet, club, and sacciform cells found in the fish epithelium (Cone et al., 2009). Mucus secreting goblet cells are abundant on all fish epidermal surfaces and particularly on gill surfaces. In this study, we will focus on the influence of a salinity and/or temperature stress on interlamellar mucus cells in sea bass gills. For that purpose, mucous cell number and area was investigated using histology and PAS staining. The mRNA expression of *hsp90b1*, *hsp90ba* was also measured at the branchial level in sea bass as a proxy of thermal stress.

Material and methods

Experimental conditions

The experimental conditions have been previously described in Masroor et al. (2018). Briefly, juvenile sea bass were obtained from the Ifremer Station at Palavas-les-Flots (Herault, France). Fish were brought to the Montpellier University and maintained for one week in 3,500 L tanks containing natural seawater (SW) form the Mediterranean Sea at 38 ‰ and 18 °C, under a 12 h light/12 h dark photoperiod. Fish were transferred to 200 L tanks (14 fish/tank, density of 6-7 kg/m3, two replicates per conditions) to be acclimated either at 18 °C

or 24 °C (with a temperature increase of 0.2 °C/h). After two weeks of temperature acclimation, fish were transferred directly either to dechlorinated tap water (fresh water, FW), or to SW (7 fish/tank, two replicates for each conditions) and maintained in this salinity two weeks before sampling. Ionic composition (in $mEq.L^{-1}$) of the FW was Na^{+} (0.12), K^{+} (0.04), Ca^{2+} (5.70), Mg^{2+} (0.29), Cl^{-} (0.98), NO^{3-} (0.06) SO_4^{2-} (0.61). Water was aerated and mechanically/biologically filtered (Eheim System, Lens, Pas-de-Calais, France). Temperature, salinity, oxygen and nitrogen levels were checked daily. A third of the water volume was changed every two days. Fish were fed twice a week with fish granules (Aphymar feed, Meze, Herault, France) until 2 days before sampling. At the end of the experiment, fish were anesthetized in a solution of phenoxy-2-ethanol (240 ppm) prior to tissue collection. The fish used for the experiment had a length of 20.77±1.32 cm (mean ± SD) and average weight was 86.87±20.23 g (mean ± SD). Four groups were compared: SW at 18 °C (temperate SW), SW at 24 °C (warm SW), FW at 18 °C (temperate FW) and FW at 24 °C (warm FW). These experiments were respected the guidelines of the European Union (directive 86/609) and of the French law (decree 87/848) regulating animal experimentation.

Periodic Acid Schiff (PAS) staining and light microscopy

Transverse sections (4 µm) were cut on Leitz Wetzlar microtome, collected on poly-L-lysine-coated glass slides. Sections were stained with Alcian Blue pH 2.5 (DIAPATH) Periodic Acid (CARLO ERBA) and Schiff (SIGMA). Acidic and neutral mucous substances were stained in magenta red and blue, respectively. Sections were incubated in Periodic Acid Schiff (PAS)-Alcian Blue for 20 min and PAS for 10 min, respectively, followed by Periodic Acid for 10 min and by Schiff for 20 min.

Mucous cell number and area

PAS stained slides were observed under a Leica Diaplan microscope, and gill lamellae sections were photographed at a magnification of x400. Interlamellar mucous cell numbers as well as area of mucus accumulation in each cell were measured using the software imageJ (ImageJ 1.51F) (https://imagej.nih.gov/ij/). The number of mucous cells located on the filaments were counted on 200 µm of filament length in each picture considering one side of the filament (n= 15 measurements per conditions, N=3 animal per conditions). The area of mucous in each mucous cell was measured and indicated as mucous cell size on 200 µm located within the interlamellar area.

RNA extraction and reverse transcription

Gill tissues were collected from gills of SW- and FW-exposed sea bass at temperate and warm conditions. The epithelium of the gills was scraped with a sterile scalpel, immerged in Trizol® reagent and flash frozen in liquid nitrogen. Tissues were then stored at -80 °C until analysis. Total RNA was extracted using Trizol® reagent according to the manufacturer's instructions. RNA quantity and purity were assessed by measuring the A260/A280 ratio using the NanoDrop® ND-1000 V3300 spectrometer (Nanodrop Technology Inc., Wilmington, Delaware, USA). RNA quality was checked using Agilent bioanalyzer (Agilent) using electrophoretic trace method. One microgram of the total RNA was treated with DNase I amplification grade (Invitrogen™, Life Technologies). Reverse transcription was performed using 200 U M-MLV reverse transcriptase (Invitrogen™) and first strand of complementary DNA (cDNA) was generated using 250 ng of random primers (Invitrogen™), dNTPs (10 mM) and 40 U of RNAse out recombinant (Invitrogen™), following manufacturer's instruction.

Relative quantification at the transcript level

The specific primers (forward and reverse) for different transporters are listed in Table 1. Quantitative PCR analyses (qPCR) were performed using the LightCycler® 480 Real-Time PCR System (Roche, Mannheim, Baden-Württemberg, Germany), with 2X LightCycler-FastStart DNA Master SYBER-Green I™ Mix (Roche), forward and reverse primers (at a final concentration of 0.5 µM) and cDNA. The qPCR conditions were: denaturation at 95 °C for 10 min, followed by 45 cycles of repeated amplification (95 °C, 10s), hybridization (60 °C, 10 s) and elongation (72 °C, 10 s), and a final step at 40 °C for 30 s. A melting curve program was performed to control the amplification specificity, and the amplification products were sequenced. Ef1α (encoding elongation factor 1 α) was used as reference gene as in previous studies performed on salinity challenged sea bass (Blondeau-Bidet et al., 2016; Lorin-Nebel et al., 2006) and as recommended by Mitter et al. (2009). Ultra-pure water was used as a no-template control in the qPCR. Efficiencies were determined and given in Table 1. The relative expression ratio of each target gene was calculated using the ΔCt method with the formula: Efficiency (E)-ΔCt (Pfaffl, 2001), using the efficiency of each primer pair previously determined.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 6, GraphPad Software Incorporated, La Jolla, CA, 268 USA). Normality and homogeneity of variance were

respectively checked using D'Agostino-Pearson test and Bartlett test. When necessary, data were log-transformed to fit homogeneity of the variance assumption. Two-way factorial analysis of variance with temperature and salinity as the main factors was performed. Critical differences between groups were appraised using the Fisher's least-square difference test. For histology data (number/size of mucous cells), a parametric ordinary one-way ANOVA test followed by a multiple comparison Tukey's test was used. Data are presented as box and whisker plots showing median, minimum and maximum values. Level of statistical significance was set at $p<0.05$.

Results

HSP90 mRNA expression

Two-way ANOVA revealed a slight significant salinity effect ($p<0.05$) and a highly significant temperature effect ($p<0.0001$) for *hsp90b1*. In case of *hsp90ba*, temperature is the only factor exerting a highly significant effect ($p<0.0001$, Fig. 1B). Interaction between both temperature and salinity did not exert a significant effect on *hsp90b1* and *hsp90ba* (two-way ANOVA, Fig. 1).

At both salinities, fish acclimated to 24 °C had a 2- to 4-fold higher expression of *hsp90b1* and *hsp90ba* compared to 18 °C (Fig. 1A, B). No difference in *hsp90b1* expression was observed between salinities in temperate conditions. At 24 °C, fish acclimated to FW had a significantly lower *hsp90b1* expression than fish acclimated to SW.

Histology

Mucous cells were identified by PAS staining and localized in interlamellar spaces of the gill filaments (Fig. 2). In temperate conditions, fish did not exhibit a change in mucous cells area between salinities (Fig. 3B). In warm conditions however, significantly higher mucous cell area was observed compared to temperate conditions at both salinities. Fish acclimated to the FW temperate condition had significantly more mucous cells on gill filaments compared to all other conditions (Fig. 2C, 3A). PAS staining was applied in order to determine if neutral or acidic glycoconjugates are present. Mucous cells were stained in blue as shown in Fig 2.

Discussion

Previous studies focusing on thermal stress have largely focused on HSP genes (Kyprianou et al., 2010; Logan and Somero, 2011; Podrabsky and Somero, 2004; Tomalty et al., 2015). Based on the literature, the aim of the present study was to analyze HSP90 as a positive

marker of thermal stress. In sea bass, due to ancestral genome duplication, two different paralogous genes encoding HSP90 protein were found in sea bass genome. As expected, both analyzed HSP90 encoding genes (*hsp90b1, hsp90ba*) were significantly upregulated in gills of sea bass maintained in warm conditions at both salinities. This increase of HSP90 encoding genes at higher temperature has been shown in gills of several other teleost species (Jensen et al., 2015; Logan and Somero, 2010) and confirms their key role in the response to thermal stress. Higher branchial expression of *hsp90* were reported in longjaw mudsucker *Gillichthys mirabilis* at higher temperature ranging from 16 °C to 38 °C (Dietz, 1994). Jensen et al. (2015) measured an overexpression (5.68-fold change) of HSP 90-α (*hs90a*) mRNA after 2 days exposure to temperature increase from 10 to 16 °C in salmon skin. In another study on longjaw mudsucker *G. mirabilis* maintained in SW at 14 °C and transferred to 9 °C, 19 °C and 28 °C, none of the genes encoding HSPs were upregulated at 28 °C except *hsp90b* (Logan and Somero, 2010). The overexpression of HSP90 encoding genes in sea bass indicated that 24 °C represents a thermal stress requiring the induction of chaperone proteins.

The interest of our study was to analyze the interactive effects of salinity decrease and temperature increase on HSPs levels. According to the literature, HSP90 seems more inducible than HSP70 following salinity stress (Deane and Woo, 2011; Palmisano et al., 2000; Peng et al., 2015). Our results show that the effects of FW transfer on HSP90 encoding genes expression were weak compared to the effects of temperature increase. We observed a decrease of expression of *hsp90b1* (not *hsp90ba*) after transfer from SW to FW at 24 °C. This is consistent with the study of Peng et al. (2015) where they measured lower mRNA levels of *hsp90* (and in a lower extent *hsp70*) at lower salinities in several tissues, including the gill.

Using SEM, we observed more mucus coverage on the gill epithelium in FW at 18 °C (data not shown here). We reported that filament thickness was significantly increased from SW to FW at 18 °C (Masroor et al., 2018) which might be linked to the observed increased of interlamellar mucous cell number, in addition to the already mentioned increase of ionocytes density. Higher mucous cell coverage in gills of fish maintained in FW probably contributes to the maintenance of ion and water balance (Blair et al., 2017; Shephard, 1994; Wendelaar Bonga, 1997).

The number of mucus cells was not significantly changed at 24 °C in SW compared to 18 °C and there is no increase in the mucus cell number following salinity transfer at 24 °C. According to the study of Jensen et al. (2015), a higher mucous cell number could have been

expected in warm conditions at both salinities. At 24 °C, an absence of increased mucus layer could induce increased passive ion losses in FW which could negatively affect ion levels as previously reported in sea bass at 24 °C *vs* 18 °C, notably regarding Na^+ levels (Masroor et al., 2018). We can hypothesize that gills exposed to warm temperatures lack the appropriate mucus layer to prevent ion loss. Interestingly, though the number of mucous cells did not change after heat stress, we observed an increase in the area of mucus stained within the goblet cells in warm conditions at both salinities. This may indicate a compensatory increase of mucus production in response to temperature increase at both salinities.

Perspectives

Teleost mucus is similar to mammalian mucus and is mainly composed of mucins expressed in mucous cells (Shephard, 1993). The mucins are high molecular weight glycoproteins that impart viscoelastic and rheological properties to the mucus. Two structurally distinct families of mucins can be distinguished: large secreted gel forming (SGFM) and membrane-bound forms, characterized by a transmembrane domain (Lang et al., 2016). The gel-forming mucins are large glycosylated proteins that are essential components of the mucus layers covering epithelial cells.

To date, gill mucins were poorly studied in fish gills. According to Micallef et al. (2013), two different mucin sequences, muc18 (membrane-bound) and muc5 (secreted and gel-forming), were consistently detected in gills of Atlantic salmon. Jevtov et al. (2014) identified the same secreted gel forming mucin MUC5 in zebra fish gill (muc5.1 and muc5.2 genes). In addition, by separating the gill lamellae from the gill arches they saw that only muc5.1 is expressed in the lamellar part. Transcript levels of mucin5 encoding genes may therefore represent good candidates to investigate for future studies, since this mucin is a constitutive component of mucus, potentially expressed by mucous cells.

Conclusion

Our results confirm that gill surface mucous layer is modified in response to salinity and/or temperature change. These modifications may have consequences on gills functioning and may potentially affect the immune response at the gill level. In the future, the role of gill mucus in sea bass should be further analyzed with regard to environmental stress and immune response.

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Table 1 Sequences and efficiencies of primers used for qPCR in this study. Sequence ID indicates ID genome sequences from the sea bass genome or Genbank identification numbers when available. F: forward primer: R: reverse primer.

Sequence ID	**Target gene** **Primer name**	**Sequence (from 5´ to 3´)**	**Amplicon size**	**Efficiency**
DLAgn_00169960	*hsp90b1 F* *hsp90b1 R*	CTACCAGCTGGCTGACACAA CCGCTTTATCCTCAGAGTCG	161	1.456
DLAgn_00070720	*hsp90ba F* *hsp90ba R*	GTGGAGAAGGAGCGTGACAA CTGAGCCCACATCCTCGATC	115	1.442
AJ866727	*ef1α F* *ef1α R*	GGCTGGTATCTCTAAGAACG CCTCCAGCATGTTGTCTCC	239	2.024

Gene	Interaction	Salinity	Temperature
hsp90b1	ns	p<0.05	p<0.0001
hsp90ba	ns	ns	p<0.0001

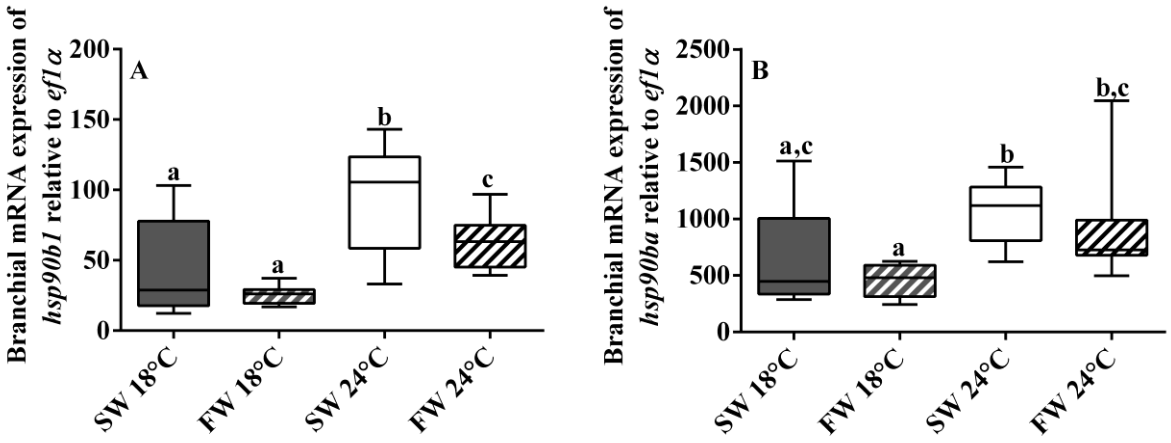

Fig. 1: Box and whisker plot showing median, minimum and maximum values of mRNA expression level of *hsp90b1* (A) and *hsp90ba* (B) in gills of sea bass exposed to seawater (SW) and fresh water (FW) at 18 °C (temperate) and 24 °C (warm). Different letters indicate significant differences between conditions (two-way ANOVA followed by a Fisher LSD post hoc test $p<0.05$, N=8-13). The table above the graphs gives the results of two-way ANOVA with temperature and salinity as the main factors.

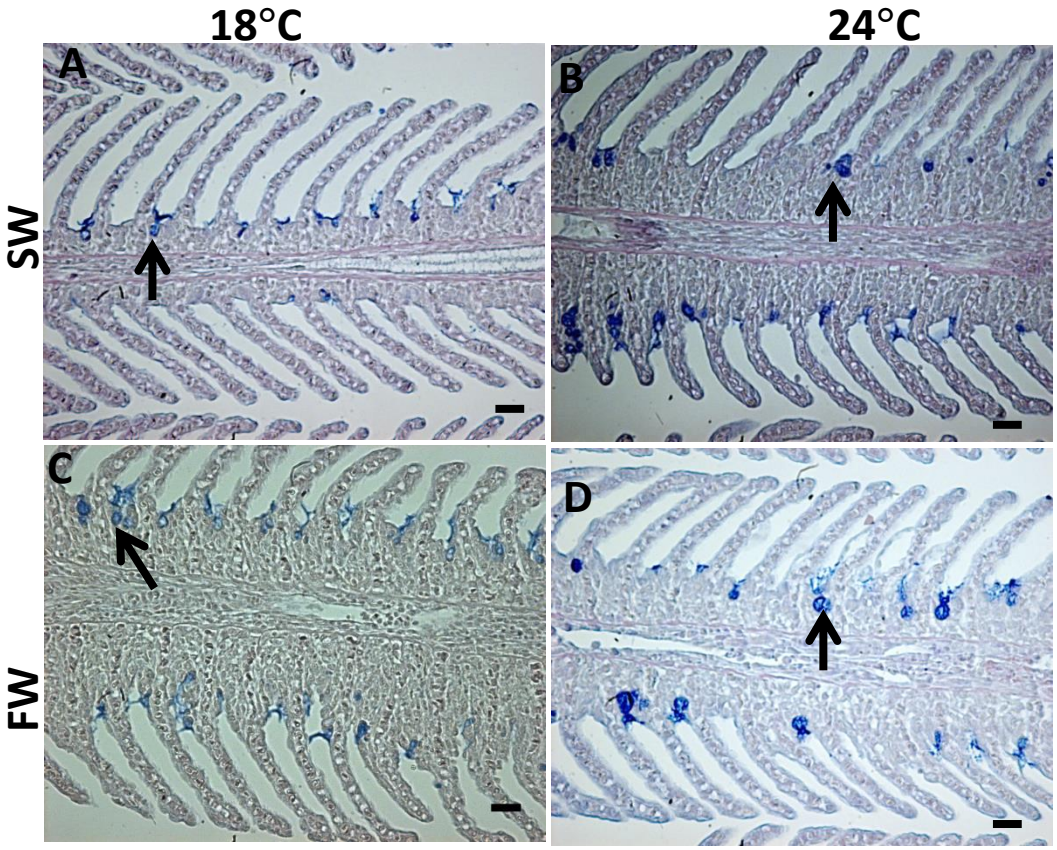

Fig. 2: Periodic Acid Schiff micrographs of sea bass gills exposed to seawater (SW) (A,B) and fresh water (FW) (C,D) at 18 °C (temperate, A,C) and 24 °C (warm, B,D). Arrows indicate mucous cells. Scale bar: 20 µm.

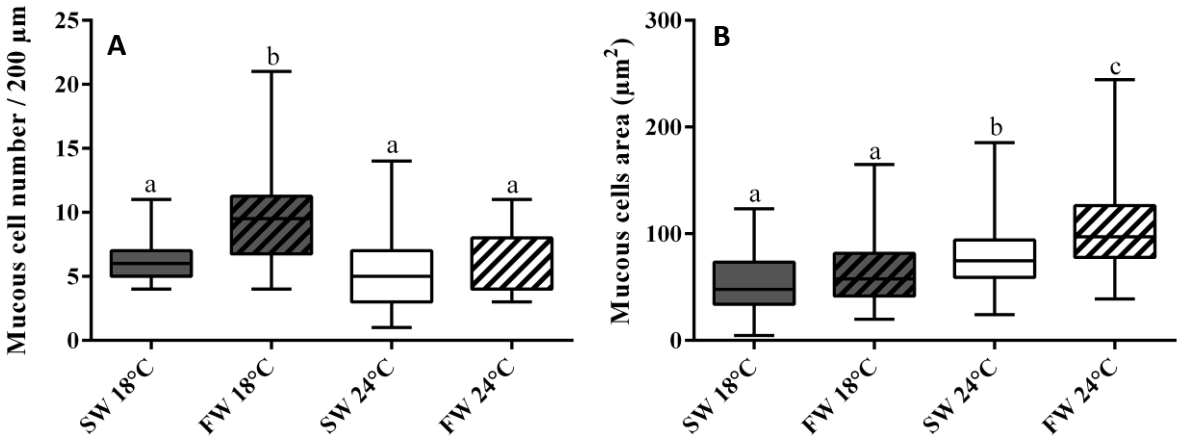

Fig. 3: Box and whisker plot showing median, minimum and maximum values of Periodic Acid Schiff (PAS) staining of mucous cells per 200 µm of gill filament (A) and mucous cell area (B) in sea bass exposed to seawater (SW) and fresh water (FW) at 18 °C (temperate) and 24 °C (warm). Different letters indicate significant differences between conditions (one-way ANOVA test followed by a Tukey's test, $p<0.05$, N=3, 5 measurements per individual). FW: fresh water, SW: seawater.

Chapter VI

General discussion

Chapter VI - General discussion

This work was designed to study the effects of salinity and temperature changes in European sea bass that may encounter these changes during their migration and their stay in lagoons and estuaries. The effects of multiple environmental stressors on the responses of fish are usually underestimated and often misunderstood (Przeslawski et al. 2015). The environmental complexity of transitory habitats is associated with global change and particularly the increase of extreme events such as summer heat waves or heavy rain events(Pörtner et al. 2001; Anthony et al. 2009; Shaltout and Omstedt 2015). In this context, there is an increasing need to study interactive effects of multiple stressors on organisms which will allow a better prediction of their responses to changing environments.

The overall goal of this thesis was to improve knowledge of sea bass osmoregulatory processes under environmentally realistic parameters (*i.e.* salinity decrease and temperature increase). The effects of theses parameters at the whole organism, tissue, cell and molecular levels have been presented in Chapter III, IV and V and are discussed here.

Hydromineral balance (blood osmolality) and circulating ion levels have been assessed in order to get insights at the whole-organism level. The gill tissue was then chosen as target tissue for histological, cellular and molecular analyses. The gills are, besides being a key site in osmoregulation, considered as a multifunctional organ in the center of main physiological functions in fish. We have analyzed gill morphology, NKA enzyme activity, and density of ionocytes and mucocytes. We also investigated molecular mechanisms by targeting genes involved in ion transport and thermal stress. The main findings will be outlined in the current chapter (each integration level is summarized in Tables 7 to 11).

Table 7 Temperature and salinity effects at the organism level in sea bass exposed to SW and transferred to FW at two temperatures (18 °C and 24 °C). * p <0.05, ** p <0.01, *** p <0.001, ****p<0.0001. N = 8-13 per condition.

Level of integration	**Parameters**	**Temperature increase from 18 °C to 24 °C**		**Salinity decrease from SW to FW**	
		SW	**[1]FW**	**18 °C**	**24 °C**
Organism	Blood osmolality	-	↑ (****)	↓ (****)	↓(****)
	Na+ levels	↓ (*)	↓ (**)	-	-
	Cl- levels	-	↑ (****)	↓ (****)	↓(****)
	Na+/Cl- ratio	↓(**)	↓(****)	↑(***)	↑(*)

[1]Note that the FW challenged fish at 24 °C have been previously pre-acclimated to SW 24 °C before salinity transfer

6.1 Effects of combined stress on physiological, biochemical and morphological parameters

Few studies are available in the literature focusing on combined stress responses in fish (Stuenkel and Hillyard 1980; Kültz and Somero 1995; Fiess et al. 2007; Sardella et al. 2008). At the **whole-organism level**, sea bass maintained higher blood osmolality in warm FW compared to temperate conditions. This indicates that sea bass are efficient hyper-osmoregulators at 24 °C. However, imbalance of plasma ion levels was demonstrated for Na^+ and Cl^-. As it is known in the literature that these two ions are absorbed by different sets of transporters and cells (Hwang and Lin 2014), it seemed interesting to focus on transporters linked to Na^+ and Cl^- uptake. Plasma Na^+ levels were decreased in warm temperature in both tested salinities (Table 7) which suggests that Na^+ transport mechanisms might be handled differently in warm conditions. Plasma Cl^- levels were significantly correlated to blood osmolality in all tested conditions. Plasma Cl^- levels did not change between the two tested temperatures in SW conditions but were significantly increased at 24 °C in FW. This and the calculated Na^+/Cl^- ratio suggest an ion imbalance under this condition. Ion homeostasis is regulated by energetically demanding ion pumps in the gills and other osmoregulatory tissues (kidney, intestine), namely Na^+/K^+-ATPase and V-type H^+-ATPase. Ionoregulatory and osmoregulatory perturbations in regard to temperature changes have been shown to alter main plasma ion levels and blood osmolality in several fishes for example goldfish *Carassius auratus* (Mitrovic and Perry 2009), common carp *Cyprinus carpio* (Metz et al. 2003),

Mozambique tilapia *Oreochromis mossambicus* (Fiess et al. 2007; Sardella et al. 2008) and rainbow trout *Salmo gairdneri* (McCarty and Houston 1977).

Table 8 Temperature and salinity effects at the organ/tissue level in sea bass exposed to SW and transferred to FW at two temperatures (18 °C and 24 °C). * p <0.05, ** p <0.01, *** p <0.001, N = 8-13 per condition.

Level of integration	**Parameters**		**Temperature increase from 18 °C to 24 °C**		**Salinity decrease from SW to FW**	
			SW	**[1]FW**	**18 °C**	**24 °C**
Organ/Tissue	Gill morphology	Lamellar length	-	↑(***)	↓(*)	↑(***)
		Filament thickness	-	↓(*)	↑(**)	-

[1]Note that the FW acclimated fish at 24 °C have been previously pre-acclimated to SW 24 °C before salinity transfer

At gill tissue level, sea bass exposed to salinity and/or temperature change showed a high plasticity in gill morphology as shown in several teleost species (Sardella et al. 2008; Mitrovic and Perry 2009; Vargas-Chacoff et al. 2009a). The filament thickness (Table 8) and lamella width significantly increased from SW to FW at 18 °C, probably linked to increased ionocyte and mucous cell number (Chapter III and V) (Table 9). In warm conditions, lamellar length increased at 24 °C *vs* 18 °C in FW conditions to optimize gas exchange (Table 8). Several studies in other fish species have demonstrated an osmorespiratory compromise between gas exchange and ion uptake mechanisms and may explain morphological changes observed in sea bass transferred to warm FW (Sollid et al., 2005). Lamellar length was not increased in warm SW conditions, which may affect oxygen uptake capacities (Ivanoff 1972; Pörtner and Knust 2007; Bowden et al. 2014). Across several studies, gill plasticity in response to various stressors is essential to cope with environmental fluctuations as also shown in sea bass in this study (Sollid et al. 2005; Sardella and Brauner 2007; Mitrovic and Perry 2009; Matey et al. 2011; Gilmour and Perry 2018). Few species develop a potentially protective interlamellar cell mass that could protect fish gills from salinity change (Blair et al., 2017), ammonia exposure combined with temperature (Perry et al., 2010), oxygen and temperature (Sollid and Nilsson, 2006), or temperature alone (Sollid et al., 2005). The change in filament thickness measured in this study upon FW transfer at 18 °C might indicate an increased interlamellar cell mass as recently shown in threespine stickleback *Gasterosteus aculeatus* and Arctic grayling *Thymallus arcticus* exposed to salinity change (Blair et al. 2017; Gibbons et al. 2018).

Table 9 Temperature and salinity effects at the cellular level in sea bass exposed to SW and transferred to FW at two temperatures (18 °C and 24 °C). * p <0.05, ** p <0.01, ****p <0.0001. N = 8-13 per condition.

Level of integration	**Parameters**		**Temperature increase from 18 °C to 24 °C**		**Salinity decrease from SW to FW**	
			SW	**[1]FW**	**18 °C**	**24 °C**
Cell	Ionocyte	Area	-	↓(*)	-	↓(**)
		Number on filament	-	↓(**)	↑(****)	↑(**)
		Number on lamellae	-	↓(*)	↑(****)	↑(****)
	Mucous cells	Number	-	↓(**)	↑(*)	-
		Area	↑(****)	↑(****)	-	↑(****)

[1]Note that the FW acclimated fish at 24 °C have been previously pre-acclimated to SW 24 °C before salinity transfer

At cellular level (Table 9), an increase in the ionocyte density has been observed following salinity transfer (to FW) in order to take up ions by highly specialized ionocytes. This was previously reported in several teleost species (Lin et al. 2003; Seo et al. 2009; Riou et al. 2012) and in sea bass (Varsamos et al. 2002; Nebel et al. 2005; Bossus et al. 2013). Interestingly at 24 °C the increase in ionocyte density is less important than at 18 °C (Table 9, Article 1, Fig. 4). This may reflect the fact that fishes exposed to thermal stress may not be able to switch from ion secretion to ion uptake as efficiently as in temperate conditions. It remains to be studied what type of ionocytes are predominant in FW warm-acclimated fish gills, as at least two types of ionocytes have been previously characterized, NHE3-expressing ionocytes and NCC2-expressing ionocytes respectively involved in Na^+ uptake/H^+ excretion or Na^+/Cl^- uptake (Blondeau-Bidet et al. 2019).

In SW warm conditions, pavement cell microridges showed a less tightly packed labyrinth than in the other treatments. As discussed in Chapter III and IV, there is evidence that blood acidosis induces changes of the surface structure and ultrastructure of pavement cells (review in Goss et al. 1998). More research is needed to link gill surface structure with acid-base imbalance in sea bass.

We observed a higher number of mucous cells following salinity transfer at 18 °C but not at 24 °C (Chapter V, Table 9). A higher mucous cell coverage in gills of FW-acclimated fish probably contributes to the maintenance of ion and water balance. Indeed, mucus recycles ions by trapping them (Shephard 1994), which may interfere with osmoregulation, acid base

regulation and ammonia excretion. Regarding osmoregulation, an increased mucous layer could prevent excessive passive ion loss in FW. An increase of mucous cell number has been shown to be a response to several types of environmental stressors such as high salinity, ammonia and pollutants (Shephard 1994; Wendelaar Bonga 1997; Blair et al. 2017). Interestingly, the area of mucus stained within each cell is significantly increased, which may suggest an increased production of mucus within the mucocytes at 24 °C at both salinities. The results of previous studies have demonstrated that the effects of temperature on mucus production are complex and subjected to the confounding influences of several environmental stressors such as hypoxia and temperature-related skin diseases (Matey et al. 2008, 2011; Ekman et al. 2015; Jensen et al. 2015). The presence of a branchial mucous layer might also act as an obstruction to gas exchange depending on thickness of the mucous coverage (Shephard, 1994; Ultsch and Gros, 1979).

Table 10 Temperature and salinity effects at the protein level in sea bass exposed to SW and transferred to FW at two temperatures (18 °C and 24 °C). * p <0.05, ** p <0.01. N = 8-13 per condition.

Level of integration	**Parameters**	**Temperature increase From 18 °C to 24 °C**		**Salinity decrease From SW to FW**	
		SW	**FW**	**18 °C**	**24 °C**
Protein	NKA activity	**-**	**↓(*)**	**↑(**)**	**↑(*)**

[1]Note that the FW acclimated fish at 24 °C have been previously pre-acclimated to SW 24 °C before salinity transfer

At protein level, NKA activity ($V_{max,}$ assayed at 37 °C) was significantly decreased in warm compared to temperate FW conditions (Table 10). Higher NKA activity was expected in FW in order to compensate passive ion loss gain of water (Metz et al. 2003; Crockett and Londraville 2006). In our study, low branchial NKA activity is putatively related to less abundant ionocyte density and decreased ionocytes area in warm FW (Table 4). This lower NKA activity might partially explain lower plasma Na^+ levels at 24 °C *vs* 18 °C FW (Table 7). In SW, the differences in gill NKA (V_{max}) were not significant between temperatures, but a lower tendency can be shown in SW warm conditions compared to temperate SW.

Table 11 Temperature and salinity effects at the gene level in sea bass exposed to SW and transferred to FW at two temperatures (18 °C and 24 °C). Analyzed genes are grouped regarding their function ion secretion (*cftr*, *nkcc1*), ion absorption (*nhe3*, *ncc2a*, *nkaα1a*), acid-base regulation (*vha-a*, *vha-b*), ammonia excretion (*rhbg*, *rhcg1*) and heat shock proteins (*hsp90ba, hsp90b1*) * p <0.05, ** p <0.01, *** p <0.001, p <0.0001. N = 8-13 per condition.

Level of integration	**Parameters**		**Temperature increase from 18 °C to 24 °C**		**Salinity decrease Form SW to FW**	
			SW	**[1]FW**	**18 °C**	**24 °C**
Gene expression	Ion secretion	*cftr* / *nkcc1*	↑(****) / -	↑(***) / -	↓(*) / ↓(**)	↓(***) / ↓(**)
	Ion uptake	*ncc2a*	↑(***)	-	↑(***)	↑(*)
		nhe3	↑(**)		↑(**)	-
		nka α1a	↑(****)		↑(****)	
	Acid secretion	*vha-a* / *vha-b*	↑(****) / ↑(**)	↑(*) / -	-	↓(***) / ↓(**)
	Ammonia excretion	*rhbg* / *rhcg1*	- / ↑(***)	- / ↑(***)	-	-
	Heat shock protein	*hsp90b1* / *hsp90ba*	↑(****) / ↑(**)	↑(***) / ↑(**)	-	↓(*) / -

[1]Note that the FW acclimated fish at 24 °C have been previously pre-acclimated to SW 24 °C before salinity transfer

6.2 Temperature stress modulates branchial mRNA levels of genes involved in diverse functions

It is worth noting that a significant increase in the expression of genes encoding for proteins involved in ion secretion, ion uptake, acid secretion and ammonia excretion were observed at warm temperature in SW compared to temperate conditions (Table 11). This suggests an overall increased metabolism in warm conditions (Clarke and Fraser, 2004; Person-Le Ruyet et al., 2004) with consequences on the transcriptionnal machinery. On the other hand, in FW conditions, almost a similar trend can be shown (increased expression at 24 °C) except for transporters involved in ion uptake (Table 11).

Cystic fibrosis transmembrane conductance regulator (CFTR) and $Na^+/K^+/2Cl^-$ contransporter (NKCC1) are involved **in ion secretion** (Table 11) in gills of sea bass. In FW conditions, at both tested temperatures, *nkcc1* mRNA expressions were lower compared to SW conditions (Article III, Fig. 1). Higher *cftr* mRNA levels were recorded in warm SW and FW conditions compared to temperate conditions at the respective salinity. An effect of temperature change

on *cftr* expression has been shown in other species (Nilsen et al. 2007; Handeland et al. 2014). CFTR is apically localized in ion secreting cells as it has been shown by Bodinier et al (2009) in sea bass in SW temperate conditions. In killifish *F. heteroclitus* gills, CFTR seems to be predominantly (in 90% of analyzed ionocytes) localized in cytoplasmic vesicles, when fish were acclimated to FW (Marshall et al. 2002). This suggests a change in CFTR localization according to salinity. The localization of this chloride channel needs to be further studied following temperature stress in sea bass, in order to better understand its role, notably in warm FW conditions.

Besides Na^+/K^+-ATPase (NKA), main transporters involved in **Na^+ (and Cl^- uptake)** are the apical Na^+/H^+-exchanger 3 (NHE3) and the Na^+/Cl^- cotransporter (NCC2a). In SW warm conditions, both genes are overexpressed which might be a response to heat stress (Table 11). The pronounced increase in mRNA expression of transporters involved in ion uptake under salinity, PCO_2 and/or thermal stress have already been shown in other teleost species (Kreiss et al. 2015b; Michael et al. 2016b; Gibbons et al. 2018). *Nhe3*, involved in Na^+ uptake and proton secretion, is not transcriptionally induced following FW transfer in warm conditions, as it is the case for *nka α1a*. This might explain low plasma Na^+ ion levels in warm FW conditions (Table 7). In contrast, transfer from SW to FW at 24 °C induced changes in the expression of *ncc2a* to enhance Cl^- uptake (Table 11). In fact, Cl^- levels are well maintained in this condition and even higher than in temperate FW conditions (Table 7). Overall, gene expression levels for key transporters involved in Cl^- and/or Na^+ uptake (NCC2, NHE3, NKA mainly) explain partially the ion levels and ion imbalance observed and discussed above.

Regarding **acid secretion,** the higher mRNA expression of *vha-a* in warm conditions at both salinities seems to be a consequence of lower plasma Na^+/Cl^- ratio that could indicate a blood acidosis (Table 7, 11). A similar response has been shown in Atlantic cod *Gadus morhua* exposed to respiratory acidosis, where *vha-v1a* mRNA expression levels were increased following heat stress in gills (Michael et al. 2016b). Apical localization of V-type H^+-ATPase (VHA) protein would generate a favorable electrochemical gradient for energizing Na^+ uptake (Sullivan et al. 1995; Yan et al. 2007). An overexpression of *nhe3*, *vha-a* and *vha-b* strongly support our hypothesis that sea bass responded to lower plasma Na^+/Cl^- ratio or possible blood acidosis at 24 °C *vs* 18 °C in SW conditions by secreting protons to the water. Blood pH should be further confirmed in sea bass. Heat stress likely indicates profound functional interactions between acid secretion mechanisms and Na^+ uptake.

Rh glycoproteins are involved in **ammonia excretion.** But they have also been shown to be indirectly involved in Na^+ uptake based on the metabolon hypothesis involving apical NHE3, Rhcg1 and VHA (see Chapter IV, Page 59.) (Hirose and Nakada, 2010; Hwang and Lin, 2014; Nawata et al., 2010). This mechanism is so far unknown in sea bass. The mRNA levels of *rhcg1* were significantly increased in warm temperatures at both salinities, primarily to excrete ammonia (Table 11). As the metabolism is generally increased in ectotherm species upon warm conditions, an increase in ammonia excretion could have been expected, as previously shown in sea bass exposed to 25 °C (Person-Le Ruyet et al. 2004). In pufferfish *Takifugu rubripes,* increased mRNA levels of branchial *rhcg1* under ammonia exposure (1 $mmol.L^{-1}$ NH_4HCO_3) (Nawata et al., 2010) are in line with our observation. The high expression of *rhcg1* might also contribute indirectly to Na^+ uptake to compensate for low plasma Na^+ levels, notably in FW.

Overall, these findings strongly suggest that warm temperatures induce an ion imbalance, and particularly decreased plasma Na^+ levels. In warm SW, most genes involved in ion transport are overexpressed as a response to heat stress. Following FW transfer, however, some genes involved in ion uptake are not induced, notably *nhe3* and *nka α1a*, which might explain partially the observed ion imbalance at this salinity.

A thermal s**tress** was shown through the expression of genes encoding HSP90 (*hsp90ba* and *hsp90b1*) that were significantly upregulated in warm conditions at both considered salinities (Table 11). HSP90 is a highly conserved, extensively studied protein in the Heat Shock protein family, which acts as molecular chaperone and is essential for newly synthesized proteins, refolding denatured proteins, and signal transduction. HSP90 was selected for this study among other HSP proteins for its involvement during heat stress in teleost species (Dietz and Somero 1992; Dietz 1994; Podrabsky and Somero 2004; Ikeda et al. 2017). According to the literature, HSP90 seems also more inducible than HSP70 following salinity stress (Deane and Woo, 2011; Palmisano et al., 2000; Peng et al., 2015). More generally, HSP induction represent an adaptive physiological response to cope with a variety of stressors (Barton, 2002; Bottoni et al., 2009). The expression of *hsp90* is regulated by various environmental stressors: benzo (a) pyrene (Liu et al., 2015), pH (Zhou et al. 2015), cadmium (Choi et al. 2008), crowding (Ni et al., 2014), hypoxia (Mohindra et al., 2015). The overexpression of these two genes (Table 11) indicates that 24 °C represents a stress factor in European sea bass requiring the induction of chaperone proteins. Regarding salinity decrease, the expression *hsp90b1* was significantly decreased after FW transfer at 24 °C and *hsp90*

transcript levels were generally lower and less variable in FW compared to SW (Article III, Fig. 1). This is consistent with the study of Peng et al. (2015) where they measured lower mRNA levels of *hsp90* (and in a lower extent *hsp70*) at lower salinities in several tissues, including the gill. This suggests that HSP90 may acts differently between SW and FW in teleost, but further studies are needed.

Chapter VII

Discussion générale

Chapter VII - Discussion générale

Le loup méditerranéen doit naturellement faire face à des changements de salinité et de température lors de ses migrations en milieu estuarien ou lagunaire. Cette étude expérimentale a été menée afin d'étudier la capacité de *D. labrax* à s'acclimater à des variations abruptes de salinité et de température de son habitat, dans un contexte de changement global au cours duquel une augmentation de la fréquence et de l'intensité des événements climatiques extrêmes est attendue (Shaltout and Omstedt 2015). En effet, en Méditerranée, plusieurs études soulignent une augmentation des fortes précipitations associées (ou non) à des vagues de fortes chaleurs, en période printanière notamment (Anthony et al. 2009). Les effets physiologiques de stress environnementaux sont souvent considérés isolément (McCarty and Houston 1977 ; Metz et al. 2003 ; Seo et al. 2013 ; Barnes et al. 2014). Les effets combinés de stress multiples ont été moins étudiés chez les poissons et sont donc probablement sous-estimés (Stuenkel and Hillyard 1980 ; Kültz and Somero 1995 ; Fiess et al. 2007 ; Sardella et al. 2008). Pour mieux anticiper les réponses des espèces à ces changements environnementaux et plus largement, l'impact des changements globaux sur la biodiversité et le fonctionnement des écosystèmes, il est nécessaire de mieux comprendre l'influence de stress multiples sur l'écophysiologie des organismes.

Ce travail de thèse a pour objectif général d'améliorer les connaissances sur l'influence de deux types de stress réalistes d'un point de vue environnemental (*i.e.* une baisse de salinité avec ou sans hausse de température) sur les processus impliqués dans l'osmorégulation. L'équilibre de la balance hydrominérale (osmolalité sanguine) et les concentrations en ions circulants ont été mesurés afin d'évaluer les effets intégrés au niveau de l'organisme entier. Étant donné que la branchie représente un organe clé pour l'osmorégulation, elle a été choisie comme tissu cible pour les analyses histologiques, cellulaires et moléculaires. De plus, la branchie présente l'avantage d'être un organe multifonctionnel jouant un rôle central pour différentes fonctions physiologiques chez les poissons (respiration, régulation acido-basique, osmorégulation et excrétion azotée). Elle permet donc d'étudier l'interaction entre la régulation ionique et les autres fonctions physiologiques assurées au sein de la branchie. Dans cette étude, nous avons analysé la morphologie branchiale, l'activité de l'ATPase Na^+/K^{+-}, la densité des ionocytes et des mucocytes. Les mécanismes moléculaires associés à trois grandes fonctions branchiales (osmorégulation, régulation acido-basique, excrétion azotée) ont été

étudiés au travers des niveaux de transcrits de gènes impliqués dans les transports ioniques. Les transcrits de gènes impliqués dans la réponse au stress thermique et la production de mucus ont également été mesurés. Les résultats obtenus ont été présentés et partiellement discutés dans les chapitres III, IV et V. L'objectif de cette discussion générale est de discuter l'ensemble des effets observés au cours de ce travail de thèse à différents niveaux d'intégration du vivant (résumé dans les tableaux 12-16) : à l'échelle individuelle, à l'échelle tissulaire (avec un focus particulier sur la branchie), à l'échelle cellulaire et moléculaire.

7.1 Effets de stress combinés sur les paramètres physiologiques, biochimiques et morphologiques

Tableau 12 Effet d'une acclimatation des loups de 18 °C à 24 °C au niveau de l'organisme en eau de mer (EM) et suite au transfert en eau douce (ED). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. N= 8-13 par condition.

Niveau d'intégration	**Paramètre**	**Hausse de température De 18 °C *à* 24 °C**		**Baisse de salinité De l'EM *à* l'ED**	
		EM	**[1]ED**	**18 °C**	**24 °C**
Individu	Osmolalité sanguine	-	**↑ (****)**	**↓ (****)**	**↓(****)**
	Concentration Na^+	**↓ (*)**	**↓ (**)**	-	-
	Concentration Cl^-	-	**↑ (****)**	**↓ (****)**	**↓(****)**
	Ratio Na^+/Cl^-	**↓(**)**	**↓(****)**	**↑(***)**	**↑(*)**

[1]Notez que les loups acclimatés à l'ED 24 °C ont préalablement été maintenus en EM à 24 °C avant transfert en ED

<u>Au niveau de l'organisme entier</u>, le loup maintien une osmolalité constante en eau de mer (EM) suite à une acclimatation de 2 semaines à 24 °C, ce qui suggère une sécrétion efficace d'ions en eau chaude, probablement au niveau branchial et rénal. Après transfert en eau douce (ED), le loup maintient une osmolalité sanguine plus élevés à 24 °C qu'à 18 °C. Cela suggère que le loup est également un bon hyper-osmorégulateur à 24 °C et qu'il est capable de tolérer des changements abrupts de salinité pendant au moins 2 semaines en eau chaude (à 24 °C). Toutefois l'analyse détaillée des ions circulants permet de mettre en évidence que la concentration de Na^+ plasmatique est significativement plus faible à 24 °C aux deux salinités testées, en ED et en eau de mer (EM), ce qui indique que les mécanismes de transport de Na^+ sont probablement modifiés à l'échelle moléculaire à 24 °C par rapport à 18 °C. Nous verrons

plus loin si cette hypothèse est confirmée par les mesures de transcrits et d'activité enzymatique de l'ATPase Na^+/K^+ et d'autres transporteurs. La concentration de Cl^- plasmatique est significativement corrélée à l'osmolalité sanguine pour toutes les conditions testées, ce qui confirme que le Cl^- est un composant majeur de l'osmolalité sanguine totale (avec le Na^+). Le niveau de Cl^- plasmatique n'est pas significativement modifié entre 18 et 24 °C en EM mais une hausse significative de Cl^- plasmatique est observée à 24 °C après transfert en ED. Ceci suggère que les loups sont capables en ED à 24 °C de mettre en place au niveau branchial et rénal des mécanismes d'absorption de Cl^- efficaces et/ou minimisent les pertes de Cl^- par voie paracellulaire. Globalement, une diminution du ratio Na^+/Cl^- est mise en évidence à 24 °C, suggérant un déséquilibre ionique au niveau sanguin en réponse à une hausse de température. D'après la littérature, les changements thermiques induisent une modification des concentrations ioniques plasmatiques et de l'osmolalité sanguine chez les téléostéens, due à une altération des processus impliqués dans la régulation ionique, comme démontré chez le poisson rouge *Carassius auratus* (Mitrovic and Perry 2009), la carpe commune *Cyprinus carpio* (Metz et al. 2003), le tilapia du Mozambique *Oreochromis mossambicus* (Fiess et al. 2007 ; Sardella et al. 2008) et la truite arc en ciel *Salmo gairdneri* (McCarty and Houston 1977). Dans les branchies et dans les autres tissus osmorégulateurs (rein, intestin), l'homéostasie ionique est régulée par deux pompes nécessitant de l'énergie sous forme d'ATP : la pompe ATPase Na^+/K^+ et la pompe à protons V-type H^+-ATPase. La mesure d'activité de la pompe V-type H^+-ATPase aux deux températures pourrait nous indiquer si ce mécanisme de régulation moléculaire est altéré en eau à 24 °C. De plus, étant donné que le Na^+ et le Cl^- sont absorbés par différents voies impliquant différents transporteurs ioniques dans différents types cellulaires en ED (Hwang and Lin 2014), il semble indispensable d'analyser l'ensemble des transporteurs impliqués dans l'absorption du Na^+, du Cl^- ou des 2 ions simultanément. Ces résultats seront présentés ci-dessous dans le paragraphe qui traite des résultats obtenus à l'échelle moléculaire.

Tableau 13 Effet d'une acclimatation des loups de 18 °C à 24 °C au niveau de l'organe et des tissus en eau de mer (EM) et suite au transfert en eau douce (ED). *p<0.05, **p<0.01, ***p<0.001. N= 8-13 par condition.

Niveau d'intégration	**Paramètre**		**Hausse de température De 18 °C à 24 °C**		**Baisse de salinité De l'EM à l'ED**	
			EM	**[1]ED**	**18 °C**	**24 °C**
Organe/Tissu	**Morphologie branchiale**	**Longueur des lamelles**	**-**	**↑(***)**	**↓(*)**	**↑(***)**
		Épaisseur des filaments	**-**	**↓(*)**	**↑(**)**	**-**

[1]Notez que les loups acclimatés à l'ED 24 °C ont préalablement été maintenus en EM à 24 °C avant transfert en ED

A l'échelle du tissu branchial, des changements abrupts de salinité et/ou de température induisent des modifications morphologiques de la branchie, comme cela a déjà été démontré chez différentes espèces de téléostéens (Sardella et al. 2008 ; Mitrovic and Perry 2009 ; Vargas-Chacoff et al. 2009). L'épaisseur des filaments et la largeur des lamelles augmentent significativement après transfert de l'EM à l'ED à 18 °C, probablement en lien avec l'augmentation du nombre d'ionocytes (cf. chapitre III) et de cellules à mucus (cf. chapitre IV). Après transfert en ED à 24 °C, la longueur des lamelles augmente significativement. Cela permet d'augmenter la surface d'échange et d'optimiser les échanges gazeux en réponse à la hausse de température. En revanche, la longueur des lamelles n'est pas significativement augmentée en EM après transfert dans l'eau à 24 °C, ce qui pourrait affecter l'efficacité d'absorption d'oxygène au niveau branchial dans cette condition (Ivanoff 1972 ; Pörtner and Knust 2007 ; Bowden et al. 2014). D'après plusieurs études, ces changements morphologiques de la branchie peuvent être associés à la notion de compromis osmorespiratoire (review Gilmour and Perry, 2018), qui correspond au compromis fonctionnel entre les échanges gazeux et la conservation des ions au niveau branchial. Plusieurs études ont montré que les branchies de certaines espèces montrent une forte plasticité dans un environnement changeant et modifient leur structure en fonction des besoins en oxygène et de maintien de l'équilibre ionique (Sollid et al. 2005 ; Matey et al. 2011). Les modifications morphologiques observées dans la branchie de loups illustrent bien la réponse intégrée mise en place pour tolérer les stress combinés au niveau de branchie, au regard des multiples fonctions physiologiques assurées par cet organe.

Tableau 14 Effet d'une acclimatation des loups de 18 °C à 24 °C au niveau de la cellule en eau de mer (EM) et suite au transfert en eau douce (ED). *p<0.05, **p<0.01, ****p<0.0001. N= 8-13 par condition.

Niveau d'intégration	**Paramètre**		**Hausse de température De 18 °C à 24 °C**		**Baisse de salinité De l'EM à l'ED**	
			EM	**[1]ED**	**18 °C**	**24 °C**
Cellule	Ionocyte	Aire	-	↓(*)	-	↓(**)
		Densité au niveau des filaments	-	↓(**)	↑(****)	↑(**)
		Densité au niveau des lamelles	-	↓(*)	↑(****)	↑(****)
	Cellule à mucus	Nombre	-	↓(**)	↑(*)	-
		Aire	↑(****)	↑(****)	-	↑(****)

[1]Notez que les loups acclimatés à l'ED 24 °C ont préalablement été maintenus en EM à 24 °C avant transfert en ED

<u>A l'échelle cellulaire</u>, une augmentation de la densité des ionocytes est observée après transfert en ED aux deux températures testées. Ce phénomène a déjà été rapporté chez différents téléostéens (Lin et al. 2003 ; Seo et al. 2009 ; Riou et al. 2012) et chez le loup (Varsamos et al. 2002 ; Nebel et al. 2005 ; Bossus et al. 2013). Il est associé à une différenciation des ionocytes impliqués dans l'absorption des ions en ED. Chez le loup, au moins deux types cellulaires ont été caractérisés en ED, l'un étant impliqué dans l'absorption de Na^+ et la sécrétion de protons (les ionocytes exprimant l'échangeur Na^+/H^+-3 (NHE3) et l'autre type cellulaire qui semble absorber à la fois le Na^+ et le Cl^- (exprimant le cotransporteur Na^+/Cl^- (NCC), voir section discussion au niveau moléculaire (Blondeau-Bidet et al. 2019). Toutefois, il est intéressant de noter que cette augmentation de la densité des ionocytes en eau douce est moins importante à 24 °C qu'à 18 °C. **L'ensemble de ces résultats suggère que les poissons soumis à un stress thermique pourraient présenter une capacité moindre à inverser leurs mécanismes osmorégulateurs pour passer de la sécrétion à l'absorption d'ions.** Au niveau moléculaire, cela pourrait se traduire par une plus faible induction du NHE3, un des transporteurs les plus exprimés dans les ionocytes localisés au niveau des lamelles branchiales en ED (Blondeau-Bidet et al. 2019). La mesure des niveaux de transcrits du gène NHE3 et d'autres gènes caractéristiques des types cellulaires caractéristique de l'eau douce permettra de confirmer cette hypothèse (voir section discussion au niveau moléculaire).

D'après l'observation de l'épithélial branchial en microscopie électronique, les crêtes des cellules pavimenteuses ont pris une apparence atypique en EM à 24 °C, caractérisée par une forme labyrinthique moins serrée (protubérances de la membrane plasmique moins abondantes) que dans les autres traitements. Comme discuté dans les chapitres III et IV, ce changement de la structure de surface et de l'ultrastructure des cellules pavimenteuses pourrait être lié à une acidose sanguine (review in Goss et al. 1998). Toutefois, il faudrait plus d'éléments pour pouvoir relier les modifications de structure et de morphologie branchiale avec le maintien de l'équilibre acido-basique chez le loup.

Le mucus est aussi considéré comme une barrière de protection contre divers stress environnementaux comme les pathogènes, les modifications thermiques, de pH, etc. (Shephard 1994 ; Beck and Peatman 2015). Une augmentation du nombre de cellules à mucus a été observée au niveau des filaments branchiaux chez les poissons transférés en ED à 18 °C (chapitre V). Cette augmentation du recouvrement branchial par les cellules à mucus en ED tempérée pourrait contribuer au maintien de l'équilibre ionique et hydrique. En effet, comme le mucus piège les ions (Shephard 1994), il pourrait interférer avec les différentes fonctions branchiales faisant intervenir des ions (*i.e.* osmorégulation, régulation acide-base). Ainsi, une augmentation de la couche de mucus en ED pourrait contribuer à diminuer la perte passive d'ions par diffusion. Mais cette augmentation du nombre de cellules à mucus pourrait aussi traduire une réponse à un autre type de stress, tels que la présence de pathogènes ou la modification du pH de l'eau (Shephard 1994 ; Wendelaar Bonga 1997 ; Blair et al. 2017). En réponse au stress thermique, bien que le nombre de cellules à mucus ne soient pas significativement augmenté, la surface de mucus marqué au sein des cellules est plus élevée aux deux salinités testées à 24 °C. Cela pourrait suggérer une augmentation de la production de mucus en réponse au stress thermique. Les résultats de précédentes études ont démontrés que les effets de la température sur la production de mucus sont complexes et potentiellement influencés par différents facteurs confondants tels que le statut nutritif, l'hypoxie ou la présence de pathogènes (Matey et al. 2008, 2011 ; Ekman et al. 2015 ; Jensen et al. 2015).

Au final, pour améliorer notre compréhension du rôle joué par les différents types cellulaires (ionocytes, cellules pavimenteuses, mucocytes) et leur fonction dans la branchie, il faudrait établir un plan expérimental dédié permettant de déconvoluer l'influence de la température, de la salinité et du pH.

Tableau 15 Effet d'une acclimatation des loups de 18 °C à 24 °C au niveau protéique en eau de mer (EM) et suite au transfert en eau douce (ED). *p<0.05, **p<0.01. N= 8-13 par condition.

Niveau d'intégration	**Paramètre**	**Hausse de température De 18 °C à 24 °C**		**Baisse de salinité De l'EM à l'ED**	
		EM	**[1]ED**	**18 °C**	**24 °C**
Protéine	Activité NKA	-	↓(*)	↑(**)	↑(*)

[1]Notez que les loups acclimatés à l'ED 24 °C ont préalablement été maintenus en EM à 24 °C avant transfert en ED

Au niveau protéique, l'activité de la pompe ATPase Na^+/K^+ (NKA, $V_{max,}$ mesuré à 37 °C) est significativement plus faible à 24 °C comparé à 18 °C après transfert en ED. Ce résultat diffère de celui attendu (*i.e.* une augmentation de l'activité NKA pour compenser la perte passive d'ions par absorption ionique active) (Metz et al. 2003 ; Crockett and Londraville 2006). Cette faible activité NKA mesurée dans les branchies peut être mise en relation avec les observations faites en histologie : une densité et une aire d'ionocytes réduites en ED à 24 °C, comparé à 18 °C. Cette plus faible activité pourrait aussi expliquer les concentrations en Na^+ plus faibles à 24 °C *vs* 18 °C en ED. En EM, la même tendance peut être notée avec une activité NKA (V_{max}) moindre dans la condition SW 24 °C *vs* SW 18 °C. Néanmoins, les différences ne sont pas significatives entre les 2 conditions thermiques testées. La mesure d'expression des transcrits du gène *nka α1a* mRNA permettra de vérifier si cette baisse de l'activité NKA est compensée par une augmentation de l'expression d'ARNm.

7.2 Le stress thermique module l'expression des chaperones et des gènes impliqués dans les mécanismes de régulation ionique

Tableau 16 Effet d'une acclimatation des loups de 18 °C à 24 °C au niveau de l'expression des gènes en eau de mer (EM) et suite au transfert en eau douce (ED). Les gènes étudiés sont regroupés par fonctions de sécrétion ionique (*cftr*, *nkcc1*), absorption ionique (*nhe3*, *ncc2a*, *nkaα1a*), régulation acido-basique (*vha-a*, *vha-b*), excrétion azotée (*rhbg*, *rhcg1*) et protéines de stress thermique 90 (*hsp90a1*, *hsp90b1)*. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. N= 8-13 par condition.

Niveau d'intégration	**Paramètre**		**Hausse de température De 18 °C à 24 °C**		**Baisse de salinité De SW à FW**	
			EM	**[1]ED**	**18 °C**	**24 °C**
Expression de gènes	Sécrétion ionique	*cftr* / *nkcc1*	↑(****) / -	↑(***) / -	↓(*) / ↓(**)	↓(***) / ↓(**)
	Absorption ionique	*ncc2a* *nhe3* *nka α1a*	↑(***) ↑(**) ↑(****)	-	↑(***) ↑(**) ↑(****)	↑(*) -
	Régulation acido-basique	*vha-a* / *vha-b*	↑(****) / ↑(**)	↑(*) / -	-	↓(***) / ↓(**)
	Excrétion azotée	*rhbg* / *rhcg1*	- / ↑(***)	- / ↑(***)	-	-
	Protéines de stress thermique	*hsp90b1* / *hsp90ba*	↑(****) / ↑(**)	↑(***) / ↑(**)	-	↓(*) / -

[1]Notez que les loups acclimatés à l'ED 24 °C ont préalablement été maintenus en EM à 24 °C avant transfert en ED

<u>Au niveau des transcrits</u>, il est intéressant de noter qu'une hausse de température en EM induit une surexpression significative de certains gènes codant pour les protéines impliquées dans la sécrétion ionique, l'absorption ionique, la régulation acido-basique et l'excrétion azotée. En ED, la même tendance est observée, excepté pour les transporteurs impliqués dans l'absorption d'ions.

Les protéines Cystic Fibrosis Transmembrane conductance Regulator (CFTR) et co-transporteur $Na^+/K^+/2Cl^-$ (NKCC1) sont impliquées dans la sécrétion ionique dans les branchies de loup en EM. L'expression du gène *cftr* est significativement diminuée en ED, aux deux conditions thermiques testées, conformément à ce qui était attendu en réponse à une baisse de salinité (Nilsen et al. 2007 ; Handeland et al. 2014). En revanche, une surexpression du gène *cftr* a été mesurée à 24 °C *vs* 18 °C en EM et en ED. Chez le loup en condition tempérée (18 °C), le CFTR est localisé en position apicale permettant aux ionocytes de sécréter des ions chlorures comme cela a été montré par Bodinier et al. (2009). En ED, par

contre, la localisation du CFTR semble être cytoplasmique dans 90 % des ionocytes branchiaux suggérant un changement de localisation intracellulaire et de fonction de cette protéine selon la salinité, comme démontré chez le choquemort *F. heteroclitus* (Marshall et al. 2002). La localisation ainsi que le rôle du CFTR en ED n'est pas clair chez le loup. L'immunolocalisation du canal à chlorures aux deux températures testées permettrait de compléter les données de transcrits. Le gène *nkcc1* ne semble pas influencé par la hausse de température. Son expression est significativement diminuée en ED, aux deux conditions thermiques testées, conformément à ce qui était attendu en réponse à une baisse de salinité (Lorin-Nebel et al., 2006).

L'expression d'un transporteur lié à l'absorption Na^+, l'échangeur Na^+/H^+-3 (NHE3), n'est pas significativement affectée par le stress thermique après transfert en ED. Cela pourrait expliquer les faibles niveaux de Na^+ plasmatique mesurés par rapport aux conditions tempérés. Au contraire, le transfert de l'EM à l'ED à 24 °C induit une augmentation de l'expression du gène *ncc2a*, codant pour le cotransporteur apical Na^+/Cl^-. Effectivement, les concentrations de Cl^- sont bien maintenues dans cette condition et même supérieurs aux conditions tempérées. Globalement, les niveaux d'expression des gènes codant pour les transporteurs clés dans l'absorption du Na^+ (NHE3) et du Cl^- (NCC2A) expliquent, au moins en partie, les résultats obtenus à l'échelle individuelle pour les ions circulants et le déséquilibre de la balance ionique discuté précédemment, qui semble être principalement dû à un déficit d'absorption du Na^+.

Si l'on considère les transporteurs ioniques liés à la sécrétion de H^+ (pompe à protons V-type H^+-ATPase (VHA), sous-unités VHA-A et VHA-B), la forte expression du gène *vha-a* à 24 °C comparé à la condition tempérée aux deux salinités testées semble indiquer une réponse à une acidose sanguine, qui a été suggérée du fait d'une diminution du ratio Na^+/Cl^-. Une acidose respiratoire combinée à une surexpression du gène *vha-a* dans la branchie ont également été observées chez la morue *Gadus morhua*, suite à un stress thermique (Michael et al. 2016b). La localisation apicale de la protéine VHA permettrait de générer un gradient électrochimique favorable pour fournir de l'énergie pour l'absorption du Na^+ (Sullivan et al. 1995 ; Yan et al. 2007). La surexpression des gènes *nhe3*, *vha-a* et *vha-b* à 24 °C supporte l'hypothèse que le loup active des mécanismes pour compenser la chute de Na^+, et limiter les conséquences dues au déséquilibre ionique, notamment une possible acidose métabolique, plus marquée en EM. Toutefois, cette probable acidose nécessiterait d'être confirmée par des mesures de pH sanguin, qui n'ont pas pu être réalisées dans cette étude. En tous cas, le

stress thermique met en exergue les interactions moléculaires existantes entre les fonctions de sécrétion de H^+ et de transport du Na^+, au sein de la branchie.

Les glycoprotéines Rh sont impliquées dans l'excrétion azotée. Mais elles sont également impliquées, de manière indirecte, dans l'absorption du Na^+ selon l'hypothèse du métabolon, qui implique les protéines apicales NHE3, Rhcg1 et VHA (Hirose and Nakada 2010 ; Nawata et al. 2010 ; Hwang and Lin 2014). Ce concept de métabolon n'a pas encore été exploré chez le loup. Les niveaux de transcrits du gène *rhcg1* sont significativement augmentés en réponse au stress thermique, aux deux salinités testées. Comme le métabolisme est augmenté en eau chaude chez les ectothermes, une augmentation de l'excrétion azotée est attendue. Chez *Takifugu rubripes,* une augmentation du niveau d'expression d'ARNm branchial de *rhcg1* a été observé suite à une exposition à 1 $mmol.L^{-1}$ de NH_4HCO_3 (Nawata et al. 2010), confirmant que l'expression du gène *rhcg1* est activée dans la branchie en présence d'ammonium. Mais cette augmentation de l'expression de *rhcg1* pourrait aussi contribuer, de manière indirecte, à l'absorption du Na^+ pour compenser la chute de Na^+ au niveau sanguin.

Globalement, les résultats de cette étude suggèrent que le stress thermique induit un déséquilibre ionique au niveau systémique (particulièrement dû au Na^+), lié à une surexpression de la majorité des gènes impliqués dans le transport ionique en EM à 24 °C. Cependant, l'augmentation attendue de l'expression des gènes liés à l'absorption ionique après transfert en ED n'est pas observée pour tous les gènes (absence d'induction du NHE3 et NKAα1a).

La mesure de l'expression de deux gènes paralogues codant pour HSP90 (*hsp90ba* et *hsp90b1*) indique que la hausse de température de 18 °C à 24 °C induit l'expression de protéines de stress thermique. Les HSP90 sont des protéines de la famille des Heat Shock Protein, fortement conservées entre les taxa et largement étudiées. En tant que protéines chaperones, elles jouent plusieurs rôles clés en intervenant dans la conformation des protéines néo-synthétisées, des protéines dénaturées, ou dans la transduction de signaux cellulaires. Dans cette étude, HSP90 a été sélectionnée parmi les nombreuses protéines de la famille des Heat Shock Protein comme un marqueur de stress thermique. L'inductibilité des gènes codant pour HSP90 en réponse à une hausse de température a déjà été largement démontrée chez les téléostéens (Dietz and Somero 1992; Dietz 1994 ; Podrabsky and Somero 2004 ; Ikeda et al. 2017). Cette surexpression indique que la mobilisation de protéines chaperones est nécessaire pour maintenir la conformation des protéines. Plus largement, les HSPs participent à la

réponse au stress et leur expression peut être régulée en réponse à une variété de stresseurs environnementaux (Barton 2002 ; Bottoni et al. 2009): benzo (a) pyrene (Liu et al. 2015), pH (Zhou et al. 2015), cadmium (Choi et al. 2008), surdensité (Ni et al. 2014), hypoxie (Mohindra et al. 2015) et salinité (Deane and Woo 2004 ; Peng et al. 2015). D'après la litérature, HSP90 est une des HSPs la plus inductible en réponse à un stress de salinité. Comme attendu, l'expression de ces deux gènes est significativement augmentée à 24 °C *vs* 18 °C en EM et en ED. Si l'on s'intéresse à l'effet de la baisse de salinité sur les niveaux d'expression de *hsp90*, les niveaux de transcrits ont tendance à être plus bas (significativement pour *hsp90b1* à 24 °C) et moins variables après transfert en ED. Cette observation est cohérente avec celle de Peng et al. (2016) qui ont également observé, chez l'esturgeon, des niveaux d'expression de HSP90 plus faible en ED par rapport à l'EM dans différents tissus, notamment la branchie. Cela suggère que HSP90 pourrait aussi jouer un rôle différent entre l'EM et l'ED, mais cela mériterait d'être d'avantage étudié. Globalement, les études dans lesquels les poissons ont été soumis séparément à des stress thermiques et des stress salins mettent en évidence que le stress thermique a plus d'influence que le stress salin sur les niveaux de transcrit d'*hsp90*.

General conclusion

General conclusion

The current work on European sea bass has been conducted at different integration levels, from the molecule to the whole-organism. We have demonstrated that increased temperature affects hydromineral balance, gill morphology, protein activity and mRNA levels of several genes involved in ion transport. We have shown that temperature affects these traits differently in fish acclimated to seawater or transferred to freshwater. From this thesis the following main general conclusion can be drawn:

- Our histology analysis of sea bass gill demonstrates a valuable, quantitative determination for assessing branchial/osmoregulatory response to a combined stress and for identifying how gill remodeling might occur with regard to different abiotic parameters. Salinity transfer as well as higher temperature affected the gill morphology of sea bass. Temperature as well as salinity impacts water oxygen concentration. Thus it can be concluded that the alteration in the gill morphological and molecular traits observed in this study have been caused either by ion homeostasis or increased oxygen consumption or by a combination of these two factors.

- Blood osmotic pressure measured in sea bass have proven to be useful in assessing osmoregulatory capacity, but did not inform about ion balance. Salinity transfer can affect ion balance without affecting blood osmolality. Our first results have shown that in warm conditions, main plasma ion levels showed an imbalance in the plasma Na^+/Cl^- ratio suggesting blood acidosis in warm conditions that was more pronounced in FW. Simultaneous salinity and temperature changes could lead to elevated physiological stress, which seems to be the case in our study as chaperone proteins are activated. The fishes used in our experiment had a similar age/size than fishes that can be found in lagoons and estuaries in spring end summer. In their natural habitat, sea bass are likely exposed to higher temperatures in some of the Mediterranean lagoons combined with salinity fluctuations, and their gills might undergo physiological stress. Overexpression of ion transport at the transcript level could be a good indication of increased metabolism in warm conditions which could affect overall energy allocation in fish. Consequent energy shortage may impair growth, reproduction and behavioral traits. Physiological stressors resulting from changing abiotic parameters may

subsequently reduce fitness and lead to relocation to preferred areas within the habitats. In both of these situations, population dynamic may negatively be affected. The shifts in distribution could have negative effects on sea bass fishery with economical consequences.

- Changing environments scenarios on ocean warming predict decreases in ocean pH because of elevated CO_2. The gill is a key target as this multifunctional organ is involved in acid-base regulation as well as respiration and overall transepithelial transport. Estuaries and lagoons are also vulnerable to changing oxygen levels and pollution. The combination of all these environmental and anthropogenic factors will affect fish populations with the gill as a key target due to its direct contact with the water. Further studies on this organ should be considered in future. However, it is important to note that biological responses to climate change will be determined by linking ecological and physiological whole-organism phenomena. Findings of my research establish data on sea bass plastic responses to overcome stressors they may encounter when exposed to environmental parameters in their habitat. However, it is important to build more knowledge on the physiology, ecology and evolution of sea bass populations. Other responses of fish related to climate change could include movements towards more favorable habitats which would lead to a change in fish population dynamics. For this, we should investigate sea bass in their habitats or in more environmental-relevant mesocosms. Here we need long term rearing experiments related to the plasticity and/or adaptability of life-history traits are now crucial to study. In such studies, a clear challenge will be to distinguish to what extent phenotypic traits can shape plasticity.

Future perspectives

Future perspectives

There is a consensus among fish physiologists that the fish gill is an essential organ in multiple physiological functions and notably through its direct contact with the environment. Knowledge on osmoregulatory processes following combined stress (salinity and temperature) is still scarce in sea bass and demands to be explored beyond this thesis. We have shown that temperature increase induces significant changes on morphological, physiological, biochemical and molecular traits. This strengthens the need to further explore phenotypic traits at different temperature regimes.

In this regard, future work on physiological traits could include the investigation of additional species from different habitats, for example gilthead sea bream that is widely farmed and it is also frequent in lagoons and estuarine habitats.

Previous studies have shown a tight link between osmoregulation and acid-base regulatory processes (Michael et al. 2016b; Hu et al. 2016) and ammonia excretion over ranges of temperatures, pH and salinity (Al-Zaidan et al., 2013; Avella and Bornancicn, 1989; Bower and Bidwell, 1978; Cameron, 1978; Shih et al., 2012) that should be analyzed in sea bass. The current study shows some evidence that there were acid secretion mechanisms activated to balance potential blood acidosis at higher temperatures. However, the interaction between osmoregulation, acid-base balance and ammonia excretion has to be more thoroughly studied. Exposure to different water pH or different ammonia levels at two different temperatures would help us to elucidate environmental effects on sea bass physiological mechanisms. Future studies could focus on the effects of elevated respiratory acidosis or metabolic acidosis on key ion transporters (NKCC1, ClC-3, VHA, HCO_3^-/Na^+ contransporters, HCO_3^-/Cl^- exchanger, NHE3) involved in osmoregulation and acid-base regulatory mechanisms and temperature in gills of sea bass. For example, in sea bass dissolved water oxygen level did not change the acid-base status exposed to environmental hyperoxia (Cecchini and Caputo, 2003).

We have analyzed two branchial Rhesus (Rh) (Rhbg and Rhcg1) glycoproteins mRNA level. The focus of future study therefore should evaluate other Rh proteins for example Rhag and Rhcg2 as being explored in pufferfish *Takifugu rubripes* (Nakada et al. 2007b) and analyzed by several other authors (Hung et al. 2007; Nawata et al. 2007; Wu et al. 2010). Rh proteins are critical proteins for their ability to which can interact with VHA and NHE2/3 and

influence Na^+ uptake (Nawata et al., 2010). Results of this study showed a higher expression of *rhcg1* which would be an indication of increased metabolism. Although the role of Rh proteins is not yet fully elucidated, identifying the localization and protein expressions will allow us to better understand the temperature-dependent nature of these proteins. Also, the kidney proximal tubules might also be involved in nitrogen excretion in fish and therefore the role of kidney in nitrogen excretion should not be overlooked (Fehsenfeld and Wood, 2018).

Image analysis through histology was in the present work limited to one tissue, the gills, and limited to Masson's trichrome, PAS staining and NKA immunolabeling. Although the choice of this tissue was relevant to assess the main functions of the gill, we still lack information on the effect of temperature on metabolism-related processes at the gill and liver. Carbohydrate metabolism plays a critical role in the energy supply for ionoregulation and in this regard the liver is the major source supplying carbohydrate metabolites to osmoregulatory organs (Tseng and Hwang, 2008). Some studies have shown effects on ion homeostasis mainly because of energy shortage (Bucking and Wood, 2006; Shrivastava et al., 2017; Uliano et al., 2010). Future studies could analyze in sea bass energy shortage effects on ion homeostasis. Additionally, oxygen consumption should also be considered in such studies because it is widely used as a primary indicator of metabolic activity.

In future studies, investigations on kidney or intestine in *D. labrax* should allow to better understand whole animal osmoregulatory processes following temperature increase. Also, a comparative study of NKA or V-type H^+-ATPase activity combined to gene expression of ion transporters at different temperatures could be analyzed in kidney and intestine.

Impact of increased temperatures on the distribution and abundance of different branchial cell types is not yet clearly understood in *D. labrax*. The use of co-immunolocalization of different transporters using specific antibodies combined to different fluorochromes could help to identify specific cell populations according to their protein content and would contribute to a better understanding of gill ion transport functions as being explored in other species (Dymowska et al., 2012).

Cellular localization for specific ion transporters would provide a powerful tool to elucidate the exact function of each of these transporters. For example, V-type H^+-ATPase is an essential pump whose localization within the gill ionocyte can be either apical or basal. An apical localization would indicate H^+ excretion from the cell to the water whereas a basal localization indicates a transport of H^+ from the cell to the blood. In our study, it is still not

clear if increased *vha-a* expression in warm temperature leads to increased H^+ excretion or increased H^+ absorption to the blood.

Another interesting parameter can also be analyzed at cellular level as temperature modulated pavement cells (PVCs) microridges. This raises the question if increased temperature changes membrane fluidity. If so, it may change intramembrane protein mobility. Changes of the fluidity of biological membranes can affect their integrity which might compromises the function of membrane proteins (ion transporters), which can affect trans-epithelial ion and organic solutes fluxes (Moyes and Ballantyne, 2011). The less developed microridges in PVCs of warm-acclimated fish gills should be investigated in future studies, notably regarding membrane fluidity.

Previous studies have shown that teleost species exhibit interspecific and intraspecific variations regarding osmoregulatory and acid-base regulatory capacities (Berdan and Fuller, 2012; Fangue et al., 2006; Scott and Schulte, 2005). This variation in the responses among fish could be linked to differential gene expression pathways and should be investigated in future studies. It would help to understand the mechanisms involved in temperature acclimation by comparing different sea bass population from Atlantic and Mediterranean Sea. Future studies might show that molecular or physiological differences exist regarding acid-base regulatory mechanism.

The time course of the sampling could also be optimized by increasing the sampling frequency, notably following short-term transfer, and extending the duration of the study. For example, it would be nice to see the gill morphology in sea bass challenged from FW to SW. Such investigation would further highlight if the observed phenomenon is reversible and how increased temperature would affect gill plasticity following a FW-SW challenge.

List of publications

and

international oral presentations

List of publications and international oral presentations

Published paper

Masroor, W., Farcy, E., Gros, R., Lorin-Nebel, C., 2018. Effect of combined stress (salinity and temperature) in European sea bass *Dicentrarchus labrax* osmoregulatory processes. **Comparative Biochemistry and Physiology Part A**: Molecular & Integrative Physiology 215, 45–54. https://doi.org/10.1016/j.cbpa.2017.10.019

Submitted paper

Masroor, W., Farcy, E., Blondeau-Bidet, E., Lorin-Nebel, C. Effect of salinity and temperature on the expression of genes involved in gill ion transport processes in European sea bass *Dicentrarchus labrax*. Submitted in **Journal of Thermal Biology** (02/2019).

Paper in preparation

Masroor, W., Lorin-Nebel, C., Hermet, S., Farcy, E. Temperature and salinity stress affect gill mucus production in the European sea bass *Dicentrarchus labrax*. (In prep)

Oral presentation

Lorin-Nebel C*., Blondeau-Bidet E., **Masroor W.**, Maugras G. How do European sea bass Dicentrarchus labrax cope with freshwater environments? Society of Experimental Biology (SEB), Gothenborg, Sweden, 3 – 6 July 2017.

Masroor W*, Farcy E., Lorin-Nebel C. How does European sea bass cope with salinity and temperature changes at the gill level? Society of Experimental Biology (SEB), Florence, Italy 3 – 6 July 2018.

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Coping with salinity and temperature changes: a focus on the gill response in European sea bass *Dicentrarchus labrax*

The European sea bass *Dicentrarchus labrax* undertakes seasonal migrations to estuaries and lagoons that are characterized by fluctuations in environmental conditions. It is unclear to what extent salinity acclimation mechanisms are affected at temperatures higher than in the sea, as usually encountered in transitional waters in spring and summer. In this study, juvenile sea bass were pre-acclimated to seawater (SW) at 18 °C (temperate) or 24 °C (warm) for two weeks and then transferred to either fresh water (FW) or SW at the considered temperatures. We have shown that sea bass are able to efficiently maintain blood osmolality at 24 °C at both salinities. However, temperature increase induced significant changes regarding several physiological traits related to osmoregulation, acid-base regulation, ammonia excretion and mucus production. This study showed that thermal acclimation at 24 °C affects gill morphology through gill remodeling and whole-organism ion balance. Plasma Na^+ levels seemed to be particularly affected leading to decreased plasma Na^+/Cl^- ratio in warm conditions, suggesting a blood acidosis. Following FW transfer, the major effects observed were a lower increase in the density of branchial ionocytes and in Na^+/K^+-ATPase activity at 24 °C compared to 18 °C. Moreover, several key ion transporters involved in ion uptake were not transcriptionally induced following FW transfer at 24 °C. These data suggest a less effective capacity to switch from hypo-to hyper-osmoregulation after FW transfer when fish are exposed to higher temperature. At the molecular level, the temperature increase affected the expression of several branchial transporters, notably in SW. At 24 °C, higher expression of transporters linked to H^+ transport might be linked to an acid-base imbalance. Moreover, the induction of ammonia-transporting channels might also indicate increased need for nitrogen excretion, potentially due to enhanced metabolism in warm conditions. Molecular chaperones HSP90 expression was strongly affected by temperature increase and to a lesser extent by salinity decrease. Finally, gill goblet cells analysis suggested that mucus production is increased in response to thermal stress. This study and additional functional studies investigating different temperature regimes provide insights on the effect of increased temperatures on fish responses and may help to predict how teleost will face the consequences of global change.

Key words: Ecophysiology, temperature, salinity, *Dicentrarchus labrax*, gills, ionocytes, ion transport, mucus, gene expression

S'acclimater à des salinités et températures changeantes : un focus sur la branchie chez le loup européen *Dicentrarchus labrax*

Le loup ou bar européen effectue une partie de son cycle de vie dans les lagunes et estuaires, des eaux de transitions caractérisées par des fluctuations environnementales plus marquées qu'en milieu marin. Les mécanismes d'acclimatation à la salinité à des températures plus élevées qu'en milieu marin sont encore relativement inexplorés. Dans cette étude, des juvéniles de loup ont été préacclimatés pendant deux semaines à l'eau de mer (EM) à 18 °C (eau tempérée) ou à 24 °C (eau chaude) puis transférés soit dans l'eau douce (ED) soit dans l'EM aux deux températures testées. À 24 °C, les loups parviennent à maintenir leur pression osmotique sanguine relativement constante, quelle que soit la salinité testée. En revanche, la hausse de température affecte significativement différents traits physiologiques liés à l'osmorégulation, la régulation acido- basique, l'excrétion azotée et la production de mucus. Cette étude a notamment montré qu'une acclimatation thermique à 24 °C modifie la structure morphologique de la branchie et induit un déséquilibre de la balance ionique sanguine. Ce déséquilibre concerne particulièrement les ions Na^+ et a pour conséquence une diminution du ratio Na^+/Cl^- plasmatique, pouvant être symptomatique d'une acidose sanguine. Après transfert en douce, une augmentation moins importante de la densité des ionocytes branchiaux et de l'activité de la pompe Na^+/K^+ ATPase a été observée à 24 °C comparé à 18 °C. De plus, certains transporteurs ioniques jouant un rôle clé dans l'absorption ionique en eau douce n'ont pas été induits au niveau transcriptionnel à 24 °C. Cela suggère une capacité réduite des poissons de passer d'un épithélium branchial hypo-osmorégulateur vers un épithélium hyper-osmorégulateur lors d'un transfert en eau douce à 24 °C *vs* 18 °C. Au niveau moléculaire, la hausse de température a affecté l'expression de plusieurs transporteurs ioniques branchiaux, notamment en EM. À 24 °C, la surexpression de transporteurs liés au transport de proton H^+ pourrait être le signe d'un déséquilibre acido- basique. De plus, l'induction de transporteurs d'ammonium pourrait indiquer un besoin accru d'excréter de l'azote par voie branchiale, potentiellement lié à un métabolisme plus élevé en eau chaude. L'expression des gènes codant pour la protéine chaperonne HSP90 a été fortement affectée par la hausse de température et dans une moindre mesure par la dessalure. Pour finir, l'analyse des mucocytes suggère que la production de mucus pourrait être induite en réponse au stress thermique. Ce type d'étude fonctionnelle sur l'acclimatation à différents régimes de température permet d'apporter des éléments de compréhension pour pouvoir prédire les réponses des téléostéens face aux conséquences du changement global.

Mots-clés : Écophysiologie, température, salinité, *Dicentrarchus labrax,* branchies, ionocytes, transport ionique, mucus, expression de gènes