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LIST OF ABBREVIATIONS

ADP	Adenosine Diphosphate
AOX	Alternative Oxidase
ATP	Adenosine Triphosphate
BN-PAGE	Blue Native-Polyacrylamide Gel Electrophoresis
CCCP	Carbonyl Cyanide m-Chlorophenyl Hydrazine
CI	Complex I, NADH dehydrogenase
CII	Complex II, Succinate dehydrogenase
CIII	Complex III, ubiquinone-cytochrome c reductase
CIV	Complex IV, Cytochrome c oxidase
COX	Cytochrome c Oxidase
CS	Citrate Synthase
CV	Complex V, ATP synthase
Cyt c	Cytochrome c
DDM	n-dodecyl-β-D-maltoside
DHA	Docosahexaenoic Acid
DMA	Dimethyl Acetals
EPA	Eicosapentaenoic Acid
ETS	Electron Transport System
FA	Fatty Acid
FAD	Flavin Adenine Dinucleotide (oxidized from)
FADH2	Flavin Adenine Dinucleotide + 2Hydrogen (reduced form)
FCCP	Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
FCR	Flux Control Ratio
GC-FID	Gas Chromatography - Flame Ionization Detection
GPDH	Glycerol-3-Phosphate Dehydrogenase
GSH	Glutathione
H2O2	Hydrogen peroxide
HHE	Hydroxyhexenal
HNE	4-hydroxynonenal
IMM	Inner Mitochondrial Membrane
IMS	Intermembrane Space (mitochondrial)
KCN	Potassium cyanide
MCA	Metabolic Control Analysis
MDA	Malondialdehyde
MFRTA	Mitochondrial Free Radical Theory of Aging
MLSP	Maximum Lifespan
MOSTA	Mitochondrial Oxidative Stress Theory of Aging
MRD	Metabolic Rate Depression
MRL	Maximal Reported Longevity
MSR	Mass-Specific metabolic Rate

mtDNA	Mitochondrial DNA
mTOR	Mammalian Target Of Rapamycin
mtROSp	Mitochondrial Reactive Oxygen Species production
MUFA	Monounsaturated Fatty Acid
NAD+	Nicotinamide Adenine Dinucleotide (oxidized form)
NADH	Nicotinamide Adenine Dinucleotide + Hydrogen (reduced form)
NADP+	Nicotinamide Adenine Dinucleotide Phosphate (oxidized form)
NADPH	Nicotinamide Adenine Dinucleotide Phosphate + Hydrogen (reduced form)
NMI	Non-Methylene-Interrupted fatty acid
OMM	Outer Mitochondrial Membrane
OXPHOS	Oxidative phosphorylation
PDH	Pyruvate Dehydrogenase
PI	Peroxidation Index
PUFA	Polyunsaturated Fatty Acid
RCR	Respiratory Control Ratio
RCS	Reactive Carbonyl Species
ROS	Reactive Oxygen Species
SC	Supercomplex
SFA	Saturated Fatty Acid
SML	Shared Maximum Longevity
SOD	Superoxide Dismutase
SUIT	Substrate-Uncoupler-Inhibitor-Titration
TCA	Tricarboxylic acid cycle
Trx	Thioredoxin
UPR	Unfolded Protein Response
UI	Unsaturation Index

RÉSUMÉ

La durée de vie des animaux varie grandement au sein du règne animal, et ce fait intrigue et captive les biologistes particulièrement depuis plusieurs décennies. Les mécanismes qui établissent et régissent la longévité sont pour la plupart méconnus, et maints efforts ont été mis en place pour mieux saisir ce qui explique le processus du vieillissement. Parmi les théories mécanistiques ayant le mieux résisté au passage du temps se situe la théorie du vieillissement par le stress oxydant mitochondrial (MOSTA de par son acronyme anglais). Celle-ci lie la détérioration progressive de cette organelle centrale à la production d'énergie de la cellule, à une augmentation non contrôlée du stress oxydant sous la forme de génération d'espèces réactives de l'oxygène, entraînant des dommages à la structure et à l'ADN mitochondriaux, entraînant le vieillissement cellulaire et de l'organisme. L'objectif global de cette thèse était de décortiquer les liens entre architecture, fonctionnement et ajustement de la respiration mitochondriale, et les différences de longévité trouvées au sein d'organismes modèles du vieillissement, les bivalves marins. Ces mollusques démontrent en effet une variabilité au niveau de la durée de vie atteinte maximale, tant au sein de leurs espèces, qu'entre celles-ci : de 28 à 507 ans de longévité maximale, cet âge exceptionnel étant atteint par l'animal le plus longévif connu à ce jour, le quahog nordique *Arctica islandica*. Le contexte et le cadre de travail théorique sont présentés dans le chapitre I de cette thèse, qui se veut une introduction générale au domaine et aux hypothèses et prédictions qui suivent dans les trois chapitres expérimentaux qui y font suite.

Dans le premier chapitre expérimental (chapitre II), nous avons questionné le lien entre la structure lipidique membranaire (conférant une résistance au stress oxydant), l'activité enzymatique des complexes du système de transport des électrons mitochondrial (ETS, acronyme anglais) et la longévité variable des populations Européennes d'*Arctica islandica*, à savoir si l'existence d'une relation entre ces paramètres correspondait aux prédictions de la MOSTA. Par l'emploi de techniques comprenant la chromatographie à phase gazeuse et détecteur à ionisation de flamme, ainsi que par de la spectrophotométrie dans les tissus et mitochondries isolées de spécimens obtenus par nos collaborateurs, nous avons démontré que ces paramètres ne variaient pas avec la longévité des populations, contrairement à ce qui est établi par le cadre théorique basé sur des analyses interspécifiques. Ainsi, toutes les populations possèdent une membrane robuste (démontré par leur faible indice de peroxydation lipidique), et aucune activité enzymatique de l'ETS ne semble liée à la longévité de celles-ci (Rodríguez et al., 2019). Par conséquent, avoir une membrane résistante au stress oxydant, ainsi qu'une plus faible activité des complexes générant les ROS ne garantissent pas à eux seuls d'atteindre la longévité maximale de l'espèce, et

l'influence d'autres facteurs tels que les conditions environnementales sont considérés et discutés.

Dans le deuxième chapitre expérimental (chapitre III), nous poussons plus loin les analyses précédentes publiées par Munro et Blier (2012) et Munro et al., 2013 par le biais de la respirométrie à haute résolution et l'analyse du contrôle métabolique pour évaluer si les différences de longévité entre espèces de bivalves sont liées à des changements dans la force de contrôle du flux d'électrons des différentes enzymes de l'ETS. La titration de la respiration mitochondriales à l'aide d'inhibiteurs spécifiques à chacune de ces enzymes chez quatre espèces de bivalves démontre ainsi que les voies d'entrée des électrons (les voies du NADH et du succinate) n'exercent presque aucun contrôle sur la respiration chez les espèces plus longévives, alors que le contraire est vrai au niveau du complexe IV de l'ETS, l'accepteur final des électrons (Rodríguez et al., 2020). Ces résultats (les premiers du genre dans une analyse comparative interspécifique) suggèrent que l'allègement du contrôle lors de l'entrée des électrons dans l'ETS, comme proposé dans différentes études précédentes, bénéficie l'atteinte d'une grande longévité, probablement via un plus faible taux de production de ROS. De plus, la somme des forces de contrôle exercées par chacune des enzymes de l'ETS dépasse la valeur théorique de 1 dans les systèmes linéaires, ce qui suggère la présence de supercomplexes, et fait le pont avec le chapitre suivant (chapitre IV).

Dans ce dernier chapitre expérimental, nous avons employé une technique d'électrophorèse sur gel de polyacrylamide (BN-PAGE) et de la spectrométrie de masse sur les mitochondries isolées des mêmes espèces, pour vérifier si l'ETS de celles-ci forme des agencements supramoléculaires appelés supercomplexes. En effet, plutôt que de former une chaîne tel qu'imaginé auparavant, il est maintenant démontré chez plusieurs espèces (mais pas pour toutes) que les complexes de l'ETS sont agencés en plusieurs copies formant des structures compactes et à poids moléculaire élevé, ce qui donnerait un avantage au niveau de la gestion de la respiration et du stress oxydant. Nous démontrons dans ce chapitre que ces agencements existent bien aussi chez les bivalves marins, mais que des différences importantes sont visibles entre espèces et suggèrent un lien entre la complexité des supercomplexes, leur robustesse et le vieillissement. Nous discutons en profondeur de ces résultats et de leurs implications dans la discussion de thèse correspondant au dernier chapitre (V).

ABSTRACT

The remarkable diversity in lifespan found among animals has captivated biologists for many decades, and considerable efforts have been put in place to decipher the intricacies of the aging process. Among the important mechanistic theories of aging, the mitochondrial oxidative stress theory of aging (MOSTA) is one that has arguably well stood the test of time. It posits that a progressive decay of this organelle's function leads to an uncontrolled increase in oxidative stress, with damaging effects on mitochondrial structure and DNA, ultimately promoting aging. In that vein, the global objective of this thesis was to dissect the links between mitochondrial architecture, function, and adjustment of respiration in relation to lifespan differences inside and between species of marine bivalves. These invertebrates are useful models for lifespan due to their wide inter- and intraspecific divergences in maximum lifespan (28 to 507 years old). In this thesis, we start by introducing the context and framework of the MOSTA (chapter I), then further pursue the aforementioned objective in three experimental chapters.

In the first experimental chapter (chapter II), we investigated whether membrane lipid structure and enzymatic activities of lifespan-diverging populations of the ocean quahog *Arctica islandica* aligned with the predictions of the MOSTA. Using gas chromatography coupled with flame ionization detection and spectrophotometric techniques on isolated mitochondria and whole-tissues in mantle and gills, we found that the result didn't follow the aforementioned predictions. In fact, all populations possessed a robust membrane (evidenced by their peroxidation index), and no enzymatic activities of the electron transport system (ETS) components showed any differences associated with population lifespan (Rodríguez et al., 2019). Reaching the species' maximum reported age therefore isn't guaranteed by robust mitochondrial membrane and protein structure and function, but seems to be linked to other parameters such as environmental conditions which are discussed in the published article and in the present chapter. The important roles of lipid metabolism and ETS enzyme activities in the aging phenotype are hereafter discussed, and despite the lack of correlations found in this thesis, we advocate for further research on these two subjects.

In the second experimental chapter (chapter III), we pushed the previous interspecies analysis further (Munro and Blier, 2012;Munro et al., 2013) using the approaches of high-resolution respirometry and metabolic control analysis to assess whether differences in lifespan were associated with changes in the control of respiration and electron flux by the different enzymes of the ETS. Using inhibitor titrations on the Oroboros O2k-respirometer (Oroboros Instruments, Innsbruck, Austria), we found that long-lived species were characterized by an almost null control at the NADH- and succinate-pathways of electron entry, while complex IV of the ETS exerted a significantly higher control than in shorter-lived species (Rodríguez et al.,

2020). It suggests that relieving control at the complexes associated with the entry of electrons into the ETS is beneficial and we suspect that this is linked with management of ROS, which should be studied going forward. These results are reminiscent of studies evidencing an increase in control by complex I (NADH-pathway) with increasing longevity inside species, and we demonstrate this link on a comparative basis on four species of bivalves ranging from 28 to 507 years MLSP, for the first time. Complex IV also appeared as an important selection site for long lifespan and related control of electron flux, which we discuss further hereafter. The sum of the various flux control coefficients from the ETS complexes was found to be superior to the theoretical value of 1 in linear systems; hence we proposed and tested for the presence of supramolecular assemblies – supercomplexes in the follow-up experiment (chapter IV).

In this final experimental chapter, we used the technique on blue-native polyacrylamide gel electrophoresis (BN-PAGE) on isolated mitochondria of the same four species and compared the resulting banding patterns, in-gel activities and mass-spectrometry profiles to assess the presence and complexity of supercomplexes. We found that marine bivalves do exhibit supercomplexes assemblies; this is to our knowledge the first report in a marine invertebrate model of aging. There were commonalities in banding patterns with a "core" supercomplex band containing assemblies of complexes I and IV, while it appeared that longer-lived species possessed more complex assemblies with various bands exhibiting arrays of complexes I, III, IV and V in different combinations. This potential association between complexity and lifespan needs to be investigated further and may be linked to a relationship between robustness, lipid environment surrounding these assemblies and longevity. The results exposed in this thesis and their overarching implications are discussed in the final chapter of this dissertation (chapter V).

CHAPTER I
GENERAL INTRODUCTION

1.1 Why do we age?

Aging is the process of becoming senescent, and understanding its roots and causes has captivated Humanity for millennia. It is defined as a progressive decay of physiological function, along with an increase in degenerative diseases, ending in death (Austad, 2005). It is an endogenous, progressive, irreversible and deleterious age-dependent increase in mortality that is especially manifest in post-mitotic cells (Barja, 2013). Longevity as an indicator of the rate of aging varies more than 10 000-fold among metazoans: from at most two days in mayflies (insects of the order Ephemeroptera), to at least 507 years in the bivalve *Arctica islandica* (the ocean quahog), the longest-lived non-colonial animal on Earth (Carey, 2002;Austad, 2005;Butler et al., 2013).

What are the mechanistic explanations for such a wide range in lifespan among animals? We know that aging is broadly distributed among phylogenetic lineages, but some species do not show age-associated increase in mortality or decline in fertility: hence, it is not completely universal (Kirkwood and Austad, 2000). As we deepen our knowledge as to why animal age in a certain fashion (or don't age at all), many questions emerge and the complexity of the problem calls for more research on the subject. Many theories have been put forward over decades of research, attempting to answer why aging is not opposed by natural selection, and how aging occurs mechanistically.

1.1.1 The evolutionary theories of aging

How can we make sense of aging evolutionarily? Why does evolution not prevent aging, considering that natural selection should cause the evolution of increased fitness; hence should favour organisms that have optimal survival and reproductive success?

Scientists have attempted to solve this apparent paradox for decades. The idea that senescence is programmed to limit population size or increase the turnover of generations (by ensuring the survival of the youthful and reproductively productive individuals) stood until well into the 20th century. However, subsequent evidence showed that at given equivalent reproductive output, longer-lived individuals have more offspring than shorter-lived ones; and a species' benefit of an individual's death is largely inferior to the individual cost, therefore selection would not favour such a mechanism (Fabian and Flatt, 2011).

By the middle of the 20th century, another explanatory framework was developed, largely based on the works of evolutionary biologists J.B.S. Haldane, Peter Medawar and George C. Williams. The emerging explanation for the existence of senescence shifted to individual, rather than group fitness and selection. The main tenet being that the force of natural selection becomes inefficient at maintaining function at old age, a hypothesis which evolved into various specific theories and is now supported by empirical data (Charlesworth, 2000). Three classic evolutionary theories of aging continue to be refined and debated to this day (reviewed in Hughes and Reynolds, 2005; Maklakov et al., 2015 Maklakov and Immler, 2016). Medawar's (1952) "mutation accumulation" theory, explains aging by the decline in natural selection with age: this allows for alleles with late deleterious effects to accumulate with little to no selection. Therefore, a trait causing a fatal effect only after an animal had viable progeny would have a relatively weak effect on fitness, a description which aging seemed to fit. Recent transcriptomic evidence from multiple mammalian species and tissues shows that genes highly expressed in old adults are under weaker purifying selection than those highly expressed in young adults (Turan et al., 2019). Medawar also suggested that alleles having early favourable effects would be preferentially selected, despite their negative effects at a later age; a concept that was further developed in Williams' (1957) "antagonistic pleiotropy" theory of aging. It therefore evokes a life-history trade-off, where a small beneficial effect early in life can outweigh a late deleterious effect, even

if it results in senescence and death of the individual. Evidence for antagonistic pleiotropy can be found for example in the negative correlation between the rate of aging and age at maturity, whereby species who mature at a later age also have a longer lifespan (Ricklefs, 2010). The third classic evolutionary theory of aging is the "disposable soma" theory (Kirkwood, 1977), which is close in many aspects to the antagonistic pleiotropy theory, but additionally takes a physiological approach to the aging question by its emphasis on the acquisition of resources by all organisms. It proposes a trade-off between somatic maintenance and reproduction in terms of allocation of metabolic resources. Indeed, an animal will benefit from investing in reproduction when resources are rare, rather than in repair mechanisms, even if this strategy is detrimental later as damage accumulates and causes aging. The disposable soma theory therefore predicts that longer-lived species invest more into somatic maintenance, which has empirical support (see for example Kapahi et al., 1999).

These classical theories of aging have paved the way for many subsequent scientific questioning and tests which have revealed their strengths and weaknesses. For example, the mutation accumulation and antagonistic pleiotropy theories were largely based on the thought that animals in the wild do not die of old age, hence natural selection could only exert limited influence over the process. However, evidence now shows that senescence in the wild is actually widespread (see Nussey et al., 2013). Some seemingly antagonistic pleiotropic genes have been found, but studies on mutant flies and worms have shown increased lifespan without any fitness costs (reviewed in Flatt and Promislow, 2007). The disposable soma theory comes in apparent contradiction with the fact that caloric restriction (not increased caloric intake) is a successful intervention to extend lifespan in a wide variety of species (Mitteldorf, 2001). In addition, another question arose following some pioneering genetic studies starting in the 1990, and remains under strong debate: is aging a programmed phenomenon? The classical theories define aging as a non-adaptive phenomenon resulting in the decline of the strength of natural selection, hence not a genetic program.

Yet, genetic studies in yeast, worms, flies and mice have uncovered common genetic pathways of lifespan extension through mutations that switch the organism into starvation-like phases; this suggests a genetic program that controls the level of protection against damage, or one that promotes ageing for the benefit of the group or closely-related individuals (see Longo et al., 2005 for a review of the "programmed and altruistic ageing theory"). Despite this genetic evidence, others argue that instead of being programmed, aging is the continuation of programmed pathways that are essential for development, growth and survival, such as the insulin or the mammalian target of rapamycin (mTOR) pathways. Aging is, as figuratively put by Blagosklonny (2013), a "shadow" of actual genetic programs: natural selection cannot eliminate this shadow, but selects the beneficial growth and development components of the genetic pathways.

The classical evolutionary theories of aging have generated important advances in the aging field throughout the years. However, they have also generated hundreds of new theories, pointing to flaws and unanswered questions (and prompting the need for a classification system, see Trindade et al. 2013). Therefore, a unified evolutionary theory of aging has yet to emerge. Moreover, these theories focus on the fundamental existence of the phenomenon, but aside from a few exceptions (such as the disposable soma theory), they do not hint at the proximate, mechanistic basis of aging. This subject has also triggered a vast amount of research in the last century, and mounting evidence points towards the involvement of oxygen, respiration and the functioning of the cell's powerhouse: the mitochondrion (Wallace, 2005).

1.1.2 The mechanistic basis of aging

The accumulation of cellular damage with time is at the very basis of the aging phenotype. The principal common denominators that manifest during aging, that accelerate senescence when aggravated, and that delay it when experimentally ameliorated have been put together in a comprehensive review by López-Otín and

colleagues (2013). They all share varying degrees of connectedness, hence experimental modifications to one of them can impact positively or negatively the others. These hallmarks of aging include genomic instability (mutation accumulation in nuclear and mitochondrial DNA, defects in nuclear architecture and in DNA repair; e.g. Kazak et al. 2012), telomere attrition (deterioration as a consequence of DNA damage accumulation with age; e.g. Blasco et al. 1997), epigenetic alterations (changes in DNA methylation patterns, post-translational modification of histones, chromatin alterations; e.g. Han and Brunet, 2012), loss of proteostasis (stability of the proteomes; see Koga et al., 2011), and deregulated nutrient sensing (involving the insulin-like growth factor 1 and the mechanistic target of rapamycin pathways, among others; reviewed in Kenyon, 2010). The hallmarks of aging also encompass cellular senescence (accumulation of senescent cells, reflecting their increase of a decrease in the rate of clearance; reviewed by Collado et al., 2007), stem cell exhaustion (decrease in regeneration potential of tissues; see Rossi et al., 2007), and altered intercellular communication (among which an increase in inflammatory responses; e.g. Zhang et al., 2013). All these processes occur during aging and are interconnected; some can be classified as being initiating the aging process ("primary hallmarks" such as genomic instability or loss of proteostasis), while others are initially beneficial but accumulate and become negative ("antagonistic hallmarks, e.g. nutrient sensing) or arise when damage caused by the other two categories cannot be repaired ("integrative hallmarks" such as stem cell exhaustion).

Understanding the relative contributions of these distinguishing features to the aging process and developing experimental interventions to improve healthy lifespan (healthspan) are major challenges of current and future research in the field (López-Otín et al., 2013). Another major hallmark discussed in the aforementioned review is the underlying theme of this thesis and has received much attention in over the past 60 years: the role played by mitochondrial dysfunction in the aging phenotype.

As early as the 4th century B.C., ancient Greek philosopher Aristotle proposed that death was caused by the destruction of "vital heat". In his view, the length of life in animals and plants was linked to the maintenance of heat and moisture, both of which dependent on the organism's nature and could be modulated to a certain extent by its environment (Woodcox, 2018). His idea is reminiscent of more contemporary theories that have been put forward to explain differences in rates of aging among species. Indeed, 18th century French naturalist Buffon linked total duration of life to the rate of growth (reviewed in Lints, 1989). In the early 20th century, Max Rubner noticed that larger animals had longer lifespans that smaller ones, and pioneer of biogerontology Raymond Pearl built on this observation to formulate the "rate of living" theory of aging (Pearl, 1928). In it, he proposes an inverse relationship between the rate of oxygen consumption and lifespan, where smaller species have higher mass-specific metabolic rates, and are shorter-lived, than larger species. This theory suffered considerable criticism, firstly due to an arguably unclear formulation of the hypothesis by Pearl, itself giving rise to further misinterpretations by researchers in the field (see Lints, 1989). Pearl proposed that lifespan was contingent on the individual's "vitality" or metabolic potential, determined by inheritance, and on its lifelong average rate of energy expenditure (Sohal and Orr, 2012). This would render a strict test of the hypothesis difficult, as individuals that are genetically identical would need to be exposed to conditions that only modify the rate of energy expenditure to assess whether their lifespans are modified, without considering the myriad of other physiological parameters that could be affected. Secondly and crucially, empirical evidence very often goes against the predictions of the "rate of living" theory. For example, birds and bats are long-lived despite their low body masses and high metabolic rates (thought to be linked to the reduced mortality rates associated with flight and efficient management of oxidative stress – see Munshi-South and Wilkinson, 2010), fruit flies show no relationship between resting metabolic rates and longevity (Van Voorhies et al., 2004), and dogs show the opposite relationship, smaller breeds being longer-lived than bigger ones (for a complete review see Glazier, 2015).

Among the many theories of aging that have been proposed over the years by biologists and more than half a century after its inception, a refined version of the Free-Radical Theory of Aging (FRTA, Harman, 1956) remains highly discussed and challenged (Sanz and Stefanatos, 2008;Barja, 2013;Stuart et al., 2014). In the original theory, Harman proposes that endogenous reactions with molecular oxygen produce free radicals that attack cell constituents and can explain aging and its associated diseases. Support for the theory was difficult to obtain at first, but luck shifted with the discovery of superoxide dismutase (SOD) by McCord and Fridovich (1969), and even more so with the demonstration of mitochondrial hydrogen peroxide (H_2O_2) formation by Chance et al. (1979). This gave an impulse to the theory that would then be known as the Mitochondrial Free Radical Theory of Aging (MFRTA, Harman, 1972). The mitochondrion is thus viewed as the biological clock of animals, and maximum longevity believed to be a result of the clock's functioning and resistance to the accumulation damage caused by free radicals generated as by-products during normal metabolism. The nature of these "free radicals" and how they are produced will be explored next. The MFRTA was later refined and expanded to encompass all aspects of oxidative stress in the Mitochondrial Oxidative Stress Theory of Aging (MOSTA, reviewed in Blier et al., 2017).

1.2 The mitochondrial oxidative stress theory of aging (MOSTA)

1.2.1 Mitochondrial respiration and oxidative phosphorylation

The role of oxidative stress is at the core of the MOSTA, and is believed to be a by-product of oxidative phosphorylation (OXPHOS). Just four years after the discovery of SOD, Chance's group demonstrated that even during normal mitochondrial respiration, the incomplete reduction of oxygen triggers the formation of highly reactive and unstable molecules termed "reactive oxygen species" (ROS). In addition to their endogenous (intracellular) origin, they can also result from exogenous factors such as pollutants, xenobiotics, or radiation (Lu and Gong, 2009). Their

intracellular origin is of particular interest in the context of aging, as mitochondria are thought to be the main producers of intracellular ROS during the process of OXPHOS (reviewed in Holmstrom and Finkel, 2014). Another important source of ROS production are NADPH oxidase (Nox) isozymes, which are plasma membrane-bound (facing the extracellular environment) with associated cytosolic components (Sahoo et al., 2016). Nox participate mainly in host defence, for example by regulating intracellular ROS levels in T cells (Chen et al., 2016), but they also play major roles in cellular signalling and regulation of gene expression (Panday et al., 2015). Other ROS sources include the cytochrome P450 superfamily of enzymes which catalyse the oxidation of various organic molecules (lipids, steroids) and xenobiotic compounds, and are located on the endoplasmic reticulum and the inner mitochondrial membranes (Bae et al., 2011); while the enzyme xanthine oxidase is an important extracellular ROS producer, for example in the pulmonary arteries (Hartney et al., 2011).

Sources and sinks of H_2O_2 are multiple, vary according to cellular and subcellular localization or cell type and are reviewed in more detail in Sies et al. (2017). Although the exact contribution of each ROS producing system in specific tissues and conditions remains to be fully elucidated (Brand, 2010), most studies on oxidative stress in aging remain centered on mitochondrial ROS production (mtROSp). Mitochondria are hubs of intermediary metabolism, and mtROS have recently been shown to play important roles in signalling pathways (Hamanaka and Chandel, 2010;Dan Dunn et al., 2015). To better understand how mtROS are implicated in senescence, a fundamental knowledge of the mitochondrial function, OXPHOS and mtROSp is necessary.

Nutrients feeding electrons to power OXPHOS (carbohydrates, lipids and proteins) are broken down in the cytosol (glycolysis, lipolysis and proteolysis), and enter the mitochondrial matrix thanks to various shuttles and transporters. Once inside the matrix, they are oxidized into acetyl-CoA with the help of coenzyme A or β-oxidation (fatty acids). Acetyl-CoA then enters the tricarboxylic acid cycle (TCA), where it is oxidized and forms the crucial reducing equivalents nicotinamide adenine

dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$, which is also formed directly by β-oxidation), along with some adenosine triphosphate (ATP), and carbon dioxide as a waste by-product. The electron acceptors NAD^+ and FAD are regenerated during OXPHOS by the removal of electrons from NADH and $FADH_2$ (hence they are oxidized) and their subsequent transfer to the enzymes of the electron transport system (ETS). Located within the inner mitochondrial membrane (IMM), the ETS creates an electrochemical gradient that is used to drive ATP synthesis and energy-dependent transport processes (see figure 2 in Gnaiger et al., 2019).

Electron transport is achieved through the action of four enzymatic complexes (complexes I, II, III and IV), two electron carriers (ubiquinone or coenzyme Q and cytochrome *c*), and the mitochondrial F_1F_0ATPase (complex V or ATP synthase). Complex I (CI, NADH dehydrogenase) collects electrons from NADH and transfers them to ubiquinone, while complex II (CII, succinate dehydrogenase) transfers electrons from succinate to FAD, forming $FADH_2$, which then feeds them to ubiquinone, which accumulation forms the so-called Q-pool at the Q-junction. Electron transport continues through the reduction of ubiquinone and transfer of its electrons to complex III (CIII, ubiquinone-cytochrome *c* reductase), cytochrome c (cyt *c*) and complex IV (CIV, cytochrome *c* oxidase). CIV accepts an electron from each of four cyt *c* and passes them on to one molecular oxygen molecule (O_2), converting it to two molecules of water (H_2O). Crucial to this mechanism is the fact that CI, CIII and CIV use the energy released from the oxidation of NADH to pump protons (H^+) out of the IMM, creating a buildup in the intermembrane space and an electrochemical gradient across the membrane. This protonmotive force is potential energy that is then used to drive ATP synthesis by complex V through phosphorylation of ADP (see figure 1.1). However, some energy gets lost in the formation of two by-products: heat and ROS (Moyes and Schulte, 2008).

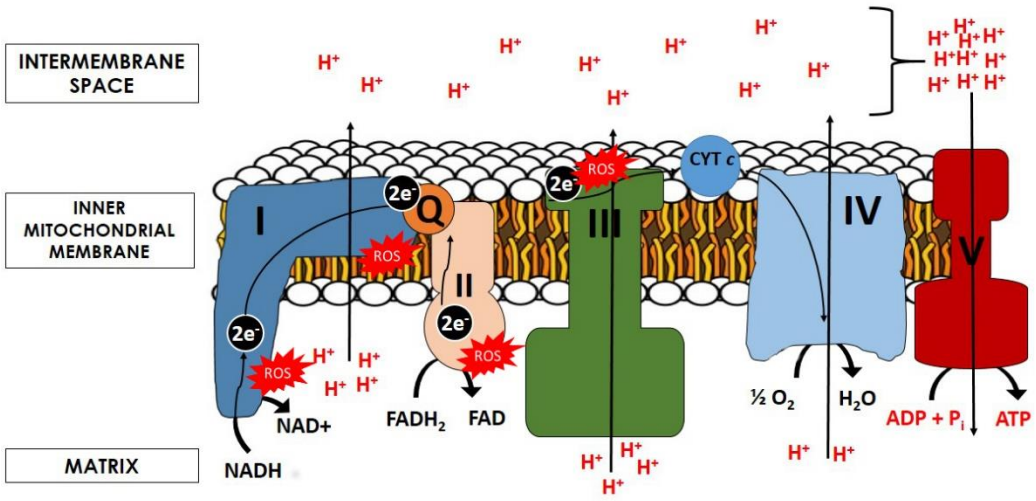

Figure 1.1 Schematic representation of the electron transport system (ETS) and the major ROS-producing sites in the mitochondrion.

OXPHOS and the functioning of the ETS complexes can be experimentally assessed using high-resolution respirometry, through the use of substrates, uncouplers and inhibitors titration protocols (SUIT) in a respiration medium. An overview of the main substrates, pathways and acronyms used in this thesis and generally in mitochondrial physiology is presented in figure 1.2. Glutamate (G) and malate (M) are some of the substrates used to stimulate respiration by feeding electrons through complex I (NADH-pathway). Malate is oxidized by malate dehydrogenase into oxaloacetate in the TCA cycle, generating reducing equivalents in the form of NADH. Oxaloacetate reacts with acetyl-CoA to form citrate, transformed in α-ketoglutarate by isocitrate dehydrogenase. Glutamate is oxidized in α-ketoglutarate by glutamate dehydrogenase, and NADH is also formed at the level of isocitrate dehydrogenase and α-ketoglutarate dehydrogenase to feed electrons to the ETS through complex I. The substrate succinate (S) is for its part oxidized in the TCA cycle, in a reaction catalyzed by succinate dehydrogenase (which is both a component of the ETS and the TCA cycle; succinate-pathway), as explained above. This reduces FAD into $FADH_2$, transferring

electrons to ubiquinone in the system. Both complexes I and II can be inhibited by the use of specific inhibitors: rotenone, which binds to the ubiquinone binding site of CI; and malonate, which binds to the active site of CII and thus competes with succinate. Another important enzyme for electron entry is the mitochondrial G3P dehydrogenase (mG3PDH), catalysing the oxidation of glycerol-3-phosphate (Gp) to dihydroxyacetone phosphate (DHAP) and transferring electrons directly to the Q-junction. This substrate is at the junction of glycolysis, OXPHOS and fatty acid metabolism, and has been relatively under-studied despite its importance in an increasing amount of models (McDonald et al., 2018). Once specific combinations of these substrates are added to the respiration medium in a respirometer together with mitochondrial isolates, permeabilized or homogenized tissue or live cells, the LEAK state (L) is reached (analogous to state 4 in the classic terminology), and addition of saturating ADP allows transition to the OXPHOS state (P, analogous to state 3). Next, carbonyl cyanide m-chlorophenyl hydrazine (CCCP) or carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) are protonophores used as uncoupler of OXPHOS to estimate the maximal activity of the ETS, not limited by ATP synthase (ET-state, E; previously state 3u). The next ETS enzyme, complex III, can be inhibited through the use of antimycin A, binding to the Q_i site of the complex and inhibiting the transfer of electrons to oxidized ubiquinone, while potassium cyanide (KCN) is a competitive inhibitor of CIV that binds to cytochrome a_3 heme group of the enzyme (Antonini et al., 1971). These substrates and inhibitors are used in chapter III of this thesis to assess the control or the involvement of each enzyme on overall respiratory flux. For an in-depth review of the different mitochondrial respiratory states and rates and the differences between the classical terminology from Chance and Williams, see Gnaiger et al. (2019).

The functioning of OXPHOS is at the core of mitochondrial respiration, and sets the basis of the MOSTA. Nonetheless, to better understand the age-related decline

in physiological function linked to mitochondrial respiration, the existence and mechanisms behind oxidative stress need to be better understood.

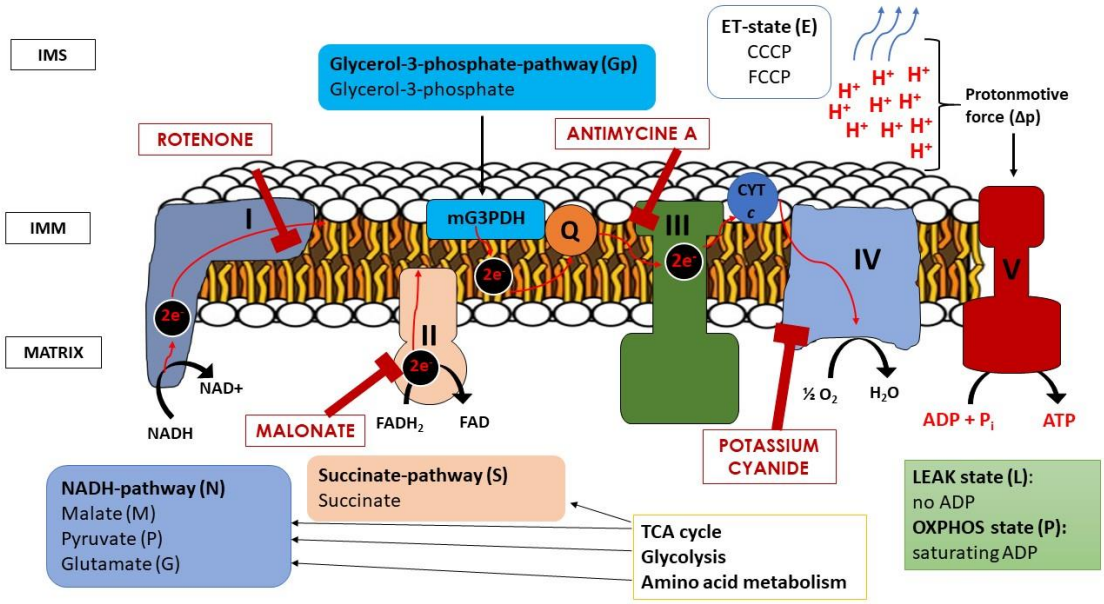

Figure 1.2 Schematic representation of the mitochondrial electron transport system (ETS) with the name and acronyms used for the various substrate pathways, uncoupler and inhibitors. Rounded boxes show the substrate pathways feeding electrons (electron pathways in red arrows) into the ETS enzymatic complexes (complex I, II and mG3PDH) studied in this thesis, either in absence (LEAK, L) or saturating ADP (OXPHOS, P) conditions. The rounded empty box shows the uncouplers used to dissipate the proton gradient and attain the ET-state, while the red boxes show the chemical inhibitors used for each specific complex. For details on pathways please refer to the main text. IMS: intermembrane space, IMM: inner mitochondrial membrane.

1.2.2 The molecular basis of oxidative stress in mitochondria

Mitochondrial ROS production mainly derives from superoxide anion ($O_2^{\cdot -}$) production in the ETS. The first evidence of mtROSp by the respiratory system came in the mid-1960s when suspension of electron-transport particles from beef hearts were found to produce hydrogen peroxide (H_2O_2), a less reactive, non-radical molecule (Jensen, 1966). As previously mentioned, the key finding in support of Harman's MFRTA was that of superoxide dismutase (SOD), the enzyme in charge of converting

superoxide to hydrogen peroxide (McCord and Fridovich, 1969). The subsequent findings that hydrogen peroxide came from the dismutation of $O_2^{\cdot}$ (Loschen et al., 1974), and that isolated mitochondria indeed produce H_2O_2 (Chance et al., 1979) provided more evidence of endogenous free radical production and detoxification mechanisms.

How is this superoxide produced? In normal conditions, molecular oxygen is reduced at CIV to produce water. Yet in particular conditions, $O_2^{\cdot -}$ is produced by a one-electron reduction of oxygen, where an enzyme of the ETS gives an electron directly to oxygen, instead of cytochrome *c*. This gives an oxygen molecule where at least one of the orbitals has an unpaired electron, fitting in the definition of a free radical. Kinetic factors such as substrate and enzyme or protein concentrations (among which $[O_2]$), pKa and pH influence this thermodynamically favoured reduction, and ultimately determine mitochondrial $O_2^{\cdot -}$ production (Murphy, 2009). However, not all potential electron donors carry out this reaction: the two main sources of superoxide production are complexes I and III of the ETS.

Complex III was first recognized as the main respiratory complex responsible for ROS production (Barja, 2013). Investigations over the years have uncovered several different sites of mtROSp in the ETS, as well as in other mitochondrial enzymes (Figure 1.1). Important sources of $O_2^{\cdot -}$ in the ETS are the ubiquinol-oxidizing site in CIII (III_{Qo}), the flavin and ubiquinone-reducing sites in CI (I_F and I_Q respectively) and electron-transferring flavoprotein ubiquinone oxidoreductase. Other sources of $O_2^{\cdot -}$ include the oxoglutarate dehydrogenase complex (OGDC), pyruvate dehydrogenase (PDH), glycerol-3-phophate dehydrogenase (GPDH), and the less characterized 2-oxoglutarate and non-mammalian NADH-Q oxidoreductases (Brand, 2010). Sites reaching maximal rates of superoxide production are III_{Qo}, I_Q, GPDH and recently, the flavin site at complex II (II_F) has recently gained interest as a potentially important ROS producer in the ETS (Quinlan et al., 2012;Quinlan et al., 2013a).

It was initially estimated that 1-4% of mitochondrial oxygen consumption was diverted into ROS in vivo, but more realistic assessments now predict a lower value of 0.15% (Brand, 2010). Nonetheless, overproduction of superoxide results in molecular damage, hence the evolution of detoxifying mechanisms by organisms. In the IMM, MnSOD converts $O2^{\cdot -}$ to H_2O_2, while Cu/ZnSOD does the same in the intermembrane space (IMS), as part of the superoxide produced by $IIIQ_o$ and all of that produced by GPDH is released in the IMS (the other sites release superoxide in the matrix). The balance between ROS production and consumption mechanisms is gaining more interest in recent years and mitochondria are now proposed to be regulators rather than simple producers of ROS (Munro and Treberg, 2017). Among the pathways for consumption of ROS, H_2O_2 produced by SOD can be detoxified into water by catalase, or by two respiration-dependent pathways: the glutathione and the thioredoxin pathways, which depend on the supply of electrons by NADPH. Despite these defense mechanisms, mtROSp impacts the principal surrounding macromolecules: lipids (which will be of particular interest in chapter II of this thesis), proteins and DNA.

ROS are often lipophilic and tend to be localized inside phospholipid bilayers, rather than in the aqueous medium; membrane lipids are therefore particularly prone to oxidative damage (Pamplona, 2008;Calhoon et al., 2015). Moreover, sensitivity to attacks by free radicals and their metabolites depends on the particular nature of the fatty acid chains forming the lipids. In particular, hydrogens atoms attached to single-bonded carbons between two double-bonded carbons (bisallylic) are more susceptible to free radical attack. Hence, fatty acid chains with no double bonds or one double bonds (saturated fatty acids, SFA and monounsaturated fatty acids, MUFA) resist peroxidation, while those with multiple double bonds (polyunsaturated fatty acids, PUFA) are very sensitive to damage by free radicals. The sensitivity of a fatty acid to ROS-induced damage increases exponentially with its number of double bonds (Holman, 1954). For example, docosahexaenoic acid (DHA, a PUFA with 6 double bonds) has ten bisallylic hydrogens and is 320 times more sensitive to lipid

peroxidation than oleic acid, a MUFA (Hulbert, 2005). Reactive free-radicals remove the hydrogen atom from the PUFA side chain, resulting in a carbon atom with an unpaired electron. Combined with oxygen dissolved inside the membrane, this carbon radical forms a peroxyl radical (ROO˙), highly reactive and prone to attack membrane proteins and oxidize surrounding PUFA chains, producing a lipid peroxyl radical (LOO˙). This radical is capable of starting another cycle of peroxidation, amplifying this free radical chain reaction, and generating lipid hydroperoxides (LOOH). Being more hydrophilic, lipid hydroperoxides move to the membrane surface and interact with water, altering membrane structure, fluidity and making it leaky. Lipid peroxidation is therefore overall an autocatalytic reaction. Endoperoxides are also generated through lipid peroxidation, and they produce a wide range of reactive intermediates called reactive carbonyl species (RCS). Among the most reactive RCS are 4-hydroxynonenal (HNE, from n-6 PUFAs), hydroxyhexenal (HHE, from n-3 PUFAs) and malondialdehyde (MDA). Chen and Yu (1994) found that liver mitochondrial membrane fluidity in rats decreased with age due to the action of lipid peroxidation products, especially HNE. Binding of HNE to membrane phospholipids was less intense in diet-restricted animals, a treatment found to increase lifespan in various organisms. Other effects of these RCS include the inhibition mitochondrial adenine nucleotide transferase, induction of the mitochondrial permeability transition, cytotoxicity and inhibition of DNA and protein synthesis. HNE has also been shown to stimulate a mild uncoupling of mitochondria, causing a decrease membrane potential and ultimately keeping mtROSp under control in a negative feedback loop (Hulbert et al., 2005).

Nucleic acids and proteins are also the targets of ROS and can accumulate damage. Mitochondrial DNA (mtDNA) is modified by ROS attacks at its sugar-phosphate backbone or at the bases, yielding different purines and pyrimidines (8-oxodG being often measured), and can also cause single or double-strand breaks as well as DNA mutations (reviewed in Pamplona, 2011). The proposed vicious cycle of

accumulation of mtDNA mutations can drive the expansion of clones containing damaged DNA, and an increase with age of the amount of cells containing these clones (Payne and Chinnery, 2015). This added burden can in turn negatively impact the function of respiratory complexes, because mtDNA encodes for 13 key proteins of the ETS, although mitochondrial biogenesis can partially compensate these effects (Kauppila et al., 2017). Proteins are also well documented to be the target of ROS with impacts such as the oxidation of amino acid residue side-chains, the creation of protein-protein cross-linkages, and oxidation of their backbone inducing protein fragmentation (Berlett and Stadtman, 1997). There is evidence for an age-related accumulation of modified enzymes linked to the oxidative modification of proteins in various species (Levine and Stadtman, 2001), impacting cellular function. Yet, recent evidence points to a role for oxidative alterations of proteins in redox signalling, hence the portrait might be, as it often is, more complicated (Wall et al., 2012). Nonetheless, lipid, DNA and protein damages caused by oxidative stress are proposed by the MOSTA to be at the basis of the age-related decline in function.

1.3 The MOSTA in comparative studies

1.3.1 Support and debate around the MOSTA

Antioxidants were initially the main focus of research on the link between oxidative stress and aging, and it was first theorized that their presence would be higher in longer-lived species. However, evidence quickly showed that long-lived species actually have lower endogenous antioxidant levels in their tissue than short-lived ones, and most importantly that diet supplementation with antioxidants does not extend lifespan (reviewed in Barja, 2013). The idea that the repair mechanisms could be the main determinant of aging is logical but flawed, as demonstrated by comparative studies. In fact, the answer seems to lie in the production and the structural protection from damage, as two factors universally correlate with longevity in animals: 1) the rate of mtROSp and 2) the degree of unsaturation of membrane fatty acids.

With the evidence on the antioxidants-longevity relationship and the idea that maintenance of high antioxidant enzyme levels would be energetically costly, Barja et al. (1994) predicted that free radical production was the key factor determining longevity. Subsequent studies showed an inverse relationship between rates of mitochondrial $O_2^{\cdot -}$ and H_2O_2 generation and lifespan in seven mammalian species, and in comparisons between two mice species spanning a 2 to 10-fold difference in lifespan (Ku et al., 1993;Sohal et al., 1993). Importantly, these differences appear not to be attributable to the higher mass-specific metabolic rates (MSR) in shorter lived species, an idea exposed by the "rate of living theory of aging" and Rubner's rule (Rubner, 1908;Pearl, 1928). In fact, when comparing species having longer lifespans than predicted by their metabolic activity (birds) to shorter-living species but with similar MSR (mice), the former produced less H_2O_2 during respiration in heart mitochondria (Herrero and Barja, 1998). To correct for the confounding effects of mass and phylogeny, Lambert et al. (2007) examined the relationship between H_2O_2 production and lifespan in 12 vertebrate species. They found the negative correlation was even more significant when correcting for body mass, and remained significant when correcting for the degree of relatedness using phylogenetic independent contrasts. Moreover, the dietary manipulations that are known to increase longevity (dietary restriction, protein restriction and methionine restriction) can all decrease mtROSp (Sanz and Stefanatos, 2008).

Rates of mtROSp depend on the physiological state of the cell: absolute and relative decreases in the rate of mtROSp occur during aerobic exercise, hence during the mitochondrial transition from respiratory state (resting state) to phosphorylating state 3 (Barja, 2013). This is explained by the fact that electron flow accelerates in the ETS, decreasing the reduction of the electron carriers and consequently of some mtROS-producing sites. Moreover, the increase in $mtVO_2$ lowers the pO_2 and the high K_M for O_2 from the ROS generators contributes to lower mtROSp. Increasing exercise activity appears not to be the only strategy for diminishing mtROSp. When comparing

two species with similar oxygen consumption rates, Barja and Herrero (1998) found that free radical production was higher in the rat (MLSP 4 years) than the pigeon (MLSP 35 years). By blocking the entry of electrons to CIII with site-specific inhibitors, they found CI to be the major site of mtROSp, and free radical production due to a higher reduction state of the ROS-generating sites of this complex. Moreover, in Wistar rats subjected to a 7-week dietary and methionine restriction (treatments known to increase maximum longevity and decrease mtROSp), the content of CI and CIII decreased remarkably (Ayala et al., 2007;Caro et al., 2008). Protection against a high reduction state of ROS-producing sites could thus be done by lowering the enzymatic activity of the complexes, but more studies are needed to assess the existence of such a relationship with species longevity.

The advantages of having a small endogenous mtROSp are easy to grasp when considering its impact on proteins, lipids and mtDNA (discussed above). Comparative studies show that the longer-living species show smaller degrees of mtDNA oxidative damage (Barja, 2013). Although the ROS-induced origin of mtDNA mutations is under debate (Kauppila et al., 2017), recent intervention improving lifespan in mtDNA mutator mice treated with a mitochondrial-targeted, ROS-scavenging antioxidant suggests a strong link between oxidative and DNA damage (Shabalina et al., 2017). Oxidative damage can be further amplified by the products of lipid degradation during oxidative stress, leading to a series of studies on the relationship between membrane composition and longevity. Evidence shows trends among species in vertebrate and invertebrate models, and stress the importance of considering lipid composition in aging studies.

1.3.2 Bridging the gap between inter- and intra-specific studies

Comparative studies show that membrane fatty acid composition varies widely among species, and is linked to body mass in mammals (Couture and Hulbert, 1995) and birds (Hulbert et al., 2002). Due to the relationship between mass and basal

metabolic rate, these findings suggested that fatty acid composition may be a determinant of metabolic rate differences among species, and gave rise to the "membrane pacemaker theory of metabolism" (Hulbert and Else, 1999). Subsequent studies showed that membrane peroxidation index (PI), a marker of susceptibility to oxidative damage, is negatively correlated to maximum longevity in mammals (including humans) and birds (Pamplona et al., 2000;Hulbert et al., 2007). This is true for whole-tissue and mitochondria of skeletal muscle, liver, heart, as well as whole-brain tissue. This finding, added to the previously discussed oxidative stress theory, devised the "membrane pacemaker theory of aging" (Hulbert, 2005), which can be seen as a corollary of the MOSTA and where the differences in propensity to peroxidative damage of membrane lipids are thought to mechanistically explain differences in longevity between species. Long-lived vertebrate species also show a low sensitivity to lipid peroxidation in vivo and in vitro, and a low steady-state level of lipoxidation-derived adducts in tissue and mitochondrial proteins from various organs (Pamplona, 2008). Despite a lack of differences in H_2O_2 production between the longest-living rodent, the naked-mole rat (*Heterocephalus glaber*; MLSP > 28 yrs) and the short-lived laboratory mice (*Mus musculus*; MLSP, 3.5 ~ yrs), they do show differences in membrane PI in line with the membrane pacemaker theory of aging.

Some evidence suggests that the same trend is found among populations and strains of a same species with different longevities. An intraspecific study comparing longer-living wild-strain versus genetically distinct laboratory mice (*Mus musculus*) showed lower PI values for wild mice skeletal muscle membranes, despite being kept in the same environment and fed the same diet as their shorter-lived counterparts (Hulbert et al., 2006). This suggest a genetic control for differences in membrane composition. In a strain of senescence-accelerated mice (SAM), Park et al. (1996) found these mice had higher levels of DHA (22:6n-3) and arachidonic acid (20:4 n-6), two PUFAs prone to peroxidation, and lower amounts of 18:2n-6, a PUFA less prone to peroxidation, compared to SAM-resistant individuals. Finally, support for a link

between fatty acid composition and aging in invertebrates has emerged. Haddad et al. (2007) studied the honeybee (*Apis mellifera*), where the MLSP of queens is an order of magnitude higher than that of workers (2-5 years versus 15-38 days), despite them being genetically identical. They found that the membranes of queen honeybees were more monounsaturated and less polyunsaturated than that of worker bees, resulting in a PI 2-3 fold higher in the shorter-lived workers, in agreement with the MOSTA.

The previously mentioned studies are among the few that have looked at filling the gap between inter- and intra-specific relationships. Should the relationships between characters linked to oxidative stress and lifespan be supported among both levels of organization, this would greatly reinforce the MOSTA. Moreover, most studies on senescence-related traits are done on individuals in tightly controlled laboratory conditions. Environmental conditions influence physiological processes, hence a more robust approach at testing theories of lifespan should ideally include natural populations in order to better understand interactions between genotype and environment (Nussey et al., 2013). This question is addressed in chapter II of this thesis, where we take a look at species and populations of short and long-lived marine bivalves that allow us to tackle these conundrums.

1.4 Bivalves as models for the study of lifespan

1.4.1 Taxonomy and life-history traits of marine bivalves

Model organisms used for aging studies are often limited by either a small range in lifespan variation, or shared phylogenetic history that pose the problem of the non-independence of data (Speakman, 2005). Clues on the aging process may be obtained by the longest-living organisms on Earth, however multi-centenarians are hardly found in scientific studies. Hence, bivalve molluscs are emerging as an important model for aging studies not only because of their often-extreme longevity, but also because of their important, gradual range in lifespan, and their relatedness allows to circumvent the problem of phylogenetic distance between species (Figure 1.3). A member of this

group of invertebrates, the ocean quahog *Arctica islandica*, is the longest-living non-colonial animal with a maximum reported longevity (MRL) of 507 years (Ridgway and Richardson, 2011;Butler et al., 2013). Although specimens close to 200 years are common in the North Icelandic shelf population (Strahl et al., 2007;Ridgway and Richardson, 2011), and individuals of more than 300 years have been found, this record longevity was reported for one particular individual; it was subsequently nicknamed "Ming", as the Chinese dynasty reigning at the beginning of its life (Butler et al., 2013). Conveniently, age determination of bivalve molluscs can be done through sclerochronology: the counting of the annual growth bands in the shell using acetate peels preparations (Ropes, 1985;Kilada et al., 2007). Bivalve molluscs have proven to be even more so polyvalent for scientists, and are used in marine paleoclimatology. Indeed, environmental conditions affect shell growth increment widths and geochemical properties, hence the stratigraphic signature of the shells are used as records of environmental and climatic changes (Wanamaker Jr et al., 2008;Butler et al., 2013;Schöne, 2013).

Ocean quahogs burrow beneath the sediment and extend their short siphon for feeding and oxygen uptakes. They regularly close their shell and burrow deeper into the sediment, facing hypoxic conditions and inducing metabolic rate depression (MRD) for 1 to 7 days (Strahl and Abele, 2010). This lifestyle implies frequent hypoxia-reoxygenation cycles, to which they have adapted, as the expected increased ROS formation upon surfacing is not observed in this species (Strahl et al., 2011a). Ocean quahogs thus seem to shut down the anticipatory upregulation of antioxidant/stress defense by limiting $O_2^{\cdot}$ formation during reoxygenation of gill tissues, maintaining high and stable antioxidant defenses during MRD and decreasing antioxidant and stress gene transcript levels in response to anoxia (Strahl et al., 2011b). Important biochemical changes occur during the first 25 years that correspond to the initial rapid somatic growth and sexual maturation stage. Stabilization of parameters such as antioxidant enzyme activities occurs once the mature stage is reached, around 32 years (Abele et

al., 2008). The positive relationship between age at maturity and lifespan is consistent with the predictions of the antagonistic pleiotropy theory of aging, as shown by the analysis of 111 species and populations of both marine and freshwater bivalves by Ridgway et al. (2011a). Hence, according to this evolutionary view of aging, natural selection would have acted to optimize fitness early in life, at the cost of the deleterious effect of a shorter lifespan (see section 1.1). Indeed, both a "traditional" analysis and one with corrections for phylogenetic distance showed that age at sexual maturity could explain between 30 and almost 50% of the variation in lifespan, with the longest-lived species maturing at older age (Ridgway et al., 2011a).

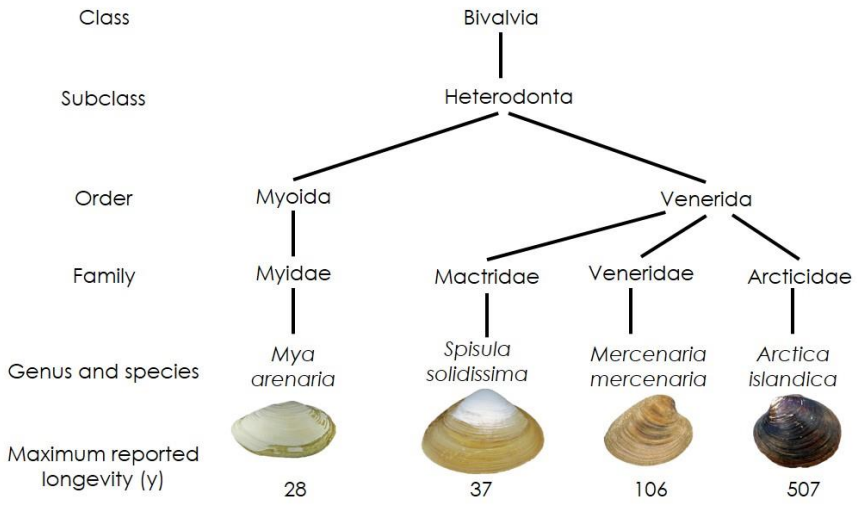

Figure 1.3 Taxonomy and maximum reported longevities of the four bivalve species used as a longevity model in this thesis (adapted from Munro and Blier, 2012).

1.4.2 MOSTA-related parameters linked with lifespan in bivalves

The membrane lipid composition of marine bivalves differs from that of insects and endotherms (figure 1.4). Indeed, they possess large amounts of plasmalogens (alk-1-enylglycerophospholipids), believed to confer resistance to peroxidation because of their vinyl ether linkage between the carbon chain at the *sn-1* position and the glycerol backbone, instead of the typical ester bond (Engelmann, 2004). Methylation of

plasmalogens gives dimethylacetals (DMA), and since most alkenyl chains are saturated or monounsaturated in bivalves, they allow to lower the PI value of the membrane. In addition, they possess non-methylene-interrupted fatty acids (NMI), where the double bonds are separated by more than one methyl group, a property that reduces sensitivity to peroxidation since the bisallylic methylene hydrogens are absent (Barnathan, 2009). In a test of the membrane pacemaker theory of aging, Munro and Blier (2012) compared the gill membrane lipid composition of *A. islandica* with four other bivalve species of the same subclass (heterochonchia): *Mya arenaria* (MRL = 28 yrs), *Spisula solidissima* (37 yrs), *Mactromeris polynyma* (92 yrs), and *Mercenaria mercenaria* (106 yrs). PI decreased exponentially with increasing longevity, in part due to a decrease in DHA in longer-living species, as predicted by the pacemaker theory of aging. They found a steeper relationship in mitochondrial membranes, the main targets of attacks by RCS, than in cellular debris. Moreover, when re-examining the data using the shared maximum longevity (SML), the maximum longevity attained in at least two populations, the exponential decrease in PI was still observed and more so with a better fit. No significant trend between NMI and MRL or SML was found, however, *A. islandica* had higher NMI levels compared to all other species. Plasmalogen abundance was lower for the short-lived *M. arenaria*, while it was among the highest in *A. islandica*. Overall, the relationship between abundance of plasmalogens and longevity wasn't significant.

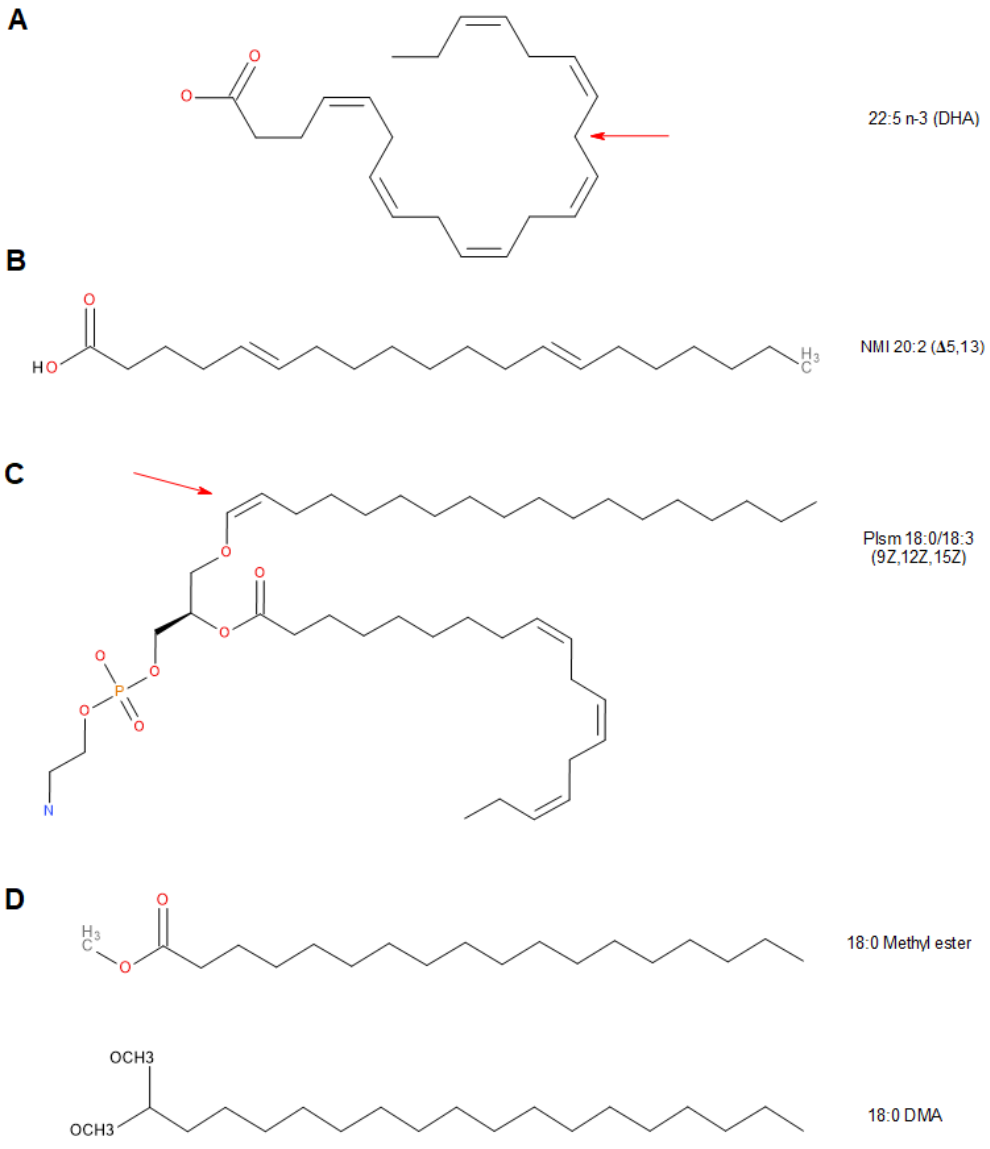

Figure 1.4 Representation of various membrane lipids found in marine bivalves. (A) Docosahexaenoic acid (DHA), a common polyunsaturated fatty acid, possessing peroxidation-sensitive methylene bonds between its double-bonds (indicated by the red arrow). (B) Example of a non-methylene interrupted fatty acid (NMI 20:2 (Δ5,13)), having more than one carbon atom between its double-bonds and making it less peroxidation-sensitive than DHA (C) A plasmalogen (PE(P-18:0/18:3(9Z,12Z,15Z)), with the vinyl ether bond at the *sn-1* position (red arrow) of the alkenyl chain. (D) Examples of a fatty acid methyl ester (18:0 methyl ester) and a dimethyl acetal (18:0 DMA), the latter being the product of plasmalogen methylation and are detected during lipid analysis.

Most intraspecific studies on PI-longevity relationships have focused on genetically manipulated strains displaying relatively narrow differences in maximum lifespan. Recent research has highlighted the importance of studying membrane composition variation with physiological traits at different evolutionary scales. For example, in a re-assessment of the membrane pacemaker theory of metabolism, membrane composition was found to vary with body size and metabolic rate in orchid bees' flight muscle, and the differences show species-specific groupings (Rodríguez et al., 2015). Their findings show that refining the analysis to go to the genus or species-specific level can reveal how malleable certain traits such as fatty acid composition are, at different phylogenetic scales. As mentioned before, should inter- and intraspecific relationships between PI and longevity be demonstrated, this would highly reinforce the MOSTA. Conveniently, population-specific variations in MLSP have been reported in specimens of northern European *A. islandica*, and whether differences in membrane composition exist among populations remains unknown.

Studies indicate that *A. islandica* is able to maintain populations across a broad range of population age structures in the North Atlantic shelf, which could explain its wide distribution throughout this region (Begum et al., 2010). Around the Icelandic shore, MLSP of the species is of 507 years, and individuals close to 200 years old are regularly encountered (Strahl et al., 2007;Ridgway and Richardson, 2011). In Canadian waters, population structure of two different locations studied by Kilada et al. (2007) with little or no fishing pressure shows frequency of age classes around 25-30 years, but higher attained longevity in the offshore Sable Bank population (210 y) compared to the inshore St. Mary's Bay (72 y). The growth curves of these two populations were found to be similar, but maximum attained size was lower in St. Mary's Bay, which was deemed to be attributable to natural mortality due to predation. Back on the European side, *A. islandica* has become rare in areas of high historical fishing pressure, such as in the Oyster Ground area of the southern North Sea, where the increased sea temperature is within the limits for larval development, and may also be responsible

for its decline (Witbaard and Bergman, 2003). Norway populations attain a maximum lifespan of 300 years, while in the German Bight (Helgoland), maximum age drops to 125 years; but more impressively low MLSP are observed in the Kattegat (71 y), White Sea (53 y), Baltic Sea and Kiel Bay (40 y) areas, where lifespan values are far from the species maximum (Begum et al., 2009;Begum et al., 2010). These sites were characterized by a wider thermal and salinity windows, potential stress factors that should increase the energetic costs and limit maximum age and size of quahogs. Maximum lifespan and mortality rates of *A. islandica* therefore seem to result of an interplay between exogenous (environmental temperature and salinity, bottom topology and sediment properties, predator and fishing pressure) and endogenous (physiological, genetic) factors. The latter are of particular interest in the context of the MOSTA and are the subject of this thesis.

The physiological basis of population-longevity differences has been explored by Basova et al. (2012) in 6 populations of *A. islandica*: Iceland, Norwegian coast, German Bight, Kattegat, White Sea and Kiel Bay. The authors found no differences in antioxidant capacity among populations, but shorter-lived populations exhibited higher mass-specific metabolic rates, and did not show a metabolic response (as measured by the effect of temperature on mass-specific respiration rate using the temperature coefficient Q_{10}) when exposed to higher temperatures, compared to their long-lived counterparts. Hence, they suggested that populations most strongly exposed to environmental fluctuations required more plasticity in their responses to thermal and salinity challenges, perhaps accounting for their earlier reproduction and their adjustment of metabolism. Indeed, in the two populations with the shortest lifespans, superoxide dismutase (SOD) and catalase (CAT) activities increased with age, and may indicate an adjustment to elevated stress. In accordance with this idea is the fact that SOD and CAT actually decrease significantly with age in the very long-lived (and perhaps less stressed) Icelandic population. An analysis of gene expression patterns in two populations of opposing longevity by Philipp et al. (2012) seems to confirm this

idea. They found a suppression of stress response and antioxidant genes in the long-lived populations, *versus* an increase in the population exposed to environmental fluctuations. Whether *A. islandica* displays such plasticity in membrane composition among populations in the context of the pacemaker theory of aging will be the main focus of the first chapter of this thesis.

The ROS-longevity relationship recently gained more support thanks to experiments on marine bivalves. Indeed, earlier studies were limited by the nonindependence of body mass and phylogenetic distances between the compared species. Lambert and colleagues' (2007) controlled for both factors in their vertebrate species analysis, but could only find significance in the relationship when succinate was used as a substrate for mitochondrial respiration, hence during reverse electron flow (REF) from complex II to complex I. This was also the case in recent studies on birds and rats where the relationship was only significant for heart mitochondria during REF (Brown et al., 2009;Montgomery et al., 2011;2012), casting doubts on the mtROSp-longevity relationship. Succinate oxidation elevates protonmotive force, forms a highly reduced ubiquinone pool that leads to REF, reducing the conversion of NAD to NADH, leaking superoxide radicals into the mitochondrial matrix. This condition is not encountered *in vivo*, hence casting doubts on its physiological significance, and stressing the need to find more adequate model organisms to support the theory (Buffenstein et al., 2008). Munro et al. (2013) used marine bivalves to test the theory, and found a reduced H_2O_2 production rate in all the conditions of mitochondrial metabolism in the mantle of the long-lived *A. islandica* when compared to the shorter-lived *Mya arenaria* and *Spisula solidissima*. Results were normalized by four important markers: citrate synthase (CS, an enzyme of the TCA cycle and hence a proxy for mitochondrial content), CIV (an indicator of ETS content), and by moles of consumed O_2 during the LEAK and OXPHOS states (hence during respiration without and with ADP, respectively). These results rule out the possibility that the differences may be due to lower amounts of mitochondria or lower OXPHOS capacity.

In addition, activities of enzymatic complexes I, II and of the ETS normalized for CS or CIV were significantly lower while the RCR was higher in *A. islandica* when compared to *M. arenaria*, indicating that the lower mtROSp is not due to a lower ATP production capacity. These findings clearly establish a ROS-longevity relationship in bivalves.

The mechanistic basis of this lower mtROSp is subject of debate in homeotherms, and studies on marine bivalves could provide clues. The existence of an AOX in marine bivalves, whereby electrons can avoid going through the ROS-producing sites of CIII, doesn't seem to explain this lower intrinsic oxidative damage, as *A. islandica* does not show a higher AOX activity than the two shorter-lived species (Munro et al., 2013). In the same study, the finding of a lower CI and ETS activity in *A. islandica* compared to *M. arenaria* was proposed to contribute to the lower mtROSp seen during REF. Although this condition does not represent physiological conditions, the authors propose that the lower catalytic capacity of CI and CIII in conditions of forward electron flow could reduce mtROSp and explain the interspecies differences. Their results also suggest an involvement of CII in modulating mtROSp between species, but expanding the analysis to a more important number of species is needed to support this link between complex activity and mtROSp.

1.5 Metabolic control analysis of the mitochondrial electron transport system

ROS-induced damage to mtDNA leads to a decrease in electron transfer and an amplified production of ROS in a vicious circle of oxidative stress and energetic decline, the latter considered the cause of aging and age-related diseases (Genova et al., 2004). How long or short-lived species face this energetic decline is not yet understood, and modulating the functioning of the ETS enzymes in terms of their relative contribution to respiration and ROS production may be an important strategy to achieve longevity (reviewed in Blier et al, 2017).

1.5.1 Theoretical underpinnings of metabolic control analysis

To assess whether this modulation characterizes species longevity, metabolic pathways can be dissected using the framework of metabolic control analysis (MCA). In it, control strength is used to measure the amount of control exerted by a step in a pathway on a particular flux through the pathway. It is defined as the fractional change in flux through the pathway induced by a fractional change in the enzyme under study (Groen et al., 1982). Behind its inception are the works of Chance and Williams (1955b), and the subsequent studies by Kacser and Burns (1973), as well as Heinrich and Rapoport (1974).

In mitochondria, respiratory control has been studied extensively, as it is a fundamental yet complex function that is shared between different bioenergetics steps. Respiration is generally controlled by the rate of re-entry of protons through ATP synthase, endogenous leak and other proton-consuming pathways (Nicholls and Ferguson, 2013). However, the activity of ATP synthase is linked to the rate of ATP synthesis and therefore to the rate of ATP turnover in the cytoplasm. The question then arises of how the respiratory chain knows how fast it has to operate, and the relative weight of each step on the global mitochondrial pathway. Metabolic control analysis is the quantitative analysis of the control exerted by the fluxes involved in the different bioenergetics steps involved in respiration (Nicholls and Ferguson, 2013). The respiratory chain works somewhat like an electrical circuit, with 3 proton-translocating complexes that act in parallel with respect to the proton circuit, and in series with the electron flow. The total proton current flowing is proportional to the rate of O_2 utilization. A basic factor controlling the rate of respiration is the thermodynamic equilibrium between the redox potential across the proton-translocating regions of the respiratory chain and protonmotive force (Δp, mV). In practice, there are many steps involved in the proton circuit: supply and transport of substrate across the inner membrane, supply of electrons thanks to the metabolite dehydrogenases, the rate of adenine nucleotide translocator, and the rate of ATP turnover. Metabolic control

analysis provides a simple method to describe how control is distributed between multiple steps. If we simplify a metabolic pathway with two enzymes, by the equation:

$$\xrightarrow{A \overset{E_1}{\rightarrow} B \overset{E_2}{\rightarrow} C} J$$

Where E_1 and E_2 are the enzymes, overall flux in a steady state is *J*, and C is the flux control coefficient. If we took a simple example of a mitochondrion in state 3 (maximum ATP synthesis), and changed the activity of a single step in the pathway by 50%, what effect would this have on overall respiration?

The flux control coefficient is defined as the fractional change in flux divided by the fractional change in the enzyme as the change tends towards 0, or:

$$C_{E_1}^{J} = \lim_{\delta E \to 0} \frac{\delta J / J}{\delta E_1 / E_1}$$

The effect of the change in activity of a single step can have two opposite and extreme effects on the overall flux of the pathway: no change at all, or an identical change to the one of the steps (here, 50%). Therefore, the flux control coefficient would either be 0 in the first scenario, or 1 for the second case. In practice, a flux control coefficient of 1 is only a hypothetical scenario, as the notion of a single-rate determining step is rarely seen in metabolic sequences. However, many steps are at play, and the sum of all the individual control flux coefficients must therefore be equal to 1. Figure 1.4 depicts the theoretical curves obtained with a decrease in flux due to the inhibition of a specific reaction.

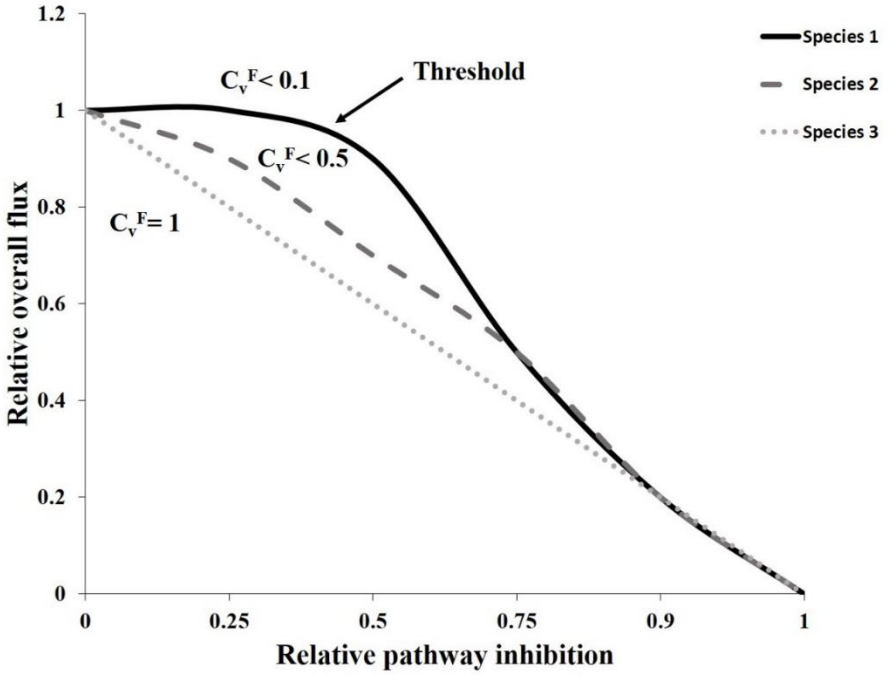

Figure 1.5 The relationship between the decrease in the flux of a network as a function of the inhibition of a reaction of the network. Different flux control coefficients (CvF) are given as examples: CvF=1 (full control on the flux), CvF<0.5 (gradual decrease in flux) and CvF<0.1 (slow linear decrease). Based on Mazat et al. (2013).

1.5.2 Metabolic control analysis and mitochondrial dysfunction

Metabolic control analysis can be applied to OXPHOS in two ways: bottom-up and top-down analysis. Bottom-up analysis allows to observe the effects of titrating particular mitochondrial enzymes and transporters with specific and irreversible inhibitors, and examining their effects on respiratory rates and ATP synthesis. This method is conditional to the knowledge of all of the system's components and the use of inhibitors (Murphy, 2001). The alternative approach, top-down (or modular) analysis, can be used to simplify the study of complex systems such as intact cells and tissues. It decomposes the system in blocks of reactions feeding or consuming a common intermediate, often the protonmotive force in the case of OXPHOS. Substrate transport, metabolism and the respiratory chain generate this protonmotive force, while

ATP synthesis, transport and turnover, and proton leak consume this common intermediate. The elasticities of these blocks to the common intermediate are then determined to calculate each flux control coefficient. Both methods have been used extensively to study the behaviour of mitochondria, cells and tissues to different metabolic situations. However, the focus has been mainly on the management of oxidative phosphorylation, rather than its by-products. For example, Groen et al. (1982) measured the distribution of control strength of respiration among various steps in rat liver mitochondria and found it dependant on the rate of respiration (state 3 or 4). In state 4, almost all control was exerted by the passive permeability of the mitochondrial membrane, whereas in state 3 control was distributed among five different steps accounting for 86% of control, with other unidentified enzymes presumed to account for the remaining 14%. At the time, these results challenged the idea that respiratory control was exerted at a single rate-limiting step.

An important application of metabolic control analysis is to elucidate mitochondrial dysfunction, or abnormalities in the processes linked to the functioning of mitochondria. These encompass of course ATP generation by oxidative phosphorylation, but also functions such as generation and detoxification of ROS, the mechanisms of apoptosis, regulation of Ca^{2+} in the IMM and cytoplasm, synthesis and catabolism of metabolites, and organelle transport (Brand and Nicholls, 2011). Mitochondrial dysfunction is increasingly linked to various pathologies (e.g.: metabolic syndrome, age-related diseases, cardiovascular diseases, diabetes and even psychiatric diseases), and can be studied in isolated mitochondria or intact cells. In the former, a general indicator of mitochondrial function is the respiratory control ratio (RCR), measured as the respiration in state 3_{ADP} (maximal respiration with ADP and substrates present) divided by that in state 4 (respiration slows as the ATP/ADP ratio reaches equilibrium). Theoretically, healthy mitochondria exhibit a high RCR, whereas dysfunctional mitochondria show a low RCR (Brand and Nicholls, 2011). However, there isn't a defined RCR value for dysfunctional mitochondria, as it is dependent on

virtually any factor linked to OXPHOS and varies with species, tissue and substrate. Fortunately, normal RCR values for various marine bivalves are well established (Tschischka et al., 2000;Munro et al., 2013). When a dysfunction is detected by measuring RCR and absolute respiration, modular kinetic analysis is the main method used to uncover its causes (Brand and Nicholls, 2011).

Examples of the application of control analysis are multiple. Kuznetsov et al. (1997) found a redistribution of flux control in muscle biopsy samples from patients with mitochondrial myopathies, and linked these changes to small non-proportional variations in enzyme activities that could be the determinant of the global energy metabolism defect in the affected tissue. Analysis of the control exerted by complex I over the respiratory chain activity in coupled liver mitochondria of rats revealed that this enzyme is strongly rate-controlling in old individuals, and more so than in younger rats (Ventura et al., 2002). Although other steps of the ETS are also proposed to be involved, it appears that aging induces an alteration of complex I that reflects on the oxidative phosphorylation system in this species. This is also seen in the decrease of rotenone (CI inhibitor) sensitivity, reflected in the increase of the I50 (the inhibitor concentration inducing half inhibition of the activities), and by the decrease of state 3 respiration in aged rats. Moreover, hepatocytes from old rats were also found to produce higher peroxide levels than those from younger animals, as well as higher complex I transcripts in response to functional alterations of the enzyme, supporting the MFRTA (Genova et al., 2004).

In the ETS, threshold values of inhibition for enzymatic complexes can vary importantly before an effect on respiration or ROS production can be appreciated. In mammalian brain mitochondria, various studies show that in complex I, a relatively low inhibition threshold (25% of complex activity) suffices to cause a decline in energy production or an increase in ROS production. On the other hand, CIII and CIV activities could be decreased by up to 80%, before major differences in oxygen consumption or ATP synthesis could be detected. This highlights the idea that deficiency in a particular

complex could favor generation of oxidative stress and sensitivity to oxidative damage, thus setting the stage bioenergetic incompetence associated with various neurological diseases (Davey and Clark, 1996;Sipos et al., 2003).

1.6 The supramolecular architecture of the electron transport system

1.6.1 From the solid and fluid models to the discovery of supercomplexes

Our understanding of the organization of the enzymatic components in the mitochondrial inner membrane has evolved over the years. From the solid model of electron transfer and oxidative phosphorylation envisioned by Chance et al. (1963) to the random diffusion (or fluid) model of electron transfer (Hackenbrock et al., 1986), the consolidating model is now that of supramolecular organization of the ETS, the supercomplex (SC). The previously accepted fluid model stated that the inner membrane proteins and the redox components diffuse constantly and independently in the membrane plane, while electron transport is a diffusion-coupled process with random collisions between electrons and redox components. Early evidence against this random distribution of respiratory complexes came from studies reporting a preferential isolation of CI and CIII or CII and CIII subunits (Yu et al., 1974). Yet, the fluid model was the textbook explanation until the studies by Schagger and Pfeiffer (2000). Their analysis by blue native polyacrylamide gel electrophoresis (BN-PAGE) in digitonin-solubilized yeast and bovine mitochondria showed multiple comigrating respiratory complexes, not linked to ATP synthase. This SC assembly was initially challenged by tenants of the fluid model, but subsequent research has led to the

discovery of such assemblies in diverse models: from yeast and fungi to plants, vertebrates, and invertebrates (Enríquez, 2016).

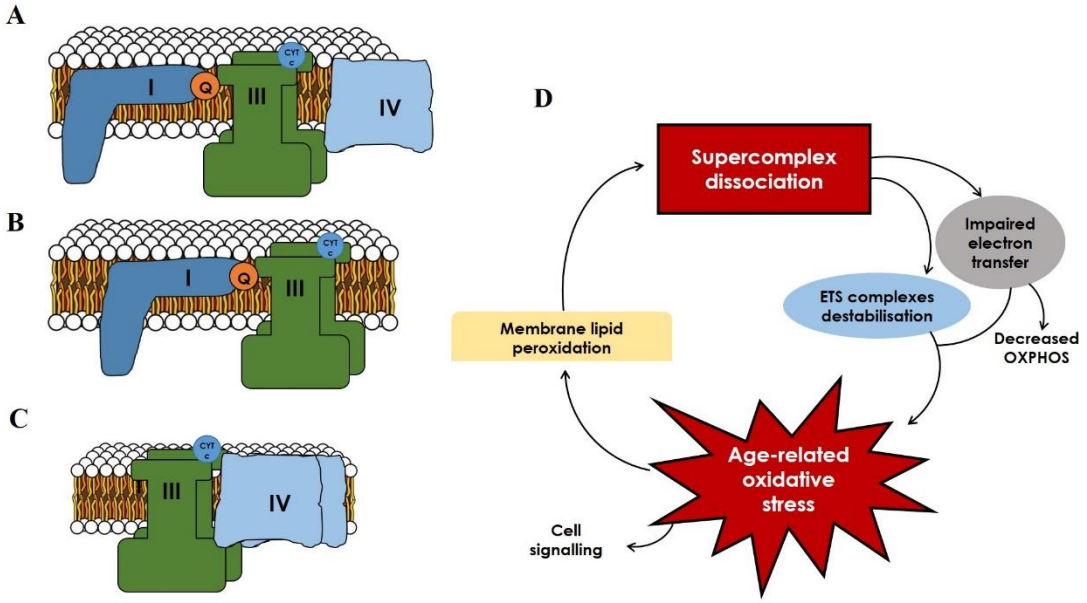

Figure 1.6 Representation of commonly encountered supercomplexes assemblies with varying stoichiometries of ETS complexes: (A) I1III2IV1, (B) I1III2 and (C) III2IV2. (D) Proposed scheme for the effect of the loss of supercomplex organisation on the "vicious circle" of oxidative stress and decreased OXPHOS efficiency (based on Genova and Lenaz, 2014)

Although the physiological occurrence of supercomplexes is still under debate, recent technological advances in methods to purify and detect complex assemblies are leading to a general acceptance of their assembly as a true phenomenon. Electrophoretic studies using BN-PAGE has led to the detection of high molecular weight assemblies, such as the SC $I_1III_2IV_1$, containing all the redox enzymes required for electron transfer from NADH to molecular oxygen. Other recurring SC assemblies are SC I_1III_2 and SC III_2IV_2 (Figure 1.5 A-C), while some studies also point to a SC formation between CII and other OXPHOS enzymes (Acín-Pérez et al., 2008). Further functional demonstration of the existence of SC came from metabolic flux control analyses, the measurement of the control exerted by individual enzymes on a pathway (reviewed earlier). The model of a respiratory chain based on random diffusion of

complexes implies different levels of rate-control by enzymes, each one controlling the pathway to a certain extent. On the opposite, the model based on SC assembly implies electron channelling between complexes and thus, a metabolic pathway behaving as a single component, where inhibition of any component would exert the same flux control. Hence, the sum of all the control coefficients would be superior to 1. Bianchi et al. (2004) found CI and CIII to be highly rate-controlling over NADH oxidation in bovine heart submitochondrial particles, evidencing a functional association between the two (C_I = 1.06, C_{III} = 0.99). On the contrary, CIV was randomly distributed (C_{IV} = 0,26), while CII was very rate-limiting for succinate oxidation (C_{II} = 0.88, C_{III} = 0.34, C_{IV} = 0.20), indicating the absence of substrate channeling to CIII and CIV. Flux control analysis can therefore serve as a detection tool for the supramolecular assembly of enzymatic complexes in the IMM.

Supercomplex formation relies on several assembly factors (proteins that enable stable interactions between complexes), but also on a specific mitochondrial shape (alteration of proteins shaping the mitochondrion can affect SC formation), and a precise lipid environment in the IMM (reviewed by Enríquez, 2016). This last finding is of special interest in the context of bivalve studies and will be discussed in more detail below. SC formation, stabilization and function appears to be fundamentally linked to the lipid structure of the IMM, particularly in the presence of cardiolipin. This phospholipid was first shown to be essential for the activity of cytochrome oxidase, CI, CIII and other mitochondrial carriers, suggesting a role for this phospholipid in the process of coupled electron transfer. Recent studies showed that cardiolipin stabilizes SC and individual complexes: a loss of cardiolipin through mutations or substitutions in yeast caused instability and dissociation of SC organization (Lenaz and Genova, 2012). Moreover, ROS production can affect the respiratory activity via oxidative damage to cardiolipin (Paradies et al., 2000;2002), and flux control analysis has shown that lipid peroxidation affects reconstitution and maintenance of a I-III SC (Genova et al., 2008).

1.6.2 Supercomplex function and its role in aging

Multiple functional advantages of supercomplex assembly are proposed. From their flux control analysis, Bianchi et al. (2004) propose that the enzyme associations taking place in the ETS allow a structural stabilization of the membrane proteins, a feature also suggested in a study on CI-CIII stability in human and mice cells (Acin-Perez et al., 2004). Another advantage of SC organization is thought to be substrate channeling, that is, the passing of an intermediate metabolite from one enzyme directly to the other, without release into the substrate pool (Bianchi et al., 2004). This bypasses enzyme competition for the substrate, increasing the efficiency of the reaction. In the respiratory chain, coenzyme Q (CoQ) and cytochrome *c* are the localized substrates, with most CoQ being free in the membrane bilayer. Kinetic evidence shows that CoQ is a homogenous diffusible pool between reducing and oxidizing enzymes, therefore the plasticity model proposes that both SCs and individual complexes coexist in the IMM, with their prevalence depending on the energetic state of the mitochondrion (Bianchi et al., 2004;Enriquez and Lenaz, 2014;Enríquez, 2016). Although it appears that SCs organize electron fluxes, the precise mechanisms involved remain unknown, and an important topic in the context of mitochondrial deficiencies.

The interactions between enzymatic complexes in SCs allow for exclusive electron pathways to form, and minimize ROS production. Considering the oxidative stress theory of aging, SC assembly is therefore potentially crucial to minimize damage to the mitochondrion. A clear indication of that is the important consequences observed after a loss in the stability of SCs. As previously mentioned, lipid peroxidation can disrupt these supramolecular structures (Genova et al., 2008), and Maranzana et al. (2013) used bovine heart mitochondria and reconstituted liposomes of CI and CIII to test whether destabilization of SCs lead to an increase in mtROSp. They found that dissociation of SC with detergent dodecylmaltoside led to an increase in hydrogen peroxide and superoxide in both models. When investigating age-related differences in the electron transport system organization of hearts of young and old rats, Gómez et al.

(2009) found that the levels of individual complexes did no vary. However, they separated and identified the SCs, and showed that their levels were diminished between 13-25% in old rats versus young individuals, especially for those SCs with higher molecular weight. These seemingly small decreases are actually compared to the levels found in pathological studies with important mitochondrial structural and respiratory chain defects, such as Barth or Leigh Syndromes. Their results ultimately suggest that there is either an impairment in formation, or an increase in the rate of decomposition of supercomplexes in aging heart mitochondria. Aging is therefore accompanied by a decrease in SC association, and an increase in ROS and oxidative damage. If SC stability is key in determining mitochondrial aging phenotype, species differences in MLSP could be related to their capacity to maintain SC integrity (see figure 1.5 D).

Studies on SC assemblies and cardiolipin content in invertebrates are scarce, but recent research in marine bivalves has shown important amounts of cardiolipin with distinct FA profiles. Kraffe et al. (2008) compared bivalves from the Heterodonta subclass (burrowers) had high levels of PUFAs EPA and DHA in their cardiolipins, while the two other subclass, Pteriomorph and Eupteriomorph (epifaunal bivalves) had significantly less EPA in their cardiolipin. One of the long-lived species of heterodonts, *Mercenaria* (MLSP: 106 y), had especially high levels of both long-chain PUFAs. They proposed some adaptive value of the OXPHOS process linked to the different environments faced by the two bivalve subclasses, although the consequences of these differences to mitochondrial functions remain to be explained. The presence of cardiolipin in marine bivalves could be a hint as to the existence of supercomplex assemblies in these species, and could confer an advantage in terms of OXPHOS efficiency, protection against oxidative stress, and ultimately, aging.

1.7 Thesis objectives

The overall objective of this thesis is to attempt to link mitochondrial structure, function and regulation of respiration to lifespan across and inside species.

Using the framework of the mitochondrial oxidative stress theory of aging (MOSTA), the **first objective** of this thesis is to investigate whether membrane lipid composition and mitochondrial enzyme activities are linked to longevity intraspecifically in the same fashion as they are interspecifically. We use six distinct populations of *Arctica islandica* from the Northern Atlantic, isolate mitochondria and perform GC-FID analysis for lipid composition, as well as spectrophotometric analyses for enzymatic activities. Our working hypotheses are that mitochondrial membrane and cellular debris lipid composition vary as a function of MLSP in six distinct populations of *Arctica islandica*. We hence expect to find 1) a decrease in membrane peroxidation index (PI) with increasing longevity, 2) a higher %PUFA and %DHA in shorter-lived populations and 3) more plasmalogens in longer-lived populations. Moreover, we expect to find a decrease in the enzymatic activities of CI+CIII, COX and ATPase with an increase in longevity. Chapter two of this thesis explores these associations in the form of a research paper published in the July 2019 edition of *Frontiers in Physiology*.

Fundamental research on aging has shown the importance of structure and regulation of the ETS in aging-related processes, but comparative studies with adequate models are still lacking. Using this approach in closely-related bivalves, the **second objective** of this thesis is to assess the control strength of the principal ETS enzymes over OXPHOS in isolated mantle mitochondria of four bivalve species (see figure 1.3). We use high-resolution respirometry and specific inhibitor titrations (see figure 1.2), coupled to protein and citrate synthase activity measurements to attain this objective. Based on the lower activity of the complexes associated with electron entry in *A. islandica*, we predict that complexes I, II and III would exert a higher control on respiration in longer-lived species. Since these also exhibit a conserved OXPHOS capacity and a similar ratio of complex IV (normalized by CS, see Blier et al. 2017 fig. 5A) as their shorter-lived counterparts, a compensating increase in the proportions of TCA cycle dehydrogenase (feeding electrons to the ETS) and downstream complex IV would need to occur. We thus expect to find a lower control at downstream complex

IV as a function of longevity. The results from this second objective are reported in chapter three as a research paper to be submitted in *Journals of Gerontology Series A.*

The **third objective** of this thesis is to delve deeper into mitochondrial structure and organization by looking at the evidence for supercomplex assemblies of the ETS. The results of the flux control analysis done in the previous chapter should give a first hint of the existence of these macromolecular structures, if the sum of the control coefficients is superior to unity. Therefore, using blue-native gel electrophoresis and working out the experimental conditions for the appropriate solubilisation of the mantle mitochondrial membrane, we evaluate the presence of a supercomplexes in four marine bivalve species. Then, we aim to compare specific patterns of organization of these assemblies and determine whether these patterns are linked to species longevity, using in-gel activity and mass spectrometry approaches. These findings are reported in chapter four as a research paper to be submitted in a peer-reviewed journal.

Globally, this thesis contributes to understanding the mitochondrial traits that dictate lifespan differences among and inside species in a model of short- to extreme longevity: marine bivalves. The results will be discussed in-depth in a fifth chapter, and put in perspective in light of the literature on the MOSTA, of comparative studies on metabolic control, supercomplexes, and potentially confounding factors such as environmental pressure.

CHAPTER II
MITOCHONDRIAL TRAITS PREVIOUSLY ASSOCIATED WITH SPECIES MAXIMUM LIFESPAN DO NOT CORRELATE WITH LONGEVITY ACROSS POPULATIONS OF THE BIVALVE *ARCTICA ISLANDICA*

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2.1 Abstract

The mitochondrial oxidative stress theory of aging posits that membrane susceptibility to peroxidation and the organization of the electron transport system (ETS) linked with reactive oxygen species (ROS) generation are two main drivers of lifespan. While a clear correlation has been established from species comparative studies, the significance of these characteristics as potential modulators of lifespan divergences among populations of individual species is still to be tested. The bivalve *Arctica islandica*, the longest-lived non-colonial animal with a record lifespan of 507y, possesses a lower mitochondrial peroxidation index (PI) and reduced H_2O_2 efflux linked to complexes I and III activities than related species. Taking advantage of the wide variation in maximum reported longevities (MRL) among 6 European populations (36 to 507y), we examined whether these two mitochondrial properties could explain differences in longevity. We report no relationship between membrane PI and MRL in populations of *A. islandica*, as well as a lack of intraspecific relationship between ETS complex activities and MRL. Individuals from brackish sites characterized by wide temperature and salinity windows had, however, markedly lower ETS enzyme activities relative to citrate synthase activity. Our results highlight environment-dependent remodeling of mitochondrial phenotypes.

2.2 Introduction

Understanding the physiological determinants of lifespan in animals has been the focus of biologists and gerontologists over the past decades. Comparative studies suggest a role for mitochondrial structure and function associated with the aging process, in particular the management of reactive oxygen species (ROS). This evidence has been integrated into one unifying hypothesis, the "mitochondrial oxidative stress theory of aging" (MOSTA, reviewed in Blier et al., 2017) . It points out, among others, the importance of cell membrane composition and particularly the susceptibility of mitochondrial membrane lipids to oxidation (Peroxidation Index, PI) as potentially causative parameters of cellular aging. In addition, modifications of the redox state and stoichiometry of mitochondrial electron transporters appear to play a crucial role for mitochondrial coupling and the rates of ROS formation (Barja, 2007), and hence are important components of the MOSTA.

Membrane lipids are particularly prone to oxidative damage (Gamliel et al., 2008), and the sensitivity of fatty acids (FA) to oxidation by ROS depends on their chemical conformation, especially with respect to the presence of conjugated double bonds. FA chains with no or only one double bond (saturated fatty acids; SFA; and monounsaturated fatty acids: MUFA) are more resistant to peroxidation than polyunsaturated FA (PUFA), the sensitivity of which increases exponentially with the number of conjugated double bonds (Holman, 1954). The products of fatty acids oxidation (reactive aldehydes) induce autocatalytic progression of lipid peroxidation that can further damage membrane proteins, PUFA chains and DNA. This leads to impaired protein functions and DNA mutations and underlines the crucial role of membrane lipid composition for cellular integrity, with implications for organismal lifespan (Hulbert, 2010).

The strongest association between a physiological trait and divergences in species lifespan has so far been observed for membrane structure. The early evidence

of a strong negative correlation between membrane PI and increasing maximum lifespan was reported in vertebrates, specifically in mammals and birds (Hulbert et al., 2007). Later, Munro and Blier (2012) took advantage of the extreme longevity of *Arctica islandica* (the ocean quahog) to corroborate the same association in five species of bivalves (Munro and Blier, 2012). Among these species, the ocean quahog *Arctica islandica* is the longest-lived non-colonial animal with a record lifespan of 507 years in the North Atlantic (Butler et al., 2013). Munro and Blier (2012) compared gill membrane lipid composition of *A. islandica* with four shorter-lived bivalve species from the shelf areas off the coasts of eastern Canada belonging to the same subclass (heterochonchia), with lifespans ranging from 28 to 106 years. Membrane PI decreased exponentially with increasing longevity, in part due to a lower DHA (docosahexaenoic acid, 22:6, n-3) content in mitochondrial membranes of longer-lived species. Other molecules protecting bivalve cellular membranes against ROS attack include plasmalogens (dimethylacetals in their methylated form, DMA) and non-methylene interrupted fatty acids (NMI). Plasmalogens act as a ROS scavenger, whereas NMI reduce susceptibility to peroxidation (Engelmann, 2004;Barnathan, 2009) while maintaining fluidity of the membrane. Although no consistent relationship was observed between NMI abundance and MRL, *A. islandica* had higher NMI and plasmalogen levels than the shortest-lived species *Mya arenaria*. The association previously observed in vertebrates therefore appears to be conserved in a wide range of animal taxa and tighter, at least in bivalves, for mitochondria.

In addition to membrane structure, Munro and colleagues (Munro et al., 2013) found *A. islandica* mantle mitochondrial isolates to exhibit lower rates of net ROS production than two shorter-lived bivalve species. These differences related to the activities of mitochondrial ETS (electron transport system) complexes, including major sites of ROS production (complex I and III) that differed between *A. islandica* and shorter-lived species (especially *M. arenaria*), despite similar oxidative phosphorylation (OXPHOS) capacities. Relatively lower activities of CI (NADH

oxidoreductase) and CII (succinate dehydrogenase) relative to downstream complex (cytochrome c oxidase) in *A. islandica* suggest a lower degree of reduction at the ETS, resulting in lower overall mitochondrial ROS production without compromising phosphorylation rates (Munro et al., 2013;Blier et al., 2017). This is reminiscent of the lower CI and CII content in calorie-restricted rats and in long-lived drosophila (Ayala et al., 2007;Neretti et al., 2009). Hence, stoichiometric arrangements between upstream and downstream ETS complexes might play an important role in lifespan determination.

From these studies, we can suspect that a low membrane PI and the associated high robustness to ROS attack are prerequisites for reaching long lifespan. On an evolutionary perspective, one obvious question arising from these observations is to which extent membrane variability among populations of one species could set the plasticity and potential evolution of longevity, or associated life history characters. At the intraspecific level, most studies linking lipid composition to metabolism or lifespan have focused on genetically manipulated strains (Brzęk et al., 2007) with relatively small differences in maximum lifespan, or on laboratory bred animal lines (Valencak and Ruf, 2013). Haddad and colleagues (Haddad et al., 2007) found a lower PI in longer-lived honeybee queens than shorter-lived workers, but otherwise no studies have looked at the importance of membrane composition inside a species in a natural setting. The bias of comparing controlled laboratory experiments with physiological processes occurring in nature, could potentially blur some of the real age-related changes (Austad, 2018). In order to test to what extent mitochondrial characters associated to lifespan divergences among species govern the rate of mitochondrial aging within a species, studying different natural populations displaying a wide range of contrasting average lifespans is necessary.

Considerable population-specific variations in MRL are seen in *A. islandica*, both in the western (Kilada et al., 2007) and eastern (Basova et al., 2012) parts of the North Atlantic. One individual of 507 y was found in Icelandic (IC) waters where

animals more than 300 years old are regularly encountered (Strahl et al., 2007;Butler et al., 2013). All other populations have much shorter reported lifespan maxima with the shortest MRL of only 40 y reported for the well-studied Baltic Sea population (Basova et al., 2012, and see Table 2.1). The different *A. islandica* habitats are characterized by wide thermal and salinity windows, but there is no consistent evidence for specific metabolic rate or antioxidant activities in animals from any population of these regions associated to environmental conditions (Basova et al., 2012). On the other hand, environmental factors such as food and sunlight availability (Moss et al., 2017), as well as dredging effort (Thorarinsdóttir et al., 2009) could help explain differences in lifespan. Begum et al. (2018) used one mitochondrial locus (cytochrome b) to prove that, in spite of their vastly differing MRL, all these populations belong to the same species and hence provide an applicable system to test longevity-associated characters at the intraspecific level.

The main objective of the present paper is to determine if characters associated to divergences in lifespan among species can dictate the pace of aging among populations of one species. We therefore compared membrane lipid composition, PI, and ETS complex activities across the same *A. islandica* populations from the Atlantic and the brackish coastal seas. We hypothesized that ETS activities and lipid parameters in the populations vary as a function of MRL in the same fashion as found between different bivalve species (Munro and Blier, 2012). We thus predicted to find 1) a decrease in mitochondrial membrane PI with increasing longevity, 2) a higher %PUFA and %DHA in shorter-lived populations, 3) more plasmalogens in longer-lived populations, and 4) a decrease in enzymatic activity for the CI+CIII relative to COX as longevity increases.

Table 2.1 Life history traits of populations of Arctica islandica sampled and their maximal reported longevities (MRL), salinity regime characterization according to mean salinity and salinity amplitude (see Basova et al., 2012, table 1): marine 33 PSU; marine/coastal 32 PSU/variable; brackish, <30 PSU

Population	Coordinates	MRL (years)	Salinity regime	References
Kiel Bay (KB)	54° 32.6N, 10°42.1E	36	brackish	(Gruber et al., 2015)
White Sea (WS)	66°18N, 33°38E	53	brackish	(Basova et al., 2012)
Kattegat Sea (KA)	56°10N, 11°48E	71	marine/coastal	
German Bight (GB)	54°10N 7°53E	150	marine/coastal	Kerstin Beyer (Alfred Wegener Institute, personal comm.)
Norwegian Coast (NC)	69°39 N, 18°57 E	300	marine	
Icelandic Coast (IC)	66°02N, 14°51W	507	marine	(Butler et al., 2013)

2.3 Materials and Methods

Specimens of *A. islandica* from six populations with contrasting MRLs were collected across a geographic gradient of European coastal seas (KB and WS) and from the North Atlantic (see table 1 for details). Approximately 500 mg of gills and mantle tissues were dissected, and immediately frozen in liquid nitrogen and kept at -80 °C before enzymatic and lipid analyses were undertaken. The mantle was chosen as it is the largest somatic tissue and as a means of comparing with a wealth of other studies, while the gills were chosen on the basis of their important respiratory and environmental sensory role. Mitochondrial isolation was carried out on 250 mg of frozen mantle and gills, as previously described (Munro and Blier, 2012). The resulting mitochondrial pellet was resuspended in 150µl of buffer, and both mitochondria and cellular debris fractions were conserved at -80ºC after nitrogen flush for lipid analysis. The abundance of mitochondrial electron transport systems in both biological fractions was assessed using the NADH-INT (iodonitrotetrazolium) oxidoreduction assay, targeting complexes I and III. The values for the cellular debris pellets were below 1/20th of those in the mitochondrial pellets, and were therefore considered essentially devoid of mitochondria.

Analysis of the phospholipid composition of the mitochondrial and cellular debris fractions was done following the protocol established by Munro and Blier (2012). Separation of phospholipids was done using solid-phase extraction columns (Bond Elut-NH_2, 500 mg, 3mL), and trans-methylated overnight at 55ºC in a 1% H_2SO_4 solution in methanol. The resulting fatty acid methyl esters (FAME) and dimethyl acetals (DMA) were recovered in 100 µl hexanes and analyzed by GC-FID (Agilent Trace Ultra 100, Thermo Fisher Scientific, Waltham, MA, USA) using a column with a high polarity stationary phase (HP-88, 60 m, 0.25 mm × 0.20 µm, Agilent Technologies Canada, Mississauga, ON, Canada). All solvents used were of ultra-pure grade. Conveniently, calibration of the system was previously done by GC-MS using common FA mix standards (SUPELCO 37 FAME) marine FA standards (SUPELCO) and DMA standards (SIGMA). FA composition-longevity relationships were tested using relative abundance in mol% of individual FA, FA classes (total saturated, monounsaturated, polyunsaturated, DMA and NMI), as well as of different indexes as per Hulbert et al. (2007). The peroxidation index (PI) was calculated as PI = (0.025 × % monoenoics) + (0.258 × % 20:2 NMI) + (0.32 × % 22:2 NMI) + (1 × % dienoics) + (2 × % trienoics) + (4 × % tetraenoics) + (6 × % pentaenoics) + (8 × % hexaenoics). The unsaturation index (UI) represents the number of double bonds per 100 acyl chains and was calculated as UI = (% monoenoics) + (2*% dienoics) + (3* % trienoics) + (4*% tetraenoics)+ (5*% pentaenoics) + (6*% hexaenoics). Results will be presented as means ± SEM.

Each gill and mantle sample was weighed and homogenized in 6 volumes of cold homogenization buffer (20 mM Tris (hydroxymethyl) aminomethane, 1mM EDTA, 0.1% Tween 20, pH 7.4) in Precellys Homogeniser 24 (Bertin Technologies) with 2 times 15 seconds at 5000 rpm at 8 ˚C. Enzymatic activities of CI+III, COX and CS were measured at 8˚C using Plate Reader TriStar (Berthold Technologies) and expressed in $U.g^{-1}$ tissue fresh mass. All assays were run in duplicate. All protocols were adapted from Breton et al. (2009). We chose to present CI+III, rather than using

the previous annotation “ETS”, since this assay does not measure COX activity, which is a major component of the system.

Differences between groups were assessed using Systat v. 13 software. Variables were first tested for normality using the Shapiro-Wilk test. A between-group principal analysis (PCA) was used to assess the variation in enzymatic activities and membrane lipid composition for individuals belonging to the six populations studied. This analysis consists to running PCA on a dataset where observations are gathered by user-defined groups to emphasize the between-groups variability in the analysis process (Dolédec and Chessel, 1989). Here groups were built by distinguishing specimens accordingly to their sampling localisation. The significance of the proportion of the variability explained by the grouping factor (*e.g.* population) in between-groups PCA was tested by Monte-Carlo permutation (999 permutations) and were considered significant when the simulated p value = 0.001. Between-groups PCA was performed using ‘ade4’ library (Chessel et al., 2004) implemented in R 3.2.1 (R Development Core Team, 2012).

2.4 Results

2.4.1 Membrane susceptibility to peroxidation (PI)

Population grouping significantly explained the variability of lipid peroxidation susceptibility markers (PI, plasmalogens, DHA, n-3 and n-6 PUFA) in the gills (observed variability between populations = 38%, simulated *p* value = 0.001), but not in the mantle tissue (simulated *p* value = 0.085; see supplementary material figure S1C). The between-population distribution pattern of the lipid markers in the gills, however, did not reflect a clear relationship between PI and population MRL. Instead, WS individuals had a significantly lower PI in the gills than KB ($p = 0.023$), KA ($p = 0.039$), and NC ($p = 0.019$) populations (Fig. 1), and did not differ from the IC populations. In all populations except IC, gill mitochondrial membranes had a lower PI than mantle mitochondria. The IC and WS populations had the lowest PIs in both

tissues. Gill PI values were 77.5 (±10.3) and 102.0 (±7.2) for WS and IC respectively, while mantle PI values were of 110.0 (±18.6) and 101.1 (±6.7) for these same populations (Fig. 2.1). These values related partly to low DHA levels: 4.6% (±1.0) and 6.2% (±0.5) in the gills, and 7.5% (±1.6) and 5.5% (±0.7) in the mantle, respectively.

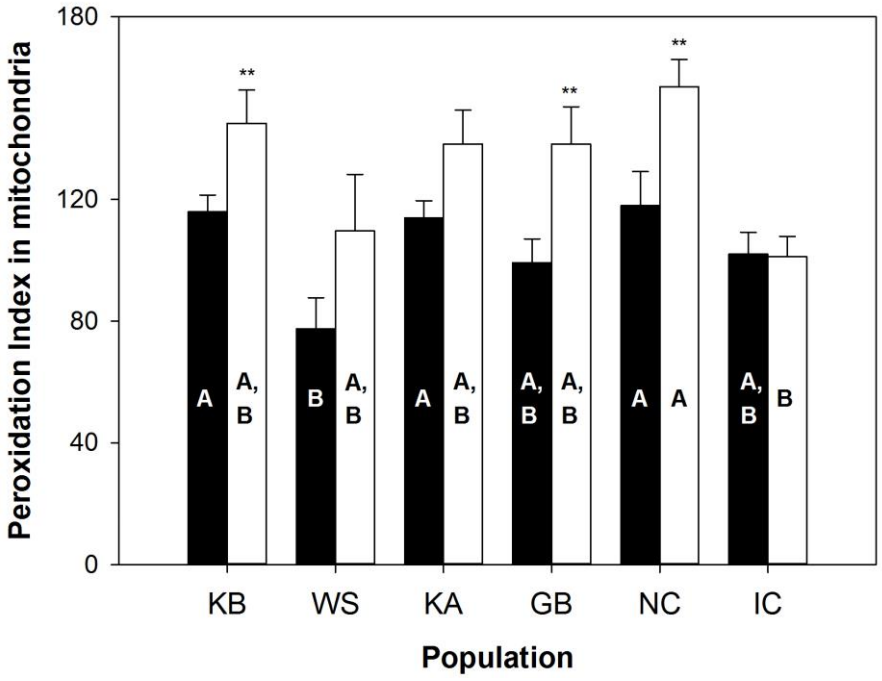


Figure 2.1 Peroxidation index in mitochondria from *A. islandica* populations ranked from shortest- to longest-lived. Values are means ±SEM. Letters denote significant ($p < 0.05$) differences between populations for gills (filled bars) and mantle (empty bars) tissues, while asterisks indicate significant differences between tissues (**: $p < 0.05$).

The abundance of plasmalogens (measured as their methylation products DMA) and NMIs did not vary significantly among populations. In gill mitochondria, DMA abundance ranged from 12.0% (±0.9) in WS to 17.7% (±2.2) in KA, whereas it fluctuated between 8.7% (±1.7) in WS and 14.0% (±1.9) in NC in mantle mitochondria. There were significant differences between tissues, as DMA abundance was higher in the gills for KA ($p = 0.018$), GB ($p = 0.018$) and IC ($p = 0.009$) compared to the mantle. Gill mitochondria NMI abundance ranged between 11.6% (±0.6) in NC to 15.8% in

WS, and from 9.1% (±1.0) in KA to 12.2% (±2.3) in IC in mantle mitochondria. NMI abundance was also significantly higher in the gills than in the mantle in WS (p =0.036) and KA (p =0.002). See tables S2.1 and S2.2 for detailed FA composition of mitochondrial membranes in gills and mantle, respectively. FA composition of the cellular debris was similar to that of mitochondria and are thus not presented as figures.

Between-group PCA did not significantly explain the distribution pattern of phospholipid classes in either gills or mantle. Moreover, the repartition of the amount of phospholipids resistant to peroxidation (NMI, MUFA, SFA, DMA) and those that are peroxidation sensitive (n-6 PUFA and n-3 PUFA) did not differ significantly between populations and could not be linked with their respective MRL (Fig. 2.2).

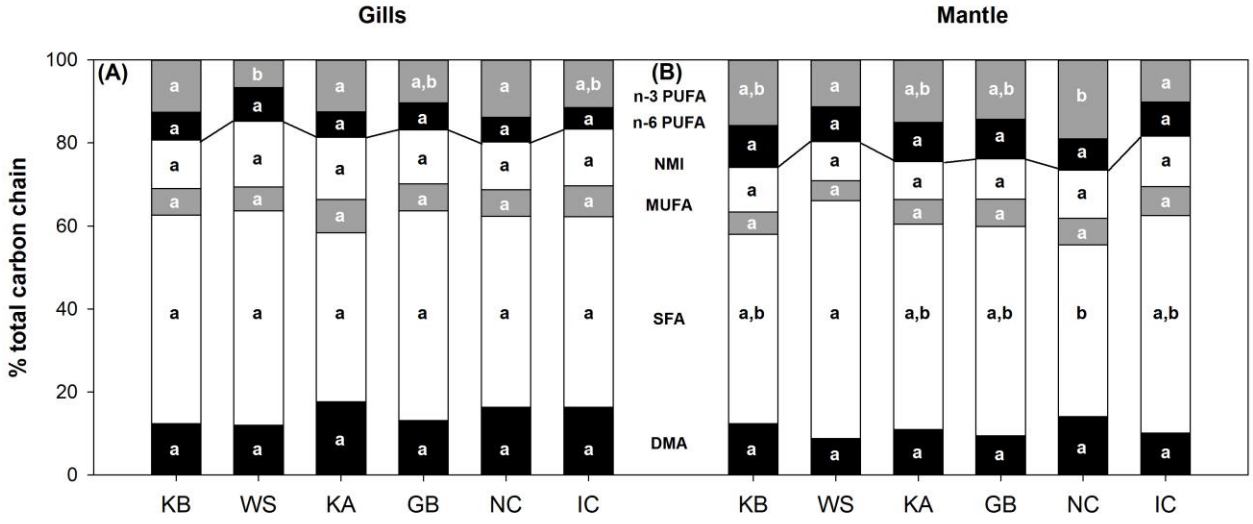


Figure 2.2 Mitochondrial phospholipid carbon chain composition from *A. islandica* populations ranked from shortest- to longest-lived, in (A) gills and (B) mantle tissues. Letters denote significant ($p < 0.05$) differences between populations for each carbon chain class: DMA, dimethyl acetals, SFA, saturated fatty acids, MUFA, monounsaturated fatty acids, NMI, non-methylene-interrupted fatty acids, and PUFA, polyunsaturated fatty acids. The line delimits peroxidation-resistant (below) and peroxidation-sensitive (above) carbon chains. Samples sizes can be found in table S1.

2.4.2 ETS enzyme activities

The between group PCAs performed on ETS enzyme activities (CI+III, COX; see supplementary material figure S1A and B) indicates that "population" factor explains significantly more variability in gills (observed variability between population grouping = 61%, simulated *p value* = 0.001; Fig. S1A) than for mantle tissue (observed variability between population grouping = 26%, simulated *p value* = 0.001; Fig. S1B). In both cases, the first PCA axis, which accounted for 72% and 84% of the total variance in gills and mantle respectively, is associated with CI+III and COX activities (normalized to CS activity). The second PCA axis represents 27% of the variability in gills and 15% in mantle tissue based on $CI+III \cdot COX^{-1}$ activity.

In both tissues, the brackish water populations from KB and WS exhibit lower CI+III and COX activities, when normalized to CS activity, compared to the fully marine KA, GB, NC and IC. Most comparisons differed significantly ($p < 0.05$; Fig. 2.3A and B), distinguishing populations along the first PCA axis for both tissues. More subtle differences separate the last four populations of which GB and IC animals had higher $COX \cdot CS^{-1}$ activity in gills than KA and NC ($p < 0.05$; Fig. 2.3B). Moreover, KA and NC exhibit higher $CI+III \cdot COX^{-1}$ ratio in gill mitochondria than all other populations ($p < 0.05$; Fig. 2.3C). These ratio differences can explain the distinction between KA and NC vs. GB and IC along the second axis of the between group PCA in gills. Even though the two shortest-lived populations (KB and WS) exhibited the lowest $CI+III \cdot CS^{-1}$ and $COX \cdot CS^{-1}$ activities (Fig. 2.3A and B), we could not find any clear correlation between ETS complex stoichiometry and MRL.

Tissue comparisons of $CI+III \cdot CS^{-1}$ and $COX \cdot CS^{-1}$ revealed significantly lower activities in gills than in mantle mitochondria, for all six populations ($p < 0.005$, Fig. 2.3A and B). As for $CI+III \cdot CS^{-1}$, only the KB ($p = 0.002$) and the NC populations ($p = 0.008$) showed significantly higher gill activities compared to the mantle (Fig. 2.3C). Much of these contrasts in metabolic organization observed in short-lived populations,

living in brackish water, appear to be driven by significantly higher CS activity (expressed by mg of tissues or proteins) in both tissues (see fig S2).

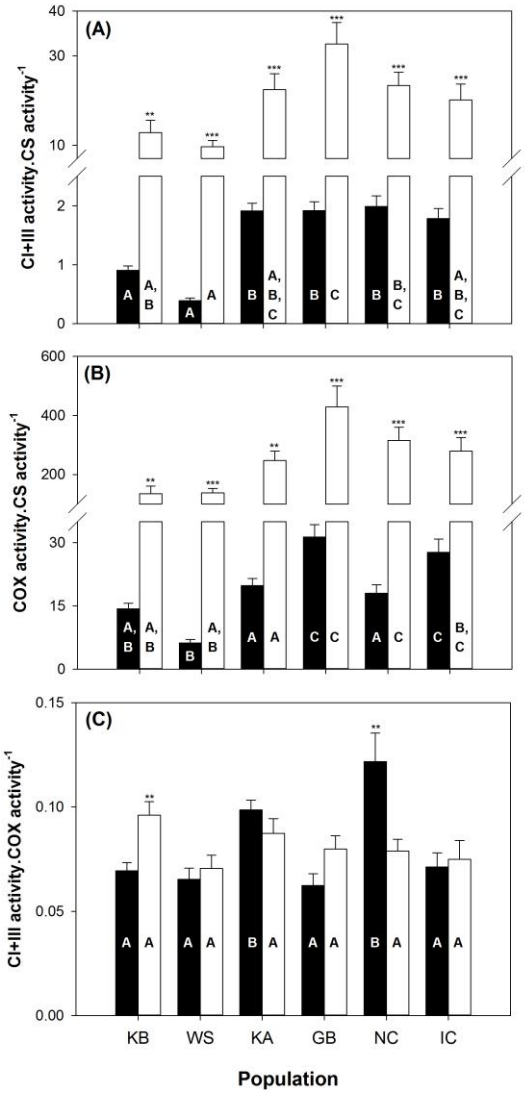


Figure 2.3 Mitochondrial enzyme activities from sampled *A. islandica* populations ranked from shortest- to longest-lived. (A) Complex I and III (CI+III) activity per moles of citrate synthase (CS), (B) Cytochrome c oxidase (COX) activity normalized by CS, (C) CI+III activity normalized by COX. Values are means ±SEM. Letters denote significant ($p < 0.05$) differences between populations for gills (filled bars) and mantle (empty bars) tissues, while asterisks indicate significant differences between tissues (**: $p < 0.05$, ***: $p < 0.001$).

2.5 Discussion

Mitochondrial resistance to peroxidation and ETS activity linked to ROS production are two important tenets of the MOSTA at the interspecific level (Barja, 2013;Blier et al., 2017). Our study aimed at assessing whether these characters were related to longevity at the intraspecific scale, by taking advantage of the extraordinary MRL diversity across well-studied regional populations of the ocean quahog, *A. islandica*.

2.5.1 Membrane lipid composition does not align with population MRL

Our results show no MRL related pattern of population specific membrane lipid composition. Instead, differences in membrane lipid composition were very subtle, especially in mantle tissue if we compare them to the interspecific differences in Munro and Blier (Munro and Blier, 2012). Likewise, no relationship between plasmalogen or NMI content and MRL emerged from our analysis. We had predicted lower PI in the longer-lived populations of *A. islandica*, whereas in fact, the short-lived, brackish White Sea population had one of the lowest PIs. Furthermore, neither PUFA nor DHA content (by %) were higher in the shorter-lived populations. Instead, both the WS population, with one of the shortest documented lifespans, and IC subarctic populations with very long lifespan had the lowest %DHA and average %PUFA. Both populations are subpolar and, hence, cold adapted and it is interesting that they have on average lower %PUFA in the population comparison instead of relatively higher unsaturation levels to maintain membrane fluidity in their low temperature habitats (see homeoviscous adaptation in poikilotherms, summarized in Storelli et al. 1998). Other important parameters that could be implicated in the aging phenotype of these populations, such as phospholipid classes, sphingolipid or cholesterol content were not included in our analysis and should be assessed in subsequent studies.

Inferring from the lipofuscin content for a standardized 50y old individual across all *A. islandica* populations, Basova and colleagues (Basova et al., 2017)

reported higher rates of fluorescent age pigment, lipofuscin accumulation in the mantle and gills of short-lived (WS, KB) individuals compared to the IC population. Since this pigment is a proxy of physiological aging (Lushchak and Semchyshyn, 2011) and the KB population showed the fastest accumulation of the pigment, followed by the WS, Basova et al. (2017) suggested a high rate of aging in these populations induced by the cellular stress response to large seasonal salinity and temperature amplitude in both brackish habitats. Our results also show similarities between these two brackish sites, with only the PI in gill membranes being markedly lower in WS than KB animals (via lower n-3 PUFA abundance), whereas for all the other longevity-linked lipids, the distribution of the two populations were overlapping in the PCA.

2.5.2 Ratios of ETS and TCA cycle components differ among populations from different salinity backgrounds

In line with our results for membrane composition, the capacities of the ETS compounds, and the ratios between different complexes showed no clear relationship with population MRL. Based on the findings from the interspecific comparison (Munro et al., 2013), we expected decreased $CI+III \cdot CS^{-1}$ or $CI+III \cdot CIV^{-1}$activities with increasing population MRL. Instead, tissues of short-lived KB and WS quahogs exhibited lower activities for both mitochondrial ETS complexes than longer-lived populations, when normalized to CS activity as a marker for mitochondrial volume density and TCA cycle units. In fact, the large diversity in enzymatic complex activities between populations (up to 3 and 4-fold difference for $CI+III \cdot CS^{-1}$ and $COX \cdot CS^{-1}$ respectively) resulted mainly from higher CS activity in short-lived KB and WS populations (Fig S2.2). Hence, contrary to the interspecific comparison, the intraspecific comparison does not support the functional concept of lower upstream (CI + III) *vs* downstream (COX) ETS activity, but revealed an altered balance between ETS and CS complexes in shorter lived *A. islandica* populations. It should however be noted that we did not assess the activity of complex II, another electron entry portal

(succinate) and potential source of ROS (Quinlan et al., 2012), which was shown to have a much lower activity in longer-lived bivalves (Munro et al., 2013).

Relatively higher mitochondrial volume density and more TCA cycle units (such as CS) in tissues of short-lived, brackish water *A. islandica* could result in a higher capacity to generate NADH in the TCA cycle in relation to the capacity to pass electrons down the respiratory system toward COX reduction. Zurburg and De Zwaan (1981) highlighted the fact that (hyper-) osmotic stress causes a general shift towards the usage of anaerobic pathways in marine bivalves. Higher aerobic capacity in brackish water could however help adjust the free amino acid pool by the TCA cycle (glutamate oxidation) and deliver NADH and aspartate for the aspartate–alanine pathway in the cytosol. Frequent alterations between aerobic and anaerobic glycolysis in bivalves exposed to the vagaries of environmental salinity fluctuations typical for Kiel Bight and the White Sea presumably cause higher metabolic demand, oxidative stress and mitochondrial turnover and may be causal for the faster rates of lipofuscin accrual from deteriorating mitochondria in these populations (Rivera-Ingraham and Lignot, 2017). It appears therefore likely that these biochemical peculiarities are determinant in the "fast aging phenotype" in both brackish *A. islandica* populations.

Thus, mitochondrial energetics may play a role in shortening lifespan of the ocean quahog in brackish water environments, and our study suggest a potential role of mitochondria in the aging process of populations, related to their role in osmoregulation. It remains to say that mitochondrial ETS parameters and membrane PI, in all populations of *A islandica* that we analyzed, are very low compared to other bivalves (Munro and Blier, 2012). In the same line of evidence, Strahl *et al.* (Strahl et al., 2007) showed that Icelandic *A. islandica* display one of the lowest growth constants in a comparison of 147 worldwide bivalve populations. This suggests that *A. islandica* has evolved as a long-lived phenotype (see also Moss et al., 2016), including a peroxidation-protected membrane lipid composition across all populations. Recent data (Begum et al., 2018) show very low genetic differences between *A. islandica* from the

same sites as in our study, suggesting an important degree of phenotypic plasticity instead of local genetic adaptation. Population-specific maximum age seems therefore to be a function environmental challenge, biotic and/or abiotic, including salinity stress in brackish populations. Similarly, Basova and colleagues failed to link population-specific antioxidant enzyme capacities and metabolic rate to MRL (MLSP in Basova et al., 2012), and instead found lower antioxidant activity (superoxide dismutase) in habitats with highly variable annual salinity amplitudes. Additionally, higher DNA damage accumulation rates have been reported in the KB compared to the IC population by Gruber et al. (2015). All these aspects underline the strong influence of adaptation to vastly differing habitat conditions across different populations of this species.

2.5.3 Tissue specific differences and dietary influence on mitochondrial membranes and ETS activities

We found important differences between the two tissues, mantle and gills. The lower PI in the gills compared to the mantle tissue in all populations, except the extremely long-lived IC, suggests that the respiratory organs, directly exposed to bottom water oxygen levels during ventilation, might be particularly protected from peroxidation. In addition, gills are exposed to fluctuations of salinity, environmental toxins and pathogens, and appear more exposed and susceptible in a general sense. Consequently, the intensity of apoptotic cell removal in the gills is higher than in mantle tissue of *A. islandica*, as found in the GB and IC populations (Strahl and Abele, 2010). Gill tissue was found to have a higher mitotic index than the mantle in the oyster (Jemaà et al., 2014), and this could translate into differences in accumulation rates of dysfunctional lipids, proteins and DNA. Hence, tissue-specific functions and turnover rates are important to consider when looking at the biochemical determinants of lifespan.

Lifespan in bivalves is strongly linked to latitude (Moss et al., 2016), with longer-lived species and populations generally found at higher latitudes. This could be explained through the physiological effects of lower temperature and light, and hence limited and seasonal food supply at high latitudes, causing prolonged periods of caloric restriction (Moss et al., 2017). As the effect of FA regime on mitochondrial membrane lipids are well established (Guderley et al., 2008;Lemieux et al., 2008), diet might also explain part of the inter-population differences we observed. Few studies have demonstrated an effect of diet on mitochondrial membrane lipid composition in bivalves. Comparing different algal diets of the Pacific oyster *Crassostrea gigas*, Dudognon and colleagues (Dudognon et al., 2014) found changes in various FA classes, especially in DHA and EPA (eicosapentaenoic acid, 20:5, n-3) in gill mitochondria, which however did not cause alterations in gill COX activity, state 3 and state 4 oxygen consumption, or ROS production between diet groups. When studying the effects of diet abundance, temperature and age on the lipid composition of *A. islandica* and the shorter-lived *Spisula solidissima* mitochondria, Munro and Blier (Munro and Blier, 2015) found that although proportions of PUFA and PI increased in both species through microalgae supplementation, the differences between the two in longevity-related parameters (PI and NMI) remained unchanged. These elements suggest that mechanisms regulating membrane composition should be important (reviewed in Hulbert et al., 2014), and the effects of membrane composition modulation on enzymatic activity are not consistently seen across phyla (Lemieux et al., 2008). In the interpretation of our data, a limitation should be considered due to the fact that mitochondria were isolated from frozen tissue (a more uncommon procedure, but see for example Hulbert et al., 2008). Nonetheless, nutrition and abiotic factors in the field likely impact mitochondrial phenotype and function, and form part of the metabolic response to environmental food levels which may also affect life-history traits in marine invertebrates.

At the interspecies level, these environmental conditions could also impact the pace of aging, but on the long term they may not be as critical in setting maximal lifespan that can be reached by a species. For example, Munro et al. (2013) compared five species living in relatively similar environments and comparable optimal and critical temperatures, but with widely divergent maximum lifespans.

2.6 Conclusions

The species *A. islandica* has evolved a long-life phenotype with adjustment of mitochondrial membrane composition, low metabolic activity and control of cellular waste products (Strahl et al., 2007;Strahl and Abele, 2010;Munro et al., 2013;Moss et al., 2016). Our results show that at the intraspecific level, there is no direct relationship between two important mitochondrial components: fatty acids composition of the membrane, enzymatic organization at the level of CI+III, COX and CS, and population-specific MRL. Nonetheless, it appears that contrary to the ratio of upstream to downstream ETS complexes (which is conserved among populations), the enzymatic activities and aerobic capacities (as citrate synthase activity per mg of proteins) are plastic traits among populations. Brackish, coastal environments in KB and WS is associated to an increase of citrate synthase maximal activity relative to ETS capacities suggesting elevated rates of substrate oxidation. This might better support both osmoregulation and anaerobic energy metabolism under fluctuant salinity and temperature conditions in these habitats.

Differences in population longevity appear to be independent of sampling effort (see for example Gruber et al., 2015) and, although no precise record of age at maturity from our sites could be found, data from the western Atlantic suggests that it is variable among populations (Thompson et al., 1980) and likely correlated to longevity as it is in different bivalve species (Ridgway et al., 2011a). As our understanding of oxidative stress increases, it appears that mitochondrial ROS management is at the center of modulation of life-history traits (such as reproduction and growth, see Costantini,

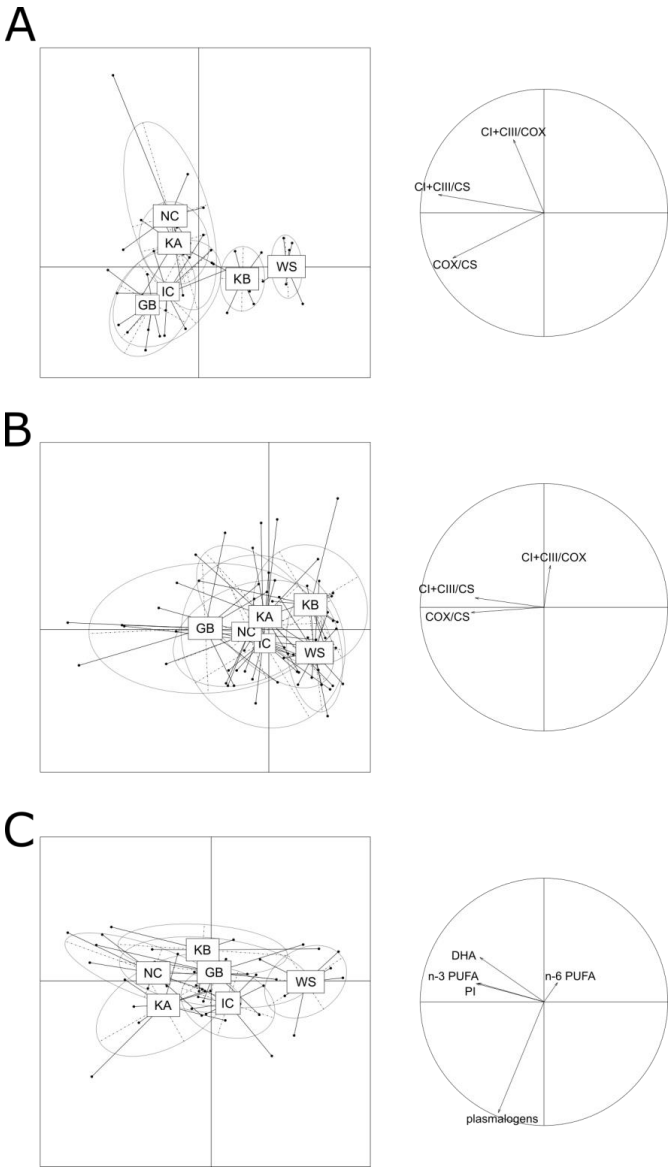
2018). We then suggest that the divergences in observed MRL among populations should not only be the consequence of detrimental effects of stressful conditions, but could also result from the adjustment of maturation to insure completion of the life cycle in the different environmental conditions experienced by the populations. It seems that *A. islandica* populations are able to deal with a large range of environmental conditions at the expense of MRL. We suspect that all *A. islandica*, independently of the population they belong to, appear to have the potentiality to reach extreme longevity. However, we cannot exclude that reaching such high longevity as 507 years old would require very special dispositions, even for a centenarian bivalve, as particular environmental or ecological conditions (such as predation pressure, see Moss et al., 2017), specific mitochondrial phenotypes and/or peculiar genetic predispositions.

The results of the present study clearly reveal that characters that have been tightly associated to divergences of lifespan among species namely PI of mitochondrial membranes cannot explain the observed divergences among populations of the longest-lived species *A. islandica*. It therefore rules out the proposal that variation in PI could alone manage pace of aging or dictate the expressed lifespan of populations. High metabolic demands requested by stressful and viable environments could partly explain these populations' longevity divergences, but they cannot only result from the impact of stress on physiological conditions, since divergences in lifespan occur with adjustments of the age at reproduction. These bivalves are thus a useful system to disentangle the metabolic characters modulating reproduction and linking maturation to lifespan and provide support or challenge aging theories. Integrating field observation and experimentation with results obtained in the laboratory is the only way to tackle this crucial question (Austad, 2018).

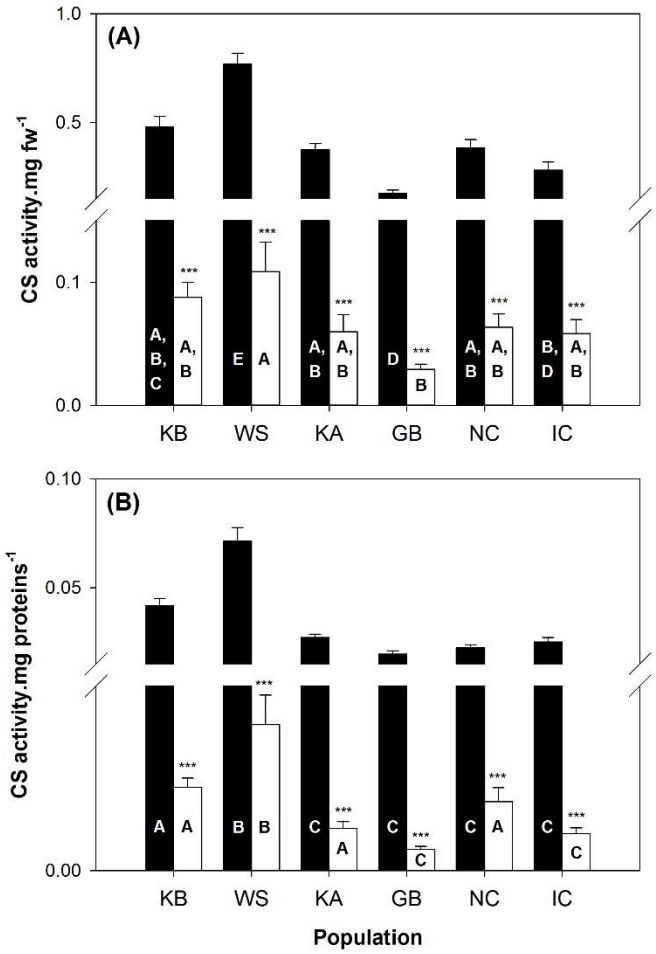
Future studies shall investigate the biochemical adjustments in mitochondria under range edge conditions, including their response to fluctuating salinities and hypoxia in terms of ROS production and the use of alternative electron transport pathways. Other mitochondrial determinants of these lifespan differences have been

proposed in recent studies and should be explored. These potential candidates include different phospholipid species such as cardiolipin (reviewed in Paradies et al., 2010), the stability of ETS supercomplexes (Gómez and Hagen, 2012), the control of electron flux and ROS management (Blier et al., 2017;Munro and Treberg, 2017), and the rates of mtDNA mutations (reviewed in Pinto and Moraes, 2015).

SUPPLEMENTARY MATERIAL FOR CHAPTER II



Supporting figure S2.1 Between group principal component analyses (PCA) on European populations (KB: Kiel Bay, KA: Kattegat, GB: German Bight, WS: White Sea, NC: Norwegian Coast and IC: Iceland) of *A. islandica* according to enzymatic activities in the gills (A), enzymatic activities in the mantle (B) and membrane lipids linked to longevity in the gills (C). Circle at the right of the plot describes the physiological meaning of axes 1 and 2 of between-groups PCA.



Supporting figure S2.2 (A) Citrate synthase activity normalized by tissue fresh weight and (B) by mg of proteins from sampled *A. islandica* populations ranked from shortest- to longest-lived. Values are means ±SEM. Letters denote significant ($p < 0.05$) differences between populations for gills (filled bars) and mantle (empty bars) tissues, while asterisks indicate significant differences between tissues (**: $p < 0.05$, ***: $p < 0.001$).

Table S2.2 Fatty acid and DMA composition (mol%) of phospholipids from gill mitochondria in European populations of *Arctica islandica*

	Kiel Bay (n = 9)	White Sea (n = 8)	Kattegat Sea (n = 9)	German Bight (n =9)	Norwegian Coast (n = 8)	Icelandic Coast (n = 8)
9:0	1.0 ± 0.3	2.1 ± 1.0	1.0 ± 0.3	2.0 ± 0.6	0.6 ± 0.1	1.0 ± 0.3
14:0	1.1 ± 0.2	1.5 ± 0.3	0.9 ± 0.1	1.3 ± 0.4	1.0 ± 0.1	0.9 ± 0.2
15:0	0.6 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.6 ± 0.2	0.5 ± 0.1	0.4 ± 0.1
16:0	21.4 ± 1.8	18.6 ± 1.9	17.3 ±2.2	20.3 ± 2.4	19.4 ± 1.7	17.3 ± 1.3
17:0	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.1 ± 0.0	1.3 ± 0.1
18:0	22.3 ± 1.4	24.8 ± 2.9	17.6 ± 1.8	22.3 ± 2.1	19.9 ± 1.9	22.6 ± 2.2
18:1 n-13	2.1 ± 0.1	2.5 ± 0.3	3.9 ± 0.7	2.7 ± 0.4	2.2 ± 0.2	3.3 ± 0.3
18:1 n-9	1.6 ± 0.2	1.5 ± 0.2	1.4 ± 0.2	1.7 ± 0.2	1.5 ± 0.2	1.4 ± 0.1
18:1 n-7	0.6 ± 0.1	0.4 ± 0.1	0.9 ± 0.1	0.4 ± 0.1	0.9 ± 0.1	1.2 ± 0.2
18:2 n-6	0.5 ± 0.2	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.2 ± 0.1
20:1 n-11	1.1 ± 0.6	0.2 ± 0.1	0.2 ± 0.0	0.6 ± 0.3	0.3 ± 0.1	0.3 ± 0.1
20:1 n-9	0.2 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.1
20:1 n-7	1.0 ± 0.0	0.8 ± 0.1	1.1 ± 0.2	0.9 ± 0.1	1.1 ± 0.2	1.0 ± 0.1
20:2 n-6	0.7 ± 0.0	0.7 ± 0.1	0.7 ± 0.2	0.5 ± 0.0	0.9 ± 0.2	0.5 ± 0.1
20:3 n-6	2.0 ± 1.0	4.6 ± 1.6	2.3 ± 0.3	2.5 ± 1.2	2.1 ± 0.3	2.1 ± 0.5
20:4 n-6	1.7 ± 0.4	1.2 ± 0.2	1.7 ± 0.3	1.6 ± 0.2	1.3 ± 0.3	1.3 ± 0.2
20:5 n-3	3.6 ± 0.3	1.2 ± 0.4	3.8 ± 0.3	3.0 ± 0.5	5.0 ± 1.2	4.0 ± 0.3
22:4 n-6	1.1 ± 0.3	0.6 ± 0.1	0.7 ± 0.1	1.0 ± 0.2	0.5 ± 0.2	0.5 ± 0.1
22:5 n-6	0.7 ± 0.3	0.8 ± 0.2	0.5 ± 0.1	0.7 ± 0.2	0.7 ± 0.2	0.6 ± 0.1
22:5 n-3	0.4 ± 0.2	0.6 ± 0.1	1.0 ± 0.2	0.8 ± 0.2	1.0 ± 0.1	0.8 ± 0.2
22:6 n-3	8.1 ± 0.5	4.6 ± 1.0	7.4 ± 0.5	6.0 ± 0.6	7.1 ± 0.9	6.2 ± 0.5
NMID 20:2 (Δ5, 11)	7.1 ± 0.5	6.3 ± 1.6	7.6 ± 1.2	7.1 ± 0.7	6.7 ± 1.0	8.2 ± 0.6
NMID 20:2 (Δ5, 13)	1.6 ± 0.1	2.2 ± 0.3	3.5 ± 0.7	1.7 ± 0.1	2.0 ± 0.5	2.1 ± 0.3
NMID 22:2 (Δ7, 13)	1.1 ± 0.1	0.6 ± 0.1	0.9 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.9 ± 0.1
NMID 22:2 (Δ7, 15)	0.3 ± 0.1	0.4 ± 0.2	0.0 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
NMIT 22:3 (7, 13, 16)	1.5 ± 0.8	4.5 ± 1.3	2.1 ± 0.8	2.5 ± 1.1	1.7 ± 0.3	1.9 ± 0.4
DMA 16:0	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.1
DMA 17:0 iso II	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
DMA 17:0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
DMA 18:0 iso I	0.7 ± 0.3	0.3 ± 0.2	0.6 ± 0.2	0.8 ± 0.2	0.7 ± 0.3	0.6 ± 0.2

DMA 18:0	1.0 ± 0.5	1.7 ± 0.3	3.4 ± 0.5	2.2 ± 0.7	2.9 ± 0.5	2.4 ± 0.6
DMA 19:0 iso	0.3 ± 0.1	0.8 ± 0.2	0.5 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
DMA 20:1 iso II	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
DMA 20:1	9.6 ± 0.5	7.6 ± 1.0	12.4 ± 1.5	9.1 ± 0.8	10.2 ± 1.0	12.0 ± 0.8
Branched FA	2.6 ± 0.3	1.9 ± 0.4	2.6 ± 0.2	2.1 ± 0.5	3.2 ± 0.5	1.7 ± 0.3
Fatty acids < 0.5%	2.3 ± 0.3	4.5 ± 1.4	3.1 ± 0.4	3.4 ± 0.9	3.4 ± 0.4	3.5 ±0.3
SFA	50.2 ± 2.3	51.7 ± 3.7	40.7 ± 3.9	50.5 ± 3.0	46.0 ± 2.8	46.0 ± 3.0
MUFA	6.5 ± 0.7	5.7 ± 0.7	8.0 ± 0.8	6.5 ± 0.5	6.4 ± 0.4	7.4 ± 0.2
PUFA	31.0 ± 1.7	30.7 ± 3.0	33.6 ± 2.1	29.9 ± 2.0	31.3 ± 1.8	30.3 ± 1.8
n-6 PUFA	6.7 ± 1.0	8.1 ± 1.5	6.1 ± 1.0	6.5 ± 1.2	5.9 ± 0.6	5.1 ± 0.5
n-3 PUFA	12.6 ± 0.8	6.7 ± 1.5	12.5 ± 0.8	10.4 ± 1.2	13.9 ± 1.7	11.5 ± 0.9
n-3 PUFA (%PUFA)	41.2 ± 2.4	22.2 ± 3.7	37.6 ± 2.4	35.3 ± 3.8	43.7 ± 3.7	38.0 ± 1.7
PUFA (without NMI)	19.3 ± 1.1	14.8 ± 1.9	18.6 ± 1.3	16.9 ± 1.4	19.8 ± 1.7	16.7 ± 1.2
NMI total	11.7 ± 0.8	15.8 ± 2.3	15.0 ± 1.2	13.0 ± 1.1	11.6 ± 0.6	13.7 ± 0.9
NMI (%PUFA)	37.6 ± 1.1	51.0 ± 4.1	44.4 ± 2.0	43.8 ± 2.7	37.5 ± 2.2	45.1 ± 1.6
DMA < 0.5%	0.1 ± 0.1	0.2 ± 0.2	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.2 ± 0.1
Branched DMA (iso)	1.2 ± 0.4	1.9 ± 0.2	1.5 ± 0.3	1.4 ± 0.4	1.7 ± 0.6	1.2 ± 0.2
DMA total	12.3 ± 1.0	12.0 ± 0.9	17.7 ± 2.2	13.1 ± 1.3	16.3 ± 1.9	16.3 ± 1.4
Unsaturation index	135.2 ± 6.3	115.9 ± 10.8	143.4 ± 7.6	125.4 ± 7.4	137.5 ± 9.0	130.6 ± 7.7
Peroxidation index	116.0 ± 5.5	77.5 ± 10.3	113.9 ± 5.7	99.2 ± 7.8	117.9 ± 11.2	102.0 ± 7.2
MRL (years)	36	53	71	150	300	507

Table S2.3 Fatty acid and DMA composition (mol%) of phospholipids from mantle mitochondria in European populations of *Arctica islandica*

	Kiel Bay (n = 9)	White Sea (n = 7)	Kattegat Sea (n = 9)	German Bight (n = 9)	Norwegian Coast (n = 9)	Icelandic Coast (n = 8)
9:0	1.2 ± 0.2	1.23 ± 0.3	1.6 ± 0.7	1.2 ± 0.3	0.7 ± 0.2	1.8 ± 0.8
14:0	1.0 ± 0.2	1.7 ± 0.3	0.8 ± 0.2	1.5 ± 0.2	1.0 ± 0.1	1.5 ± 0.2
15:0	0.6 ± 0.1	0.7 ± 0.2	0.5 ± 0.1	0.9 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
16:0	18.7 ± 2.0	25.1 ± 3.1	18.6 ± 1.2	19.9 ± 1.5	17.4 ± 1.6	20.5 ± 2.0
17:0	1.0 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
18:0	20.4 ± 2.0	25.3 ± 3.1	24.6 ± 1.9	23.2 ± 2.4	18.5 ± 1.2	25.4 ± 2.4
18:1 n-13	1.6 ± 0.2	0.9 ± 0.2	1.5 ± 0.2	1.3 ± 0.1	1.9 ± 0.2	1.5 ± 0.3
18:1 n-9	2.0 ± 0.2	1.9 ± 0.2	2.0 ± 0.1	2.8 ± 0.4	2.2 ± 0.2	1.8 ± 0.1
18:1 n-7	0.6 ± 0.1	0.6 ± 0.2	0.8 ± 0.1	0.7 ± 0.3	1.0 ± 0.1	0.8 ± 0.1
18:2 n-6	0.4 ± 0.2	0.3 ± 0.1	0.5 ± 0.3	0.3 ± 0.1	0.6 ± 0.2	0.5 ± 0.2
20:1 n-11	0.2 ± 0.2	0.5 ± 0.4	0.4 ± 0.2	0.6 ± 0.3	0.2 ± 0.1	1.0 ± 0.9
20:1 n-9	0.4 ± 0.2	0.2 ± 0.0	0.4 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0
20:1 n-7	0.7 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
20:2 n-6	1.0 ± 0.3	0.5 ± 0.1	1.8 ± 0.9	0.3 ± 0.1	1.1 ± 0.4	0.7 ± 0.1
20:3 n-6	4.6 ± 1.3	4.1 ± 1.3	2.3 ± 0.8	4.2 ± 1.8	2.8 ± 0.7	3.8 ± 1.1
20:4 n-6	1.6 ± 0.4	1.7 ± 0.5	3.3 ± 0.7	1.5 ± 0.4	1.5 ± 0.3	1.5 ± 0.4
20:5 n-3	4.0 ± 0.7	2.8 ± 0.6	4.7 ± 0.6	4.0 ± 0.6	6.8 ± 0.6	2.9 ± 0.5
22:4 n-6	1.2 ± 0.5	0.6 ± 0.2	0.8 ± 0.4	1.3 ± 0.6	1.1 ± 0.4	0.4 ± 0.1
22:5 n-6	1.2 ± 0.2	1.1 ± 0.3	0.8 ± 0.2	1.9 ± 1.0	0.6 ± 0.3	1.5 ± 0.6
22:5 n-3	0.9 ± 0.1	0.7 ± 0.2	1.0 ± 0.2	0.5 ± 0.1	0.9 ± 0.2	0.7 ± 0.2
22:6 n-3	10.2 ±1.1	7.4 ± 1.6	8.7 ± 1.2	9.3 ± 1.3	10.3 ± 0.8	5.6 ± 0.7
NMID 20:2 (Δ5, 11)	4.5 ± 0.8	3.1 ± 0.8	3.7 ± 0.5	4.1 ± 0.7	7.5 ± 0.8	3.8 ± 0.8
NMID 20:2 (Δ5, 13)	1.7 ± 0.3	1.3 ± 0.3	2.2 ± 0.5	1.3 ± 0.2	1.4 ± 0.1	1.4 ± 0.2
NMID 22:2 (Δ7, 13)	0.7 ± 0.2	0.6 ± 0.1	0.9 ± 0.6	0.3 ± 0.1	0.7 ± 0.3	0.4 ± 0.1
NMID 22:2 (Δ7, 15)	0.1 ± 0.1	0.3 ± 0.2	0.3 ± 0.1	0.4 ± 0.2	0.0 ± 0.0	0.8 ± 0.7
NMIT 22:3 (7, 13, 16)	3.1 ± 1.3	3.6 ± 0.9	1.7 ± 0.7	3.5 ± 1.7	1.6 ± 0.5	4.2 ± 1.1
DMA 16:0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1
DMA 17:0 iso II	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
DMA 17:0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
DMA 18:0 iso I	1.0 ± 0.4	1.0 ± 0.3	0.9 ± 0.3	0.7 ± 0.4	0.7 ± 0.3	0.1 ± 0.0
DMA 18:0	1.8 ± 0.4	1.1 ± 0.3	1.7 ± 0.4	0.9 ± 0.3	2.3 ± 0.5	1.7 ± 0.5
DMA 19:0 iso	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.1 ± 0.0	0.5 ± 0.1	0.2 ± 0.1

DMA 20:1 iso II	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.3 ± 0.3
DMA 20:1	8.0 ± 1.1	5.0 ± 1.2	7.2 ± 0.9	6.8 ± 1.0	9.3 ± 0.9	6.0 ± 0.8
Branched FA	2.5 ± 0.39	2.0 ± 0.3	2.1 ± 0.2	2.2 ± 0.4	1.7 ± 0.2	1.4 ± 0.2
Fatty acids < 0.5%	3.4 ± 0.57	2.7 ± 0.4	4.0 ± 1.2	2.2 ± 0.4	3.6 ± 0.6	4.6 ± 1.4
SFA	45.6 ± 3.4	57.4 ± 6.3	49.5 ± 2.7	50.4 ± 2.0	41.5 ± 2.2	52.8 ± 3.5
MUFA	5.3 ± 0.4	4.8 ± 0.5	5.9 ± 0.4	6.6 ± 0.7	6.4 ± 0.3	6.3 ± 1.0
PUFA	36.6 ± 2.2	29.1 ± 4.2	33.6 ± 1.7	33.6 ± 1.9	38.1 ± 1.1	30.8 ± 3.0
n-6 PUFA	10.0 ± 1.5	8.4 ± 1.3	9.4 ± 1.4	9.5 ± 1.6	7.6 ± 0.9	8.3 ± 0.8
n-3 PUFA	15.8 ± 1.5	11.3 ± 2.3	15.1 ± 1.5	14.3 ± 1.7	19.0 ± 1.4	9.7 ± 1.2
n-3 PUFA (%PUFA)	44.3 ± 4.4	37.9 ± 4.6	45.0 ± 3.9	43.5 ± 5.0	49.7 ± 3.1	32.3 ± 4.1
PUFA (without NMI)	25.9 ± 1.5	19.7 ± 2.9	24.5 ± 1.1	23.8 ± 1.5	26.6 ± 1.4	18.0 ± 1.4
NMI total	10.8 ± 1.2	9.4 ± 1.3	9.1 ± 1.0	9.8 ± 1.1	11.5 ± 1.0	12.8 ± 2.0
NMI (%PUFA)	29.0 ± 2.0	32.7 ± 1.7	26.8 ± 2.2	29.1 ± 2.6	30.3 ± 2.8	40.4 ± 3.6
DMA < 0.5%	0.0 ± 0.0	0.2 ± 0.2	0.1 ± 0.1	0.2 ± 0.1	0.0 ± 0.0	0.1 ± 0.1
Branched DMA (iso)	2.0 ± 0.6	2.0 ± 0.4	1.7 ± 0.4	1.2 ± 0.5	1.7 ± 0.5	1.7 ± 0.3
DMA total	12.4 ± 2.1	8.7 ± 1.7	10.9 ± 1.3	9.5 ± 0.6	14.0 ± 1.9	10.1 ± 1.5
Unsaturation index	160.9 ± 8.5	125.2 ± 19.2	148.4 ± 8.2	151.8 ± 8.0	170.1 ± 5.5	125.8 ± 9.9
Peroxidation index	145.0 ± 10.9	109.6 ± 18.6	138.1 ± 11.2	138.2 ± 12.2	157.0 ± 8.9	99.1 ± 6.9
MRL (years)	36	53	71	150	300	507

CHAPTER III
DIVERGENCES IN THE CONTROL OF MITOCHONDRIAL RESPIRATION ARE ASSOCIATED WITH LIFESPAN VARIATION IN MARINE BIVALVES

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3.1 Summary

The role played by mitochondrial function in the aging process has been a subject of intense debate in the past few decades, as part of the efforts to understand the mechanistic basis of longevity. The mitochondrial oxidative stress theory of aging (MOSTA) suggests that a progressive decay of this organelle's function leads to an exacerbation of oxidative stress, with deleterious impact on mitochondrial structure and DNA, ultimately promoting aging. Among the traits suspected to be associated with longevity is the variation in regulation of oxidative phosphorylation, potentially impacting the management of oxidative stress. Longitudinal studies using the framework of metabolic control analysis have shown age-related differences in flux control of respiration, but this approach has seldom been taken on a comparative scale. Using four species of marine bivalves exhibiting a large range of maximum lifespans (from 28y to 507y), we report lifespan-related differences in flux control at different steps of the electron transfer system. Increased longevity was characterized by a lower control by NADH- (complex I-linked) and Succinate- (complex II- linked) pathways, while respiration was strongly controlled by complex IV when compared to shorter-lived species. Complex III exerted a strong control over respiration in all species. Furthermore, high longevity was associated with higher citrate synthase activity, and lower ATP synthase activity. Relieving the control exerted by the electron entry pathways could be advantageous for reaching a higher longevity, leading to an increased control by complex IV, the final electron acceptor in the electron transfer system.

3.2 Introduction

The fundamental mechanisms associated with longevity have captivated ancient philosophers and modern biologists alike, and their intricacies are still subject of intense debate. Mounting evidence from longitudinal and comparative studies point toward mitochondrial structure and function, particularly the electron transfer system (ETS) and its involvement in reactive oxygen species production (ROSp). The mitochondrial oxidative stress theory of aging (MOSTA) posits that ROS-induced damage to lipids, proteins and mitochondrial DNA ultimately lead to accumulation of dysfunctional macromolecules and attendant deficits in mitochondrial function. Accumulation of oxidative damage is not only associated with ROS appearance but also with attenuated repair mechanisms and species-dependent loss of antioxidant defenses (Deepashree et al., 2019;Munro et al., 2019). Ultimately, a vicious cycle of increasing damage leading to morbidities and mortality ensue (reviewed in Blier et al. 2017). Despite suffering from criticism often linked to the role of antioxidants and ROS in cellular function (Stuart et al. 2014, Barja 2013) , evidence points toward a link between mitochondrial membrane resistance to oxidative insult, regulation of ROS, mtDNA damage control, and lifespan in a variety of species (Hulbert, 2010;Shabalina et al., 2017;Munro et al., 2019).

Recent support of the MOSTA comes from studies comparing the longest-lived non-colonial metazoan, the ocean quahog *A. islandica*, to other shorter-living marine bivalves. *A. islandica* has a maximum reported longevity (MRL) of 507 years in the North Atlantic (Butler et al., 2013). This is far above the MRLs of three closely related and similar-sized species: *Mya arenaria* (28 y), *Spisula solidissima* (37 y), and *Mercenaria mercenaria* (106 y, Munro and Blier, 2012) . The ocean quahog's case of extreme longevity comes with a more peroxidation-resistant mitochondrial membrane and lower ROSp (measured as H_2O_2 efflux) compared to the other bivalves, but similar capacities for oxidative phosphorylation (OXPHOS, Munro and Blier 2012, Munro et al., 2013). Interestingly, the low ROSp of *A. islandica* is not the result of low OXPHOS

capacity or reduced protonmotive force, because it occurs in substrate conditions associated with high respiratory coupling efficiency, i.e., high respiration rates and low proton leak. This suggests that this peculiar species has not only evolved a strong antioxidant system (see Abele et al. 2009), but also a mitochondrial phenotype where a low proportion of oxygen is diverted to ROSp. The exact mechanisms behind this feat, however, remain obscure.

A possible mechanism by which long-lived species may be able to maintain low rates of ROSp without compromising respiration may be through a rearrangement of the stoichiometry of the ETS and the enzymes of the tricarboxylic acid cycle (TCA). Indeed, activities of key ETS complexes normalized to mitochondrial content differed clearly between the short-lived *M. arenaria* and the long-lived *A. islandica*. Complexes I (NADH:ubiquinone oxidoreductase), I-III segment (coenzyme Q – cytochrome c oxidoreductase), and II (succinate dehydrogenase) were all markedly lower in the longest-lived species, while a similar activity was found for the terminal electron acceptor enzyme, complex IV (cytochrome *c* oxidase). Complexes I and II are especially relevant as the important electron entry complexes into the NADH- and Succinate-pathways, respectively. Depending on the substrate oxidized, the flavin and quinone reduction sites of complex I (I_F and I_Q), and quinol oxidation site in complex III (III_{Qo}) are among the sites reaching maximal rates of superoxide production leading to maximal rates of H_2O_2 efflux, while evidence points to a substantial contribution of the flavin site at complex II (site II_F, Quinlan et al. 2013). Hence, the lower activities of complexes I, II and complexes I-III segment in longer-lived species could entail a lower diversion of electrons to these ROS-producing sites and explain the smaller rates of ROSp (Lambert et al., 2010;Miwa et al., 2014).

Investigating the topologies of the ETS complexes and the enzymatic control of electron flow among species of diverging lifespans could prove useful to determine the mitochondria involvement in animal longevity (Blier et al., 2017). This can be achieved using the framework of metabolic control analysis (MCA), which measures

the specific control strength by a step in a pathway (see Groen et al., 1982 and references therein). Increased control strength by a specific step is thus associated with a higher sensitivity of the overall pathway flux to the reduction in the capacity of this step (Letellier et al., 1993). This method has been useful in detecting mitochondrial dysfunctions in the context of pathologies and age-related diseases; a redistribution of control can be linked to the onset of disease or development of aging (reviewed in Murphy, 2001 and Mazat et al., 2013). Complex I is often found as a determinant site: an increase in control by this step has been shown to cause OXPHOS dysfunction and mitochondrial ROSp (Kuznetsov et al., 1997;Ventura et al., 2002;Sipos et al., 2003). Nonetheless, the understanding of the regulatory mechanisms of OXPHOS under physiological conditions remains incomplete (Arnold, 2012). To our knowledge, no comparative studies have investigated the control of respiration in relation to lifespan differences among species.

We used the framework of metabolic control analysis to study the control of electron flux in the mitochondrial ETS and its possible link with lifespan in the mantle, a predominantly aerobic tissue composed of secretory, sensory and muscular cells (Gosling, 2015b). Based on the lower activity of the complexes and enzymes associated with electron entry in *A. islandica*, compared to short-lived species *M. arenaria* (Munro et al., 2013), but conserved OXPHOS capacity and a similar ratio of complex IV to CS, we predicted that the distribution of control on respiration should diverge in longer-lived species.

3.3 Experimental procedures

3.3.1 Bivalve collection and mitochondrial isolation

Bivalve species were either sampled at low tide (*Mya arenaria*, *Mercenaria mercenaria*), or collected by professional divers (*Spisula solidissima, Arctica islandica*). They were maintained in flow-through tanks at 8 ºC for at least a month before experiments. Mitochondrial isolation was carried out as previously described

(Munro & Blier, 2012) with a modified isolation buffer (Moyes et al., 1985) in which KCl was replaced with NaCl. Approximately 8 g of mantle were pooled from 2-4 individuals, and rinsed in the isolation buffer (400 mM Sucrose, 70 mM HEPES, 50 mM NaCl, 6 mM EGTA, 3 mM EDTA, 10 mg/ml aprotinin, 1% [w/w] bovine serum albumin), minced and homogenized using a glass-Teflon potter on ice. The homogenate was centrifuged at 4 ºC twice at 1250 *g* for 10 min, and the supernatant was centrifuged at 10500 *g*. The resulting mitochondrial pellet was resuspended with a 0.5% BSA isolation buffer, centrifuged again and resuspended in about 500 µl of buffer per 4 g of tissue wet weight. Protein concentration was immediately determined using the Biuret test prior to respirometry.

3.3.2 Pathway control of mitochondrial respiratory capacity

Mitochondrial respiration was quantified at 10 ºC using a substrate-uncoupler-inhibitor-titration (SUIT) protocol in an Oxygraph-2K (Oroboros Instruments Inc., Innsbruck, Austria). The maximal capacity of each specific pathway (NADH-, succinate-, or complex III-pathways) or step (complex IV) was measured under electron transfer (ET) state after uncoupling in one chamber of the O2k. Once the flux was stabilized to the maximal capacity, a titration with a specific inhibitor of the pathway or step was performed. Simultaneously, the effect of the same inhibitor titration was measured in the other chamber on the combined pathways flux under coupled OXPHOS state. All the substrate concentrations were previously tested in bivalves to ensure saturation (maximal respiration rates).

Combined pathways flux under coupled OXPHOS state (NSGp-pathway)

Flux thorough the combined pathways was measured with successive addition of substrates feeding three pathways: NADH-pathway (25 mM glutamate and 2 mM malate), succinate-pathway (10 mM succinate), and glycerol-3-phosphate-pathway (10 mM glycerol-3-phosphate). ADP (5 mM) was added immediately after the NADH-pathway substrates. The integrity of the outer mitochondrial membrane was diagnosed

by adding cytochrome *c* (10 µM) after ADP. The specific inhibitor titration, as described for each pathway and steps below, were then applied to that maximal combined pathways flux.

NADH-pathway under ET state

The maximum NADH-pathway (N-pathway) capacity was measured in the presence of glutamate (25 mM), malate (2 mM) and ADP (5 mM). Uncoupling with an optimal concentration of FCCP was performed (final concentration between 0.5 and 0.75 µM) to ensure maximal flux through the pathway. Rotenone (0.01 – 600 nM) was then added in successive steps until maximal inhibition of the N-pathway.

Succinate-pathway under ET state

Succinate-pathway (S-pathway) was measured in the presence of succinate (10 mM), rotenone (1 µM) and ADP (5 mM). Maximum pathway capacity was obtained by titration up to an optimum concentration of FCCP (0.5 - 0.75 µM). Inhibition of S-pathway was done by successive addition of malonate (0.5 – 11 mM).

Complex III-pathway under ET state

Complex III-pathway was measured with ADP (5 mM) and reduced coenzyme Q_2 (CoQ_2, 15 $\mu g.ml^{-1}$, Mathers et al., 2017). Maximum CIII-driven ET capacity was obtained by titration up to an optimal concentration of FCCP (0.5 - 0.75 µM). Inhibition was achieved through stepwise additions of antimycin A (1.25 nM – 3.31 µM).

Complex IV under ET state

Complex IV capacity was measured following the same protocol as in the combined pathways flux under coupled OXPHOS state (above), followed by titration with optimal FCCP concentration (0.5 - 0.75 µM; ET state), inhibition of complex III (2.5 µM antimycin A), alternative oxidase (100 µM n-propyl gallate), and complex I (1 µM rotenone), and addition of ascorbate (2 mM) and N,N,N',N'-Tetramethyl-p-

phenylenediamine dihydrochloride (TMPD, 0.5 mM). Complex IV inhibition was done by titration with freshly prepared potassium cyanide (KCN, 0.05 – 102 µM).

Enzymatic activities of citrate synthase, CIV and ATP synthase

Spectrophotometric measurements of the enzymatic activities were done on frozen aliquots of mitochondrial isolates from the same individuals used for the respirometry assays. CS and free CIV activities were measured at 10 ºC in a UV/VIS spectrophotometer (Ultrospec 2100 pro, Biochrom Ltd, Cambridge, UK) with an adapted version of the protocols established by Thibault et al. (1997). For the CIV assay, cytochrome *c* was reduced in 4.5 mM sodium dithionite. ATP synthase activity was done using a modified version of the protocol developed by Barrientos et al. (2009). The medium contained 250 mM sucrose, 20 mM HEPES and 5 mM $MgSO_4$ (pH 8.0) to which 0.35 mM NADH, 2.5 mM phosphoenolpyruvate, 2.5 mM ATP, 5 µM antimycin A, 4 units/ml each of lactate dehydrogenase and pyruvate kinase, and 3 µM oligomycin were added. The absorbance was read a 340 nm for 4 minutes.

3.3.3 Data analysis and calculation of flux control ratios

Datlab software was used for data analysis (Oroboros Instruments, Austria). Instrumental and chemical backgrounds were calibrated as a function of oxygen concentration and subtracted from the oxygen fluxes. Preliminary experiments validated the replacement of KCl by NaCl in the isolation buffer and the quality of mitochondrial isolates obtained, as respiration never increased over the 15% threshold upon cyt*c* addition (*A. islandica* NaCl = 10.39% [5.37-14.01], KCl = 6.32% [0.94-11.78]; *M. arenaria* NaCl = 1.06% [0-2.37], KCl = 5.87% [0-14.92]; n=4 per species).

We calculated flux control coefficients for each individual and species as the ratio of the initial slope of the global flux (J) over the initial slope of the isolated pathway activity (v_i) titrated with inhibitor I $\frac{(\frac{dJ/J}{dI})}{(\frac{dvi/vi}{dI})}$ (Groen et al., 1982).

3.3.4 Statistical analyses

Differences between groups were assessed via ANOVAs with Tukey's post-hoc HSD using Systat v. 13 (Systat Software, San Jose, CA). Variables were first tested for normality using the Shapiro-Wilk test. Figures were made using Sigmaplot v.12 (Systat Software, San Jose, CA) and Statistica 64 (Statsoft, Tulsa, OK).

3.4 Results

3.4.1 Pathway control of mitochondrial respiratory capacity

We measured respiration in mantle mitochondrial isolates using a SUIT protocol and calculated the flux control ratios (FCRs) normalized for maximal uncoupled respiration (ET capacity). Because the FCRs of the various steps did not significantly differ among species, we grouped the median responses for all 4 species in fig. 3.1A. The FCR for the N-pathway was 0.38 (0.24-0.87). Adding the succinate pathway to the N-pathway had a strong stimulatory effect on respiration, reaching a FCR of 0.56 (0.43-0.93). The effect was even stronger by the addition of the Gp pathway ($NSGp_P$), with a FCR of 1.00 (0.38-1.34). The addition of the uncoupler FCCP produced either very little or no increase in respiration, suggesting low or no limitation of OXPHOS by the phosphorylation system (ATP synthase, ADP/ATP transport, or phosphate carrier) in these bivalves. The contributions of N-, S- and Gp-pathways in the OXPHOS state, calculated as N/NSGp, NS-N/NSGp, and NSGp-NS/NSGp, are detailed in fig. 3.1B. *M. arenaria* and *S. solidissima* display a significantly higher N-pathway contribution compared to either *M. mercenaria* or *A. islandica* (MA vs AI: $p = 0.008$; SS vs AI: $p = 0.016$; MA vs MM: $p = 0.001$; SS vs MM: $p = 0.003$). The S-pathway contribution to maximal OXPHOS was similar among all species. The Gp-pathway contribution was stronger in *A. islandica* and *M. mercenaria* compared to *S. solidissima* ($p = 0.016$ and 0.008, respectively).

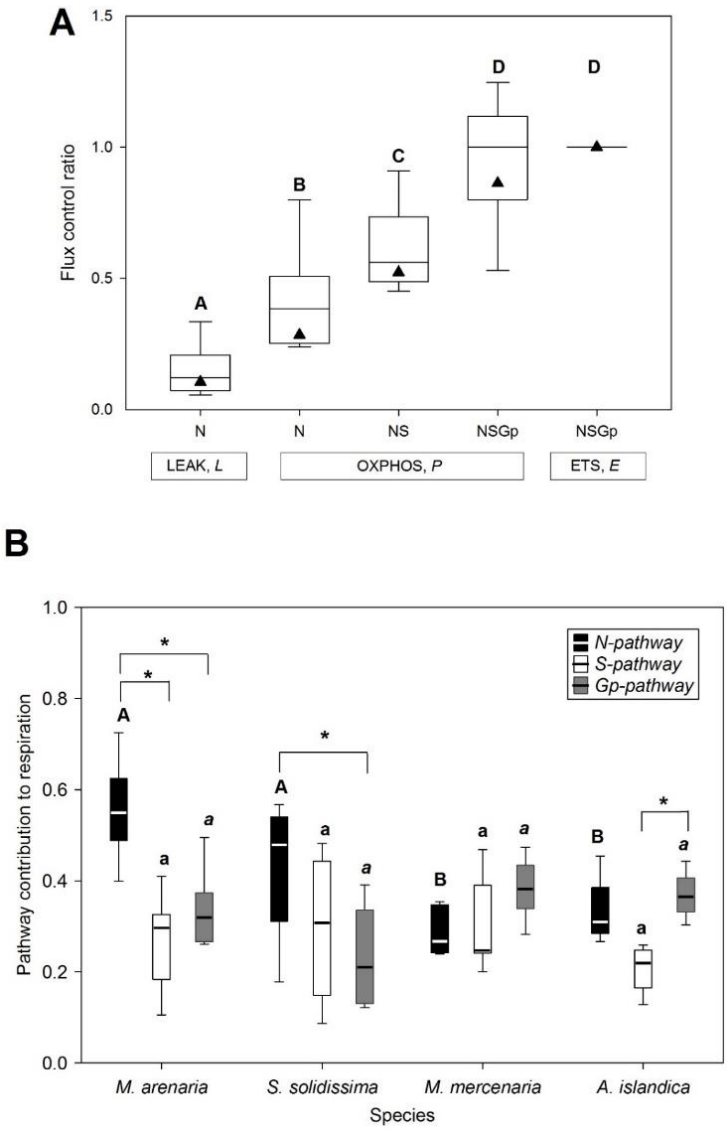

Figure 3.1 (A) Flux control ratios normalized for NSGp-linked pathway capacity in isolated mitochondria from 4 species of marine bivalves. Box plots show minimum, 25th percentile, median, 75th percentile, and maximum for 3 species: *M. arenaria*, *S. solidissima*, and *M. mercenaria*, while *A. islandica* means are represented as filled triangles (n = 3-5 individuals per species). N: NADH-pathway, S: succinate-pathway, Gp: glycerol-3-phosphate-pathway. (B) Contribution of NADH (N-), succinate (S-) and glycerol-3-phosphate (Gp-) pathways to oxidative phosphorylation in 4 species of marine bivalves. Box plots show minimum, 25th percentile, median, 75th percentile, and maximum. Contributions are calculated for N: N/NSGp, S: (NS – N)/ NSGp, and for Gp: (NSGp – NS)/ NSGp (n = 4-5 individuals per species). Letters denote significant ($p \leq 0.05$) differences among states-FCRs.

N-pathway. Titration with rotenone to inhibit the N-pathway had different effects on NSGp-sustained flux among species (Fig.3.2A-D). The threshold plots display the NSGp-pathway flux as a function of the relative inhibition of the N-pathway (Fig. 3.2E-H). Calculated flux control coefficients for the N-pathway were 0.41±0.12 for *M. arenaria*, 0.41±0.06 for *S. solidissima*, 0.52±0.1 for *M. mercenaria*, and 0.04±0.01 for *A. islandica*. The N-pathway thus showed markedly lower control over NSGp-driven respiration in *A. islandica* compared with the 3 other species (Tukey's post-hoc test $p = 0.047$ between *A. islandica vs. M. arenaria* and *S. solidissima* and $p = 0.007$ between *A. islandica vs. M. mercenaria*). At maximal N-pathway inhibition, OXPHOS capacity of the NSGp–pathway was inhibited by 11% in *A. islandica*, while it was inhibited by up to 58% in *S. solidissima*.

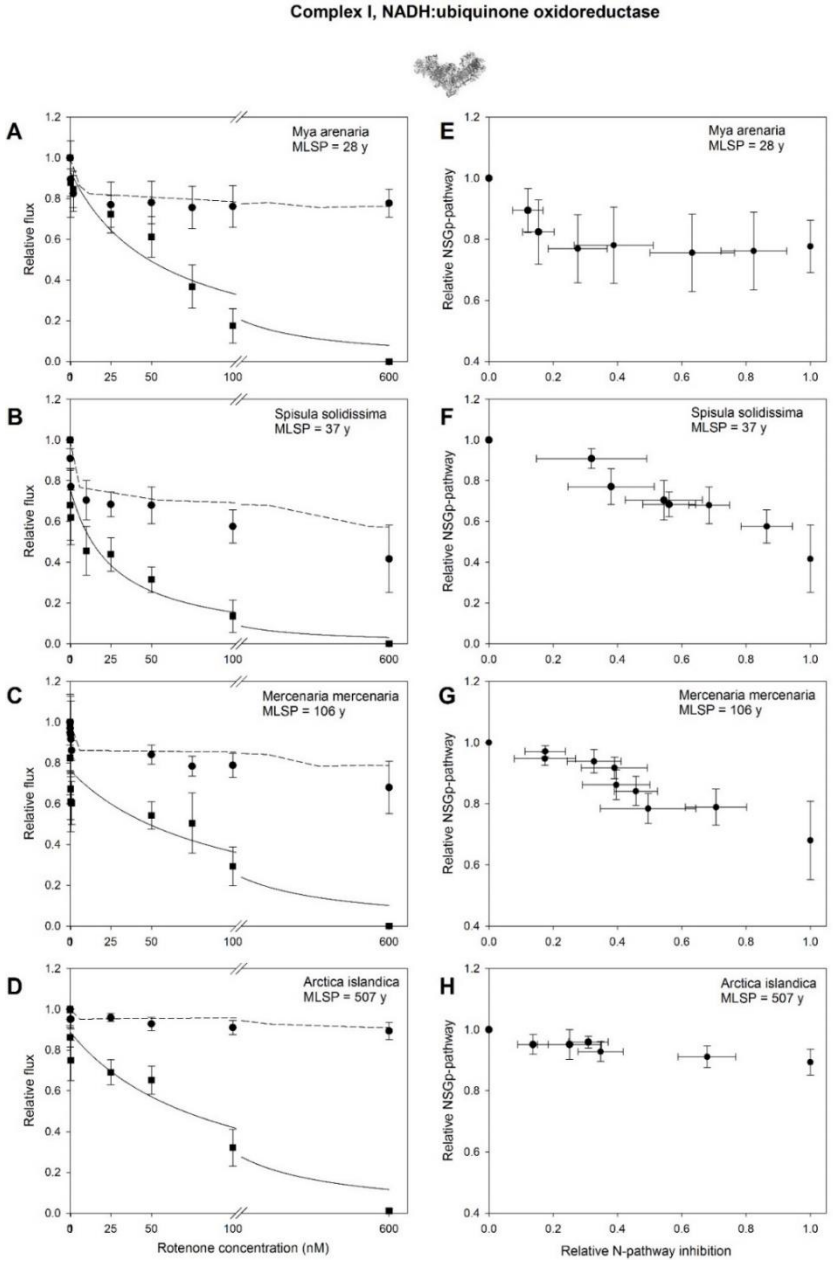

Figure 3.2 Rotenone titration and N-pathway threshold in isolated mitochondria from 4 species of short- to long-lived marine bivalves. (A-D, left graphs) Circles show the effect of rotenone titration on relative NS-pathway flux under OXPHOS state (dashed line: linear interpolation), while squares show the effect on inhibition of the N-pathway under OXPHOS state (solid line: hyperbolic fit). (E-I, right graphs) Relative NSGp-pathway flux as a function of relative N-pathway inhibition. Where applicable, open circles show data up to the threshold. Data are presented as means ±SEM (n = 4-5).

S-pathway. Inhibition of the succinate pathway with malonate (Fig. 3.3A-D) had different effects on NSGp-sustained flux among species (Fig. 3.3E-H). Calculated flux control coefficients for S-pathway were 0.15±0.01 for *M. arenaria*, 0.42±0.04 for *S. solidissima*, 0.25±0.06 for *M. mercenaria*, and 0.05±0.01 for *A. islandica*. S-pathway in *A. islandica* had lower control over combined pathway OXPHOS capacity (NSGp) compared to *S. solidissima* (Tukey's post-hoc test $p \leq 0.001$) or *M. mercenaria* ($p = 0.007$), but not compared to *M. arenaria* ($p = 0.422$). Flux control coefficient for the succinate pathway was significantly higher in *S. solidissima* compared to both *M. arenaria* ($p = 0.001$) and *M. mercenaria* ($p = 0.042$). At maximal S-pathway inhibition, combined OXPHOS pathway flux (NSGp) was inhibited by 11% in *A. islandica*, while it was inhibited by 43% in *S. solidissima*. Fig. S3.2 shows N- and S-oxidative phosphorylation pathways capacity normalized by maximal N and S uncoupled pathways in isolated runs for each pathway. The flux through the N-pathway did not vary among species and was lower than the maximal ET flux. But the flux through S-pathway was significantly closer to maximal uncoupled capacity in *A. islandica* (1.11±0.06) and *M. mercenaria* (1.00±0.03) compared to the two shorter-lived species (0.55±0.1 and 0.62±0.03 for *S. solidissima* and *M. arenaria*, respectively; $p < 0.05$). Inside species, no difference was found between both pathways for short-lived bivalves, whereas S-pathway during oxidative phosphorylation was much closer to the maximal uncoupled capacity than the N-pathway in *A. islandica* (1.11±0.06 vs. 0.74±0.04; $p = 0.028$) and *M. mercenaria* (1.00±0.03 vs. 0.68±0.06).

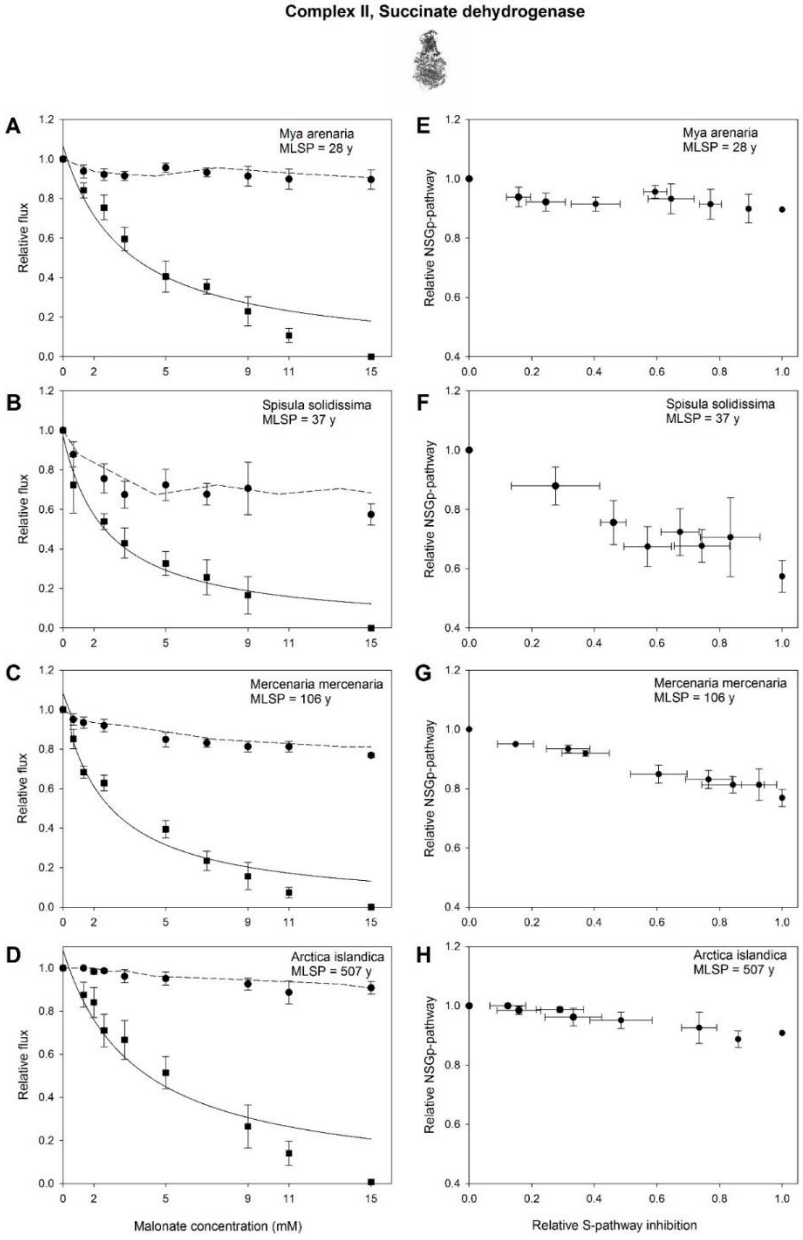

Figure 3.3 Malonate titration and S-pathway threshold in isolated mitochondria from 4 species of short- to long-lived marine bivalves. (A-D, left graphs) Circles show the effect of malonate titration on relative NSGp-pathway flux (dashed line: linear interpolation), while squares show the effect on inhibition of S-pathway (solid line: hyperbolic fit). (E-I, right graphs) Relative NSGp-pathway flux as a function of relative S-pathway inhibition. Where applicable, open circles show data up to the threshold. Data are presented as means ±SEM (n = 4-5).

Complex III-pathway. Antimycin A titration of complex III (Fig. S3.1A-D) had a strong effect on the NSGp-sustained flux among species (Fig. S3.1E-H). Calculated flux control coefficients for CIII were 0.76±0.1 for *M. arenaria*; 0.92±0.05 for *S. solidissima*; 0.90±0.13 for *M. mercenaria*, and 0.91±0.1 for *A. islandica*. No statistically significant differences were found among species for the control of respiration by CIII.

Complex IV. Cyanide titration of complex IV had different effects on NSGp-sustained flux among species (Fig. 3.4 A-H). Calculated flux control coefficients for complex IV and initial linear regressions were 0.25±0.09 for *M. arenaria* [y = 0.962 – (0.354x); r^2=0.75]; 0.19±0.04 for *S. solidissima* [y = 1.015 – (0.324x); r^2=0.9]; 0.46±0.08 for *M. mercenaria* [y = 1.009 – (0.457x); r^2=0.97], and 0.70±0.05 for *A. islandica* [y = 0.975 – (0.633x); r^2=0.97]. Complex IV in *A. islandica* exerted a higher control over combined OXPHOS pathways flux (NSGp) compared to *S. solidissima* (Tukey's post-hoc test $p = 0.001$) and *M. arenaria* ($p = 0.002$), and a trend for a higher, albeit non-statistically significant, control compared to *M. mercenaria* ($p = 0.103$). Complex IV excess capacity (CIV_E/NSGp flux ratio), calculated as the intercept at zero complex IV inhibition of a linear regression through the points after the inhibition threshold varied among species: *M. arenaria* = 2.404 (1.110-3.847), *S. solidissima* = 3.106 (1.771-4.256), *M. mercenaria* = 2.224 (1.305-3.865), *A. islandica* = 1.339 (1.065-1.637); $r^2 \geq 0.8$ for all species. Fig. S3.3 shows the activities of each pathway combined, normalized by maximal ET state. Whereas N-pathway activity did not vary significantly among species, the combined NS-pathway activity was significantly higher in *S. solidissima* (0.60±0.17) compared to *M. arenaria* and *A. islandica* (0.51±0.04 and 0.50±0.03; $p < 0.05$), but not to *M. mercenaria* (0.57±0.04). Similarly, NSGp-pathway activity was significantly higher in *S. solidissima* (1.06±0.16) than in *M. arenaria* and *A. islandica* (0.81±0.03 and 0.89±0.08; $p < 0.05$), but not *M. mercenaria* (1.01±0.02). Inside species, sequential addition of S- and Gp-pathway substrates significantly elevated activity comparatively to the previous step ($p < 0.05$

for all comparisons), except for the addition of Gp-pathway substrates in *S. solidissima* compared to NS-pathway.

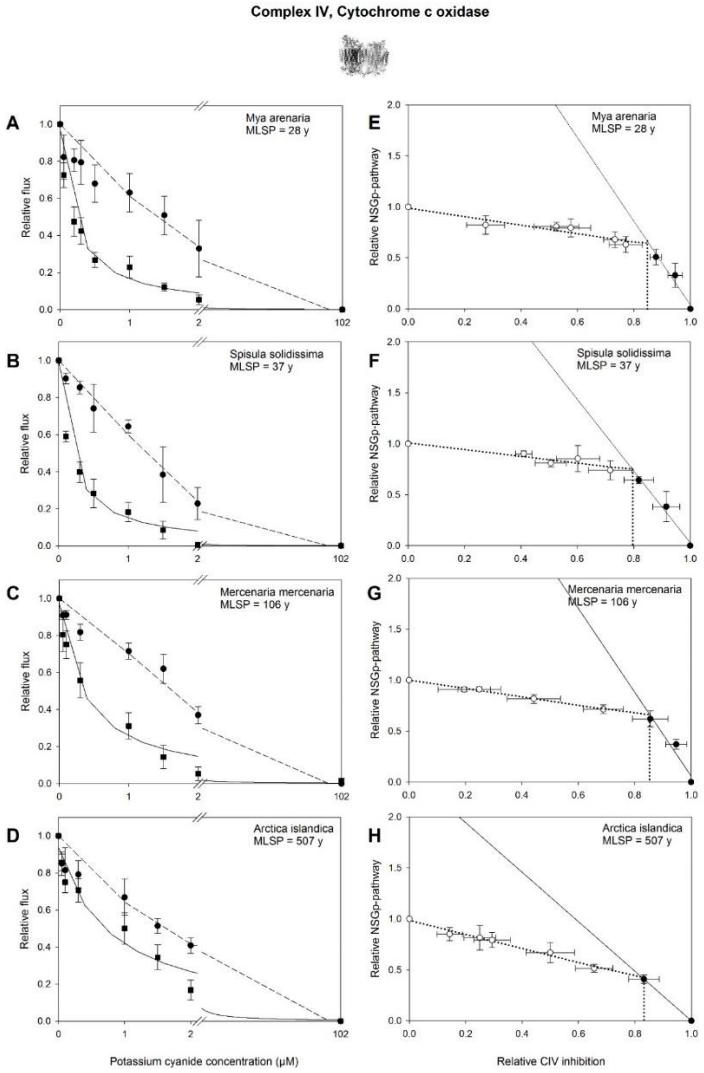

Figure 3.4 Potassium cyanide titration and complex IV single step threshold in isolated mitochondria from 4 species of short- to long-lived marine bivalves. (A-D, left graphs) Circles show the effect of potassium cyanide titration on relative NSGp-pathway flux (dashed line: linear interpolation), while squares show the effect on inhibition of single enzyme cytochrome c oxidase (complex IV, solid line: hyperbolic fit). (E-I, right graphs) Relative NSGp-pathway flux as a function of relative complex IV inhibition. Open circles show data up to the threshold of inhibition, and the CIVE / NSGp flux ratio is calculated as the intercept at zero CIV inhibition of a linear regression through the points after the inhibition threshold. Data are presented as means ±SEM (n = 4-5).

Sum of flux control coefficients. Fig. 3.5 shows the control coefficients calculated for N-, S-, CIII- pathways and CIV single step (and sums thereof) in each species (*n = 4-5* individuals per pathway/step and species), as explained above. It showed significant ($p < 0.05$) differences among species. The sum of single step control coefficients varied between *M. arenaria* (1.57) and *M. mercenaria* (2.13).

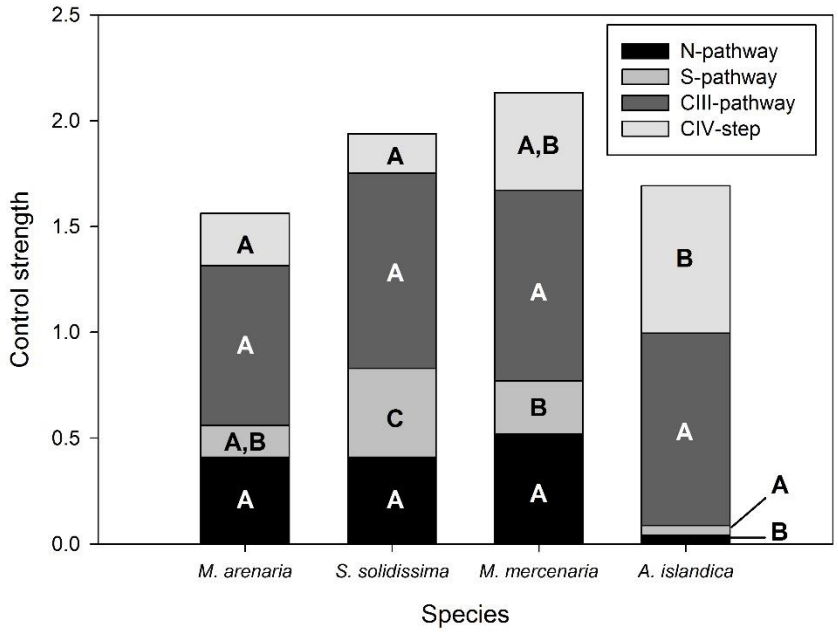

Figure 3.5 Stacked bars showing flux control coefficients for N-, S-, CIII- pathways and CIV single step (and sums thereof) in 4 species of short- to long-lived marine bivalves. Letters denote significant ($p \leq 0.05$) differences among species. Data are presented as means (n = 4-5 individuals per calculated coefficient, per species).

3.4.2 Enzymatic activities of CS, CIV and ATPase.

Fig. S3.4 shows the activities of 3 key enzymes in mitochondrial aliquots of the same individuals used for the high-resolution respirometry runs. CS activity (in U by mg of mitochondrial proteins) was significantly higher in longer-lived *M. mercenaria* and *A. islandica* (0.28±0.05 and 0.28±0.02 respectively) than in the shorter-lived species *M. arenaria* and *S. solidissima* (0.10±0.01 and 0.08±0.04; $p < 0.05$). CIV

activity (normalized by mg of mitochondrial proteins) was significantly higher in *A. islandica* than *M. arenaria* (0.007±0.02 vs 0.001±0.0003; $p = 0.028$), but not significantly different between other species. ATPase activity, when normalized by CS was lower in the longer-lived species *M. mercenaria* and *A. islandica* (0.1±0.04 and 0.05±0.01) compared to the shorter-lived species *M. arenaria* and *S. solidissima* (0.45±0.13 and 0.28±0.03; $p < 0.05$). When normalized by CIV activity, ATPase activity remained significantly higher in *M. arenaria* (20.02±2.49), but not *S. solidissima* (4.27±0.29), compared to longer-lived bivalves (9.33±0.89 for *M. mercenaria* and 3.24±0.89 for *A. islandica*; $p \leq 0.001$).

3.5 Discussion

We provide evidence for longevity-related differences in the control of respiration by the pathways and enzymatic complexes of the ETS in marine bivalves. In saturating substrate condition, i.e., when electrons are supplied to the N-, S- and Gp-pathways simultaneously, the respiration rate reaches close to its maximum capacity and is only slightly stimulated by the addition of an uncoupler. This indicates that ATP synthase or nucleotide transport does not exert any limitation on OXPHOS capacity. Using a physiologically relevant approach in intact mitochondria, we found that the upstream electron entry pathways (NADH and succinate) exert a lower control on respiration in the extremely long-lived species *A. islandica*, when compared with three related species of shorter lifespans. Longer lifespan is further characterized by a higher control of OXPHOS by the terminal electron accepting enzyme complex IV (Fig. 3.4), while control by complex III was found to be high and similar among species (Fig. S3.1). As predicted, upstream contribution of the TCA cycle (in the form of CS activity) was higher in longer-lived species. On the other hand, the activity of the ATP synthase (complex V) was lower in long-lived than in shorter-lived species.

Our study points to an interspecific relationship between N-pathway control and longevity; the N-pathway exerts a stronger control over combined pathways

respiration in shorter-lived than in longer-lived species. This is reminiscent of a previous study on rat liver mitochondria, where complex I was found to be strongly rate-controlling in old individuals, but to exert little control in younger rats (Ventura et al., 2002). Although other steps of the ETS were also proposed to be involved, it appears that aging induces an alteration of complex I that reflects on the decreased OXPHOS capacity (measured with glutamate, malate and succinate) in aged rats. Hepatocytes from old rats were also found to produce higher peroxide levels than those from younger animals, as well as higher complex I transcripts in response to functional alterations of the enzyme, again in support of the MOSTA (Genova et al., 2004). In planarian flatworms, animals considered immortal (Sahu et al., 2017), the contribution of the N-pathway to maximal OXPHOS capacity is very minor compared to other species (Scott et al., 2019), again supporting a low contribution of the N-pathway capacity as a potential advantage for longevity. Taken together, these results suggest that relieving part of the control exerted by the N-pathway might give an advantage in terms of longevity. A link between aging and N-pathway sensitivity is also reported in invertebrates; a higher resistance to the complex I inhibitor tebufenpyrad was found in lines of beetles with an increased longevity-related nuclear background (Jovanović et al., 2014). These previous studies suggest a link between alteration of CI and the aging process. The impact of these alteration would be clearly affected by the importance of CI on the control of respiration and ROS production. We could therefore hypothesize that the high rates of H_2O_2 production reported in *M. arenaria* and *S. solidissima* (but not in *A. islandica*) in the LEAK (with glutamate and malate) and the OXPHOS states (with succinate and rotenone, Munro et al., 2013) are related to the high control strength of respiration by CI in these two species. This high control would result in a high susceptibility to alteration at this step, and a faster aging process. Furthermore, if complex I is a sensitive step that accumulates dysfunction during aging, it would make sense that long-lived species minimise control strength at this level to ensure that any alteration of complex I would have a minimum impact on the overall oxidative capacity or fine regulation of respiration. This is further supported by experiments on *C. elegans*

showing a relationship between the structural stability of complex I and the increased lifespan, improved mitochondrial function and decreased oxidative stress (Pujol et al., 2013). The stability and associated robustness of the ETS components as a function of longevity remains to be fully assessed in bivalves, but our results and other studies of both vertebrate and invertebrate models (Lenaz et al., 1997;Copeland et al., 2009;Baumgart et al., 2016) suggest a key role of the N-pathway in the aging process.

We report longevity-related differences in succinate-pathway control over respiration in marine bivalves, echoing the result found for the N-pathway. We found that, among the four species, long-lived *A. islandica* exerts a significantly lower control of respiration through the S-pathway compared two out of three shorter-lived species (excluding *M. arenaria)*. Nonetheless, a lack of sensitivity of this pathway to malonate-mediated inhibition could help explain the low H_2O_2 production found for the longest-lived bivalve (Munro et al., 2013). The same study reported a seven-fold lower ratio of complex II/complex IV activity in *A. islandica* compared to *M. arenaria*, reinforcing the idea of a role for this enzyme in ROS-mediated longevity. The link between enzyme activity and control strength does not appear straightforward, and may depend on the elasticity of the complex to its substrate (i.e., its change in capacity in response to changes in substrates or other effectors, see Murphy, 2001) . Moreover, measuring the maximal catalytic activities of each enzyme in homogenized mitochondria yields different information than respiration as a proxy for electron flux in the intact system. Indeed, while the relative contribution of the S-pathway in the present study was similar among species, the OXPHOS capacity in isolated runs was closer to maximal ETS capacity in *A. islandica* and *M. mercenaria* compared to the two shorter-lived species. Taken together, our current and previous results (Munro et al., 2013) suggest that the flux generated through the S-pathway in long-lived species is reached with a lower relative catalytic capacity of CII. Although neglected at first, increasing evidence points to the S-pathway as an important regulator of mitochondrial ROSp given its role in both the ETS and TCA cycle, which depends on substrate concentrations and on the

activities of other enzymatic complexes (reviewed in Dröse, 2013). Quinlan et al. (2012) demonstrated that complex II is an important site for ROS appearance in rat skeletal muscle in both forward and reverse reactions. Increased longevity through methionine restriction in rats was accompanied by a substantial decrease in CII concentration in the liver (Caro et al., 2008), while longer-lived strains of *Drosophila* also had lower complex II content (Neretti et al., 2009). Here, it appears that this pathway is less sensitive to inhibition in longer-lived species, hence partial dysfunction would have a milder negative impact on overall respiration and ROSp, much like the N-pathway.

The complex IV pathway exerted a higher control over respiration in *A. islandica* than in shorter-lived bivalves. To our knowledge, this is the first report of longevity-linked control of respiration by this complex. Indeed, complex IV catalytic activity normalized by citrate synthase activity (a proxy of mitochondrial content) was not different between *A. islandica* and *M. arenaria* in Munro and colleagues' study (Munro et al., 2013). The current results show that its control over respiration in coupled conditions increases in longest-lived species. Complex IV is known to play a role in the regulation and control of metabolic state, with various studies suggesting that it exerts a high control over respiration (Groen et al., 1982;Dalmonte et al., 2009). Moreover, its activity can be regulated by substrate and cofactor availability, among which NADH and ADP, but also by oxygen and inorganic phosphate. The complex isoform IV-1 binds ATP causing allosteric inhibition of the enzyme, a way to detect cellular ATP levels and adjust ATP production to demand. This is proposed to help keep the membrane potential at low values in conditions of high matrix ATP/ADP ratios, thus preventing excessive ROS production (discussed in Arnold, 2012 and see Vogt, 2016). Limitation of the electron flow by complex IV could keep the ubiquinone pool in a highly reduced state, thereby limiting ROS production at complex III (shown to play an important role in hypoxia-induced redox signalling), since it has been shown that superoxide formation by CIII is stimulated by the presence of oxidized ubiquinone

pool rather than fully reduced (Dröse and Brandt, 2008). A decrease in both complex IV activity and ATP producing capacity with age has also been reported by a number of studies comparing young and older animals in vertebrate and invertebrates models (Ren et al., 2010;Petrosillo et al., 2013). Therefore, age-related changes in CIV activity would considerably impact oxidative capacities; it would be of interest to further evaluate how these changes vary longitudinally among individuals of bivalve species, and how differences in control affect rates of ROS efflux by upstream complexes.

In addition to respiration, we also measured the catalytic activity of TCA cycle enzyme citrate synthase as a means of evaluating the capacity of pathways upstream, as well as downstream of the electron transport system, by measuring ATP synthase activity. In line with our initial prediction, we found that longer-lived species are also characterized by a higher CS activity per mitochondrial protein. Thus, the capacity to generate reducing equivalents that enter the ETS could be more important in longer-lived bivalves, perhaps echoing the maintenance of a high CS activity throughout lifespan that has previously been evidenced (Short et al., 2005). However, this would need more investigation as Munro et al. (2019) found a lower CS activity in the naked-mole rat, a murine model of longevity, compared to mice. Enzymatic activity of free cytochrome *c* oxidase (CIV) was slightly higher in *A. islandica* than *M. arenaria*, contrary to other reports (Munro et al., 2013), but not when compared to *S. solidissima* or *M. mercenaria*. Contrary to our initial predictions, we found that ATPase activity was lower in longer-lived species when normalized by CS activity, but less so when normalized by CIV. Reports of studies in *C. elegans* worms have shown that ATP synthase inhibition extends lifespan, although the mechanism has not been fully elucidated and is proposed to be linked to mTOR activity (Chin et al., 2014;Xu et al., 2018). Nonetheless, we show for the first time interspecies variation in ATPase activity linked to lifespan, a finding which warrants further investigations.

In this study, we measured respiration at NADH, succinate, and Gp pathway fluxes at substrate and ADP concentrations ensuring saturation. We tested various

substrate combinations in preliminary experiments and decided to use Gp as a substrate based on the high respiration rates obtained here and previously in permeabilized tissue (Bettinazzi et al., 2019) to ensure maximal saturation of ETS. We found differences among species in the contribution of this Gp-pathway to respiration, with a higher contribution in *A. islandica* and *M. mercenaria* compared to *S. solidissima*. It appears possible that there is different organizations of electron entry, with Gp varying among species, hence partly explaining the differences in control strength. A possibility is that this site of electron entry (relative to the N-pathway) is more important in *A. islandica*. This would potentially help alleviate the reduction state of CI and the potential electron "slips" to molecular oxygen and ROSp. Gp is a physiologically relevant substrate in bivalves. The mitochondrial glycerol-3-phosphate dehydrogenase (mG3PDH) reduces Gp derived from DHAP (a metabolite from glycolysis), or from glycerol derived from triglyceride or diglyceride catabolism (McDonald et al., 2018). Bivalves diet mainly consists of phytoplankton (Gosling, 2015a), often rich in lipids, potentially explaining the high rates of respiration through the mG3PDH. Studying this type of "alternative" pathway of electron transport is relatively novel, but brings forth the diversity of bioenergetic strategies in animals, and questions the classical view of a linear electron transport system and its associated beliefs (McDonald et al., 2018). Since mG3PDH has also been shown to be a relevant site of ROS production (Orr et al., 2012), it will be of interest in the future to assess the rates of ROSp in the presence of Gp and compare it to the rates with other substrates combinations in these species.

The sum of the various flux control coefficients for the ETS complexes of marine bivalves found in this study is well over 1. Although a maximal summation of one was theorized for linear pathways, we are here facing a model with multiple electron entry enzymes, which can be in excess, and with an obligatory step at complexes III and IV. Thus, adding these multiple steps and since most of the control is located upstream of phosphorylation (as uncoupling did not substantially increase respiration), this result above 1 should not come as a surprise. It is besides reminiscent

of the pivotal studies by Bianchi and colleagues, who found the sum of controls exerted by complex I and III to be over 1, suggesting that they work as a single entity, and providing some of the first evidence for supercomplex assemblies (Bianchi et al., 2004). The existence of these supramolecular assemblies is now well established, although thus far only in a limited number of species. To our knowledge, supercomplex organization in marine invertebrates has not yet been studied. Since their composition, formation and stability appear to decrease with age (Gómez and Hagen, 2012;Fischer et al., 2015), supercomplexes are thought to play roles in the regulation of electron flux, as well as in ROS production and longevity (Kauppila et al., 2017). Supercomplexes are dynamic assemblies that are specific to the cell type and physiological conditions (Lapuente-Brun et al., 2013;Greggio et al., 2017). Pharmacological inhibition of their constituent complexes (especially of complex I) can disrupt their assembly and affect ROS efflux and ATP production (Jang and Javadov, 2018). It is therefore conceivable that longer-lived species have less control (or less "sensitivity") at the level of complex I in order to avoid supercomplex breakdown, and its detrimental consequences in terms of ATP and ROS production. This potential robustness of supercomplex assemblies and lower control of electron entry complexes over the ETS may also explain the maintenance of low rates of ROS production in *A. islandica* and its extreme longevity compared to closely-related bivalves, in line with the MOSTA. The relationship between complex I content and control over respiration, their role in supercomplex assembly and robustness, and the modulation of ROS production remain to be established; our results, however, suggest that bivalves are a powerful model to explore this question.

In conclusion, we demonstrated that differences in respiratory control by complex I, II, and IV in aquatic invertebrate species expressing different lifespans. To our knowledge, this study is the first to investigate the distribution of control strengths of key enzymatic complexes over mitochondrial respiration in four different species of bivalves from different taxonomic groups and longevity. Our results suggest a fine

regulation of the ETS in these animals, and further reinforce the idea of key roles for complexes I and IV in the aging phenotype. Indeed, we show that a long lifespan is associated with low control of respiration at the level of complex I, possibly conferring more structural stability and avoiding excessive ROS production. Furthermore, complex IV appears as an important regulator of electron flux in the extremely long-lived bivalve *A. islandica*, again possibly affecting ROS regulation. Therefore, the logical next step should involve measuring the effect of ETS complex inhibition on ROS production, i.e. their individual control strength on ROS generation.

SUPPLEMENTARY MATERIAL FOR CHAPTER III

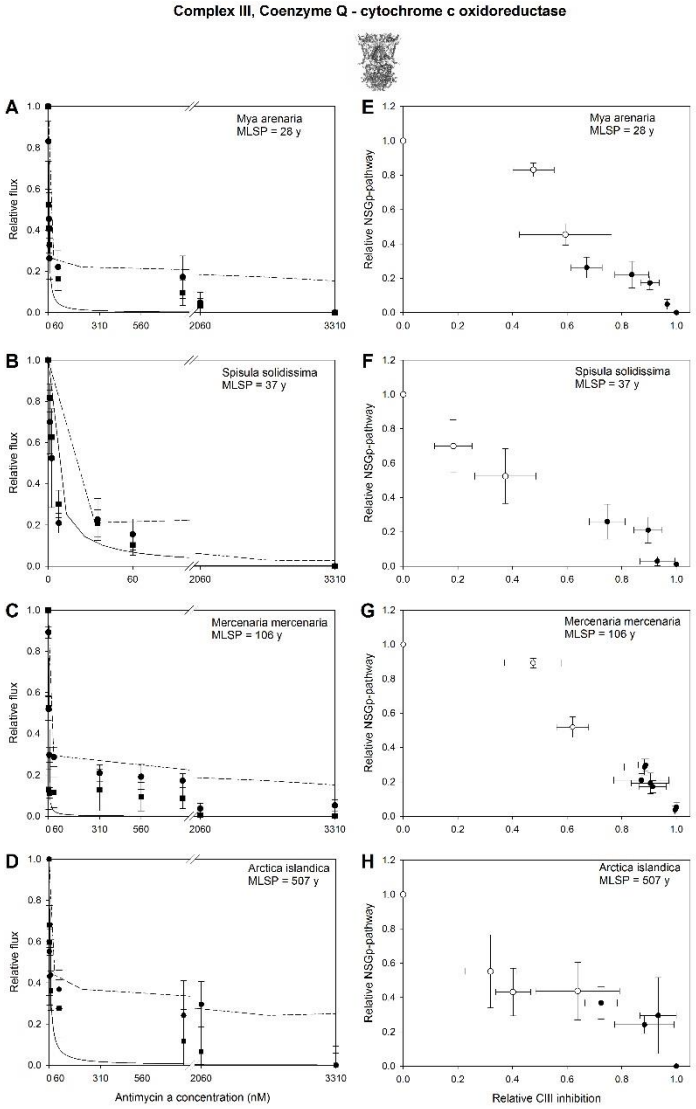

Figure S3.1 Antimycin a titration and complex III-pathway inhibition in isolated mitochondria from 4 species of short- to long-lived marine bivalves. (A-D, left graphs) Circles show the effect of antimycin a titration on relative NSGp-pathway flux (dashed line: linear interpolation), while squares show the effect on inhibition of complex III-pathway (solid line: hyperbolic fit). (E-I, right graphs) Relative NSGp-pathway flux as a function of relative complex III-pathway inhibition. Where applicable, open circles show data up to the threshold. Data are presented as means ±SEM (n = 4-5).

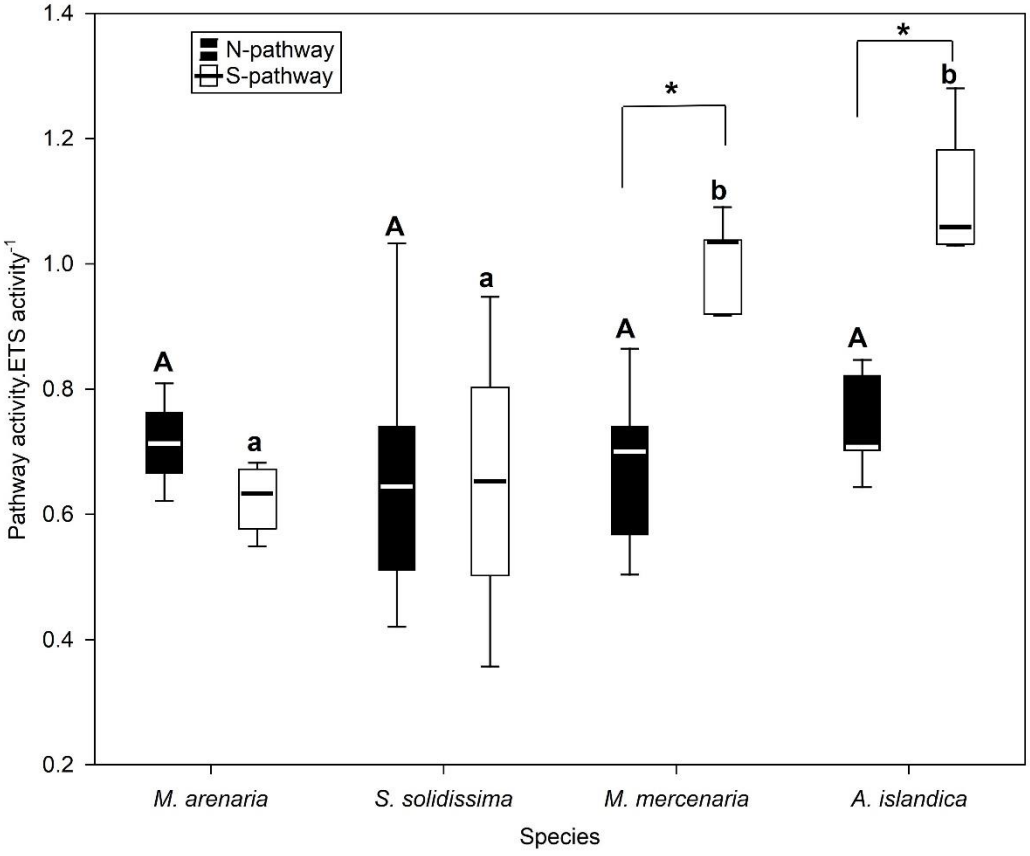

Figure S3.2 N- and S-pathways activities normalized for maximal uncoupled ETS activity in four species of short- to long-lived marine bivalves. Box plots show minimum, 25th percentile, median, 75th percentile, and maximum. (n = 4-5 indiv. *per* species). Capital and lower-case letters denote significant ($p < 0.05$) differences between species for each pathway, while asterisks denote significant differences between pathways within the same species.

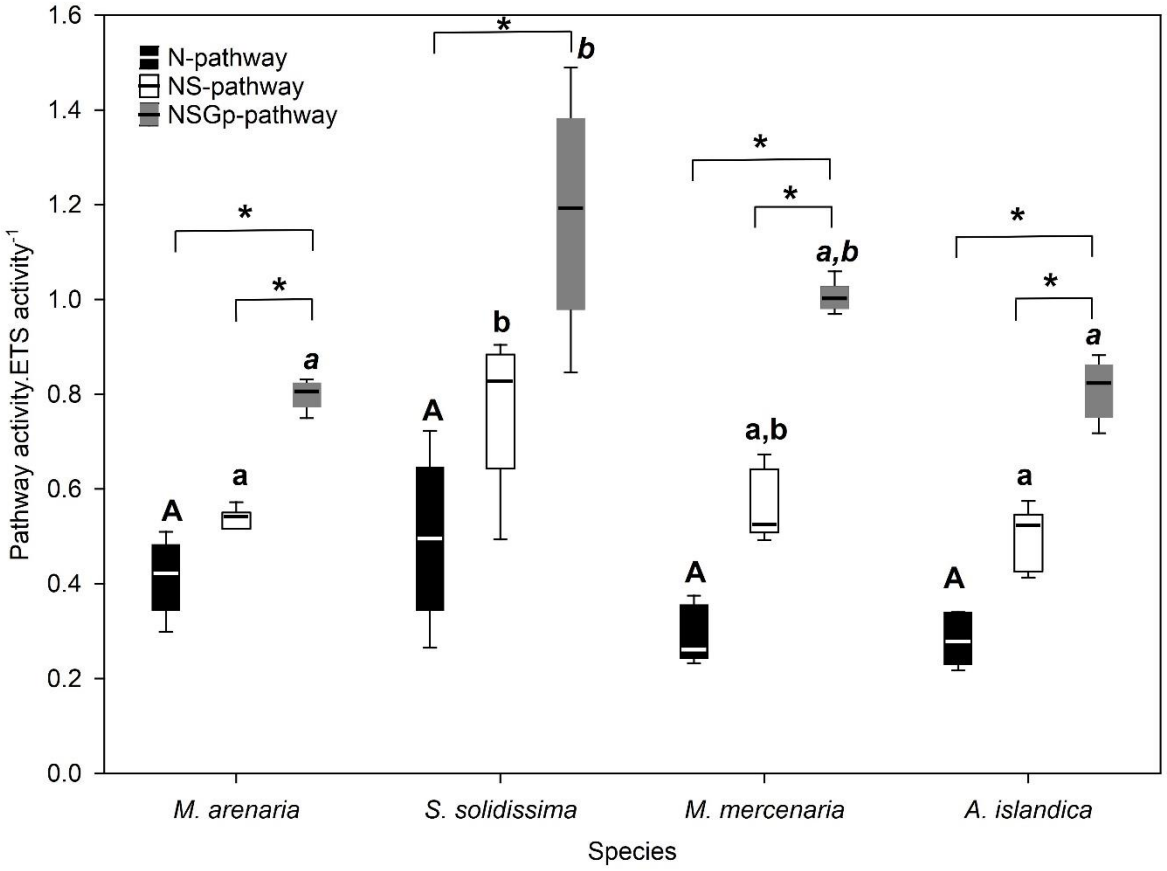

Figure S3.3 N-, NS- and NSGp- pathways activities normalized for maximal uncoupled NSGp under ET state in four species of short- to long-lived marine bivalves. Box plots show minimum, 25th percentile, median, 75th percentile, and maximum (n = 4-5 indiv. *per* species). Capital, lower case and italic letters denote significant ($p < 0.05$) differences between species for each pathway, while asterisks denote significant differences between pathways within the same species.

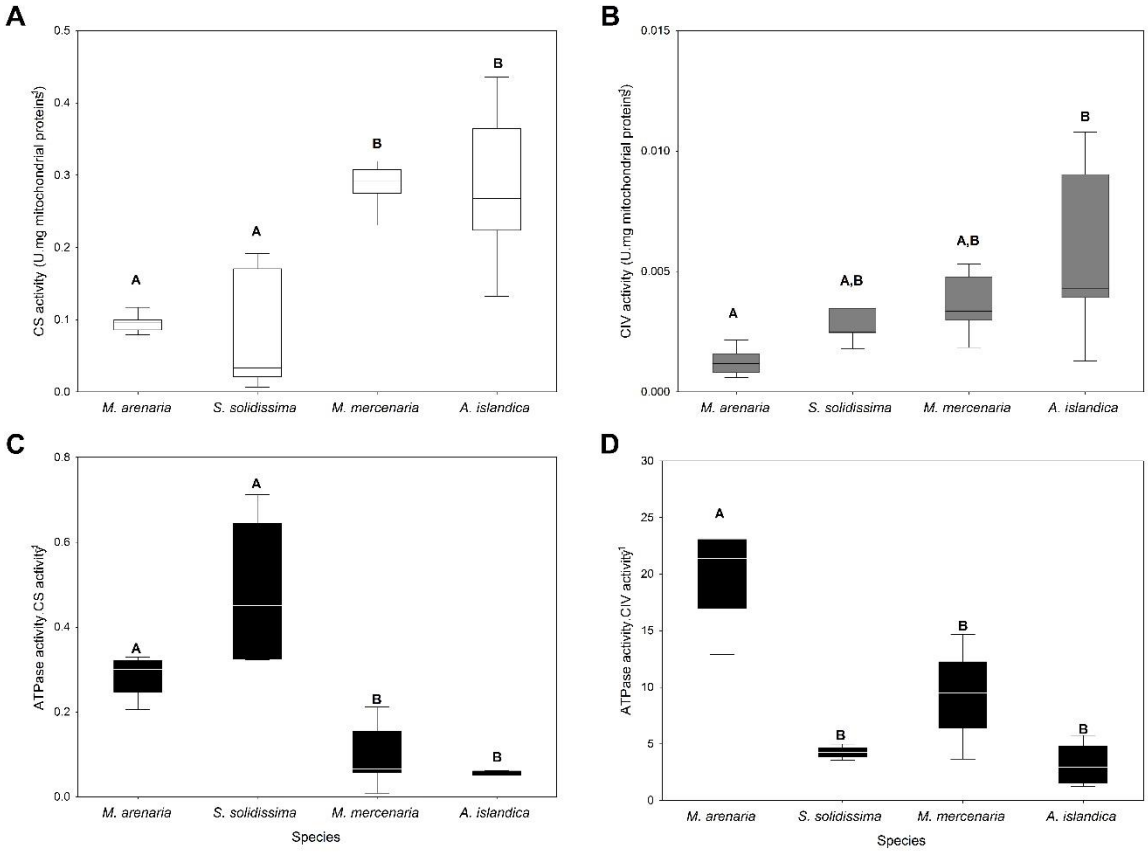

Figure S3.4 Enzymatic activities of (A) citrate synthase (in moles of product), (B) CIV (in moles of electron transferred), normalized by mg of mitochondrial proteins; (C) ATPase activity and (D) CIV activity, normalized by CS activity, in four species of short- to long-lived marine bivalves. Box plots show minimum, 25th percentile, median, 75th percentile, and maximum. Different letters denote significant differences ($p < 0.05$) among species.

CHAPTER IV
MITOCHONDRIAL ELECTRON TRANSFER SYSTEM ENZYMES SHOW VARIOUS SUPERCOMPLEX PATTERNS IN MARINE BIVALVES: A LINK WITH LONGEVITY?

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4.1 Abstract

The mitochondrial oxidative stress theory of aging (MOSTA) suggests that the organelle's decay contributes to the aging phenotype *via* exacerbated oxidative stress and loss of activity and integrity. Recent advances in understanding the organization of the mitochondrial electron transfer system (ETS) show that the enzymatic complexes responsible for oxidative phosphorylation are organized in supramolecular structures called supercomplexes, although the exact role of these and their universality among organisms is still under debate. Here, we take advantage of marine bivalves as a model to compare the structure of the ETS among species ranging from 28 to 507 years in maximal lifespan, and demonstrate that they are indeed organized as supercomplexes. We discuss this comparative model in light of differences in the nature and stoichiometry of these complexes, and highlight the potential link between the complexity of these superstructures and longer lifespans.

4.2 Introduction

Mitochondrial dysfunction is believed to be a key factor in the aging phenotype and the onset of age-related diseases (López-Otín et al., 2013). Indeed, mounting evidence from longitudinal and comparative studies point toward a progressive loss of mitochondrial integrity, linked to the activities of the electron transfer system (ETS) complexes, and the management of reactive oxygen species production (ROSp); in line with the mitochondrial oxidative stress theory of aging (MOSTA, reviewed in Blier et al. 2017) . Mitochondrial morphology is intimately linked to mitochondrial function (reviewed in Guo 2018), and loss of membrane architecture is observed at increasing age (Daum et al., 2013). In this light, the basic understanding of the physical organization of the enzymatic components in the mitochondrial inner membrane has evolved.

The ETS consists mainly of 4 multisubunit complexes: NADH:ubiquinone oxidoreductase (complex I), succinate dehydrogenase (complex II), coenzyme Q – cytochrome c oxidoreductase III (complex III), the terminal electron acceptor enzyme, cytochrome *c* oxidase (complex IV,), plus an ancillary electron transfer flavoprotein complex that augments electron flow into complex III. ETS complexes were first proposed to be assembled into large, polymeric-protein assemblies within the inner mitochondrial membrane in Chance and William's pioneering studies (Chance and Williams, 1955a). Later, purification and reconstitution of single enzymatic complexes and kinetic studies led to the proposal of a random diffusion (or fluid) model of electron transfer (Hackenbrock et al., 1986), where the complexes move randomly and independently within the inner mitochondrial membrane, with electrons flowing between them through mobile carriers coenzyme Q and cytochrome c (for a review see Lenaz and Genova, 2007).

Although the fluidity model dominated the mitochondrial field for decades, more recent evidence from new kinetic and electrophoretic studies suggest a different

organizational model may occur in vivo where a supramolecular organization of the ETS predominates: the supercomplex (SC). Schägger and Pfeiffer (2000) first isolated and resolved these high molecular weight structures composed of varying stoichiometries of complexes I, III and IV using blue native polyacrylamide gel electrophoresis (BN-PAGE). Initially dismissed as artefacts of the solubilisation method, SC assemblies have now been thoroughly demonstrated to occur *in vivo*, to be fully functional entities, and have been imaged to considerable precision (Enríquez, 2016;Guo et al., 2018). A prime example is the so-called respirasome, a SC ($I_1III_2IV_1$) containing all the redox enzymes required for electron transfer from NADH to molecular oxygen. Other recurring SC assemblies are SC I_1III_2 and SC III_2IV_2. Very few studies report the presence of complex II-containing SCs and hence its participation in these supramolecular assemblies is highly debated (Acín-Pérez et al., 2008;Guo et al., 2017;Matsubayashi et al., 2019).

Preliminary functional hints to the existence of SC came from metabolic flux control analyses, the measurement of the control exerted by individual enzymes on a pathways (reviewed in Lenaz and Genova, 2007). The model of a respiratory chain based on random diffusion of complexes implies different levels of rate-control by enzymes, each one controlling the pathway to a certain extent. In contrast, the model based on SC assembly implies electron channelling between complexes and thus, a metabolic pathway behaving as a single component, where inhibition of any component would exert the same flux control. Hence, the sum of all the control coefficients is predicted to be superior to 1, as found by Bianchi et al. (2004) who demonstrated a functional association between complexes I and III.

Supercomplexes have been proposed to play a role in the management of electron flux and substrate channelling (Lapuente-Brun et al., 2013) as well as regulate the rate of ROS generation (Lopez-Fabuel et al., 2016). Substrate channeling allows the passing of an intermediate metabolite from one enzyme directly to the other, without release into the substrate pool (Bianchi et al., 2004). This bypasses enzyme

competition for the substrate, increasing the efficiency of the reaction. However, there appears to be no confining structure for ubiquinol or cytochrome *c* molecules to guide them to their respective binding sites, and the substrate seem to be freely moving despite the fact that the active sites are in close proximity to each other, in sharp contrast with the typical enzymes that channel substrates (see Milenkovic, 2017). The grounds for substrate channelling by SCs have therefore been fiercely debated (Lobo-Jarne and Ugalde, 2017;Fedor and Hirst, 2018). Nonetheless, a recent cryo-EM resolution of respirasomes and megacomplexes appears to show a more constraining structural conformation of SCs, reviving the idea of substrate channeling as a primary function of SCs (Guo et al., 2018). Such a structural organization could be beneficial by enhancing electron transfer and limiting reverse electron flow, thus avoiding excessive ROSp at the sensitive ubiquinol-oxidizing site Q_O. This could hence be advantageous in the context of oxidative stress management and the MOSTA. Some hints at a relationship between the supramolecular structures and the rate of aging have emerged: most notably, there is increasing evidence for an age-related decline in SC integrity in rat hearts (Gómez et al., 2009). Whether the robustness of these supramolecular assemblies are linked to lifespan divergences remains unknown (Blier et al., 2017).

The presence of SC appears not to be universal, and before the relationship between their structure and lifespan divergences is scrutinized, their presence in various species needs to be assessed. So far, they have been discovered in diverse models: from yeast and fungi to plants, vertebrates, and invertebrates (reviewed in Enríquez, 2016). They are however notably absent in *E. coli* where different complexes do not co-localize (Llorente-Garcia et al., 2014). ETS complexes appear to be tightly packed, but not forming SC assemblies in the fruit fly *Drosophila melanogaster* (Shimada et al., 2018), although this result contradicts a previous report in flies with dysfunctional ATP synthase and where these assemblies were found (Celotto et al., 2011). Therefore, assessing the universality of these structures and their possible link to lifespan divergences appears necessary.

Various studies on mitochondrial structure and function in marine bivalves, peculiar invertebrate models of longevity, have recently given support to the MOSTA. Multiple bivalve species were compared, among which the longest-lived non-colonial metazoan, the ocean quahog *A. islandica*, which has a maximum reported longevity (MRL) of 507 years in the North Atlantic (Butler et al., 2013). This is strikingly higher than the MRLs of three closely related and similar-sized species: *Mya arenaria* (28 y), *Spisula solidissima* (37 y), and *Mercenaria mercenaria* (106 y, Blier et al., 2017). The mitochondrial parameters that seem to allow the ocean quahog to reach its exceptional longevity include more peroxidation-resistant mitochondrial membranes and lower rates of ROSp (measured as H_2O_2 efflux) than other bivalves, despite similar capacities for oxidative phosphorylation (OXPHOS, Munro and Blier 2012, Munro et al., 2013). Moreover, the sum of individual control coefficients by the ETS enzymes in these 4 species of marine bivalves has been recently shown to exceed 1 (Rodríguez et al. 2019, *submitted for review*), hinting at the presence of SC. Whether these structures are indeed found in bivalves and whether their composition might relate to lifespan differences has yet to be assessed. Therefore, we aimed to fill this gap in knowledge by deciphering the architecture of the ETS and their organization in species of short- to long-lived bivalves.

4.3 Experimental procedures

4.3.1 Bivalve collection and mitochondrial isolation

Bivalve species were either sampled (*Mya arenaria*, *Mercenaria mercenaria*) at low tide, collected by professional divers (*Spisula solidissima*, *Arctica islandica* from the Magdalen Islands), or by trawling (*A. islandica* from the Gulf of Maine). They were shipped in coolers with damp rags and transferred to two separate flow-through tanks upon arrival. Individuals were maintained at 8ºC for several weeks before the experiments were carried out. Shell length was recorded after tissue extraction to estimate individual age. Mitochondrial isolation was carried out as previously

described (Munro & Blier, 2012) with a modified mitochondrial isolation buffer (Moyes et al., 1985) in which KCl was replaced with NaCl to avoid Coomassie dye precipitation caused by potassium ion (Wittig et al., 2006). The replacement of KCl by NaCl was assessed in a previous study and yielded fully functional mitochondria (Rodríguez et al., 2019 *submitted*). Approximately 4 g of mantle and 2 g of gill tissues were pooled from 2-4 individuals, and rinsed in the isolation buffer (400 mM Sucrose, 70 mM HEPES, 50 mM NaCl, 6 mM EGTA, 3 mM EDTA, 10 mg/ml aprotinin, 1% bovine serum albumin), minced and homogenized using a glass-Teflon potter on ice. The homogenate was centrifuged at 4ºC twice at 1250 g for 10 min, discarding the cellular debris fraction and keeping the supernatant. The latter was then centrifuged at 10500 g, and the resulting mitochondrial pellet was resuspended with a 0.5% BSA isolation buffer, centrifuged another time and resuspended in about 500 µl of buffer. Protein concentration was immediately determined using the Biuret method, and mitochondrial isolates were divided in 500 µg fractions, freeze-dried in liquid nitrogen and stored at -80ºC until BN-PAGE analysis.

The respiratory function of the remaining mitochondrial isolate was assessed for each sample through high-resolution respirometry at 10ºC using an Oxygraph-2K (Oroboros Instruments, Austria) (Gnaiger, 2014). We assessed the integrity of the outer mitochondrial membrane by the addition of cytochrome *c* (10 µM) during state 3 respiration fueled by substrates glutamate (25 mM) and malate (2 mM) with saturating ADP (5 mM). The activity of cytochrome oxidase (CIV) was also assessed by adding electron donor TMPD (0.5 mM) and ascorbate (2 mM) to avoid the auto-oxidation of TMPD.

4.3.2 Blue-Native PAGE

We performed Blue-Native polyacrylamide gel electrophoresis (BN-PAGE) on isolated mitochondria by adapting the protocols from Wittig et al. (2006) and Gómez et al. (2009). 175 µg of frozen mantle or gill mitochondria were slowly thawed on ice,

and centrifuged at 10,000 g for 10 min at 4ºC. The supernatant was discarded, and the pellet was resuspended in a solubilisation buffer (750 mM 6-aminohexanoic acid, 50 mM Bis-Tris, 0.5 mM EDTA disodium salt, pH 7.0). To solubilize mitochondrial membranes and verify that individual ETS complexes could be obtained, *n*-dodecyl-β-D-maltoside (DDM) was used at a 2:1 detergent-to-protein ratio in preliminary gels. In gels assessing the presence of supercomplexes, a 20% (w/v) solution of digitonin was added at an empirically determined detergent-to-protein ratio of 8:1 (w/w). After 1h incubation on ice, samples were centrifuged for 35 min at 21,100 g at 4ºC, and the resulting supernatant was transferred to a new Eppendorf tube. A 5% Coomassie G-250 solution was added to a detergent-to-dye ratio of 8:1, after which the samples were loaded on NativePAGE™ 3-12% Bis-Tris Protein Gels (ThermoFisher). Samples were run in a cathode buffer (50 mM Tricine, 15 mM Bis-Tris, pH 7.0) with 0.02% Coomassie G-250 for 1h at 55V, then voltage was increased to 120V for another 1h. The cathode buffer was replaced by a clear-cathode buffer with 0.002% Coomassie and samples were run for another 3h at 120V. The apparatus was kept cold to avoid distortion of lanes. After electrophoresis, gels were de-stained with water. In-gel activity assays were then performed, or gels were stained using Bio-Safe Coomassie G-250 Stain and imaged with a Bio-Rad ChemiDoc MP system.

4.3.3 In-gel activity assays

Assays were run according to the protocol devised by Nijtmans et al. (2002). Gels were pre-incubated in 5 mM Tris-HCl (pH 7.4) for 15 min. Complex I activity was assayed by adding 0.1 $mg.ml^{-1}$ β-NADH and 2.5 $mg.ml^{-1}$ p-nitroblue tetrazolium chloride (NBT) and incubating for 10 min at room temperature. For complex II, 0.2 mM phenazine methosulfate (PMS), 84 mM succinic acid, 50 mM NBT, 10 mM potassium chloride and 4.5 mM EDTA were added and the gel incubated at room temperature for 15 min. In the complex IV assay, 20 mg 3,3'-diamidobenzidine (DAB), 20 mg cytochrome *c*, and 480 units catalase were added and the gel was incubated for 3 h at 37 ºC.

4.3.4 Gel band extraction and mass spectrometry analysis

Gels rinsed with deionized water were used for band extraction and subsequent analysis by mass spectrometry. Gel strips of 2mm x 5mm were carefully cut so that cross-band contamination was avoided. The strips were washed with water and destained twice with methanol in 50 mM ammonium bicarbonate (NH_4HCO_3). Samples were dehydrated with acetonitrile, then dried in a Speed Vac centrifuge and rehydrated in 25 mM dithiothreitol, after which they were incubated for 20 min at 56ºC. After incubation, the supernatant was discarded, and the samples were incubated in the dark at room temperature with 55 mM freshly prepared iodoacetamide. Then, the supernatant was discarded, the samples rinsed twice with water and the dehydration process with acetonitrile was repeated once more. After centrifuging in the Speed Vac, samples were this time rehydrated with a 12 $ng.ml^{-1}$ Trypsin Gold™, MS grade solution in ProteaseMAX™ (Promega, Madison, WI, USA) surfactant and incubated at 50 ºC for 1 h. Tubes were centrifuged at 16,000 g for 10 min, and the extracted peptides were transferred to a new tube where a 0.5% solution of trifluoroacetic acid was added to inactivate trypsin. Samples were stored at -80ºC until MS analysis was performed. They were analyzed by the Oregon State University Mass Spectrometry center in an Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer (ThermoFisher).

4.4 Results

4.4.1 Bivalve respiratory function

We assessed the coupling and integrity of each mitochondrial extract by high-resolution respirometry, and found the respiratory rates to be in line with previous studies (Munro et al., 2013 and Rodríguez et al. 2019, *submitted*). Mean NADH-pathway linked OXPHOS coupling efficiency, calculated as 1-(LEAK state/N-coupled state) in all species varied between 0.35 (*M. mercenaria*) and 0.55 (*A. islandica* from the Gulf of Maine), reflecting the generally low contribution of this pathway to respiration in marine bivalves. The addition of cytochrome *c* as a measure of outer

mitochondrial membrane integrity (quality control test) gave mean increases of coupled N-pathway respiration ranging from 0.49 to 11.33% (often with no increase), hence below the 15% threshold which indicates no substantial damage to mitochondria. Complex IV activity varied among species, with *M. mercenaria* showing the highest (106.42±42.73) activity, *A. islandica* with varying activity depending on the population (Magdalen Islands: 23.04±1.95, Gulf of Maine: 76.70±13.43), while both *M. arenaria* and *S. solidissima* showed the lowest activities (12.45±1.72 and 12.50±4.06, respectively). See table 4.1 for detailed results and the age of the individuals included in this study, estimated from shell length.

Table 4.1 Summary of mitochondrial respiratory function parameters and integrity of the outer mitochondrial membrane (cytochrome *c* test) in the three marine bivalve species used for supercomplex analysis. $j_{\approx P}$ is the OXPHOS coupling efficiency for N-pathway linked respiration. Maximum reported longevities for the species (MRL) and range of estimated ages based on shell height and length-at-age models (Pace et al. 2017, Ridgway et al. 2011, Giguère et al. 2005, Brousseau 1979, Filippenko and Naumenko, 2014) are also reported. Values for respirometry are ±SEM, except for the cytochrome *c* tests where maximal increase in respiration is reported.

Species (n)	MRL (yrs)	Estimated age (yrs)	$j_{\approx P}$	Cyt. *c* test (max. % increase in respiration)	Complex IV activity ($pmolO_2.s^{-1}.mg^{-1}$)
M. arenaria (3)	28	8-10	0.44 (±0.19)	0.76	12.45 (±1.72)
S. solidissima (3)	37	10+	0.38 (±0.09)	0.52	12.50 (±4.06)
M. mercenaria (2)	106	30-40	0.35 (±0.14)	0.49	106.42 (±42.73)
A. islandica from the Gulf of Maine (3)	507	50-75	0.55 (±0.04)	11.33	76.70 (±13.43)
A. islandica from the Magdalen Islands (3)	507	140-160	0.45 (±0.08)	6.82	23.04 (±1.95)

4.4.2 Blue-Native PAGE on mitochondrial extracts reveals supercomplex assemblies

Figure 4.1 shows representative results of the empirical determination of the detergent-to-protein ratio for an optimal BN-PAGE analysis. Different separation patterns are visible from 2:1 to 10:1 digitonin:protein (w/w) ratios for the shortest-lived

species *M. arenaria*. This determination was repeated twice for species *M. arenaria* and *A. islandica* in preliminary analyses, and the ratios showing excessive protein smearing and suboptimal bands separation and resolution were deemed unsuitable. The optimal ratio was set at 8:1 digitonin:protein (w/w), and was used to make further species comparisons.

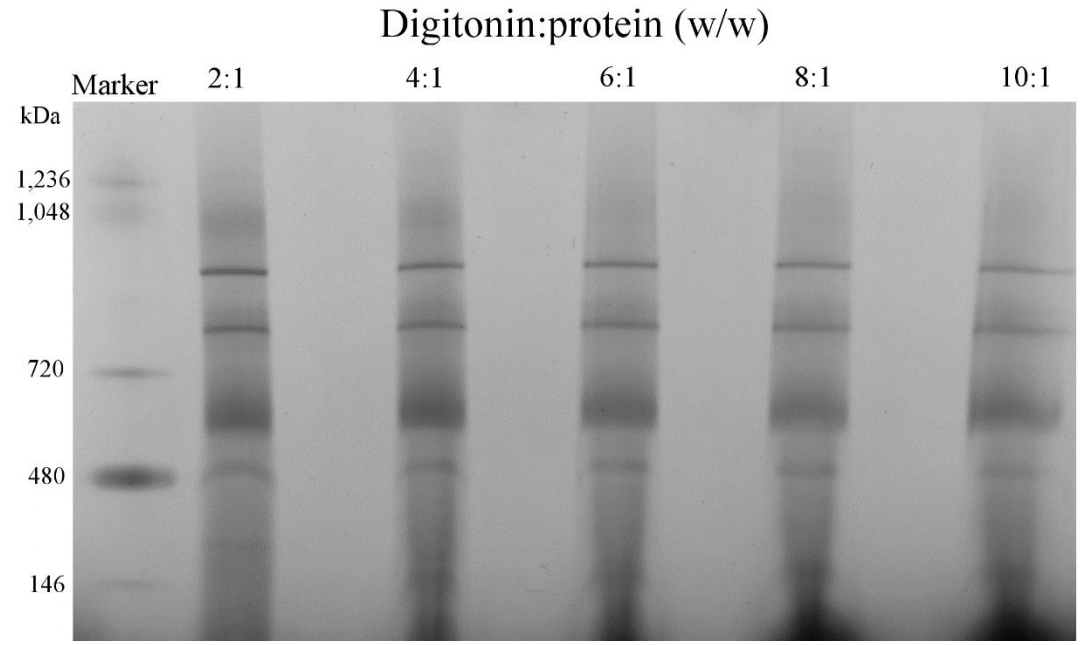

Figure 4.1 Empirical determination of the suitable digitonin:protein ratio for supercomplex analysis. Shown is *M. arenaria* (MLSP 28 yrs) in mantle mitochondria, imaged after Coomassie staining.

In-gel activity assays for complexes I and IV among our four species of interest are shown in figure 4.2. Species are presented from left to right in order of increasing maximum lifespan and show commonalities and differences in banding patterns. The activity assays demonstrated the existence of active ETS complexes in different assemblies of distinct molecular weights, with various bands in the 1,236 kDa-720 kDa area. Crucially, they showed that the digitonin solubilisation conditions did not substantially damage the ETS complexes, and differences in color intensity suggested variations in the amount of complexes in particular bands. In all our species, the activity assays evidenced cases of co-localization of complexes I and IV within the same band.

Complex IV activity was qualitatively more pronounced in *M. arenaria* than in the other three species, while *S. solidissima* exhibited very faint bands for both complexes. *M. mercenaria* and *A. islandica* showed more intense complex I activity in the high molecular weight bands than the two shorter-lived species. Combined together, these elements suggested the existence of supercomplexes in marine bivalves, which was further assessed in by mass spectrometry. The first prominent band below the 1,048 kDa marker appeared common to all species, and the in-gel activity of complex I suggested more presence of this complex in this band.

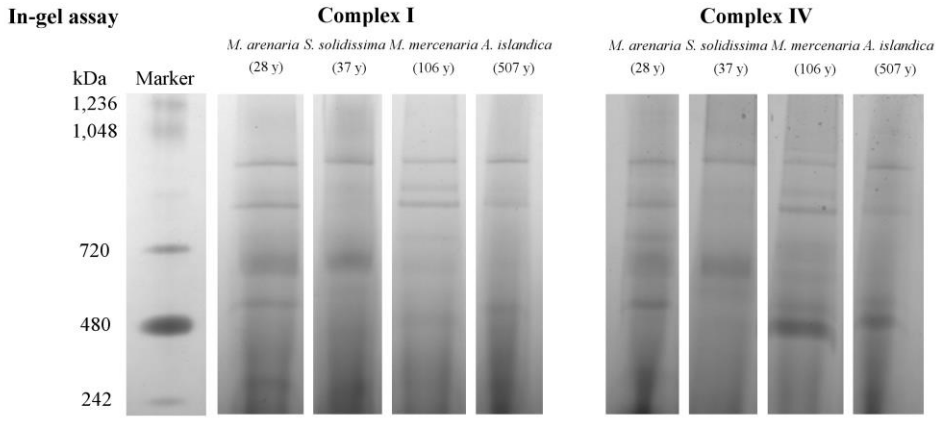

Figure 4.2 In-gel activity assays of complexes I and IV in mantle mitochondria of 4 marine bivalves ranked from shortest- to longest-lived, and separated by BN-PAGE. Mitochondria were solubilized with a digitonin-to-protein ratio of 8:1 (w/w) and a NativeMark molecular weight standard was added as a means of estimating molecular weight of the resulting bands.

4.4.3 Patterns of supercomplexes assemblies and identification of proteins by Lumos MS

Detailed patterns of mitochondrial electron transport system complexes assemblies among the four species studied and stemming from the MS analysis are shown in figure 4.3. First, figure 4.3A shows the whole gel staining with Coomassie G-250 Stain, with the presence of high molecular weight bands close to and around the

1,048 kDa marker, emphasizing the existence of supercomplex assemblies in all the species. For ease of analysis, bands are hereafter named S1 to S5. In the expanded area presented in fig. 4.3B, band S1 appeared present in *A. islandica* but less so in other species, while bands S2 and the aforementioned S3 were present in all species. Band S4 and the band presumably containing free complex I (determined from in-gel activity and position respect to the marker) were common to all species, but much fainter in *S. solidissima*, a species which showed a strikingly different banding pattern. Band S5 was only apparent in the two longest-lived species *A. islandica* and *M. mercenaria*. No differences were found in the banding pattern between the two *A. islandica* populations sampled, and the results shown throughout this study are for the Magdalen Islands population.

Results from the mass spectrometry analysis revealed the proteins contained in each band and are presented in figure 4.3C. Band S1 only contained complex I in shorter-lived species (presumably in multiple units), whereas in *M. mercenaria* it contained complexes I, III and IV, hence forming a respirasome containing all the complexes for electron transfer to molecular oxygen. In *A. islandica*, no complex III was detected, and only complexes I and IV appeared in band S1. Band S2 contained a respirasome (I, III and IV), as well as complex V in *M. arenaria*. In *S. solidissima* and *M. mercenaria*, only complexes I and IV were detected in this band, while in *A. islandica*, it was composed of I, IV and V. Band S3 also varied in composition between species, containing complexes I and IV in *M. arenaria*, while only complex I was found in *S. solidissima* and *M. mercenaria*. In *A. islandica* however, this band was similar to S2 but composed of two different subunits of complex I (NAD 3 and 5, see figure for details). Composition of band S4 varied from containing complex III (*M. arenaria*) to complex I (*S. solidissima* and *M. mercenaria*) while it was a SC composed of I, IV and V in *A. islandica*. The band presumed to contain free complex I (see paragraph above) was actually found to be composed of complexes I and IV in all species, except *M. mercenaria* where it also contained complex V. Finally, band S5 only contained

complex I (*M. arenaria*) or complex IV (*M. mercenaria*), while in *S. solidissima* it was composed of complexes I and IV, and in *A. islandica* contained complexes III and IV, the only such arrangement among all bands across species. For better contrast of the bands also see figure S4.1.

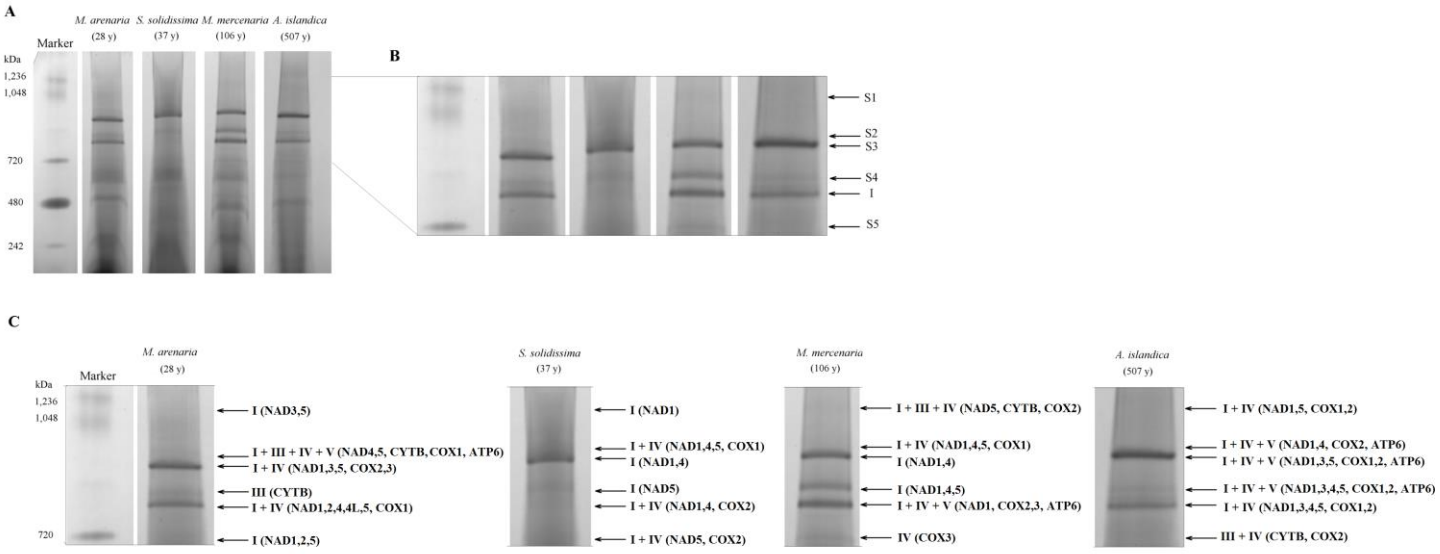

Figure 4.3 Mitochondrial electron transport supercomplexes show different patterns in four marine bivalve species with increasing longevities. (A) Mantle mitochondria solubilized with a digitonin-to-protein ratio of 8:1 (w/w) and a NativeMark™ molecular weight standard separated by BN-PAGE and stained with Coomassie. (B) Blow-up region evidencing different SC assembly patterns. (C) Blow-up regions with detailed composition of each resulting band, as obtained by Lumos MS analysis. The genes corresponding to each subunit are shown in parenthesis. See also fig S4.1 for better contrast pictures of the bands.

4.5 Discussion

We report for the first-time evidence for supercomplex organization in marine invertebrates, increasing the generality of these structures among metazoans. Furthermore, we describe differences in these arrangements, with an apparent increase in complexity with increased lifespan. In *A. islandica*, the six bands in the high-molecular weight region contained multiple enzyme complexes, while in all other species, only three of those bands had SCs. This long-lived species showed various

SCs containing complexes I, IV and ATP synthase (complex V), rarely encountered in the other three species. Interestingly, the respirasome (a SC containing all the complexes for electron transport to molecular oxygen) was found in *M. mercenaria* and *M. arenaria*, but not *A. islandica*. It is also of note that we could not find complex II in our SCs, as generally reported (Dudkina et al., 2010;Jang and Javadov, 2018). Beyond these general qualitative differences in complexity among species, whether supercomplex integrity is associated with individual age in bivalves and whether longer-lived species have more robust structures as seen in aging rat hearts (Gómez et al., 2009) remains to be meticulously evaluated.

We were first able to work out the empirical conditions to find supercomplex assemblies. We chose to work on the mantle tissue since mitochondrial function (flux control coefficients) and rates of H_2O_2 efflux have been extensively characterized in the past, and it is rich in mitochondria (Munro et al., 2013, Rodríguez et al., *in preparation*). The mantle is a tissue displaying low metabolic activity, and is composed of three folds with cells of various functions: shell secretion (outer fold), sensory cells (middle fold), and muscular (inner fold, see Gosling, 2015). Therefore, it is composed of various cell populations with presumably different energetic requirements, and our analysis represents a pool of all these different mitochondrial types. Compared to the gills (a respiratory tissue often studied), respiration rates, phosphorylation efficiency and anaerobic enzyme activities are low in the mantle (Strahl et al., 2011a;Strahl et al., 2011b). This tissue is also characterized by low rates of proliferation and apoptosis (compared to the gills), more so in *A. islandica* compared to a short-lived species (Strahl and Abele, 2010). The mantle is particularly geared towards resisting hypoxic and anoxic conditions in *A. islandica*. Indeed, phosphorylation efficiency was higher at low oxygen conditions than at normoxic PO_2 in *A. islandica* (Tschischka et al., 2000), suggesting that these bivalves strive to maintain ATP production in aerobic conditions, avoiding the switch to anaerobiosis in this tissue. In contrast, there was succinate accumulation (signalling the onset of anoxia) in the adductor muscle tissue

during anoxia in the same species (Strahl et al., 2011), and opine dehydrogenases have elevated activities, suggesting glycolytic ATP production in the foot, an organ used for burrowing (Oeschger and Storey, 1993). To our knowledge, such metabolic analysis of the mantle is not available for the three other species used in this study; hence, it is possible that these could be more reliant on anaerobic pathways, especially in an intertidal and more active species such as *M. arenaria*. For example, the mantle of cephalopod molluscs appears to be a glycolytic tissue (Storey and Storey 1979), but in these species it also serves in locomotion, contrary to bivalves.

Is the complexity of the SC assemblies linked to lifespan? This could explain the differences in the control strength among complexes found in our previous study (Rodríguez et al., 2020), where the complex I-linked NADH-pathway had no control over respiration in longer-lived species, while the opposite was true for the CIV-pathway. This question should be investigated further and the analysis be refined to compare activity patterns of in-gel activities among species, as well as quantify the abundance of protein complexes in the bands, which were not done in this study. However, we could appreciate a higher intensity of CIV banding in *M. arenaria* compared to the other species, which does not reflect its activity as measured by respirometry (table 4.1). This could be due to the difference between the free activity of the enzyme in the in-gel assay, and the measurement in functional mitochondria. Additionally, it would be important to look at the presence of SC and their exact nature in another tissue such as the gill, harboring respiratory and feeding functions. Moreover, a limitation in studying the mantle tissue is the important concentrations of mucus, secreted by the abundant acid mucopolysaccharides mucocytes. This mucus has viscous and lubricant properties that aids water flow by reducing friction (Gosling, 2015a). Hence, it is important yet challenging to separate this mucus when isolating mantle mitochondria in bivalves, and purification of mitochondrial isolates would improve ETS and SC enzymes separation and in-gel resolution. Careful separation and resuspension of mitochondrial pellets allowed us to standardize the methods and to

compare patterns of SC distribution between species in this study, but improving the mitochondrial extraction could prove useful for future analyses.

Specific age determination of the specimens included in this study *via* band-counting on the shells was not performed. However, we measured shell length and used previously published length-at-age models for each population (or nearby populations when specific population's data was not available) to estimate the age range of our individuals. In *A. islandica* from the Magdalen Islands, individuals used were at least 140 years old based on these estimates (Pace et al., 2017b), which means they had entered the phase of asymptotic growth, but were middle-aged compared to the MLSP of the closest population for which data is available (Sable Bank population, see Kilada et al., 2007). *M. mercenaria* were 30-40 years old based on reports by Ridgway et al. (2011b). *S. solidissima* were over 10 years old (Giguère et al., 2005), and *M. arenaria* are estimated to be between 8-10 years, based on Brousseau (1979) and Filippenko and Naumenko (2014); however estimating age from shell length is more challenging in this species. Therefore, we can postulate that all individuals used in this study were in the adult phase of their life-cycle, and at -or close to- mid-age. It would therefore be necessary to go further and explore SC dynamics linked to age on a longitudinal basis within and between these species to be able to assess the relationship, or lack thereof, between SC complexity and lifespan.

The current understanding of the nature of supercomplexes has evidenced assemblies of varied composition and important diversity both among and within species, tissues and cellular type. For example, rat neurons which energetically depend on oxidative phosphorylation, have significantly more complex I assembled into supercomplexes than astrocytes who rely on glycolysis, as well as more free complex I and produce more ROS (Lopez-Fabuel et al., 2016). Knockdown of a major complex I subunit (NDUFS1) disassembled this enzyme from supercomplexes, impairing electron transfer as shown by the decrease in respiration, and increased ROS production. This is consistent with a study showing that a lower abundance of free

complex I was associated with more efficient assembly of the complex, increased efficiency of substrate utilization, minimal ROS production and increased longevity (Miwa et al., 2014). Anoxia-resistant turtles have been shown to possess SCs that are more stable when exposed to detergent dodecyl maltoside than those of mammalian models, and were proposed as a putative mechanism of protection against ROS burst upon reoxygenation (Bundgaard et al., 2018). However, in a follow-up study (Bundgaard et al., 2019), the low aerobic capacity and ROSp in anoxic conditions was shown to be due to a reduction in substrate oxidation, possibly via downregulation of ETS complexes activities, rather than mitochondrial morphology. As such, neither mitochondrial content, morphology (volume density or cristae surface area) nor SC composition or stability changed between warm-acclimated normoxic or cold-acclimated normoxic and anoxic treatments. While exposure to low temperature and anoxia does not change SC distribution, perhaps the important SC robustness previously shown in this species (Bundgaard et al., 2018) partly explains their tolerance to such conditions and reoxygenation, a feat that could be verified by comparing this tolerant species' SC architecture to other less-tolerant related species. Given the exceptionally high resistance to low oxygen conditions in *A. islandica* (Strahl et al., 2011a) and the differences in the environmental conditions (marine *versus* coastal and estuarine habitats) faced by the species studied herein, it would be interesting to assess whether the differences in SC assemblies are linked to oxygen tolerance rather than longevity.

Previous research on lipid composition in marine bivalves hinted at the possible presence of supercomplexes. Indeed, Kraffe et al. (2008) found important amounts of cardiolipin in various species of bivalves, a diphosphatidyl glycerol lipid found in the inner mitochondrial membrane and essential for supercomplex assembly and stability (Szeto and Liu, 2018). The nature of the cardiolipin differed among species and these exhibited distinct FA profiles. Bivalves from the Heterodonta subclass (burrowers) had high levels of PUFAs EPA and DHA in their cardiolipins, while the two other subclass,

Pteriomorph and Eupteriomorph (epifaunal bivalves, which live attached to a surface rather than burrowed) had significantly less EPA in their cardiolipin. One of the long-lived species of heterodonts, which is also used in our study, *M. mercenaria*, had especially high levels of both long-chain PUFAs. The authors proposed an adaptive value of the OXPHOS process linked to the different environments faced by the two bivalve subclasses, although the consequences of these differences to mitochondrial functions remain to be explained. It therefore appears that studying the cardiolipin composition of our bivalve species and the potential links to SC nature is the next logical step in understanding the role of these supramolecular assemblies.

The interactions between enzymatic complexes in SC assemblies are proposed to allow the formation of exclusive electron pathways, and minimize ROS production (Guo et al., 2018). Considering the MOSTA (Blier et al., 2017), SC assembly is therefore potentially crucial to minimize damage to the mitochondrion. A clear indication of that is the important consequences observed after a loss in the stability of SCs. Lipid peroxidation can disrupt SCs: Maranzana et al. (2013) found that dissociation of SC with dodecylmaltoside led to an increase in hydrogen peroxide and superoxide in bovine heart mitochondria and reconstituted liposomes of complex I and III. When investigating age-related differences in the electron transport system organization of hearts of young and old rats, Gómez et al. (2009) found that the levels of individual complexes did no vary. However, they separated and identified the SCs, and showed that their levels were diminished between 13-25% in old rats versus young individuals, especially for those SCs with higher molecular weight. These seemingly small decreases are actually compared to the levels found in pathological studies with important mitochondrial structural and respiratory chain defects, such as Barth or Leigh Syndromes. Their results ultimately suggest that there is either an impairment in formation, or an increase in the rate of decomposition of supercomplexes in aging heart mitochondria. Aging is therefore accompanied by a decrease in SC association, and an increase in ROS and oxidative damage. If SC stability is key in determining

mitochondrial aging phenotype, species differences in MLSP could be related to their capacity to maintain SC integrity.

In conclusion, we demonstrate the existence of SC in marine invertebrates, further proving their ubiquitous nature in animals. The implications of the differences in the nature and distribution of SCs among species of varying lifespan only allow us to speculate as to their potential role in oxidative stress management, and warrants further research.

SUPPLEMENTARY MATERIAL FOR CHAPTER IV

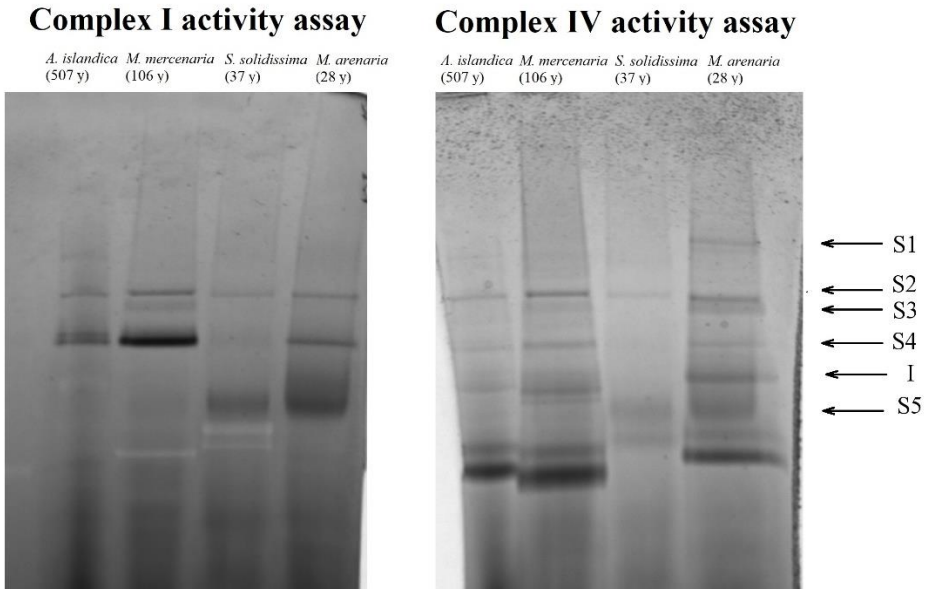

Figure S4.1 In-gel activity assays of complexes I and IV in mantle mitochondria of 4 marine bivalves ranked from longest- to shortest-lived, and separated by BN-PAGE. Mitochondria were solubilized with a digitonin-to-protein ratio of 8:1 (w/w), and band annotations are indicated as per figure 4.3.

CHAPTER V
GENERAL DISCUSSION

The overall objective of this Ph.D. thesis was to decipher the links between mitochondrial membrane and ETS structure, function and regulation of respiration inside and among species of marine bivalves in relation to lifespan differences in the framework of the MOSTA. This research model was used because it encompasses the longest-lived non-colonial animal known to science, the ocean quahog *A. islandica*, and an important range in reported MLSPs among its populations in the Northern Atlantic Ocean, but also a wide range in longevities among species of the subclass Heterodonta. To undertake this objective, I first reviewed the literature on the evolutionary and mechanistic theories of aging, further focusing on the mitochondrial basis of the phenomenon, the MOSTA and its associated evidence in comparative studies. Then, I presented marine bivalves as models of longevity, as well as the metabolic control analysis framework to understand the control of electron flux, and closed the chapter introducing the supramolecular organisation of the mitochondrial ETS in the form of supercomplexes, with a final detailed statement of the thesis objectives and hypotheses (chapter I).

In chapter II, I used lipid analysis *via* GC-FID and spectrophotometry techniques to show that among populations of the ocean quahog *A. islandica*, mantle and gills mitochondrial membrane composition and ETS enzyme activities do not align with the predictions of the MOSTA (Blier et al. 2017). The results provide further insight into the characteristics of this species in light of the previous interspecific relationships found in bivalves (Munro and Blier, 2012;Munro et al., 2013), and are published in the journal *Frontiers in Physiology* (Rodríguez et al., 2019). Further lipidomic analyses and the study of respiration in isolated functional mitochondrial extracts from these populations should provide insight into the basis for these longevity differences. These novel findings are discussed hereafter in light of the roles of

mitochondrial enzymes and lipids in the management of age-related oxidative stress, and in natural populations' environmental conditions (section 5.1).

In chapter III, I used high-resolution respirometry on mantle mitochondrial isolates from four species of marine bivalves and show that long lifespan was characterized by an almost null control from the NADH- and succinate-pathways of electron entry (complexes I and II), and a higher control by terminal electron acceptor complex IV (Rodríguez et al., 2020). I propose that these differences are linked to a favorable control of oxidative stress achieved by relieving the control at the electron entry complexes, and the sum of flux control coefficient being higher than unity hint at supercomplex assembly. I then advocate for a further exploration of the control of ROS management at these different pathways and complexes in marine bivalves. This discussion chapter explores in further details the evidence for a differential control of mitochondrial respiration in various comparative studies, as well as the role of cytochrome *c* oxidase in longevity and the various mechanisms involved in respiratory control (section 5.2).

Chapter IV expands this analysis to the supramolecular architecture of the marine bivalve ETS in relation to aging. I used BN-PAGE, in-gel activity and mass spectrometry on mantle isolated mitochondria from the same four species of short to long-lived bivalves, and found presence of supercomplex assemblies, the first report of the sort in a marine invertebrate (Rodríguez et al., *in preparation*). There were common banding patterns, with all species possessing a "core" supercomplex with complexes I and IV, and long-lived species appeared to have more complexity in supercomplex assemblies containing complexes I, III, IV and V in various combinations. These results need further investigation into the potential relationship between supercomplex robustness, complexity and longevity. The implications of supercomplex assemblies for longevity, their dynamic nature and possible control of electron flow are discussed in detail in this last chapter (section 5.3).

I finish this discussion chapter by opening up on the role and regulation of oxidative stress in cellular signalling, life-history strategies employed by animals and mitonuclear communication in the context of aging (section 5.4).

5.1 Lipid function in aging, population and evolutionary implications, and the role of the environment

5.1.1 Membrane composition, population genetics and the environment

Despite the lack of differences in membrane peroxidation index among populations of different reported lifespan (see Chapter II), all populations have a low PI, which in theory should indicate a robust membrane geared for a long lifespan. However, reported longevities are often far from the records found in Norway and Iceland, hence some other factor is interfering, and membrane composition is not sufficient *per se* to guarantee a long lifespan. It is well established that not only the genotype, but also the environment influence growth in bivalves (Filippenko and Naumenko, 2014), and growth is itself closely linked to the aging process via the mTOR pathway (Blagosklonny and Hall, 2009). As suggested in the discussion of chapter II, environmental conditions could therefore partly explain differences in population lifespan. Indeed, both Begum et al. (2010) and Basova et al. (2017) proposed that the faster aging rate in both Kiel Bay and White Sea populations could be induced by the cellular stress response to the large seasonal salinity and temperature amplitudes in these two brackish habitats. Frequent alterations between aerobic respiration and anaerobic glycolysis in bivalves exposed to the vagaries of environmental salinity fluctuations typical for these two sites presumably cause higher metabolic demand, oxidative stress and mitochondrial turnover and may be causal for the faster rates of lipofuscin accrual from deteriorating cellular components in these populations. In the soft-shell clam *M. arenaria* (MLSP of 28 y), variations in growth rates between geographical sites can be explained by differences in salinities and tide frequencies, and lifespan differences have also been reported in this species, notably

for the White Sea population which is lower than the species MLSP (Filippenko and Naumenko, 2014). What role may membrane composition play in the interplay between aging and the environmental pressure? Because controlling ionic flux across biological membranes is essential for osmoregulation, the implications of having a specific membrane composition warrants further investigation. Much like hypoxia, osmoregulation can trigger moments of metabolic arrest, and a consequent burst of ROS production upon reoxygenation after this arrest is expected. Moreover, antioxidant pathways are upregulated in different species when transitioning between stable and unstable salinity conditions (reviewed in Rivera-Ingraham and Lignot, 2017). However, data from the medium and long-lived German Bight (Helgoland) and Iceland populations suggest that no antioxidant upregulation nor increase in ROS levels takes place after hypoxia/reoxygenation events (Strahl et al., 2011a). It would be of interest to verify whether this response is retained by shorter-lived populations, or if their premature aging stems from an inability to deal with these events and their energetic costs.

Sampled individuals from the six populations used in chapter II come from locations with potentially different causes of intrinsic and extrinsic mortality. Extensive sampling efforts over the years (hundreds of individuals for the Icelandic, German Bight and Kiel Bay populations) provide a high level of confidence in the reported MLSPs (Strahl, 2011 and references therein). Nonetheless, extrinsic causes such as predation, fishing pressure or eutrophication are distinct from site to site and will potentially affect population structure. For example, the German Bight area (Northern Sea) has historically high fishing pressure (Witbaard and Bergman, 2003), especially in the Oyster Grounds region (which is geographically distinct from the region used in this and other studies), whereas the Icelandic or White Sea populations have had lower fishing activities (see table 6.1 in Strahl, 2011 and references therein). There appears to be no obvious direct link between anthropogenic pressure and lifespan, based on the data compiled by Strahl, 2011 (*ibid*). Indeed, the German Bight population has a mid-

range lifespan, and Norwegian coast has a mid to high-range lifespan (previous estimates of 93 years have been updated to 300 years, see table 1 in Rodríguez et al., 2019) but high anthropogenic impact notably from fishing pressure. On the other hand, the White Sea population has one of the lowest MLSP but also low anthropogenic impact. These ideas are corroborated by the report of a negligible influence of fishery in western Atlantic sites, where fishing mortality appears to have been below natural mortality of the species (Pace et al., 2017a;Pace et al., 2018). This suggests once again that other potential environmental factors also mentioned in Strahl's (2011) dissertation contribute to interpopulation variation in lifespan.

Considerable efforts have been put in place to evaluate the genetic background of ocean quahog populations and potential intrinsic factors for differential aging. A phenotypic analysis done by Witbaard (1997) showed that the different populations could be distinguished based on their shell morphology. Later, Holmes (2003) confirmed this using another set of populations, and prompted them to look at whether these variations were due to genotypic differences or plasticity. No direct relationship was found between geographical distance and morphology, however distinct populations were found to adopt different growth strategies (Holmes et al., 2003). Contrary to the results reported by Dahlgren et al. (2000), who found the variability inside each population to be higher than that between them, genotypic analysis showed differences between populations were higher than previously estimated (Holmes et al., 2003). However, both studies looked at considerably distinct populations and different markers. Holmes and colleagues compared four North Sea populations (two in Sweden, one each in Scotland and Holland) to a Canadian population (Nova Scotia), whereas Dahlgren and colleagues (Dahlgren et al., 2000) studied seven US (Maine, Georges Bank, Cape Cod, Eastern Long Island, Northern New Jersey, Delaware, Virginia), one Canadian (Nova Scotia) and three eastern Atlantic (Sweden, Norway and Faroe Islands) populations. Begum and colleagues (2018) assessed the mitochondrial haplotype distribution (using the *cyt b* gene marker) of *A. islandica* as well as

morphological differences in the six populations that were also used in chapter II of this thesis. They found within population variance to be much higher than between populations (80 and 2%, respectively), in agreement with Dahlgren et al. (2000), and contrasting with Holmes et al. (2003). Discrepancies between the methods may explain these diverging conclusions, which suggest that phenotypic plasticity is the main driver of morphological differences. The duration of the larval stage in *A. islandica* (30 to 60 days) is believed to favour dispersal by ocean currents, and therefore gene flow between populations; the dispersal of the species appears to have occurred from the western to the eastern Atlantic (Dahlgren et al., 2000). Moreover, an analysis of the whole mitochondrial genome showed that the northernmost population of Iceland had a higher mitochondrial genome diversity compared to the southern German Bight and Kiel Bay populations (Glöckner et al., 2013). Therefore, based on the considerable gene flux between populations, we can posit that the genomes may not have diverged and positive selection may not have yet acted and resulted in differences in lipid composition.

There is an urgent need to better assess the effect of different stressors (salinity, temperature, oxygen) on mitochondrial physiology and the link with population-specific patterns and the rate of aging. Indeed, some investigators have proposed that the evolution of hypoxia resistance and long lifespan come hand in hand (Pamenter and Munro, 2019). Most studies are based on murine models such as the naked-mole rats, but marine invertebrates offer interesting avenues to understand the interplay between the two, and the role of membrane structure in this context. The relative weight of the frequencies of salinity fluctuations versus hypoxia/reoxygenation events in marine bivalves' energetic constraints are not entirely established and studies with clear hypothesis and precise measures of mitochondrial physiology are needed. We can presume that populations of *A. islandica* with shorter lifespans face more hypoxia/reoxygenation events based on the fluctuations of their habitats, but this remains to be verified. By extension, it would be interesting to assess whether these

also have reduced hypoxia tolerance, hence if a compromise between hypoxia resistance and longevity exists in these animals. Overall, studies linking osmoregulation, energetics and oxidative stress in estuarine or coastal marine invertebrates are scarce, and the interplay between these parameters and the rate of aging therefore warrants further investigation (Freire et al., 2011).

5.1.2 Fluidity, peroxidation resistance and evolutionary implications

Maintenance of membrane fluidity is fundamental for cellular function, and the effects of adding double bonds are varied. While inserting a first double bond triggers fluidity, the addition of multiple double bonds increases permeability and proton leak across the mitochondrial membrane, but do not add more fluidity (Brenner, 1984;Brand et al., 1994). Therefore, the effect of the kink generated by the first double bond on membrane fluidity and cell functionality suggests that global unsaturation level could be beneficial to lifespan. Accordingly, in a study on 107 species of birds, Galván et al. (2015) showed that FA unsaturation level in the liver is positively, rather than negatively, correlated with lifespan. This apparently contradictory finding is due to the beneficial effect of having more MUFAs, while PUFAs did indeed decrease with increasing longevity (as predicted by the MOSTA), suggesting that resistance to peroxidation is associated with bird longevity. Most interestingly, FA length was the main determinant of variation in maximum lifespan among species, independent of degree of unsaturation. This was also suggested by Bozek et al. (2017), who showed that in multiple mammalian tissues, structural lipids are more likely to be saturated in long-lived species (which also implies a higher MUFA:PUFA ratio). However, their findings also suggest that energy-related lipids are more likely to be unsaturated in longer-lived species. Therefore, it appears that the relationship between unsaturation and lifespan is more complex and could go beyond the simple link with peroxidability, to the maintenance of adequate cellular homeostasis (i.e. fluidity and proper functioning of membrane-bound proteins). However, in our analysis of *A. islandica*

populations (chapter II), we didn't find any differences in MUFA abundance associated with lifespan at the intraspecific level.

Considering the important implications of membrane lipid structure on organismal function, the FA composition of membranes was suggested to be evolutionarily optimized (Pamplona, 2008;Naudí et al., 2013). To further evaluate this hypothesis, Galván (2018) tested for stabilizing selection on fatty acid composition in 107 species of birds previously investigated (see previous paragraph and Galván et al., 2015). The author's results show strong evidence for optimization of fatty acid length at 18 carbons, as well as stabilizing selection for the proportion of MUFAs, but not PUFAs nor SFAs. Nonetheless, it appears that at least in vertebrates, membrane FA composition is optimized by evolution to a certain chain length and degree of unsaturation, with strong implications for organismal fitness and the evolution of higher lifespan. It would be of interest to extend this analysis to non-vertebrate models, as the findings from our group (Munro and Blier, 2012) suggest that it is also the case for bivalve molluscs. Our results suggest that the FA composition of membranes might be optimized by evolution at the species level, but that it may not be the case at the population scale (Rodríguez et al., 2019).

Interspecific relationships generally point to a link between membrane lipid composition and lifespan in a variety of models, and the lack of association among populations of *A. islandica* (both in mitochondrial and cellular debris membranes, see tables S5.1 and S5.2) does not disprove this intimate link between lipids and aging. Rather, it is possible that other more finicky aspects of lipid composition have to be scrutinized at the population level, such as phospholipid classes (among which cardiolipin levels), specific polar head groups, pairing of FA into the phospholipid, etc. Future studies involving the emerging field of lipidomics (the characterization of all lipid species and their roles in gene regulation and protein expression) should help solve some of the conundrums surrounding the role of lipids in aging. For example, applying "shotgun lipidomics" to the analysis of membrane composition in the long-

lived naked-mole rats, revealed a particular phospholipid/fatty acid profile. They possess up to 28-fold lower amounts of DHA (PUFA) than short-lived mice, depending on the tissue (Mitchell et al., 2007). However, the fine-scaled distribution of FA in different phospholipids showed for example a complete absence of di-polyunsaturated phospholipids in the long-lived animals, contrary to mice. This could be explained by the doubly dangerous implications of this configuration, as having two highly susceptible FAs next to each other could exacerbate the propagation of lipoxidative damage. Moreover, *de novo* DHA synthesis appears to be down-regulated in naked-mole rats a mechanism which relies on peroxisomal metabolism: the implication of the peroxisome in lipid metabolism and aging has been overlooked and warrants further investigation. MLSP in mammals and birds correlates directly with 18:1 n-9 levels and inversely with 22:6 n-3, which is 320 times more susceptible to peroxidation than the former; however both have good fluidity properties: it therefore seems that an appropriate balance between fluidity and peroxidability is key (Hulbert, 2005). Accurate measurements of membrane fluidity in populations of *A. islandica* may reveal patterns not apparent from analysis of FA abundance.

The recent methodological developments in lipid analysis involving techniques such as ionization, quadrupole or nuclear magnetic resonance and the framework of lipidomics (reviewed in de Diego et al., 2019) should provide powerful tools to better assess potential lifespan-lipid relationships. While identification and quantification of very low concentrations of lipid classes is now feasible, the wide variety of existing lipid species represents a substantial challenge for both the experimental (improvement of the analytical equipment, choice of the extraction and detection methods) and bioinformatics (classification systems, databases, efficient software analysis, integration into signalling pathways) aspects of lipidomics. Nonetheless, lipidomics promises great advancements in knowledge of the global lipidomes and their contextualization in physiological and pathological settings (Naudí et al., 2015). The power of such studies is exemplified in Bozek et al. (2017), where the concentrations

of more than 20,000 lipid compounds were analyzed in relationship with MLSP, in 6 different tissues from 35 mammalian species. Their findings point to various lipid classes and associated pathways that underlie maximal lifespan variation. It would be of interest to extend these analyses to molluscan species and populations to assess the similarities and differences in pathways, in relation to lifespan.

5.1.3 Lipid metabolism and aging are tightly linked

Although spanning beyond the realm of membrane composition, it is now well established that lipid metabolism plays a central role in healthy aging, with direct relationships to reproduction and lifespan. For instance, studies on *C. elegans* worms and fruit flies have managed to uncover intimate relationships between lipids that act as signalling molecules, and aging modulation in interventions such as dietary restriction or intermittent fasting, which are known to promote lifespan extension and healthy aging (reviewed in Bustos and Partridge, 2017). At the basis of this longevity extension by lipid signals are nuclear receptors transcription factors, such as the dauer formation (*daf*) gene *daf-12* and its ligands dafrachronic acids (DA), which are involved in the insulin/insulin-like growth factor signalling pathway (IIS), a nutrient-sensing network modulating development and lifespan (*ibid*). The positive relationship between lifespan and age at reproductive maturity has been acknowledged for years (Prothero, 1993 and for bivalves see Ridgway et al., 2011). This intimate link between reproduction and aging is further demonstrated by the life-extending effect of germline ablation, which also increases lipid storage and promotes starvation resistance, and it appears again that *daf* genes are mediators of these effects (reviewed in Hansen et al., 2013). Lipids could thus act as "switches" between somatic maintenance and reproduction (Papsdorf and Brunet, 2019). Moreover, it appears that supplementation with lipids can have health and lifespan benefits (Bustos and Partridge, 2017, and see Morin et al., 2017). For instance, MUFAs such as oleic and palmitoleic acids can extend worm lifespan (Han et al., 2017), while n-6 PUFAs also produce the same life-extending effect by activating autophagy (O'Rourke et al., 2013). This last effect

appears to be mediated by the mammalian target of rapamycin (mTOR) pathway, a protein kinase of the phosphatidylinositol 3-kinase-related kinase (PI3K) family regulating growth via various anabolic and catabolic pathways (Blagosklonny and Hall, 2009). Modulation of transcription factors by the mTOR-autophagy pathway regulates lipid metabolism enzymes, and lifespan extension can be achieved by inhibiting the pathway (reviewed in Papsdorf and Brunet, 2018). Surprisingly, very little is known about the mTOR, PI3K and IIS pathways in bivalves. A few studies have reported their involvement in the autophagy process in response to environmental stressors and the immune response (Clark et al., 2013;Picot et al., 2020). One particular study reported maintenance of the TORC1 protein complex genes during aging in the short-lived (4 yrs MLSP) scallop *Chlamys farreri*, while insulin/growth factor genes (including PI3K) had an aging-related increase in expression (Lian et al., 2019). Future research should aim at deciphering these nutrient-sensing pathways, their regulation and their involvement in lipid metabolism using a comparative approach in short and long-lived bivalves such as *M. arenaria* and *A. islandica*.

Another striking example of the interplay between FAs, genetic background and longevity comes from a study by Shmookler Reis et al. (2011). The authors analyzed the fatty acid composition and transcripts of genes involved in FA biosynthesis in strains of *C. elegans* worms with almost identical genetic background, where the longest-lived strains have mutations in the IIS pathway. Crucially, they went further and evaluated the consequences of knocking down lipid-biosynthesis gene expression on peroxidation resistance (measured in duration of survival in a medium with hydrogen survival at a toxic level), and the effect of different FA supplementation on survival. They found that longer-living strains have more short- than long-chain saturated FAs, more MUFAs than PUFAs and shorter mean chain length, less EPA, lower PI, lower n-3/n-6 ratio. Transcript levels in these long-lived strains showed reduced elongases and n-3 desaturases, which are implicated in PUFA formation, while delta-9 desaturase transcripts increased, contributing to MUFA formation.

Supplementation with either EPA or DHA produced a significant reduction in lifespan compared to unsupplemented controls, or to those supplemented with saturated fatty acid palmitic acid (16:0, where a lesser important decrease in lifespan was reported). Moreover, RNAi knockdown of genes encoding for two elongases and a delta5 desaturase increased lifespan and peroxidation resistance of wild-type worms, while knockdown of delta9-desaturase genes had the opposite effect. Overall, these results show that modulation of FA composition and increased lipoperoxidation resistance is a potent mechanism of lifespan extension not only in IIS mutants, but also in wild-type worms. As we suggested in our interpopulation analysis in marine bivalves (see Chapter 2), they note that a reduced PI should be potentially detrimental in that it lowers membrane fluidity; however, increasing saturation is a mechanism that stabilizes membrane fluidity as reported in nematodes challenged by temperature fluctuations (Murray et al., 2007). Yu et al. (1992) showed that membrane fluidity decreases with age, and lifespan extension through dietary restriction protects from this decrease. It could be hypothesized that the maintenance of adequate fluidity is a characteristic of long-lived species, and by extension populations.

More pathways and mechanisms than those aforementioned are affected by or influence lipid metabolism. For example, epigenetic alterations of chromatin state (e.g. *via* histone acetylation) induce changes in the expression of desaturating genes, and lifespan extension is linked to an increased MUFA abundance (reviewed in Papsdorf and Brunet, 2018). Moreover, acetyl-CoA as a product of fatty acid oxidation can act on epigenetics (again via histone acetylation), while epigenetics can also modulate lipid metabolism (reviewed in de Diego et al., 2019). During aging, mitochondrial lipid cardiolipin decreases while its oxidized product accumulates; this important lipid species is implicated in the mitochondrial-cytosolic stress response and strongly linked to the assembly of supercomplexes (Paradies et al., 2010;Kim et al., 2016). Interestingly, a study on 21 species of birds found that cardiolipin content and ROS production in red blood cells, but not peroxidation resistance, was linked to maximum

longevity (Delhaye et al., 2016). It will thus be of interest to look at the role of these specific lipids in marine bivalves, especially given the results of the supercomplex analysis in chapter IV (further discussed below). In conclusion, most of the studies demonstrating the implications of these various pathways and specific lipids have been done on classic models of aging, from nematode worms and fruit flies, to laboratory mice and sometimes humans. Unraveling the importance of genetic determinants of FA composition, the link with reproductive status and the implications of growth and nutrient-sensing pathways such as mTOR and the IIS in marine invertebrates will be paramount to better understand why ocean quahogs attain such high lifespans.

5.1.4 ETS activity-longevity associations and beyond

The lack of reported differences between either ETS activities or membrane lipid composition associated to population longevity inside the species *Arctica islandica* should not be seen as a complete surprise, as many examples of relationships that held true among species are not found within a particular species (see for example mice selected for basal metabolic rate in Brzek et al., 2007). An exception is the honey bee study by Haddad and colleagues (2007), but in that case two genetically programmed castes (queen and worker) were compared, which is not exactly the same as comparing populations or individuals; or the orchid bee study on membrane composition where different genera were compared, and relationships resembled those between species, albeit weaker (Rodríguez et al., 2015). The reason behind this might be that scaling down in the tree of life to the population or individual level is not as straightforward and simple as it can be imagined, it is not a "fractal-like" relationship. Therefore, we are perhaps not as likely to find these wide relationships at a smaller scale as at the wider species or groups scale. Other factors have to be accounted for, that make relationships more complex, for example, the fact that ROS are actually signalling molecules (discussed further below), and that ETS complexes need to perform adequately to cover ATP demands. For this, a certain fatty acid composition is needed to maintain proper membrane fluidity, and as elegantly demonstrated recently

by Budin et al. (2018), the nature of fatty acids and the viscosity of the membrane directly control ETS function by mediating collisions between ubiquinone and ETS complexes. It follows from this that unless other regulatory mechanisms were involved (such as phosphorylation), the very similar activities of the ETS enzymes are not surprising, given their similar lipid compositions.

It should be noted that we could not assess the activity of complex II, another electron entry portal (succinate) and potential source of ROS (Quinlan et al., 2012), which was previously shown to have a much lower activity in longer-lived bivalves (Munro et al., 2013). The very low activity of this complex in this species reported in Munro et al.'s (2013) study and the low quantity of tissue at our disposal explains this missing information. Nonetheless, given the absence of differences associated to lifespan found for other complexes of the ETS, and the environmental factors discussed above, it appears unlikely that complex II activity remains the only parameter linked to longevity in these populations. Given the central role of complex I in aging-related phenotypes found in a variety of species (see for example Baumgart et al., 2016 and the discussion hereafter), we would have predicted to find a relationship at the level of this enzyme rather than at the level of complex II. One way that ETS function and lifespan may be related in populations could involve divergences in the control of electron transport or ROS production by the different complexes, as suggested in chapter III of this thesis using an interspecific approach.

5.2 Control of mitochondrial respiration by the ETS as a means of attaining a high longevity

Using the framework of the MOSTA and the suspected link between aging and the contribution to respiration by the ETS components, we found longevity-related differences in the control exerted by the different enzymes and pathways in four species of marine bivalves (chapter III and Rodríguez et al., 2020). More specifically, we found that the N- (complex I-linked) and S- (complex II-linked) pathways exerted very little

control of oxidative phosphorylation in long-lived species, while the opposite was true in short-lived ones. On the other hand, the terminal electron acceptor complex IV exerted a strong control over respiration in long-lived bivalves, which were also characterized by lower ATP synthase and higher citrate synthase activities. We chose to assess the effect of control by these different pathways on OXPHOS respiration with a combination of substrates achieving maximal rates of coupled respiration, rather than on the maximal uncoupled ETS activity. This is due to the fact that there was almost no limitation by the phosphorylation system, and we believe that this method is a better approach to evaluate control in physiological conditions. These results have implications on the management of electron transport and oxidative stress geared towards a high lifespan.

5.2.1 Control of electron entry into the ETS by the NADH- and succinate-pathways and oxidative stress management

By titrating the N-pathway's maximal activity and maximal OXPHOS capacity simultaneously with complex I inhibitor rotenone, we found that it exerted a stronger control over respiration in shorter-lived than in longer-lived species. Various studies have evidenced a role for this pathway in aging-related phenotypes, but it is to our knowledge the first time that such an association emerges at the interspecific level.

Aging is generally accompanied by alterations in the activity and the control over OXPHOS of complex I. Since the mitochondrial DNA codes for seven out of the 13 polypeptides pertaining to complex I, it was proposed that this should be the enzyme most affected by aging (Lenaz et al., 1997). In line with the MOSTA, complex I activity was reduced in the heart, brain and liver mitochondria of old rats (Castelluccio et al., 1994;Ventura et al., 2002). Moreover, as rats grew older and complex I activity decreased, this pathway became strongly rate-controlling over respiration in liver mitochondria, contributing to a global decrease in OXPHOS capacity (Ventura et al., 2002). The lipid environment was also found to be altered in aged individuals, with a

decrease in mitochondrial cardiolipin content (Paradies et al., 2002). A compensatory increase was seen in mtDNA transcripts levels of subunits ND1 and ND5 of complex I in older rats, and old individuals were also found to produce more hydrogen peroxide than their younger counterparts (reviewed in Genova et al., 2004). These results clearly point to the interrelatedness of complex I activity and stability, the mitochondrial lipid environment and ROS production in the aging phenotype. Similar links between aging and N-pathway sensitivity are also reported in invertebrates. Inhibition of complex I subunits in the fruit fly *D. melanogaster* abolished the lifespan-extending effects of dietary restriction (Zid et al., 2009). In lines of the seed beetle *Acanthoscelides obtectus* selected for longer lifespans (and later reproduction), inhibition of complex I by the inhibitor tebufenpyrad had less effect than in shorter-lived beetles (Jovanović et al., 2014). Since both the mitochondrial and nuclear genomes encode subunits of this complex, the authors wanted to disentangle the contribution of both genetic backgrounds. By doing crosses with combinations of short and long-lived mitochondrial or nuclear genomes, they found that the observed effect was associated with the high longevity nuclear background. Other studies in different models report the widespread occurrence of the link between N-pathway control, stability and longevity. Short-lived *C. elegans* mutants with dysfunctional ETS had a major loss of complex I together with a compensating increase in complex II activity (Pujol et al., 2013). When compared to a species with similar body mass but shorter longevity, the pigeon showed lower rates of hydrogen peroxide efflux, and this appeared to be directly linked to its lower complex I content (Lambert et al., 2010).

Analogous to the N-pathway control over respiration, titration of OXPHOS with complex II inhibitor malonate had almost no effect in long-lived *A. islandica* compared to its shorter-lived counterparts, although not when compared to *M. arenaria*, our shortest-lived bivalve. This revealed that the S-pathway tends to exert lower control in longer-lived species. Some studies report decreases in complex II activity with lifespan, although less so than for complex I. For example, suppressing

genes encoding for complexes I, III and IV in *C. elegans* increases lifespan (Dillin et al., 2002), while it does not when targeting genes encoding for complex II (Kuang and Ebert, 2012). There was however a decrease in respiration of about 40% when suppressing complex II genes, but the magnitude of the effect was much lower than when targeting genes encoding for the other complexes. Three important differences separate complex II from the other main ETS components: one is the fact that the polypeptides forming this complex are encoded by the nuclear genome only, hence it may be under less intense selection, as mutation rates of the mitochondrial genome are an order of magnitude higher than for the nuclear one (Gershoni et al., 2014). The second distinction is that complex II does not directly pump protons, and its inhibition has a very low effect on membrane potential. It has previously been proposed that mitochondrial membrane potential acts like a switch that initiates lifespan extension: long lifespan in *C. elegans* is associated with lower membrane potential (Lemire et al., 2009). Finally, since complex II participates in the TCA cycle as well as the ETS, it could be proposed that its inhibition affects reactions beyond electron transfer down to complex IV, and it may be that feedback mechanisms in place may complicate the picture seen for more integral proteins such as complex I, III and IV.

5.2.2 Cytochrome *c* oxidase control in longevity and excess capacity

Control over respiration by the complex IV pathway (cytochrome *c* oxidase) was higher in longer-lived species *A. islandica*, making it the first report of the sort for this terminal electron acceptor. Despite finding no correlation between longevity and maximal catalytic activity of this complex (normalized by citrate synthase activity) in a previous study on marine bivalves (Munro et al., 2013), studies in other models have however highlighted the importance of this pathway in the aging phenotype. An age-dependent cytochrome *c* oxidase deficiency has been reported in houseflies and fruit flies (Sohal, 1993;Ren et al., 2010), but also in various human tissues (Müller-Höcker, 1992), among others. This characteristic should impact the control of respiration by the enzyme and subsequently the upstream production of ROS.

Various studies report an important function for the control of respiration by complex IV. In rat liver mitochondria, both adenine nucleotide translocator (ANT) and complex IV were found to be rate-controlling over respiration, with control strength values of 0.29 and 0.17, respectively (Groen et al., 1982). In intact human hepatoma HepG2 cells, complex IV control coefficient was even higher (0.73) under OXPHOS conditions. However, adding the ionophore valinomycin collapsed the electrical component of the membrane potential and gave significantly lower control values (0.30), showing that membrane potential is responsible to a great extent for the control by this enzyme complex on respiration (Dalmonte et al., 2009). To our knowledge, the role of mitochondrial membrane potential remains unexplored in marine bivalves and should be further investigated. There can however be another "second mechanism of respiratory control" by complex IV that is independent of membrane potential: allosteric inhibition by ATP (Arnold, 2012). Pioneering studies by Arnold and Kadenbach (Arnold and Kadenbach, 1997;1999) in tuna and bovine hearts revealed this mechanism, whereby the complex isoform IV-1 binds ATP (instead of ADP), decreasing the affinity of the enzyme for cytochrome *c*. This allows detection of cellular ATP levels hence fine-tuning production of ATP to demands. In the context of oxidative stress this inhibition of the complex would have the benefit of keeping the membrane potential at low values when ATP/ADP ratio is elevated in the matrix, hence limiting ROS production (reviewed in Vogt, 2016). Inhibition of complex IV would thus keep the upstream ubiquinone pool in a reduced state, which would be advantageous since complex III has been shown to produce superoxide when the ubiquinone pool is oxidized (Dröse & Brandt, 2008). This shows the salubrious effect of controlling respiration at the level of complex IV; it appears that long-lived bivalves take advantage of the powerful role of the terminal oxidase. However, much more work is needed to decipher the role of allosteric regulation of electron transfer and acceptor proteins in bivalve mitochondria and the putative links to longevity.

In addition to evidencing tight control over respiration by complex IV, we found a lower excess capacity of the enzyme in *A. islandica* than in other shorter-lived bivalve species. This is illustrated by the intercept at zero complex IV inhibition of the linear regression through the points after the inhibition threshold for each species. Previously, a high complex IV excess capacity has been linked to oxygen affinity and suggested to help maintain the ETS in an oxidized state, allowing adaptation to fluctuating conditions such as oxygen concentration, energy demand, substrate availability, or temperature (Gnaiger et al., 1998;Harrison et al., 2015). However, measurements in a variety of human intact cells have suggested that this high excess capacity may be overestimated in isolated mitochondria models, as complex IV was found to be in low excess or nearly limiting in the OXPHOS state (coupled respiration with glutamate and malate, see Villani et al., 1998). Moreover, at lower oxygen concentrations, there is a significant increase in flux control by complex IV in mice (Wiedemann and Kunz, 1998), with the enzyme being almost entirely rate-controlling below 50 µM oxygen. As discussed in the previous paragraph, modulators of complex IV activity such as nitric oxide (Brown, 1995), adenine nucleotides (Kadenbach et al., 2013) and other allosteric regulators could contribute to OXPHOS regulation even more so at low excess capacity of the enzyme. In an anoxia tolerant species such as *A. islandica* and with a capacity to depress metabolic rate to low levels, as well as low energy demands (Strahl et al., 2011a), a low excess capacity of this enzyme is in line with the idea of a tight control of flux through the ETS.

In a recent study, Bettinazzi et al. (2019) determined that *A. islandica* expressed different gender-linked mitochondrial phenotypes. Indeed, two sex-linked mitochondrial haplotypes are transmitted separately through male and female gametes in this species, and can also coexist in the somatic tissue: this is the doubly uniparental inheritance mode of mitochondrial DNA inheritance (DUI), as opposed to the classic strict maternal inheritance (SMI) found in almost every multicellular eukaryote (Breton et al., 2007). Comparing two DUI species (among which *A. islandica*) to two

other SMI species (including *M. mercenaria*) revealed grouping of the species sharing the same mode of parental inheritance. Specifically, DUI species shared lower OXPHOS/ETS rates, stronger limitation by the phosphorylation system, and higher flux control by complex IV (hence an almost null complex IV excess capacity) in male compared to female-type mitochondria. These differences were important in male sperm compared to female oocytes, but they were low in the somatic tissue (gills) even if heteroplasmy (presence of the two different mtDNAs in the same tissue) can occur. SMI species exhibited, in striking opposition, similar phenotypes in all cellular types. Knowing these crucial differences in mitochondrial metabolism, in chapter III we determined the sex of sexually mature specimens of *A. islandica* prior to mitochondrial isolation, in order to always pool 1 male and 1 female together (and as our 3 other species were SMI). The low complex IV excess capacity found is reminiscent of the male mitotype (95% excess capacity in gills), rather than the female one. The different functions of the mantle (connective tissue, nerves and muscle; our study) compared to the gills (respiration and feeding, see Gosling, 2015; Bettinazzi et al. 2019) could also lead to different excess capacities of complex IV. Sex-linked differences in respiratory control by complex IV could be further explored, as they could lead to divergences in the rate of aging between males and females.

Could a low excess capacity allow for “healthier” mitochondria to remain in long-lived *A. islandica*? These bivalves are known to sustain long bouts of hypoxia, *via* metabolic rate depression and importantly no oxidative burst upon reoxygenation, for which the mechanism remains unknown (Abele and Philipp, 2013). A proposed mechanism involves nitric oxide, produced by the haemocytes and located in the mantle fluids during hypoxia (Strahl and Abele, 2020). Nitric oxide is a known inhibitor of complex IV (Brown, 1995), and it could be speculated that this inhibition combined to a low excess capacity could allow for release of cytochrome *c* and the onset of apoptosis. The apoptotic pathway has recently been evidenced to be more than merely a death mechanism: it can also release proliferation signals such as

mitochondrial biogenesis (mitogenesis), mediated by caspases (reviewed in Pérez-Garijo, 2018). Thus, apoptosis would have the salubrious effect of activating mitogenesis, maintaining fully coupled mitochondria and keeping ROS production at a minimum. This idea would need to be further tested and the links between ROS, apoptosis and longevity be explored (Hekimi et al., 2016). The complexity and diversity of mechanisms regulating the activity of the ETS complexes is only starting to be unravelled and could provide further insights into the causes of aging.

5.2.3 Other regulatory mechanisms and factors involved in respiratory control

Various metabolites and other key processes participate directly or indirectly in OXPHOS and exert a certain degree of control of respiration. Some of these have been implicated in longevity in one way or another. For instance, the adenine nucleotide translocator (ANT) was found to exert a high control over respiration in rat liver mitochondria (Groen et al., 1982). It is possible that this protein, responsible for translocation of free ATP/ADP across the inner mitochondrial membrane also controls respiration to a certain extent in marine bivalves, and it could be assessed in future experiments by titrating its effect with its inhibitor (atractyloside). It appears that the activity of the ANT can be affected by lipid peroxidation products (Chen et al., 1995), and modification of its properties could partly explain the aging-related decrease in OXPHOS (Gouspillou et al., 2010).

Apart from the TCA cycle enzyme citrate synthase (found to have a higher activity in longer-lived bivalves), the contribution of key metabolites and enzymes upstream of the ETS has not been assessed in the present study. For example, the pyruvate dehydrogenase complex (PDHC) links glycolysis to the TCA cycle by decarboxylating pyruvate to produce acetyl-CoA and thus plays a central role in feeding electrons to the ETS (Ogasawara et al., 2007). Acetyl-CoA acts as a regulator of autophagy through protein acetylation, and modifying pyruvate flux affects age-related autophagy; hence nutrient-sensing and degradation pathways are tightly linked

to longevity (Stacpoole, 2012;Schroeder et al., 2014). The PDHC is also known to be a major source of H_2O_2 production under conditions of high nutrient load and when the mitochondrial glutathione pool is in an oxidized state (Fisher-Wellman et al., 2013), hence its role in mediating oxidative stress and respiratory rates warrants further scrutiny. Another crucial component is the malate-aspartate shuttle, enabling entry of electrons from glycolysis into the mitochondrial matrix through the otherwise impermeable inner mitochondrial membrane. In yeast, overexpressing genes for components of the malate-aspartate, as well as the glycerol-3-phosphate shuttle extends lifespan through increasing the NAD/NADH ratio (Easlon et al., 2008). In both these cases, the genes involved were from the mitochondrial components of these two shuttles, hence the control of respiration by these. In the case of glycerol-3-phosphate shuttle, it is composed of the cytosolic glycerol-3-phosphate dehydrogenase (cGPDH), which reduces dihydroxyacetone phosphate to glycerol-3-phosphate (Gp), and the one embedded in the inner mitochondrial membrane (mGPDH) which catalyzes the reverse reaction, thereby providing electrons directly to the Q-pool (for a complete review see McDonald et al., 2018). In chapter III of this thesis, we measured respiration with Gp as a substrate since it increased oxygen consumption substantially. mGPDH has been characterized as an important site of superoxide production (Orr et al., 2012), yet it is relatively understudied. Using the mGPDH-specific inhibitor iGP (Orr et al., 2014), should allow evaluation of the control strength exerted by this pathway on respiration and ROS flux in bivalves, and the putative link to the aging process.

In addition to the longevity-flux control differences reported in chapter III, it is noteworthy to mention that the distribution of control of various steps can change according to temperature. For our experiments, we chose a temperature within the natural habitat range of all species (10°C), and they were kept in common garden conditions. However, we cannot rule out the possibility that the temperature used from the analyses deviates from the optimal temperature for each individual species. We chose 10°C as a compromise, as it is well within the range for all 4 species. It is the

mid-range temperature for *A. islandica*, but is in the lower end of the spectrum for the other species, and is below the minimum spawning temperatures for all 4 of them (see figure 3.5 in Gosling, 2015). Because metabolic enzymes and catalytic capacities have different thermal sensitivities, temperature could affect the regulation of the whole pathway as shown by the increased excess capacity of complex IV at low temperatures in *D. melanogaster* (Pichaud et al., 2010). In mouse heart, Lemieux et al. (2017) showed that the N-pathway (specifically the pyruvate dehydrogenase complex) and the phosphorylation system were the two strongly limiting steps at low temperature, although there seems to be considerable interspecies variation in what controls OXPHOS at varying temperatures (see Blier and Guderley, 1993 and Lemieux et al, 2010 for differences in fish species). In the eurythermal freshwater planarian *Dugesia tigrina*, temperature also affects the control of mitochondrial respiratory capacity: at lower temperature, the contribution of the N-pathway to maximal ET capacity was reduced, with partial compensation by the increased contribution from the S- pathway (Scott et al., 2019). The authors suggest that the N-pathway may be more thermosensitive than the S-pathway (again agreeing with reports in fish and mammals, see Christen et al., 2018 and Lemieux et al., 2017, respectively), the latter's increase compensating for a decline in ATP production, but likely contributing to an increase in ROSp. The effect of a four-week cold acclimation was an increase in the contribution of the N-pathway (via an increase in complex I activity), which may serve to alleviate the higher oxygen consumption stemming from the S-pathway (which provides energy at a lower P/O ratio). Also of note in the planarian study was an increased limitation by the phosphorylation system with decreasing temperature and acclimation to lower temperature resulted in a better match between OXPHOS and phosphorylation systems, possibly through and adjustment in coupling efficiency (Scott et al., 2019). Moreover, no complex IV excess capacity was found in *D. trigrina* at physiological temperature, and only a slight excess at colder temperature, suggesting that this complex is a crucial regulator of flux and redox state of the ETS (discussed above). This is in contrast with studies on mammals and fruit flies of increasing excess at colder temperatures (Pichaud

et al., 2010;Lemieux et al., 2017), but not with cold-adapted species such as the wolfish (Lemieux et al., 2010), where the thermal sensitivity of complex IV was high compared with that of the N-pathway. It suggests that the later pathway could be a key site of adaptation for mitochondrial capacity in ectotherms at the expense of a high complex IV excess capacity (Blier et al., 2014;Scott et al., 2019). For example, we could hypothesize that the sensitivity of the N-pathway is lower in long-lived species, owing to a more "robust" complex I (discussed above), and these would not necessitate compensation by the S-pathway and the incumbent increases in ROSp and oxygen consumption. The phosphorylation system seemed not to be limiting, except slightly in *A. islandica*, and it would be of interest to see how its control is affected in this and other shorter-lived bivalves.

Beyond these regulatory mechanisms potentially involved, the actual physical organization of the ETS and the possible link with longevity warranted further scrutiny. This was reinforced by our finding that the sum of control coefficients for the ETS components was well above 1, suggesting that these enzymes could behave as units. This hinted at the presence of supercomplex assemblies in marine bivalves.

5.3 Supercomplexes structure and assembly's roles in lifespan variation

ETS complexes have been known to be organized in a more complex fashion that in a "chain" of proteins as proposed by the fluid model, and the structural evidence this was in great part evidenced by the works of Schagger and Pfeiffer (2000) and Wittig et al. (2006). In chapter IV, we reported for the first time that marine bivalves possessed ETS enzymes organized as supercomplexes with different banding patterns that suggest a link between complexity of arrays and longevity, although further experiments are needed to support such claim. However, these results confirm the quasi-ubiquitous nature of these structures, and the differences among species further raise the question of their functional significance. Supercomplex assembly and function

is tightly linked with the two other parameters studied in this thesis: lipid composition and control of electron flow.

5.3.1 Supercomplex assemblies and the possible links to longevity

In rat cardiac mitochondria, the higher molecular weight assemblies show the greatest decline with age, in the first observation of such a link between supercomplex integrity and aging (Gómez et al., 2009). Further investigations into the relationship between longevity and supercomplex robustness in the same species (but different tissues) have given support to the idea. In skeletal muscle, mitochondria from older individuals contained lower amounts of complexes I, III and V, and lower weight supercomplexes were significantly reduced (Lombardi et al., 2009). In particular, the band containing complexes I and III in a ratio of 1:2 was reduced by around 40% in old rats versus their younger counterparts, while two major supercomplexes containing complexes I, III and variable copies of complex IV were increased. This points at a possible compensatory mechanism in the face of lower individual complex activities and overall reduced oxidative capacity of the skeletal muscle mitochondria. Interestingly, in rat cortex, not only did the lower-weight supercomplex I_1III_2 show the greatest decline, but the ATP synthase monomer also decreased significantly, leading to an increase in oligomeric forms of the enzyme (Frenzel et al., 2010). Rows of ATP synthase dimers shape the membrane's curvature into cristae, in which space protons are pumped from the matrix (Cogliati et al., 2013). This could potentially affect ATP synthase activity and oxidative capacity, but the authors of the rat cortex study were unable to report such parameters. No change in ATP synthase activity with age was found in a study of rat hearts (Castelluccio et al., 1994), but this contrasts with the finding of a lower activity in older rat brain and heart (Guerrieri et al., 1992). It is moreover intriguing that aging led to the opposite effect on ATP synthase in the model fungus *Podospora anserina*, where older individuals had dissociated dimers with important changes in the molecular structure of the enzyme, more monomers in senescent mitochondria, and a complete collapse of the cristae structure slowly leading

to apoptosis (Daum et al., 2013). Thus, both ETS complexes and the ATP synthase organized in supramolecular structures are modified during the aging process.

The mechanisms involved in these structures' formation and in the adequate maintenance of their assemblies are only beginning to be unraveled and could help understand their role in aging. Supercomplex assembly requires coordination of key processes: nuclear DNA–encoded transcripts are translated by the ribosome close to the outer mitochondrial membrane, imported into the intermembrane space by the TOM (translocase of the outer membrane) complex and into the mitochondrial matrix or directly in the inner membrane (in the case of single complexes and ATP synthase) by the TIM23 (translocase of the inner membrane) complex. Then, the matrix nucleoid (containing mtDNA) initiates transcription of proteins which are inserted in the lipid bilayer, thereby assembling with the nuclear-encoded subunits with the help of various assembly factors such as SCAF1 or HG2A, leading to ETS complexes and supercomplexes (reviewed in Cogliati et al., 2018). Therefore, it is clear that proper coordination and communication between nuclear and mtDNA are key in the process of supercomplex assembly. If as predicted by the MOSTA, longer-lived organisms accumulate less mtDNA damage (as seen in mammalian tissues for example, see the review by Pamplona, 2011), these would have the ability to retain more robust and proper functioning assemblies, thereby leading to lower rates of ROS production (although the link between mtDNA and ROS production is under question, see Trifunovic et al. 2005 and Shabalina et al., 2017). Interestingly, it appears that the expression of complex IV subunits may explain differences in stoichiometries and assembly of supercomplexes, and lead to tissue metabolic specificities (Cogliati et al. , 2018). Indeed, the supercomplex assembly factors Rcf1 and Rcf2 interact with complexes III_2 and IV, and are necessary for the latter's full assembly: depletion of Rcf1 in the aging fungus *P. anserina* affects the assembly and decreases the amount of complex IV-containing supercomplexes. In turn, this impairs mitochondrial activity and reduces lifespan of the fungi (Fischer et al., 2015), and in *S. cerevisiae*, deletion of

Rcf1 also causes increased ROS production (Chen et al., 2012). It has been proposed that a particular class of phospholipids, cardiolipin, may be key in the Rcf-regulated complex IV assembly (Garlich et al., 2017).

Lipid-protein interactions appear to be a fundamental in maintaining supercomplex stability. Cardiolipin is a diphosphatidylglycerol particularly susceptible to peroxidation due to its important content in unsaturated fatty acids (especially linoleic acid, 18:2 in a tetralinoleoyl cardiolipin), and because of its locale in the inner mitochondrial membrane, close to the sites of ROS production (Paradies et al., 2010). Cardiolipin was first shown to be essential for the activity of cytochrome oxidase, complexes I and III, as well as other mitochondrial carriers, suggesting a role for this phospholipid in the process of coupled electron transfer (reviewed in Enríquez and Lenaz, 2014). Recent studies showed that cardiolipin stabilizes supercomplexes and individual complexes: a loss of cardiolipin through mutations or substitutions in yeast caused instability and dissociation of SC organization (Lenaz and Genova, 2012). Moreover, ROS production can affect the respiratory activity via oxidative damage to cardiolipin (Paradies et al., 2000, 2002), and flux control analysis has shown that lipid peroxidation affects reconstitution and maintenance of a I-III supercomplex (Genova et al., 2008). More precisely, it seems that cardiolipins with symmetrical acyl side-chains promote interactions between proteins and influence ETS function, and specific cardiolipin species are associated with particular supercomplexes (reviewed in Gómez and Hagen 2012). Alterations in the acyl side-chains are linked to age-related deterioration of supercomplexes (Frenzel et al., 2010), adding to the complexity of the already appreciated decrease in cardiolipin content with age reported in various studies (reviewed in Paradies et al., 2010). In this thesis, we could not measure cardiolipin content of our bivalve species and populations, which could echo the differences found in the nature of supercomplexes in chapter IV. We know thanks to Kraffe et al. (2008) that some of the bivalves in our study possess cardiolipins, but the findings related to aging warrant more precise and refined. For instance, could the composition of

cardiolipins and their susceptibility to peroxidation explain longevity differences in natural populations of *A. islandica*? Could the differences in assembly patterns among species be linked to the presence of different acyl side-chains in the cardiolipins and could these in turn determine the robustness of supercomplexes? The use of bivalves as a study model within a comparative framework should prove powerful in answering these questions. Finally, it is of note that the role of this phospholipid in maintaining complex stability may go beyond supercomplexes, as the activity and stability of complex II (traditionally not found in supercomplexes, see the discussion of chapter IV) has been found to be cardiolipin-dependent (Schwall et al., 2012).

Beyond supercomplex stability, there appears to be a relationship between proteostasis (protein stability) and longevity, as observed in four species of marine bivalves, among which *M. mercenaria* and *A. islandica*. Indeed, Treaster et al. (2013) investigated basal protein homeostasis and responses to various stressors (urea, tert-butyl hydroperoxide and high temperature) in the foot and adductor muscle, and found that longer-lived bivalves performed better than shorter-lived ones in maintaining enzyme function (creatine kinase), resisting protein unfolding and temperature-induced protein aggregation. They suggested that a superior chaperone system could explain this feat, but were unable to demonstrate it due to technical constraints. These results are consistent with the low levels of protein carbonyl in the mantle and gills of *A. islandica* compared to shorter-lived bivalves (Strahl et al., 2007;Ungvari et al., 2011) and the lack of increase of these damaged proteins in the cardiac muscle as a function of age (Sosnowska et al., 2014). Further investigation into this exceptional proteostasis in *A. islandica* by Treaster et al. (2015) using the glyceraldehyde-3-phophate dehydrogenase (GAPDH) enzyme as a reporter revealed that the quahog retained 45% of its basal function at high urea concentrations, while mice and shorter-lived bivalves lost all function at lower concentrations. The underlying mechanism to this stress resistance remained unidentified, as the removal of post-translational glycosylation and most heat-shock proteins had no important effect on the long-lived bivalve's stress

resistance. Overall, redox homeostasis and proteostasis seem to play crucial roles in determining species lifespan, and supercomplex integrity may also be an important factor in the equation.

5.3.2 Supercomplexes: dynamic assemblies regulating electron flow?

The exact advantages of supercomplex with regards to the dynamics and management of electron transport are still under debate. From their flux control analysis, Bianchi et al. (2004) proposed that the enzyme associations taking place in the ETS allow a structural stabilization of the membrane proteins, a feature also suggested in a subsequent study showing that complex III assembly is essential for the stability of complex I in human and mice cells (Acin-Perez et al., 2004). Among the multiple functional advantages of supercomplex assembly that were initially proposed, this structural stability, the role for supercomplexes as "scaffolds" for complete complex I assembly, the management of oxidative stress, and the regulation of respiration are most widely accepted agree upon (reviewed in Milenkovic et al., 2017). Another proposed advantage of supercomplex organization is thought to be substrate channeling, that is, the passing of an intermediate metabolite from one enzyme directly to the other, without release into the substrate pool (Bianchi et al., 2004). This theoretically bypasses enzyme competition for the substrate, increasing the efficiency of the reaction. In the respiratory chain, coenzyme Q and cytochrome c are the localized substrates, with most of coenzyme Q being free in the membrane bilayer. Kinetic evidence shows that coenzyme Q is a homogenous diffusible pool between reducing and oxidizing enzymes, and the plasticity model proposes that both supercomplexes and individual complexes coexist in the IMM, with their prevalence depending on the energetic state of the mitochondrion (Enríquez, 2016; Enriquez and Lenaz, 2014). This idea of coenzyme Q channeling has gained experimental support from various studies. These include flux control analysis (reviewed in Lenaz and Genova, 2007), pharmacological inhibition and spectrophotometric/chromatographic measurements (Benard et al., 2008), genetic ablations of complexes (Lapuente-Brun et al., 2013) and

cryo-EM resolution of megacomplex $I_2III_2IV_2$ where the potential compartment for coenzyme Q was evidenced (Guo et al., 2017). Nonetheless, the existence of such a physical compartment between complex I and monomeric complex III remains to be strongly established, as the recent structural resolution of supercomplex $I+III_2$ suggests limited complex I activity when coenzyme Q trapping occurs (Letts et al., 2019). This evidence casts serious doubts on the idea of substrate channeling as a function of supercomplexes (reviewed in Milenkovic et al., 2017 and Guo et al., 2018), although other studies also point at a channeling of cytochrome *c* (Pérez-Mejías et al., 2019). This fierce debate may only be solved with accurate resolution of the molecular structures of supercomplexes coupled with enzymatic assays in conditions as close as physiological as possible, and in comparative studies going beyond the "traditional" model species. Therefore, although it appears that supercomplexes organize electron fluxes, the precise mechanisms involved remain to be fully elucidated.

One under-studied aspect of supercomplex function is the potential link between flux control at the supercomplex and its own stability. For example, Jang and Javadov (2018) studied the effects of pharmacological inhibition of complex I and II on rat cardioblast cells and isolated heart mitochondria on the supercomplexes containing complexes I, III and IV in different stoichiometries. They found that inhibition with rotenone and malonate disrupts respirasome assembly and decreases their levels by up to 25% compared to untreated controls, with important rotenone-mediated increases in H_2O_2 production. Likewise, knockdown of complex I subunit NDUFA11 induced dissociation of the respirasome, reduced complexes I, III and IV activities and ATP production. Knockdown of complex II subunit SDH in turn didn't affect respirasome assembly, but decreased complexes II and IV activities, ATP production and increased ROS production. Given the apparent important role of complex I (and complex II to a lesser extent) and in light of our results, it is conceivable that longer-lived species have less control at this complex (or less "sensitivity") to avoid supercomplex breakdown, reduced ATP production and increased in ROS

production. This potential robustness of supercomplex assemblies and lower control of electron entry complexes over the ETS may explain the maintenance of low rates of ROS production in *A. islandica* and its extreme longevity compared to closely-related bivalves, in line with the MOSTA. The relationship between complex I content and control over respiration, their role in supercomplex assembly and robustness, and the modulation of ROS production remain to be established; our results on the present study suggest that bivalves are powerful model to explore this question. Moreover, the increasingly clear dynamic aspect of supercomplexes warrant to question whether these assemblies can respond to parameter such as temperature or oxygen concentration, and the diversity in environmental conditions faced by bivalves may prove useful in exploring this question.

Supercomplexes appear to be dynamic and modifiable structures according to particular conditions. In plant mitochondria, hypoxic and low pH conditions reduce complex I activity in supercomplexes, while individual complex I activity is increased, and this is reversed during recovery to normoxic levels (Ramírez-Aguilar et al., 2011). Moreover, it appears that substrate conditions can modify supercomplex architecture in a dynamic way. Indeed, Guarás et al. (2016) showed that switching from glucose to fatty acid oxidation can induce a reconfiguration of supercomplex assemblies, whereby there is a degradation of complex I and an increase in the proportion of free complex III. This mechanism is controlled by the redox status of the Q-pool, and has the salubrious effect of allowing the increase in FAD-linked respiration (at the expense of the NADH pathway), and limiting ROS production induced by reverse electron transport to complex I. Furthermore, glucose deprivation leading to endoplasmic reticulum stress and unfolded protein response (UPR) triggers an upregulation of mitochondrial respiration via the formation of supercomplexes, and the protein kinase involved (protein-kinase R-like ER kinase, PERK) also helps rescue bioenergetic defects in patients with complex I mutations (Balsa et al., 2019).

Beyond the involvement of substrates and oxygen, it appears that other external signals can change supercomplex architecture. Indeed, Greggio et al. (2017) showed that endurance exercise in humans leads to a general increase in ETS complexes, and most importantly a shift from free to supercomplex-assembled complexes, leading to increases in muscle respiration. These effects especially concerned (yet again) complex I, whereby it was redistributed from supercomplex $I+III_2$ to $I+III_2+IV_n$, as well as individual complexes III and IV to functional supercomplexes. This plasticity in ETS assemblies thus seem to respond to increased energy demands. In both these cases, the changes in supercomplex assemblies mainly concerned complex I distribution, reaffirming the importance of the N-pathway exposed in chapter III. Clearly, the N-pathway is not only involved in aging (see chapter III and section 5.2), but also seems to influence supramolecular assembly of ETS complexes; future investigations should focus on understanding the exact involvement of this pathway in the control of supercomplexes' configuration, and the role of other less-studied pathways (such as the glycerol-3-phosphate pathway discussed earlier) in supercomplex dynamics.

This plasticity of mitochondrial ETS complexes could explain some apparent contradictory results reporting presence or absence of supercomplexes in the same species. Indeed, Celotto et al. (2011) used *D. melanogaster* mutants harboring a missense mutation in the ATP6 gene (resulting in an almost complete loss of ATP synthase activity) and found that complex I-containing supercomplexes formation was disrupted, while glycolytic flux, ketogenesis and activity of the TCA cycle were upregulated compared to wild-type flies. In contrast, Shimada et al. (2018) could barely detect any supercomplex in whole wild-type *D. melanogaster*. The preparation methods are very similar preparation among the two studies, hence the differences in banding pattern appear surprising: could potential differences in genotype among the wild-type strains be responsible for this disparity? A detailed characterization of the mitochondrial architecture in *D. melanogaster* lines with different nuclear and mitochondrial DNA therefore appears reasonable and could explain intraspecies

differences. Another curious result that seems to be universal is the apparent lack of assembly of complex II into supercomplexes. The reason why such complex should be organized separately from the rest is intriguing, especially given that it is both part of the ETS and the TCA cycle. Guo et al. (2018) explain that cryo-EM reconstitution of supercomplexes suggests that the physical space for complex II exists, but its low abundance or the conditions of sample preparation could account for its loss and explain its absence from BN-PAGE gels. To date, the most convincing piece of evidence for a complex II-containing supercomplex comes from a study on an apicomplexan parasite, where a supercomplex II-III-IV has been found (Matsubayashi et al., 2019).

Overall, multiple metabolic conditions and the aging phenotype are accompanied by changes in the architecture and functioning of the ETS, among which supercomplex assembly, but also cristae remodeling (the folds in the IMM increasing surface area for chemical reactions) and mitochondrial fission and fusion dynamics (reviewed in Baker et al., 2019). Beyond the role in the aging phenomenon, the nature and dynamics of the ETS and oxidative stress appear to be linked to life history strategies, which could explain some of the disparities in the classic studies on ROS, longevity and the MOSTA.

5.4 Oxidative stress and beyond: cellular signalling, life history strategies and mitonuclear interactions in aging

5.4.1 Regulation of ROS by mitochondria and the role of oxidative stress in cellular signalling

Oxidative stress and ROS production were originally strictly seen as harmful "by-products" of OXPHOS, but more recent and accurate reports show the important role that these play in cellular signalling pathways (reviewed in Holmstrom and Finkel, 2014). Additionally, some studies show beneficial effect of mitochondrial ROS *via* mitohormesis (reviewed in Bárcena et al., 2018), while others suggest a harmful role.

In *C. elegans*, increased lifespan through glucose deprivation happens concomitantly with augmented respiration and ROS levels (Schulz et al., 2007), but harboring a mutation increasing ROS levels in the same species accelerates aging (Ishii et al., 1998). This echoes the absence of increased ROS in mice with somatic mtDNA mutation and decreased lifespan (Trifunovic et al., 2005), and the increased ROS production in mutated mice with increased lifespan (Lapointe and Hekimi, 2008). Overall, the "harmful" view of ROS is constantly put into question, and these studies show species-specific effects and the confounding role for mitohormesis in the aging process.

The way ROS are traditionally measured is also subject of recent critique. The classic approach for quantifying ROSp relies on the Amplex Ultra Red (AUR) assay (Invitrogen, OR, USA), whereby a fluorogenic substrate for horseradish peroxidase reacts with H_2O_2 in a 1:1 stoichiometric ratio, producing Amplex UltroxRed, a fluorescent reaction product with excitation/emission maxima of 568/581 nm. There is, however, a fundamental problem with this classical approach of assessing ROSp: it only effectively measures H_2O_2 diffusing into the reaction medium, hence the ROS escaping the whole array of antioxidants in the mitochondrial matrix that convert H_2O_2 into H_2O. These are mainly the thioredoxin (Trx)- and glutathione (GSH)-dependent pathways, both respiration-dependent: they rely on electron supply via NADPH. Oxidation of respiratory substrates reduces $NADP^+$ into NADPH, maintaining reduced intermediates GSH or Trx, essential for peroxidases activity that effectively reduce H_2O_2 to H_2O. Hence, their activity also depends on the energetic status of the cell, and the substrates contributing to mitochondrial respiration (reviewed in Munro and Treberg, 2017). Ultimately, this means that the assay on which many studies have been based has the potential to underestimate actual rates of ROSp by overlooking the contribution of ROS consumption (ROSc) pathways (Munro et al., 2016). To overcome this issue, Munro et al. (2016) developed an assay whereby isolated mitochondria in different respiratory substrates and inhibitors conditions were challenged by addition

of exogenous H_2O_2, and the decrease in fluorescence over time was measured using the AUR detection system. This assay also allowed to disentangle ROSp from ROSc, from either of the two aforementioned respiration-dependent pathways GSH and Trx, by the use of specific inhibitors. Their results in rat skeletal muscle mitochondria reveal four important factors: 1) ROSc is indeed respiration-dependent, 2) ROSc is more rapid than ROSp, 3) ROSp is underestimated by up to an astonishing 80% in the traditional assay, and 4) respiratory substrates influence the efflux of H_2O_2 detected. These results effectively redefine mitochondria as regulators, rather than strictly producers, of ROS (Munro and Treberg, 2017).

Applying this methodology to models of longevity reveals new patterns linking ROS metabolism to the aging process. Reconciling the apparent contradictory observation that naked-mole rats and laboratory mice (30 vs 3 years MLSP) don't differ in ROSp despite their 10-fold difference in lifespan (Lambert et al., 2007), Munro et al. (2019) demonstrate that these species actually differ in their ROSc capacities. Indeed, the two species have similar rates of H_2O_2 production, but the naked-mole rat consume H_2O_2 at two to fivefold higher rates in the skeletal muscle and heart of naked-mole rats, respectively. These results were consistent at two different temperatures, and rates were dependent on substrates combinations. Moreover, this increase in rates of consumption were related to major contributions of the Trx- and GSH-dependent pathways. The concentration and activities of antioxidants were seldom found to correlate with longevity among species (reviewed in Hulbert et al. , 2007), but mitochondrial matrix enzymes were not generally the focus of those studies and studying their isolated activities may have hidden their actual contribution evidenced in Munro et al's (2019) study. Hence, further demands for more accurate estimations of ROS metabolism have been made (Pamenter and Munro, 2019). In accordance with this idea, we compared rates of ROSc in three species of marine bivalves using the protocol developed by Munro et al. (2016). Our results show that long-lived *A. islandica* consumes more H_2O_2 than short-lived *S. solidissima* and *M. arenaria* in

various substrate combinations, and in both the mantle and gill tissues (Munro, Rodríguez, Blier, *in preparation*). Although further experimental evidence is required, it appears that the GSH-dependent pathway is especially active, since suppression of the Trx-dependent pathway with inhibitor Auranofin does not seem to affect ROSc significantly. Moreover, H_2O_2 consumption without substrates was higher in *A. islandica* than in the shorter-lived species, suggesting a non-negligible role for antioxidant enzyme catalase. This is in agreement with Abele et al. (2008) who found a 4-fold higher catalase activity in ocean quahog gills, compared to a range of temperate and polar molluscs (among which *M. arenaria*, see Abele and Puntarulo, 2004 for detailed results). It is nonetheless in disagreement with Ungvari et al. (2011), who did not find differences in catalase nor glutathione peroxidase (GPX) activities in gills of *A. islandica* and shorter-lived *M. mercenaria*, both declining in older individuals of these species. They however found *A. islandica* to be more resistant to stress induced by exposure to *tert*-Butyl hydroperoxide (an organic peroxide that induces apoptosis and is commonly used to evaluate resistance to oxidative stress), but could not conclusively associate this resistance to either antioxidant capacities (via the use fluorescence probes that are quenched by hydroxyl and peroxyl radicals), specific antioxidant activities, or protein recycling (via proteasome activity). This type of result stresses the need for an accurate and unifying measurement of ROS management capacities in animals in lifespan-related comparative studies. We believe that our (yet to be published) results on isolated mitochondria in bivalves and the previous murine studies (Munro et al., 2016;Munro et al., 2019) make a solid case for a link between the robustness of mitochondrial ROS management and longevity, in agreement with the MOSTA (also discussed in Blier et al., 2017).

Beyond looking at the activities of ROS detoxifying pathways, an integrative approach to the interplay between energy metabolism and oxidative stress needs to be taken. Indeed, the GSH- and Trx-dependent pathways are intimately connected to the TCA cycle in their dependency on the phosphorylated form of NADH, NADPH

(Treberg et al., 2015). This molecule is the building block for many others, and pathways linking substrate oxidation to NADPH generation include the mitochondrial malic enzyme, isocitrate dehydrogenase and nicotinamide nucleotide transhydrogenase which uses the protonmotive force to generate NADPH and NADH from $NADP^+$ and NAD^+ (Munro et al., 2016). It would therefore be necessary to understand the controls exerted at each step of the pathway generating matrix antioxidant enzymes (GSH and Trx), and the wider contribution of the TCA cycle and associated enzymes (Munro et al., 2019). This falls into the increasingly recognized role of the TCA cycle in aging. For example, dysfunctions in the TCA cycle flux can lead to accumulation of important intermediates which control DNA and histone methylation, such as α-ketoglutarate, succinate and fumarate, impacting gene expression (Salminen et al., 2014). These intermediates of energy metabolism (as well as cofactors such as FAD^+ and acetyl-CoA) are also crucial messenger molecules in the signal transduction from the mitochondrion to the nucleus: it is not surprising that they play an important role in controlling gene expression, including those genes associated to aging (*ibid*). These are therefore worthy of more investigation to understand the aging process at the inter- and intraspecific scales.

What is the evolutionary driver behind the putatively beneficial roles of ROS management in long-lived species? The recent discovery that ROS levels are required for various downstream signalling pathways, among them growth factor stimulation or kinase signalling (reviewed in Holmstrom and Finkel, 2014) presses the question of understanding how evolving high ROS consumption capacity can promote longevity without affecting these other important mechanisms. A clue may lie in the remarkable fact that various species that are long-lived have also evolved hypoxia tolerance, especially compared to closely-related species within each lineage (such as *A. islandica* but also in naked-mole rats, turtles or bats, see Pamenter and Munro, 2019). Hence, the mechanisms behind hypoxia tolerance and longevity seem to be closely related, and

the better understanding of their molecular basis and the influence on oxidative stress management may bring important pieces to the aging puzzle.

5.4.2 Life history strategies and aging

A survey of the aging literature in animals suggests that different species sometimes adopt distinct longevity strategies, and this could be important to understand the aging phenomenon as a whole (reviewed in Speakman et al., 2015). In an elegant study, Brown and colleagues (2009) compared respiration, proton leak and H_2O_2 release in two long-lived (house sparrow and big brown bat) and one short-lived (house mouse) endotherms of comparable size and metabolic rate. They found that brown bats, but not sparrows had lower basal and maximal ROS efflux rates than mice. This was attributed to a higher oxygen consumption rate and higher substrate oxidation capacity in the birds. Interestingly, when using close to resting physiological levels of the substrate succinate, sparrow ROS efflux decreased significantly, and free radical leak was lower than in mice, suggesting that the high oxidative capacity of complex III allows the birds to produce fewer ROS per O_2. It is tempting to suggest that the high rates of ATP production during flight in these animals (and low ROS at rest) may have allowed for the evolution of a long lifespan, or vice-versa. In the case of bats, they were shown to possess leakier membranes than mice, and lower membrane potential: these suggest that they use the so-called "uncoupling to survive" strategy (Brand, 2000), whereby increasing the proton conductance of the IMM decreases ROS production. Sparrows on the other hand, appear to use the "spare oxidative capacity" strategy, whereby an increase in the activity of ETS complexes lowers the reduction state of the different ROS-producing sites, since the capacity to transfer electrons past these sites would be higher than to pass it to the sites (Brown et al., 2009). However, these two strategies do not take into account possible differences in the capacity to detoxify ROS, as discussed previously and shown by Munro et al. (2019). As for *A. islandica*, it appears that the presence of the alternative oxidase (AOX) allows for partial uncoupling of respiration and phosphorylation, allowing more O_2 uptake without the

need to produce ATP and providing a potential protection mechanism against oxygen burst (Tschischka et al., 2000). Hence, this mechanism together with the particular architecture of the ETS could constitute yet another strategy for reaching a high longevity, this time potentially linked to resistance to hypoxic conditions (see previous section).

Considering and integrating the life-history events that underpin the physiology of animals is another important challenge for future research into the biology of aging. As highlighted in the introduction of this thesis, the balance between somatic maintenance and reproduction appears crucial for determining the allocation of metabolic resources. This "disposable soma" theory of aging (Kirkwood, 1977) implies that an individual will benefit from investing in reproduction rather than in repair mechanisms when resources are rare, even if this strategy is detrimental later as damage accumulates and causes aging. This is increasingly seen as a mechanistic explanation for the antagonistic pleiotropy theory of aging whereby a gene giving an early favourable effect will be preferentially selected, regardless of its negative effects later in life (Williams, 1957;Maklakov and Chapman, 2019). If this theory holds true, then extracting animals from their natural setting becomes problematic, and measurements of oxidative stress parameters may be altered by the change in their housing conditions. Indeed, studies in the wild not only show that aging is common in nature, and that trade-offs and the early-life environment determine individual life histories (Nussey et al., 2013). This could for instance explain differences in maximum lifespans among populations of *A. islandica* living in stable versus highly variable environments, as discussed in chapter II and the present chapter. As shown by Salmon and colleagues (2010), in mice housed in a laboratory environment with minimal stress, oxidative stress plays a minimal role in aging, while it plays a major role in senescence when animals are subjected to chronic stress. Working on natural populations could therefore be an important first step to better address the predictions of the different aging theories, although other challenges have to be overcome, such as the need to develop

non-intrusive methods to measure oxidative stress in the field (reviewed in Speakman et al. 2015). In the case of our marine bivalves, we could not measure our targeted physiological parameters without ultimately killing the specimens, but in the future measurements *via* biopsies or through the haemolymph may provide less intrusive and more accurate portraits of oxidative stress.

As discussed in the previous section, there is a pressing need to more accurately measure ROS balance (production and consumption) and actual damage (rather than extrapolating putative damage), and most importantly their functional outcomes on aspects such as organ function and fitness, to survival and future reproductive performance (Speakman et al., 2015). Although studies on single tissues or using single damage or protection parameters have proven useful and are sometimes the only possible strategy, tissue-specific patterns of physiological parameters must be measured and compared (Selman et al., 2012). Moreover, and in close link with reproduction, separating male and female individuals when reporting measurements appears important given the often-different investments by both sexes into reproduction and offspring. Recent analysis has shown an important link between oxidative stress and the cost of reproduction: in various species, oxidative damage was positively associated with the reproductive effort of females, yet the transition to sexual maturity was associated with a reduction in damage in various tissues (Blount et al., 2016). This gives weight to the "oxidative shielding" theory, whereby a priority of the parents lies in avoiding the transfer of somatic damage to their offspring, therefore oxidative defense mechanisms could be upregulated during reproduction, despite the incurring costs (*ibid*). An illustration of this theory and linked to lipids, can be seen in a recent study on lipidomics at different life stages in honeybees. In the larval stage, all castes have similar membrane fatty acid composition, characterized by low levels of peroxidation-prone PUFAs, and high levels of monounsaturation. However, from late-larval stage to emergence, the levels of these fatty acids prone to peroxidation increases in short-lived drones and workers (MLSP ranging from days to a few weeks), but not

in queens (MLSP of 3 years). This is linked to differences in diet between the PUFA-rich pollen consumed by workers, versus the royal jelly which contains low levels of these fatty acids (Martin et al., 2019). It could be interpreted from these findings that queens "shield" their membranes to oxidative stress to avoid damage to their progeny. Going forward, and as discussed in section 5.1.3, given the intricate links between the germline and lipid metabolism (Bustos and Partridge, 2017), it would be of interest to explore the relationship between sexual maturation, oxidative stress and lipid membranes. For example, do ROS or products of lipid peroxidation act as signals for the regulation of pathways inducing sexual maturation?

As exposed by Speakman and colleagues (2015), one of the important challenges faced by the biogerontologists is to expand the variety of animal models and experimental conditions, to go beyond the traditional models such as mice and other vertebrates, to better understand the diversity and complexity of aging. We believe that marine bivalves serve this purpose well and are promising models for aging and biomedical research as a whole (Bodnar, 2009;Bodnar, 2016;Blier et al., 2017). Understanding the biology of less common, long-lived animal models such as bivalves can contribute to fundamental knowledge of the aging process and lead to new interventions aimed at ameliorating human healthspan (Stenvinkel and Shiels, 2019).

5.4.3 Aging and the two genomes

One of the central tenets of the MOSTA is the link between ROS production and damage to mtDNA, triggering the "vicious cycle" of damage and inefficient repair, leading to decreased function and the aging phenotype. Although studies point at an increase in oxidative damage to both mitochondrial and nuclear DNA with age (Hamilton et al., 2001), confounding results put in doubt the cause and effect relationship between ROS and DNA damage. Indeed, it appears that ROS per se do not cause the increase in DNA damage, and thus it is proposed that ROS production is not the initial cause, but rather a consequence, of aging (Heikimi et al., 2011). Given the

aforementioned roles of ROS in signal transduction and the stress response, this is not surprising; it remains that a more robust management of oxidative stress is beneficial to attain a long lifespan: mice with mutated mtDNA treated with the mitochondrial ROS scavenger SkQ1 helps preserve the ultrastructure of mitochondria and increase lifespan (Shabalina et al., 2017). Therefore, perhaps the MOSTA needs refining in the directionality of the relationship with DNA damage, rather than complete dismissal. Notwithstanding, if ROS are not causing of mutations in DNA, then what is the culprit?

The circular, double-stranded mitochondrial genome is composed of 16,569 base pairs in humans and encodes 37 genes: 22 transfer RNAs, 2 mitochondrial rRNAs, and 13 proteins. These 13 proteins are only about 1% of the mitochondrial proteome, but they are all components of the ETS complexes essential for OXPHOS (reviewed in Pinto and Moraes, 2015). Rates of mtDNA mutations can be of several orders of magnitude above that of nuclear DNA (nDNA, Linnane et al., 1989), and these point mutations can be inherited and present in a subset of all mtDNA copies in a cell (heteroplasmy) or in all copies (homoplasmy). Low levels of heteroplasmy can be pathogenic in some cases, but these have various levels of penetrance (Payne and Chinnery, 2015;Gorman et al., 2016). It appears that the origin of these mutations lies in errors in replication rather than oxidative damage, particularly in the only mtDNA polymerase, Polγ (reviewed in Kauppila et al., 2016). This was first shown in a mouse with a proof-reading-deficient Polγ showing an important increase in levels of point mutations, increased deleted mtDNA, reduced lifespan and various signs of premature aging (Trifunovic et al., 2004). However, a subsequent study showed at the time that despite this linear accumulation of mtDNA mutations, no increase in ROS production was reported in the animals, and it was proposed that ETS components' dysfunction was the reason for the premature aging phenotype (Trifunovic et al., 2005). A more recent study on these mice has shown that cardiolipin depletion and accumulation of hydroxynonenal protein adducts, oxidative stress parameters not assessed in the original studies, were rescued by mitochondrial-targeted antioxidants and lifespan

increased (Shabalina et al., 2017). Consequently, it appears that the mechanism linking mtDNA to aging is as follows: replication errors in mtDNA start appearing due to defects in Polγ, the resulting point mutations are amplified by the clonal expansion of mtDNA, which when reaching a threshold can lead to OXPHOS dysfunction, mismanagement of oxidative stress in postmitotic tissues, reduced fitness, and aging (Pinto and Moraes, 2015).

Because ETS components are encoded for by both the mitochondrial and the nuclear genomes (about 70 nuclear-encoded subunits), and given the former's high mutation rate and generally maternal inheritance, breaks in co-adaptation between the two can arise and impact mitochondrial function. Earlier, we have hinted at the important role of TCA cycle metabolites in controlling gene expression and impacting the aging phenotype (see section 5.4.1 and Salminen et al., 2014). The likes of α-ketoglutarate, succinate and fumarate are important actors in the signal transduction from mitochondria to nuclei, and regulation of TCA cycle flux may therefore play an important role in aging. Indeed, mitochondria are increasingly viewed as "flux capacitors", i.e. they signal the physiological status of the cell and coordinate nuclear gene expression according to the needs (Wallace and Fan, 2010). There is now evidence that even low levels of mismatch can impact normal function, such as that compiled by Burton and colleagues (Ellison and Burton, 2008;Barreto and Burton, 2013) studying the copepod *Tigriopus californicus*. In this intertidal crustacean, hybridization between individuals from populations with divergent mtDNA results in fitness breakdown of the F_2 generation, with reductions in viability, fecundity, larvae survival, slower development, reduced complex IV activity and increased oxidative damage linked to differences in mitochondrial and nuclear OXPHOS genes (reviewed in Wolff et al., 2014). Similar results were found in mice with introduced mtDNA to the same nuclear background, showing that mtDNA haplotype strongly influences ROS production, mitochondrial proteostasis, and other age-associated parameters (Latorre-Pellicer et al., 2016). In *Drosophila*, a mismatch between mitochondrial and nuclear

genomes results in a decrease in mitochondrial respiration, in a compensatory but insufficient increase in relative mtDNA copy number, augmented ROS production by 50%, while mitochondrial dysfunction was aggravated by aging (Pichaud et al., 2019).

The need for coadaptation between the two genomes for cellular respiration is apparent across different biological scales, at the inter- and intrapopulational levels (Wolff et al., 2014), and the mechanism signalling this poor matching may be in free-radical leak and apoptosis threshold (Lane, 2011). Furthermore, genomes coadaptation is proposed to underlie various evolutionary mechanisms, spanning from the evolution of the two sexes in eukaryotes and of the germline, adaptation to climate and resources, sexual selection and even speciation, in the so-called and currently debated "mitonuclear species concept" (reviewed in Hill et al., 2019). Communication between the organelle central to energy metabolism and the nucleus of the cell is thus paramount in many processes implicated in healthy aging (among which, maintenance of proteostasis) and the onset of mitochondrial diseases (reviewed in Molenaars et al., 2020).

The emerging view of mitochondria as metabolism's "flux capacitors" agrees with the mounting evidence that these organelles act as sensors of metabolic imbalance and mediate cellular stress responses impacting longevity (Karpac and Jasper, 2013). However, the relatedness between mitochondrial and longevity metabolic networks is only beginning to be unravelled. Two mitochondrial pathways may play a role in the MOSTA, one being the ROS-mediated pathway discussed in this thesis. The second pathway may involve the mitochondrial unfolded protein response (UPR^{mt}), which is thought to be activated by the imbalance between the expression of nuclear and mtDNA-encoded mitochondrial proteins, or "mitonuclear imbalance" (akin to mitonuclear mismatch discussed above; Houtkooper et al., 2013). In worms and mice, knockdown or pharmacological inhibition of mitochondrial ribosomal proteins triggers this mitonuclear imbalance, reduces mitochondrial respiration and activates the UPR^{mt}, which in turn extends lifespan (*ibid*). This effect is also achieved in worms where the

gene coding for complex IV subunit COX4 is knocked-down activating the UPR^{mt}. Intriguingly, this activation could be triggered by a knockdown of the gene in a different tissue (eg. activation in the intestine from a knockdown in the nervous tissue): this implies the existence of a mitochondrial signal mediating the response and warrants further investigation ("mitokine" signal, Durieux et al., 2011). Moreover, restoring cofactor NAD^+ levels (which decline with age in various models) *via* precursors such as nicotinamide riboside extends longevity. This is shown to be done through the activation of deacetylating protein Sir2, increasing mitochondrial biogenesis and function, as well as inducing mitonuclear imbalance, which in turns activates the UPR^{mt} response (Mouchiroud et al., 2013). Although ROS may not be directly involved in the induction of UPR^{mt}, blocking this response inhibits the induction of antioxidant mechanisms. It is therefore likely that the ROS-mediated pathway is a delayed mitohormetic response (see section 5.4.1) functioning through FOXO transcription factor DAF-16 and promoting expression of SOD-3 (Karpac and Jasper, 2013;Mouchiroud et al., 2013). Fundamental knowledge about these molecular pathways and their associated actors is crucially missing in bivalves. For instance, do NAD^+ levels decrease in aging *A. islandica*? How do the UPR^{mt} and the mitohormesis responses function in these marine invertebrates? These should be addressed sooner rather than later in future endeavors.

Studies on the genetic basis of natural lifespan variation have shown that genes involved in mitonuclear balance are linked to the evolution of lifespan. Indeed, comparison of short-lived (annual, from arid environments) and longer-lived (nonannual, from humid environments) African killifishes by Sahm et al. (2017) showed positive selection in genes involved in various mitochondrial biogenesis processes, including assembly of respiratory complexes and the ETS. Importantly, some of these genes were also under positive selection in long-lived bats (Shen et al., 2010) and ants (Roux et al., 2014), stressing the evolutionary importance of mitonuclear interactions in lifespan determination among clades (Houtkooper et al.,

2013). Expanding this analysis to less-common aging models such as bivalves is necessary, as genomic data of a wide range of species becomes increasingly available. For example, it will be of interest to know if genes associated with longevity are also under positive selection in long-lived bivalves, and which pathways are preferentially selected. Are those genes linked to ETS complexes and mitochondrial biogenesis, or rather (as in long-lived naked-mole rats) are mTOR, ROS defense and metal ion homeostasis under positive selection (Sahm et al., 2018)? It is also possible that other genes not previously associated to aging may also arise in bivalves and advance our understanding of the aging process. Finally, lifespan is known to be strongly influenced by sex (reviewed in Singh et al., 2019); bivalves could also be a suitable model to understand the role of sexual dimorphism in longevity differences, more so due to the distinct metabolic properties of M- and F- phenotypes uncovered in some DUI species, among which *Arctica islandica* (see section 5.2.2 and Bettinazzi et al., 2019).

Although the extremely long-lived nature of the ocean quahog renders longitudinal studies more challenging than in classical shorter-lived models of lifespan such as worms or *Drosophila*, short-lived bivalves such as the freshwater species *Corbicula fluminea* (MLSP 1-5 yrs, Sousa et al., 2008) could be of interest. For example, experimental manipulation of NAD^+ levels, or knockdown of ETS complexes could be done to evaluate the UPR^{mt} response, mitohormesis and the effect on lifespan in this species closely related to *A. islandica* (Levivier, 2016). Research on such species should also help in discerning between early-events that cause a decrease in cellular or organ function, and the later events that are the consequence of physiological decline; while also studying multiple tissues, an aspect that is also often overlooked in aging studies (Cellerino and Ori, 2017). The "-omics" approaches that are increasingly used in biological research allows for a slow but steady uncovering of the genomic, transcriptomic, proteomic, lipidomic and metabolomic basis of aging in multiple species, and bivalves need to be integrated in such endeavors. The recent shift in focus towards non-classical models of longevity reveals that a high lifespan may be achieved

through lineage-specific adaptations or common mechanisms applying across species (Ma and Gladyshev, 2017). Disentangling both these approaches and understanding which aging strategies are used by bivalves will be of utmost importance in future studies. The in-depth characterization of the molecular actors and important pathways appears urgent to better decipher aging, refine its associated theories (such as the MOSTA), and to ultimately understand other evolutionary associated phenomena such as age-related diseases.

SUPPLEMENTARY MATERIAL

Table S5.4 Fatty acid and DMA composition (mol%) of phospholipids from gill cellular debris in European populations of Arctica islandica

	Kiel Bay (n = 8)	**White Sea (n = 8)**	**Kattegat Sea (n = 9)**	**German Bight (n =8)**	**Norwegian Coast (n = 10)**	**Icelandic Coast (n = 9)**
9:0	1.5 ± 0.6	0.4 ± 0.1	2.0 ± 0.1	1.3 ± 0.5	0.5 ± 0.1	0.5 ± 0.2
14:0	1.2 ± 0.2	0.8 ± 0.2	0.7 ± 0.2	0.7 ± 0.1	0.8 ± 0.1	0.6 ± 0.1
15:0	0.6 ± 0.1	1.4 ± 1.0	0.3 ± 0.1	0.5 ± 0.1	0.5 ± 0.03	0.4 ± 0.0
16:0	20.8 ± 3.8	17.3 ± 2.5	14.2 ± 1.2	18.1 ± 2.6	18.6 ± 0.8	14.4 ± 0.7
17:0	0.8 ± 0.1	0.9 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.5 ± 0.1
18:0	18.8 ± 4.3	18.9 ± 2.4	14.6 ± 1.4	20.0 ± 2.8	15.4 ± 1.9	14.1 ± 1.5
18:1 n-13	2.1 ± 0.4	3.5 ± 0.5	3.9 ± 0.3	3.1 ± 0.4	2.6 ± 0.2	4.3 ± 0.3
18:1 n-9	1.7 ± 0.2	1.3 ± 0.3	1.1 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.3 ± 0.1
18:1 n-7	0.6 ± 0.1	0.6 ± 0.1	1.4 ± 0.1	0.7 ± 0.1	1.2 ± 0.1	2.2 ± 0.2
18:2 n-6	0.2 ± 0.1	0.3 ± 0.2	0.3 ± 0.1	1.1 ± 0.8	0.5 ± 0.1	0.2 ± 0.1
20:1 n-11	1.6 ± 0.7	0.2 ± 0.1	0.4 ± 0.2	0.5 ± 0.2	0.4 ± 0.1	0.3 ± 0.1
20:1 n-9	0.2 ± 0.1	0.2 ± 0.0	0.5 ± 0.2	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.1
20:1 n-7	0.8 ± 0.2	0.8 ± 0.2	1.9 ± 0.2	1.5 ± 0.2	1.2 ± 0.2	1.3 ± 0.1
20:2 n-6	0.7 ± 0.2	0.6 ± 0.2	0.9 ± 0.1	0.7 ± 0.1	1.4 ± 0.2	0.7 ± 0.1
20:3 n-6	2.0 ± 0.6	1.7 ± 0.5	1.8 ± 0.4	1.4 ± 0.4	1.5 ± 0.3	1.0 ± 0.2
20:4 n-6	2.5 ± 0.5	1.4 ± 0.4	2.6 ± 0.2	1.9 ± 0.4	1.8 ± 0.3	1.3 ± 0.2
20:5 n-3	3.9 ± 0.5	1.8 ± 0.4	4.3 ± 0.4	3.7 ± 0.5	5.6 ± 0.4	6.0 ± 0.4
22:4 n-6	2.1 ± 0.7	2.3 ± 1.0	0.9 ± 0.1	1.0 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
22:5 n-6	0.3 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.2	0.7 ± 0.3	0.4 ± 0.0
22:5 n-3	0.9 ± 0.2	0.9 ± 0.3	1.1 ± 0.2	1.1 ± 0.3	1.4 ± 0.2	1.3 ± 0.1
22:6 n-3	8.9 ± 1.6	7.2 ± 1.1	8.6 ± 0.8	7.8 ± 0.7	10.4 ± 0.5	9.0 ± 0.7
NMID 20:2 (Δ5, 11)	6.6 ± 1.3	6.9 ± 1.7	7.2 ± 0.9	8.2 ± 0.7	6.9 ± 1.2	9.8 ± 0.6
NMID 20:2 (Δ5, 13)	1.6 ± 0.1	4.0 ± 0.9	3.7 ± 0.5	2.1 ± 0.2	2.9 ± 0.8	2.5 ± 0.3
NMID 22:2 (Δ7, 13)	1.5 ± 0.3	0.8 ± 0.2	1.2 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	1.4 ± 0.1
NMID 22:2 (Δ7, 15)	0.3 ± 0.1	0.4 ± 0.3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
NMIT 22:3 (7, 13, 16)	0.9 ± 0.5	1.1 ± 0.4	1.5 ± 0.5	1.1 ± 0.4	1.2 ± 0.3	0.9 ± 0.1
DMA 16:0	0.0 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
DMA 17:0 iso II	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
DMA 17:0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
DMA 18:0 iso I	0.1 ± 0.1	1.0 ± 0.6	0.7 ± 0.2	0.5 ± 0.3	0.5 ± 0.1	0.7 ± 0.1

DMA 18:0	1.9 ± 0.7	2.7 ± 0.5	3.0 ± 0.6	2.9 ± 0.9	2.9 ± 0.5	3.4 ± 0.5
DMA 19:0 iso	0.5 ± 0.2	1.5 ± 0.4	0.6 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
DMA 20:1 iso II	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
DMA 20:1	9.4 ± 1.9	12.2 ± 1.8	13.3 ± 0.8	10.9 ± 0.9	11.2 ± 1.0	14.9 ± 0.7
Branched FA	2.6 ± 0.5	2.9 ± 0.7	2.9 ± 0.5	2.8 ± 0.5	2.7 ± 0.3	2.3 ± 0.2
Fatty acids < 0.5%	2.3 ± 0.6	3.8 ± 0.7	4.2 ± 0.6	2.7 ± 0.2	4.7 ± 0.4	4.5 ±0.2
SFA	46.6 ± 7.4	43.4 ± 5.4	36.4 ± 2.8	44.8 ± 4.5	39.9 ± 2.6	33.9 ± 1.8
MUFA	7.0 ± 0.5	6.6 ± 0.4	9.1 ± 0.6	7.6 ± 0.6	7.3 ± 0.3	9.7 ± 0.4
PUFA	33.0 ± 4.2	31.4 ± 2.5	35.9 ± 1.5	32.5 ± 2.3	37.2 ± 1.4	36.1 ± 1.1
n-6 PUFA	7.8 ± 1.3	6.8 ± 0.6	7.0 ± 0.5	6.7 ± 1.1	6.2 ± 0.4	3.9 ± 0.2
n-3 PUFA	14.1 ± 2.4	10.1 ± 1.6	14.5 ± 1.2	13.4 ± 1.3	18.4 ± 1.7	16.9 ± 1.1
n-3 PUFA (%PUFA)	40.7 ± 3.1	32.0 ± 3.9	40.2 ± 2.3	41.2 ± 2.2	49.3 ± 1.3	46.6 ± 2.3
PUFA (without NMI)	22.0 ± 2.5	16.9 ± 1.5	21.5 ± 1.3	20.1 ± 1.8	24.6 ± 0.9	20.8 ± 1.0
NMI total	11.0 ± 2.0	14.5 ± 2.3	14.5 ± 0.7	12.4 ± 1.1	12.6 ± 0.8	15.3 ± 0.8
NMI (%PUFA)	31.8 ± 2.9	44.7 ± 4.6	40.4 ± 1.8	38.5 ± 3.0	33.8 ± 1.4	42.6 ± 2.0
DMA < 0.5%	0.1 ± 0.1	0.2 ± 0.2	0.2 ± 0.1	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.1
Branched DMA (iso)	1.8 ± 0.7	3.0 ± 1.0	1.7 ± 0.4	1.0 ± 0.4	1.2 ± 0.2	1.2 ± 0.2
DMA total	13.5 ± 3.3	18.6 ± 2.9	18.5 ± 1.4	15.2 ± 2.1	15.7 ± 1.3	20.3 ± 0.7
Unsaturation index	146.8 ± 18.8	131.4 ± 10.3	158.9 ± 7.6	141.4 ± 10.3	167.5 ± 6.5	163.2 ± 5.8
Peroxidation index	131.0 ± 17.4	103.6 ± 10.8	132.9 ± 9.1	119.6 ± 9.9	153.5 ± 6.8	136.9 ± 7.5
MRL (years)	36	53	71	150	300	507

Table S5.2 Fatty acid and DMA composition (mol%) of phospholipids from mantle cellular debris in European populations of *Arctica islandica*

	Kiel Bay (n = 9)	**White Sea (n = 7)**	**Kattegat Sea (n = 9)**	**German Bight (n = 7)**	**Norwegian Coast (n = 9)**	**Icelandic Coast (n = 8)**
9:0	0.8 ± 0.2	1.5 ± 0.4	1.4 ± 0.4	1.8 ± 1.0	0.7 ± 0.2	0.9 ± 0.3
14:0	0.9 ± 0.1	1.8 ± 0.3	0.9 ± 0.1	1.3 ± 0.2	0.9 ± 0.0	0.9 ± 0.3
15:0	0.6 ± 0.0	0.8 ± 0.2	0.5 ± 0.1	0.7 ± 0.1	0.9 ± 0.5	0.6 ± 0.1
16:0	18.5 ± 1.8	25.5 ± 2.5	18.8 ± 1.7	19.4 ± 2.1	19.2 ± 2.2	21.0 ± 2.3
17:0	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.0
18:0	19.9 ± 3.0	26.2 ± 3.0	21.3 ± 2.4	24.2 ± 2.3	19.7 ± 1.9	22.2 ± 2.6
18:1 n-13	1.7 ± 0.2	0.9 ± 0.2	1.6 ± 0.2	1.5 ± 0.2	1.5 ± 0.1	1.7 ± 0.3
18:1 n-9	2.1 ± 0.1	2.0 ± 0.3	2.2 ± 0.1	2.3 ± 0.2	2.5 ± 0.2	2.5 ± 0.7
18:1 n-7	0.8 ± 0.1	0.6 ± 0.2	1.3 ± 0.2	0.7 ± 0.1	1.2 ± 0.2	1.5 ± 0.1
18:2 n-6	0.3 ± 0.1	0.2 ± 0.2	0.4 ± 0.2	0.2 ± 0.1	0.6 ± 0.2	0.3 ± 0.2
20:1 n-11	0.3 ± 0.2	2.5 ± 2.3	0.7 ± 0.6	0.1 ± 0.0	0.2 ± 0.1	0.3 ± 0.1
20:1 n-9	0.2 ± 0.0	0.6 ± 0.3	0.3 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.0
20:1 n-7	0.9 ± 0.1	1.3 ± 0.5	1.2 ± 0.2	1.1 ± 0.2	0.9 ± 0.1	1.1 ± 0.0
20:2 n-6	1.2 ± 0.4	0.4 ± 0.1	0.7 ± 0.4	0.4 ± 0.1	2.1 ± 0.3	0.5 ± 0.1
20:3 n-6	2.6 ± 0.4	2.9 ± 0.9	1.7 ± 0.4	2.2 ± 0.7	2.2 ± 0.4	1.8 ± 0.2
20:4 n-6	1.3 ± 0.4	1.4 ± 0.4	1.9 ± 0.5	1.4 ± 0.2	0.9 ± 0.3	1.0 ± 0.3
20:5 n-3	4.2 ± 0.5	2.1 ± 0.6	6.3 ± 0.6	4.3 ± 0.7	6.3 ± 0.5	4.9 ± 0.3
22:4 n-6	0.5 ± 0.1	0.9 ± 0.4	1.0 ± 0.1	0.8 ± 0.1	1.0 ± 0.4	0.5 ± 0.1
22:5 n-6	0.9 ± 0.1	1.3 ± 0.4	0.6 ± 0.1	2.0 ± 1.4	0.5 ± 0.2	0.5 ± 0.1
22:5 n-3	1.1 ± 0.1	0.7 ± 0.2	1.0 ± 0.2	1.2 ± 0.3	1.0 ± 0.3	1.0 ± 0.2
22:6 n-3	11.9 ± 1.2	5.5± 1.9	10.5 ± 0.9	8.3 ± 1.0	10.0 ± 0.8	8.2 ± 0.5
NMID 20:2 (Δ5, 11)	4.6 ± 1.0	2.4 ± 1.0	4.8 ± 0.5	4.0 ± 0.9	6.9 ± 0.7	5.9 ± 1.6
NMID 20:2 (Δ5, 13)	2.1 ± 0.4	1.2 ± 0.4	1.8 ± 0.2	1.7 ± 0.3	0.9 ± 0.1	1.8 ± 0.4
NMID 22:2 (Δ7, 13)	0.7 ± 0.2	0.6 ± 0.2	0.3 ± 0.1	0.5 ± 0.1	0.7 ± 0.3	0.6 ± 0.1
NMID 22:2 (Δ7, 15)	0.1 ± 0.1	0.5 ± 0.2	0.1 ± 0.1	0.2 ± 0.2	0.1 ± 0.0	0.2 ± 0.1
NMIT 22:3 (7, 13, 16)	2.0 ± 0.4	2.9 ± 0.8	1.3 ± 0.3	1.9 ± 0.7	1.4 ± 0.4	1.5 ± 0.3
DMA 16:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.2	0.2 ± 0.1	0.1 ± 0.0
DMA 17:0 iso II	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0
DMA 17:0	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
DMA 18:0 iso I	1.2 ± 0.4	1.0 ± 0.4	1.1 ± 0.3	0.9 ± 0.1	0.6 ± 0.3	0.8 ± 0.2
DMA 18:0	2.3 ± 0.5	1.1 ± 0.3	2.1 ± 0.5	2.5 ± 0.6	2.5 ± 0.6	2.1 ± 0.6
DMA 19:0 iso	0.6 ± 0.1	0.5 ± 0.2	0.5 ± 0.1	0.3 ± 0.1	0.5 ± 0.2	0.5 ± 0.2

DMA 20:1 iso II	0.0 ± 0.0	0.4 ± 0.4	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
DMA 20:1	8.8 ± 1.2	3.8 ± 1.3	8.8 ± 1.1	7.3 ± 0.9	9.2 ± 1.3	9.7 ± 1.8
Branched FA	2.7 ± 0.5	2.8 ± 0.8	2.1 ± 0.4	1.8 ± 0.3	2.2 ± 0.3	1.8 ± 0.3
Fatty acids < 0.5%	4.5 ± 0.9	2.3 ± 0.3	3.0 ± 0.5	3.8 ± 0.9	3.8 ± 0.6	3.9 ±0.8
SFA	44.8 ± 3.9	60.3 ± 4.9	46.2 ± 3.8	51.1 ± 3.6	44.9 ± 3.8	48.7 ± 4.5
MUFA	6.0 ± 0.4	7.8 ± 1.8	7.3 ± 0.6	6.0 ± 0.5	6.7 ± 0.4	7.3 ± 0.8
PUFA	35.6 ± 2.2	22.5 ± 3.4	33.1 ± 1.9	30.9 ± 2.6	34.7 ± 1.9	30.2 ± 1.9
n-6 PUFA	6.7 ± 0.6	7.4 ± 1.2	6.3 ± 0.7	6.9 ± 1.2	6.2 ± 0.7	4.6 ± 0.6
n-3 PUFA	18.1 ± 1.7	8.6 ± 2.6	18.2 ± 1.4	14.4 ± 2.0	18.1 ± 1.4	14.6 ± 0.9
n-3 PUFA (%PUFA)	50.6 ± 2.9	34.5 ± 7.7	54.6 ± 1.3	46.1 ± 4.1	52.1 ± 2.7	49.0 ± 2.9
PUFA (without NMI)	24.8 ± 1.8	14.7 ± 2.3	24.5 ± 1.5	21.3 ± 2.0	24.3 ± 1.6	19.11 ± 1.2
NMI total	10.8 ± 0.8	7.8 ± 1.1	8.6 ± 0.8	9.6 ± 1.4	10.4 ± 1.2	11.1 ± 1.6
NMI (%PUFA)	30.6 ± 1.9	35.2 ± 2.7	26.0 ± 2.0	30.8 ± 3.7	29.8 ± 2.9	35.9 ± 3.1
DMA < 0.5%	0.0 ± 0.0	0.3 ± 0.3	0.2 ± 0.1	0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
Branched DMA (iso)	2.1 ± 0.5	2.5 ± 0.4	2.0 ± 0.4	1.5 ± 0.2	1.5 ± 0.6	1.6 ± 0.3
DMA total	13.7 ± 2.0	8.2 ± 1.9	13.5 ± 2.0	12.1 ± 0.9	13.7 ± 2.4	13.9 ± 2.5
Unsaturation index	162.5 ± 11.2	104.8 ± 17.1	157.9 ± 9.9	141.9 ± 11.8	157.7 ± 8.4	137.3 ± 7.8
Peroxidation index	153.3 ± 13.1	88.1 ± 19.1	152.4 ± 10.4	132.3 ± 12.4	146.1 ± 9.1	120.9 ± 7.0
MRL (years)	36	53	71	150	300	507

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