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Bivalve molluscs as model systems for studying mitochondrial biology

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Abstract

The class Bivalvia is a highly successful and ancient taxon including ~25,000 living species. During their long evolutionary history bivalves adapted to a wide range of physicochemical conditions, substrates, habitats, biological interactions, and feeding habits. Bivalves can have strikingly different dimensions, and despite their apparently simple body plan, they evolved very different shell shapes, and complex anatomic structures. One of the most striking features of this class of animals is their peculiar mitochondrial biology: bivalves have facultatively anaerobic mitochondria that allow them to survive prolonged periods of anoxia/hypoxia; moreover, they show the only known evolutionarily stable exception to the strictly maternal inheritance of mitochondria. Such phenomenon is called doubly uniparental inheritance, and has been reported in 100+ species, so far. Mitochondrial activity is fundamental to eukaryotic life, and thanks to their diversity and uncommon features, bivalves represent a great model system to expand our knowledge about mitochondrial biology, which right now is limited to a few species. In this review we highlight recent works studying mitochondrial biology in bivalves at either genomic or physiological level. A link between these two approaches is still missing, and we believe that an integrated approach and collaborative relationships are the only possible ways to be successful in such endeavour.

Keywords: Doubly Uniparental Inheritance, mitochondrial genomics, mitochondrial inheritance, heteroplasmy, mitochondrial bottleneck, physiological adaptation.

1. Why Bivalves?

The paradigm of mitochondrial genome (mtDNA) as a single clock-like-evolving molecule, stable in both its content and organization has definitely fallen in the last decades. MtDNA shows a considerable variability across Eukaryota (Kolesnikov and Gerasimov 2012; Sloan et al. 2018), but it was once considered having consistent genomic features across Metazoa. As new taxa are investigated, it became increasingly clear that even within animals the level of clade-specific shifts from the “*Homo-Drosophila* paradigm” is tremendously high (Gissi et al. 2008; Breton et al. 2014; Lavrov and Pett 2016; Milani and Ghiselli 2020), also in taxa previously considered as stable in their mtDNA gene order and evolutionary rates (see for instance ascidians, holometabolous insects, but also vertebrates: Gissi et al. 2010; Kaltenpoth et al. 2012; Rubinstein et al. 2013; Macey et al. 2021; Zhang et al. 2021). Molluscs in general, and bivalves in particular, show a great diversity in numerous mtDNA features, and peculiar deviations from the “textbook descriptions” (Ghiselli et al. 2021; see Box 1 for details about bivalve mtDNA peculiar features). The most peculiar feature concerning mitochondrial biology of bivalves is the Doubly Uniparental Inheritance (DUI), a unique mitochondrial inheritance system that has been reported, so far, in more than 100 species (Capt et al. 2020). Bivalves showing DUI have two mtDNAs following two separate, sex-specific inheritance routes: one lineage, called F-type, is inherited matrilineally, the other, called M-type, is inherited patrilineally. Eggs are homoplasmic for the F-type, sperm for the M-type; adult tissues show various degrees of heteroplasmy (see Section 2.1). F-type and M-type represent two completely segregated lineages and can reach considerable levels of nucleotide and amino acid divergence (respectively up to ~45% and ~53% in Unionida (Doucet-Beaupré et al. 2010); up to 53% amino acid divergence in Venerida: Capt et al. 2020). Moreover, the two genomes can differ in their gene order and/or gene content. So far, DUI represents the only known evolutionarily stable exception to the Strictly Maternal Inheritance (SMI) typical of Metazoa. DUI bivalves represent a unique model for the study of mitochondrial dynamics: having two highly divergent mtDNA populations, characterized by different dynamics, selective pressure, and segregation pattern, can provide insights into mito-nuclear co-evolution and mitochondrial inheritance.

One of the most asked questions about DUI is: why bivalves? Is there a reason why DUI has been observed so far only in bivalves? We actually do not know if DUI or some similar mechanism exists outside bivalves, because mitochondrial genetic variation and heteroplasmy

have been not extensively investigated across invertebrates. Finding DUI is not a straightforward process, and it requires dedicated efforts and specific analyses (discussed in: Theologidis et al. 2008; Ghiselli et al. 2017). That said, there must be some reason behind the impressive mtDNA plasticity of bivalves and we think it has something to do with the likewise remarkable plasticity that they show at the physiological level. Indeed, bivalves can resist to air exposition during cycles of hypoxia-reoxygenation by changing mitochondrial metabolism, they have alternative oxidase (AOX) proteins on inner mitochondrial membrane that allows them to limit reactive oxygen species (ROS) production during stressful fluctuations of environmental parameters, they can live exceptionally longer than any other extant taxon (in particular, a DUI species is the longest-lived animal documented so far), and some of them (DUI species) are subject to sex-specific mitochondrial evolutionary pressures with female and male gametes showing different mechanisms of energy production when compared to SMI gametes (see Table 1 and Section 4).

But which is the cause, and which is the effect? Are bivalves physiologically plastic because of their genomic diversity, or is their peculiar physiology that allows the great variability at the genome level and the remarkable features described above? So far, a great deal of work was done at the mtDNA level, trying to unravel the features that could be associated with such unique traits, but much less work has been carried on at the level of nuclear genomes, transcriptomes, proteomes, and physiology. It is becoming clear how a single-discipline perspective is weak in unravelling such complex and diverse evolutionary mechanisms, so it is now necessary to build a bridge between genetics/genomics and physiology—namely between the genotype and the phenotype—to shed light on some of the most exciting and promising biological systems among animals (Ghiselli and Milani 2020). This is true not only for mitochondrial biology, but also for many other fields of life sciences.

In this work we highlight some interesting topics concerning genomics and physiology that have been previously investigated, and some others that may provide interesting starting points for future directions. The two fields are still distant and not well-connected, and prompted by the SICB2021 Symposium “Genomic Perspectives in Comparative Physiology of Mollusks: Integration Across Disciplines”, we wanted to review some potentially interesting features of bivalve mitochondrial biology for future integrative, multidisciplinary research.

2. Within-individual variability of mtDNA

MtDNA is present in multiple copies within cells and, consequently, within individuals. Such copies are not identical to each other, therefore organisms—to various degrees—show a heteroplasmic population of mtDNA (Payne et al. 2013; Aryaman et al. 2018). The presence of different mitochondrial variants results in a competition among mitochondrial genomes (Klucnika and Ma 2019): if, on the one hand, selection may favor some variants over others (Fan et al. 2008), on the other hand, stochastic events may also have a crucial role in affecting the variability of the mitochondrial population (Wonnapijit et al. 2008). As a matter of fact, both selection and genetic drift shape the within-individual mitochondrial variability (Milani and Ghiselli 2015), which in turn impacts the dynamics of mitonuclear coevolution (Rand et al. 2004), mtDNA inheritance, and mtDNA segregation during embryo development (Johnston and Burgstaller 2019). One of the first predictions for mitonuclear coevolution was the fact that the two coexisting genomes have to coadapt because their products have to cofunction in order to provide proper cellular metabolism (Rand et al. 2004). Indeed, the strict coadaptation coupled with the crucial importance of the metabolic mechanisms involved were invoked to explain the high degrees of homoplasmy observed in animal cells, and the detrimental effects of heteroplasmy (Lane 2011, 2012). Experiments on xenomitochondrial cybrids revealed a direct relationship between the efficiency of cellular respiratory capacity, and the evolutionary divergence between the nuclear background and the foreign mtDNAs (McKenzie et al. 2003; Bayona-Bafaluy et al. 2005; Niehuis et al. 2008; Wang et al. 2017; Kong, Xiang, et al. 2020). This reduced mitonuclear fitness was observed also in natural interpopulation hybrids, allowing to hypothesize a key-role of cytoplasmic incompatibilities in speciation and hybrid breakdown (Ellison and Burton 2006; Burton et al. 2013; Hill 2017; Hill et al. 2019).

Variability in the mitochondrial population can be assessed in terms of SNPs, genome structure/architecture, and pattern of presence/absence. Even if only a few works investigated the within-individual variability of mtDNA in bivalves, the results reported so far are peculiar, and deserve attention. Here we briefly highlight some of the within-individual variability patterns observed in bivalves.

2.1 Distribution and abundance of M-type in male tissues

An intriguing characteristic found in some DUI species is the plastic pattern of presence/absence of the M-type mitochondrial genome in somatic tissues. It is indeed well

established that some DUI species commonly possess the paternally-inherited mitochondrial genome not only in male gonads, but also in male and female somatic tissues (Obata et al. 2006; Kyriakou et al. 2010; Ghiselli et al. 2011, 2019; Breton et al. 2017). Still, this pattern shows both between and within species variability: the presence of M-type in somatic tissues can vary from being the predominant mitochondrial genome to being not detectable, and everything in between (see for example: Ghiselli et al. 2011; Breton et al. 2017; Iannello et al. 2021). The possibility for DUI species to manage not only two different populations of mtDNA (F- and M-type), but also variable patterns of presence/absence of M-type DNA in different tissues is fascinating. The main implication is that, differently from gonads—which are mostly homoplasmic for either F- or M-type—, somatic tissues are effectively heteroplasmic. The coexistence in the same tissue of two very divergent mtDNA populations opens questions about how replication, transcription, and translation of different mitochondrial genomes are coregulated by the nuclear genome (see Section 3).

2.2 Intraindividual mtDNA Structural Variations

In bivalves we find also examples of variability in the mtDNA structure (see for example: Ghiselli et al. 2013, 2017; Guerra et al. 2014). More in detail, mtDNA length polymorphism has been reported across different individuals from the same species, and the differences are likely due to a variable number of repeated elements in the control region. Moreover, variability in mtDNA length spanning thousands of bases was reported within the same individual of *R. decussatus* (Ghiselli et al. 2017). The reason beneath such differences is unclear, especially the maintenance in the mtDNA population of longer variants. Indeed, it was reported that mtDNA length plays a role in mitochondrial dynamics: shorter genomes should be advantaged in transmission, since these variants would require a shorter time to replicate themselves (Diaz et al. 2002). One intriguing explanation could lie in the nature of the repeated elements: if these elements could bring some sort of advantage in mtDNA replication—for example by carrying polymerase-binding motifs or secondary structures—this would explain the presence of longer mtDNA variants. In this case, such variants would not bring benefits in terms of fitness and what we observe would be the result of replication advantage allowing longer variants to be preferentially transmitted to the offspring. A similar scenario was reported in *Drosophila melanogaster* (Rand 2011). The presence of structural variants in bivalves has not been deeply investigated yet, and approaches like High-Throughput Sequencing (HTS) of mtDNA using long reads, will allow to better explore such interesting feature.

2.3 SNPs

The within-individual variability at the SNP level has been so far investigated only in the DUI species *R. philippinarum*. A recent work analyzed the within-individual mtDNA variability (Iannello et al. 2021) by Illumina HTS: while the same pattern of shared SNPs was found for the F-type among tissues of the same individual, a different situation was observed for the M-type. Indeed, a high variability was found between somatic tissues (i.e.: male adductors) and gonads: with up to 200 different M-type SNPs, this is possibly the highest within-individual mtDNA variability observed so far. This particular pattern will be recalled in the next paragraph.

2.4 Mitochondrial bottleneck

A peculiar feature of the paternally-inherited mtDNA population is that it comes from few (usually 4-5) sperm mitochondria (Cao et al. 2004; Milani et al. 2012). This implies that—even if there are no precise indications of the total number of mtDNA in spermatozoa yet—the population size of paternally-inherited mtDNA is likely considerably lower than the maternally-inherited one (Ghiselli et al. 2011). In addition, the population size of M-type does not increase during the first phases of embryo development (Guerra et al. 2016), so the M-type mtDNA faces a very narrow bottleneck during embryo development, narrower than that experienced by female-inherited organelles. This feature makes M-type mtDNA particularly suited for studying the effect of population size on genetic bottlenecks.

The within-individual variability in the mtDNA population depends on the genetic variation and sample size of the inherited mtDNA population (i.e.: the variability and size of mtDNA population in the oocyte for almost all Metazoans) (Johnston 2019). In particular, the higher the number of mtDNA that segregates in different cells during embryo development, the lower will be the cell-to-cell and tissue-to-tissue variability of mtDNA populations in the adult (Johnston and Burgstaller 2019). This was shown by mathematical simulations by Radzvilavicius et al. (2016), and the authors hypothesized that the reason behind the high number of mitochondria in oocytes is to reduce mitochondrial variance across tissues.

In DUI species, having the paternally-inherited mtDNA such a low population size, there is the unique opportunity to investigate the above mentioned prediction about mitochondrial bottleneck. Iannello et al. (2021) analyzed the within-individual mtDNA variability in the DUI species *R. philippinarum* and found a remarkable difference in M-type SNPs across adductors and gonads in male samples, possibly the highest within-individual variability in a mtDNA

population analyzed so far. Intriguingly, such variability was not detected in the F-type of the same male samples, nor in female samples. The authors argued that such variability is a consequence of the very small population size of the paternally-inherited mtDNA populations—which profoundly impact the segregation of mitochondrial variants in different tissues—and confirmed what mathematically predicted in Radzvilavicius et al. (2016) and in Johnston et al. (2019).

Such results lay the foundation for future stimulating research directions: deep HTS of mtDNA in sperm and oocytes from DUI species could provide an exact indication of both the mtDNA population size and mtDNA variability in gametes. Similarly, HTS of mtDNA in both male and female adult tissues would give a more precise indication of the variability of paternally- and maternally-inherited mitochondrial genomes in the whole mtDNA population. If such works confirmed what observed in Iannello et al. (2021), the high level of M-type variability would add to the co-existence of paternally- and maternally-inherited mtDNA populations, increasing the complexity of the heteroplasmic condition in these animals. How the same nuclear genome can handle all such mtDNA variability is an open question that will be discussed in the next section.

3. Mitonuclear coevolution in bivalves

MtDNA variance within an individual seems to be an unfavourable condition that is suppressed in multiple ways (Lane 2012). First, the exclusion of the paternal contribution to the mtDNA pool in the zygote should ensure a higher level of homoplasy in the individual (Sato and Sato 2017); in addition, the high number of mtDNA in the oocyte is believed to greatly reduce mitochondrial genetic variation across different tissues (Radzvilavicius et al. 2016; Johnston and Burgstaller 2019). So, homoplasy seems to be a favourable trait (Lane 2012), even if low levels of heteroplasy are commonly detected across animals (Dowling 2014). One reason could be that orchestrating multiple, very different mtDNA variants by the same nuclear genome, would be challenging (Lane 2011). Accordingly, the presence of heteroplasmic mtDNA population results in a reduction of fitness in mice (Sharpley et al. 2012), and a strict coevolution between nuclear and mitochondrial genomes is thought to be necessary for healthy organisms (Rand et al. 2004; Bar-Yaacov et al. 2012). That said, most of the investigations have been conducted on a few mammals and on a handful of model species, so how mtDNA heteroplasy affects organisms with a quite different mitochondrial dynamics and physiology is still unknown. If on the one hand, the evolution and variability of mitochondrial genomes have been deeply investigated in bivalves, on the other hand studies on the nuclear counterpart are limited. Nowadays, sequencing is easier and cheaper: an increase in bivalve genomes (both showing DUI and not) in public databases will

allow to investigate the evolution of nuclear genes involved in mitochondrial functions—among them oxidative phosphorylation (which will be discussed in the following sections), mtDNA replication, transcription, translation, and mito-nuclear signaling. In addition, in DUI species such data could help to understand how the three genomes (F-, M-type and nuclear) coexist. In the same way, a comparative analysis of DUI vs SMI genomes will allow to detect duplications and gene family expansions that could have a role in the functional interactions with multiple divergent mtDNA variants. Finally, transcriptomes, which are much more abundant in public databases than genomes, may be useful tools as well to investigate the nuclear response to the peculiar mitochondrial scenario we observe in DUI species. Indeed, RNA-Seq data provide information about differential transcription and alternative splicing, which are known to have a main role in evolution and adaptation (Romero et al. 2012). Specific works on DUI species are still not available, but a comprehensive investigation of RNA-Seq data from DUI species could reveal a role of differential expression in handling different mitochondrial genomes in different tissues. In a still unpublished work on *R. philippinarum*, we found that F- and M-type mtDNAs are co-transcribed with different nuclear genes (Xu et al., in preparation).

3.1 First molecular mitonuclear coevolutionary data in bivalves

Given the features discussed above, it is clear how bivalves represent an exceptional resource for mitonuclear coevolutionary investigations. Different molecular aspects are involved in coevolutionary mitonuclear mechanisms, including heterogenomic protein-protein, protein-RNA, and protein-DNA interactions (Bar-Yaacov et al. 2012). The high evolutionary rates, the size variation, gene duplication events that happen at all genic (tRNAs, rRNAs, and protein coding genes) and taxonomic (up to genus) levels, and the length variability of some OXPHOS proteins, they all represent natural experiments to test the coevolutionary dynamics between nuclear and mitochondrial genomes. Moreover, to this striking mitogenomic diversity, it has to be added the widespread distribution of DUI, that represents a natural model of high-level heteroplasmy well-suited to test for mitonuclear coevolutionary dynamics.

To our knowledge, a direct investigation on mitonuclear coevolution in bivalves was covered only in two recent works. Iannello et al. (2019) investigated the molecular evolution of mtOXPHOS and 59 nuclear OXPHOS (nuOXPHOS) subunits in two congeneric bivalve species (one of which shows DUI), reporting striking deviations from the evolutionary patterns observed in other animals. In metazoans, high nuOXPHOS dN/dS ratios are interpreted as signals of accelerated evolution driven by compensatory fixations in the nuclear genes to compensate for

the faster-mutating mtDNA (nuclear compensation hypothesis; e.g.: Osada and Akashi 2012; Havird and Sloan 2016; Barreto et al. 2018; Hill et al. 2019). Interestingly, Iannello et al. (2019) observed very high dN/dS (among the highest in animals) for mtOXPHOS subunits supporting a faster evolutionary rate of mitochondrial genes in bivalves, but these were not associated with a higher dN/dS of nuOXPHOS subunits. A second work analyzed OXPHOS genes in 31 bivalve species and retrieved similar dN/dS values, thus extending the evolutionary trends observed in Iannello et al. (2019) to the whole class (Piccinini et al. 2021). Moreover, strong signals of mitonuclear coevolution were assessed, but no unambiguous indication of nuclear compensatory evolution was observed, not even at the residue-specific level.

Most of the nuclear compensatory predictions were built on model taxa to explain how the higher mtDNA mutation rate could coexist with the preservation of the OXPHOS function: in Vertebrata, the ratio between mitochondrial and nuclear mutation rate can indeed be as high as 32 (Allio et al. 2017). In bivalves, however, the mitochondrial mutation rate is just around twice the nuclear one (Allio et al. 2017; Piccinini et al. 2021). Given the fast mtDNA evolutionary rate typical of the class, the lower mitonuclear mutational ratio might be due to a faster-mutating nuclear DNA (Plough 2016). This might be the reason behind the apparent lack of nuclear compensatory signals (Iannello et al. 2019; Piccinini et al. 2021): the mutational pressure acts on two genomes, therefore the need for compensation, or at least for a one-way nuclear compensation, is much lower (Havird and Sloan 2016). However, other non-mutually exclusive reasons could be behind it, including relaxed selection on OXPHOS genes. Bivalves are indeed sedentary, mostly filtering animals. Bivalves show the capability to survive in hypoxic states thanks to alternative acceptors pathways (Müller et al. 2012), and the presence of an Alternative Oxidase (AOX) (McDonald and Vanlerberghe 2004; see Section 4.2). Thanks to such physiological features, the selective pressure on the respiratory complexes might be lower, allowing for higher degrees of mitonuclear mismatches in their structural assembly (see for instance similar conclusions on the plant genus *Silene* in Weaver et al. 2020).

Bivalve molecular coevolutionary data are quite intriguing and meet the expectations for such a mito-diverse taxon, but the surface of the matter has been just scratched. Class-wide deviations from animal mitonuclear coevolution patterns have been observed, however an OXPHOS-centric perspective might exclude the possibility to retrieve different and potentially interesting signals at other levels of coevolution. Protein-protein interactions are just one of the aspects involved (Bar-Yaacov et al. 2012), but, up to now, the only one investigated in bivalves.

Also interactions between the nuclear-encoded ribosomal machinery and the mitochondrial-encoded RNAs, interactions between the mtDNA and the nuclear-encoded transcriptional/replicational complexes, and interactions between mtDNA and nuclear-encoded proteins involved in localisation and stability, they all represent promising research subjects (see for instance: van der Sluis et al. 2015; Sloan et al. 2017; Barreto et al. 2018). However, to address the question thoroughly, both higher-quality data and an integrative approach have to be implemented. More and better genomic data, but mostly protein 3D structures of (at least) more closely related taxa are crucial to study the actual sites of coevolution.

3.2 DUI: a potential frontier for mitonuclear coevolution

The high degree of heteroplasmy in bivalves represents an interesting scenario for mitonuclear coevolutionary studies, especially regarding the compensatory role hypothesized for nuclear subunits: the fixation of compensatory variants in the nuclear genome might be advantageous for one of the two mtDNAs, but detrimental to the other. As mentioned above, bivalve metabolic plasticity might allow for a looser selection on OXPHOS proteins, and some degrees of mitonuclear mismatches could be tolerated because of a non-significant impact on the organism fitness. However, DUI can persist for hundreds of million years while keeping functional male gametes that rely exclusively on M-type mitochondria, therefore other hypotheses should be taken into account to explain the DUI three-level scenario of mitonuclear coevolution.

One conclusion could be that the M-type is simply not selected for high efficiency of OXPHOS functions. In this case, the mitonuclear coevolution would be biased toward the F-type genome, leading to the accumulation in nuclear genes of F-type-specific compensatory fixations that would eventually disrupt cytonuclear complexes built with M-type subunits. However, M-type genomes do are under selection, they are functionally active, genes are transcribed and translated, sperm swimming rely on them, and M-type transcripts/proteins have been observed also in male somatic tissues (Ghiselli et al. 2013, 2019; Milani et al. 2014; Milani and Ghiselli 2015; Breton et al. 2017; Lubośny et al. 2018; Bettinazzi et al. 2019, 2020; 2021). Spermatozoa in DUI species rely on OXPHOS for energy production (Bettinazzi et al. 2020; see Section 4.4) therefore there should be a certain level of constraints on M-type genes. The difference observed in OXPHOS performance of sperm mitochondria when compared to egg mitochondria could be due to sperm-specific selective pressures resulting in a modified mitochondrial metabolism (Bettinazzi et al. 2019, 2020), and/or to a nuclear compensatory mechanism “prioritizing” the functional efficiency of F-type complexes while keeping a sufficient level of functionality of M-type

ones. A scenario involving exclusively the latter explanation, however, would probably be unstable and it would hardly fit with the fact that in some lineages the two mitochondrial genomes have been separated for hundreds of million years (e.g.: in Unionida): after so much time of F-type-prioritized compensatory evolution the M-type complexes could become completely nonfunctional, and conflicts between the two genomes could seriously compromise either fitness or DUI itself.

Another explanation could be found in the relative contributions of the genomes to the fixation of compensatory alleles. As said before, according to the nuclear compensation hypothesis, the nuclear genome is generally identified as the primary site of compensatory substitutions balancing the fast-mutating mtDNA. Osada and Akashi (2012) observed that, in primates, nuclear substitutions usually appeared later in time with respect to the mitochondrial mutations they should putatively compensate, therefore supporting the nuclear compensation hypothesis. However, other works on that subject lack the temporal factor, therefore they cannot precisely address a specific form of coevolution (e.g.: Gershoni et al. 2010; Levin and Mishmar 2017; Li et al. 2017; Barreto et al. 2018). On the other hand, recent *in vivo* evidence in *Caenorhabditis elegans* suggested how the mitochondrial compensatory variants were able to retrieve functional recovery after deleterious nuclear gene mutations in 60 generations (Wernick et al. 2019). Mitochondrial compensation might not be uncommon in animals and it could represent a relatively fast mechanism to maintain function after a deleterious nuclear mutation. If this was the case in bivalves, the two lineages of mitochondrial genomes in DUI species could independently compensate for mutations in the nuclear genome, therefore relieving the latter of a two-way compensatory “strabismus”, and representing, in our opinion, a more parsimonious hypothesis. Works directly addressing the direction of the compensations—either *in silico* with sequence reconstructions and investigations on recent DUI-loss/DUI-gain events, or with *in vitro* enzymatic assays of characterized mitotypes—will likely solve this issue.

A third explanation for the evolutionary stability of DUI might lie in the existence of a duplicated set of nuclear genes that specifically coevolve with the M-type genome and are differentially transcribed in a sex-specific manner. Examples of sex-specific transcription of nuOXPHOS genes are known in mammals and *Drosophila* (Gallach et al. 2010; Eslamieh et al. 2017; Havird and McConie 2019), and tissue-specific splice variants are present in humans (Barshad et al. 2018). However, no data suggested the presence of a complete set, or subset, of OXPHOS nuclear paralogues in bivalves, so far. Nuclear sequences available for DUI species

are few and high-quality annotated genomes are lacking, but a couple of duplicated OXPHOS genes were reported (e.g.: *cox5b* in *C. gigas*: XP_011413812.1 and XP_011413810.1; *cox4* in *Mytilus coruscus*, a non-DUI Mytilidae: CAC5406076.1 and CAC5401226.1). Transcriptomic data in *R. philippinarum* did not show evidence of duplicated nuOXPHOS genes with sex-specific transcription (Maeda et al. submitted; Xu et al. in preparation). To accept or refuse this hypothesis more high-quality genomic data will be crucial. However, only “canonical” nuOXPHOS proteins have been investigated so far (Iannello et al. 2019; Piccinini et al. 2021); Maeda et al. submitted; Xu et al. in preparation). It was proposed that the mitochondrial mutational load could be the reason behind the evolution of supernumerary lineage-specific nuclear-encoded proteins included in OXPHOS complexes, that would therefore represent adaptive recruitments for the functional maintenance of the complexes (van der Sluis et al. 2015; Hill et al. 2019). Within-metazoan lineage-specific novel proteins involved in OXPHOS complexes and mitochondrial ribosomes have been indeed cautiously associated with different mtDNA evolutionary patterns (van Esveld and Huynen 2018). It could be worthwhile to look for similar lineage-specific acquisitions either across all bivalves, or in long-lasting DUI lineages, since that could be a way to “patch” for highly diversifying mtDNAs.

In the end, no interpretation on the mitonuclear coevolutionary mechanism assuring DUI maintenance can be confidently excluded so far, but data are partial because the matter has not been directly approached yet. Coupling respirometric assays with molecular sequencing and the detection of co-varying residues in mitochondrial and nuclear genes could shed light on the nature of this DUI tri-genomic coevolution, yielding fruitful contributions to the defining of mitonuclear coevolutionary mechanisms in general (see next section).

4. From genotype to phenotype: mtDNA and physiological traits

In bivalves, the mostly investigated phenotypic traits related to mitochondrial function include tolerance to hypoxia, thermal tolerance, and longevity (Table 1). What makes these animals so suitable to investigate resistance to a large variety of stressors? Is it the fluctuating environmental parameters under which they evolved that may have promoted the maintenance of plastic traits for oxygen consumption and energy production? Can this resistance be linked to the presence in bivalves of the highest level of heteroplasmy observed so far in living beings?

Unfortunately, to date there is no study addressing phenotype descriptions coupled with a precise genetic characterization of the mitonuclear genetic variation from the same sample (being it specimen, population, or species), and a lack of studies about presence/quantification of mtDNA heteroplasmy. Some studies so far compared physiological traits in DUI and SMI species but without any information about the presence and the level of heteroplasmy (Table 1). Often such studies were performed on somatic tissues, thus being subject to the high variability in heteroplasmy described above (see Section 2.1). Without the analysis of mtDNA sequences, it is impossible to understand which genetic variants are associated with the observed phenotype because it is the result of a cumulative effect due to the whole mitochondrial population, with no means of clarifying the origin of a diverse organelle performance.

We think there is the need of the simultaneous investigation of genetic variation and associated phenotypic traits, of expanding the research beyond correlational data gaining functional clues, to understand the causal relationship among the different observed features. Interestingly, in some plants, a high mutation rate appears to cause no more than subtle differences in mt physiology: lineages with dramatically different mt mutation rates may indeed underlie similar mitochondrial phenotypes at higher levels of biological organization (Table 1). It would be interesting to see if this can be the same in bivalve species with diverse levels of heteroplasmy.

Here follows a selection of studies in which bivalves, given their characteristics, were used to analyze mitochondrial features, including studies that investigated the link between genotype and phenotype.

4.1 Tolerance to fluctuating environmental factors

The regulation of mitochondrial processes in animals living in highly stressful environments allows them to maintain mitochondrial integrity and function despite intense and rapid fluctuating conditions. For example, oxygen fluctuations are an important mitochondrial stressor to aerobic organisms and can cause severe damage to mitochondria, energy deficiency and cell death, thus having major consequences for organism fitness (Sokolova 2018; Sokolova et al. 2019). In hypoxia-tolerant organisms, mitochondrial tolerance appears to be achieved with the regulation of many processes, and even distantly related animal species revealed common physiological responses, including upregulation of electron transport system (ETS) capacity, suppression of ROS-producing processes, reversible suppression of OXPHOS, and the

upregulation of mitochondrial quality control mechanisms (Sokolova 2018; Sokolova et al. 2019). In the case of bivalves, for example, modification in energy metabolism appears to allow the adaptation to a wide variety of environments, including intertidal zones, cold Arctic and Antarctic waters, estuaries and coastal waters, temporary freshwater bodies, and also areas with high anthropogenic impact (Sokolova et al. 2012). Mitochondria of animals living in the intertidal zone regulate energy production preventing cellular damage during the cycle of hypoxia and reoxygenation (H/R) (Ivanina et al. 2016; Sokolova 2018). Indeed, relevant differences were found when comparing bivalve species that live in intertidal zones with species in subtidal environments (Table 1): the former showing hypoxia-tolerance, the latter being hypoxia-sensitive.

Hypoxia-tolerant intertidal bivalves (e.g.: *Mercenaria mercenaria*, *Crassostrea virginica*, see Table 1) suppress ATP-demanding cellular functions during hypoxia, contributing to their high tolerance to H/R stress (Ivanina et al. 2016). Then, during reoxygenation, mitochondrial reorganizations sustain high oxidative phosphorylation flux and ETS capacity (Kurochkin et al. 2009; Sussarellu et al. 2013; Ivanina et al. 2016). Instead, in subtidal hypoxia-sensitive species (e.g.: *Argopecten irradians*, Table 1), mitochondrial ETS, OXPHOS capacity, and mitochondrial membrane potential collapse during reoxygenation (Ivanina et al. 2016), similarly to what observed during hypoxia-reoxygenation in mammalian tissues (Kalogeris et al. 2014; Korge et al. 2015; Sokolova 2018). In hypoxia-sensitive species, exposure to hypoxia followed by reoxygenation led also to elevated levels of oxidative stress in mitochondria (Ivanina et al. 2016). Notably, *Mytilus edulis* intertidal populations that experience H/R stress showed higher antioxidant defense capacities than their subtidal steadily submerged counterparts (Letendre et al. 2009; Sokolova et al. 2012).

Another kind of stress acting on mitochondrial metabolism is that due to temperature fluctuations. In intertidal bivalves (e.g.: *Crassostrea virginica*, *Mya arenaria* and in the Antarctic clam *Laternula elliptica*), an increased temperature resulted in reduced mitochondrial coupling and excess ROS generation (Sokolova 2018) (Table 1). Also a comparison between freshwater unionids (*Dreissena bugensis* and *Elliptio complanata*) indicated that high temperature is linked to a similar impairment of the OXPHOS machinery, characterized by a lower coupling and an increased proton leakage, maybe associated with an increase in ROS production, with a lower tolerance linked to a higher aerobic metabolic depression (Hraoui et al. 2020). These data indicate that mitochondrial thermal sensitivity may affect the survival and distribution of animal species, and highlight the basic role played by mitochondria in determining thermal tolerance; also in this

case, as for hypoxia, this is true not only for aquatic ectotherms, but also, for example, for mice and humans (Lemieux et al. 2017; Mitov et al. 2017), indicating that differential thermal sensitivity of the mitochondrial OXPHOS and proton leak may be common in animal mitochondria (Sokolova 2018).

Organism resilience to stresses caused by temperature changes and hypoxia are not fully clarified in bivalves, although rapid modulation of the ETS capacity, upregulation of antioxidant defenses, and fast degradation of damaged mitochondrial proteins appear to be involved in these processes (Sokolova 2018). As suggested by many authors, the study of additional bivalve species with specific mitochondrial adaptations will help revealing the molecular pathways involved in tolerance capacity to environmental stress; at the same time, the concurrent analysis of mitochondrial genetic variability is fundamental to link the mitochondrial genotype to phenotype.

4.2 Alternative oxidase and protection from oxidative stress

The AOX enzyme is highly conserved in many non-vertebrate taxa and may protect cells against hypoxia and oxidative stress, decreasing the production of ROS during stress conditions (Liu and Guo 2017). AOX is a mitochondrial inner-membrane oxidase that accepts electrons directly from ubiquinol and it reduces oxygen to water without involving cytochrome-linked electron transport chain (Liu and Guo 2017). Given the proposed role of AOX in mediating the transition from sulfide-rich to oxygen-rich environments, it is not surprising that many extant animals that experience hypoxic and/or sulfidic conditions have an AOX (Weaver 2019). For example, burrowing and benthic organisms, such as marine bivalves, have AOX sequences in their genomes (Tschischka et al. 2000; Munro et al. 2013; Robertson et al. 2016; Weaver 2019). It was proposed that animals that occupy environments with fluctuations in oxygen content have retained AOX for the protective role against ROS-based damage during the hypoxic–reoxygenation transition (McDonald and Gospodaryov 2019). This appears confirmed in the intertidal copepod *Tigriopus californicus*, whose AOX transcription is upregulated under stress conditions, resulting in increased AOX protein levels (Tward et al. 2019; Weaver 2019).

Interestingly, two AOX mRNAs were identified in an intertidal bivalve, the oyster *Crassostrea virginica* (Liu and Guo 2017). The gene *CvAOX* has 10 exons with a tandem duplication of exon 10, that, with alternative splicing, can produce the two variants *CvAOXB* or *CvAOXA*, using either the first or second exon 10, respectively. The second exon 10 is the most conserved across taxa, while the first exon 10, present in *CvAOXB*, shows novel mutations close

to functional sites. Under stress (i.e.: air exposure) *CvAOXB* showed significantly higher transcription than *CvAOXA*. Interestingly, these data documented the evolution of a novel AOX variant adaptive to stress in a bivalve (once again, a novelty regarding mitochondrial biology discovered for the first time studying bivalves).

Notably, AOX increased activity has been reported in some plant species characterized, like bivalves, by an elevated rate of mtDNA mutations. In angiosperm plants of the genus *Silene*, ecologically similar, closely related species have either 'fast' or 'slow' mtDNA mutation rates, with "fast" species in which mtDNA evolves faster than NuDNA (as in mammals), and 'slow' species in which mtDNA evolves slower than NuDNA (similar to typical angiosperms) (Weaver et al. 2020). Interestingly, *Silene* mitochondria showed similar respiration profiles among species, regardless of them having 'fast' or 'slow' mtDNA mutation rates. Indeed, despite elevated mt mutation rates the overall mt function appears minimally impacted in fast *Silene* species. Also, some fast species with lower respiration efficiency relied on AOX increased activity. Thus, *Silene* lineages with highly different mt mutation rates may underlie similar phenotypes at higher levels of biological organization (Weaver et al. 2020) and this can be due also to an increased reliance on AOX. These data on plants suggest the importance of coupling mtDNA sequence modification to the phenotype (e.g.: physiological outcomes) to make predictions on the effects of mtDNA mutations.

4.3 Ageing and lifespan: membrane peroxidation and ROS generation

Membrane susceptibility to peroxidation was supposed to be one main driver of lifespan (Rodríguez et al. 2019). That is why mitochondrial membrane composition has been studied in bivalves, given the extremely wide range of longevity inside the class. Indeed, the release of reactive carbonyls upon oxidation of polyunsaturated fatty acids of biological membranes are believed to foster cellular ageing. This was suggested by comparative studies in mammals and birds, in which membrane lipids peroxidation index (PI) resulted negatively correlated with longevity (Munro and Blier 2012). Interestingly, a PI decrease with increasing longevity was also documented in marine bivalves. The PI-longevity relationship was addressed by comparing the clam *Arctica islandica*, having maximum reported longevity (MRL) of 507 years, to other sympatric bivalves with different longevity (in the range of 28-106 years). Membrane PI was actually found to decrease with increasing longevity, with PI decreasing faster in mitochondrial membranes than in other membranes (Munro and Blier 2012).

We discussed in previous sections that bivalves exposed to environmental stressors such as hypoxia/reoxygenation and temperature variations experience oxidative stress and that the presence of AOX can modulate ROS production. In this connection, the production of the ROS H_2O_2 in mitochondria isolated from mantle tissue was compared among the long-lived *A. islandica* and two short-lived species, *Mya arenaria* and *Spisula solidissima* (MRL = 28 and 37 years, respectively), two species of similar size and taxonomically related (Munro et al. 2013). *A. islandica* mitochondria were found to produce significantly less H_2O_2 than those of the two short-lived species. While AOX activity does not seem to explain the observed differences, the lower ROS production in *A. islandica* mitochondria was suggested to be due to reduced complex I and III activity (Munro et al. 2013). This is actually similar to what proposed for long-lived species that lack an AOX (e.g.: homeotherms), in which the rate of ROS production was supposed to be lowered by maintaining a lower steady-state degree of electronic reduction of complexes I and III (Barja and Herrero 1998; Gredilla et al. 2001).

Population studies were also carried out to search for correlation between PI, ETS enzyme activities, and MRL. Six European populations of *A. islandica* showing a wide variation in MRL (from 36 to 507 years) were analyzed, but no relationship was found between membrane PI and MRL, as well as between ETS activities and MRL (Rodríguez et al. 2019). However, individuals from sites with a wide range of temperature and salinity showed markedly lower ETS enzyme activity (relative to citrate synthase activity), for which the authors suggested the presence of environmentally dependent remodeling of mitochondrial phenotypes (Rodríguez et al. 2019).

Future perspectives to clarify physiological adjustments in mitochondria under fluctuating environmental conditions should pertain ROS production and management, the control of electron flux, the use of alternative electron transport pathways, as well as the rates of mtDNA mutations and differences in heteroplasmy levels in different populations, especially when considering DUI species, as *A. islandica* among the others.

4.4 DUI vs SMI phenotypes

Few studies so far investigated the link between mitochondrial genotype and phenotype, taking advantage from the use of the natural heteroplasmic state of DUI species. For example, OXPHOS activity of different tissues in different species were compared through respirometry (Bettinazzi et al. 2019), and taxa were chosen in order to compare DUI and SMI species (Table

1). The data obtained showed that DUI species express sex-linked mitochondrial phenotypes. Specifically, eggs, homoplasmic for the F-type mtDNA, and female somatic tissues (i.e.: gills) expressed a common 'F-phenotype', whereas sperm, homoplasmic for the M-type, express a 'M-phenotype'. The M-phenotype was characterized by low OXPHOS/ETS rates, a strong limitation by the phosphorylation system, and a high flux control of complex IV over the upstream ETS complexes (with an almost null excess capacity of complex IV) (Bettinazzi et al. 2019). This represents the first description of a mitochondrial phenotype resulting from male-driven evolution of mtDNA, the first case of an mtDNA that can specifically adapt for male functions. Complex IV excess capacity was exclusively observed in the F-phenotype and was supposed to provide a way to sustain changes in the ETS performance deriving from the presence of a specialized M-phenotype (of another specialized mtDNA, with M-phenotype) (Bettinazzi et al. 2019). The observed metabolic remodelling could represent an adaptive trait and the evolution of two sex-linked mitochondrial lineages could have potentially allowed to fulfill the different requirements of different gametes. Indeed, the existence of an M-type mtDNA, the only mtDNA present in DUI sperm, allows the study of a M-phenotype, possibly resulting from direct selection on sperm performance and potentially linking male-energetic adaptation with mitotype preservation and inheritance (Bettinazzi et al. 2019).

The comparison of DUI and SMI species was also extended to the measure of sperm performance and sperm response to the presence of eggs (Bettinazzi et al. 2020). The sperm of DUI species showed low speed and low linearity, a strict dependence on OXPHOS for energy production, and a partial shift towards glycolysis and fermentation in the presence of eggs (Bettinazzi et al. 2020). In the absence of egg chemical cues, DUI sperm strictly rely on OXPHOS, whereas sperm of SMI species combine both aerobic and anaerobic pathways of energy production. Instead, in the presence of egg chemoattractants, a partial metabolic shift in the DUI sperm was produced but did not affect SMI sperm. These results are based on distantly related bivalve species and are shown to be consistent across the DUI species analysed, suggesting a convergent evolution of sex-linked mtDNAs in DUI systems (Bettinazzi et al. 2019, 2020). Of course, further investigations are necessary, possibly extended to other DUI species, to further test the hypothesis of a link between male-specific mtDNA energetic adaptation, paternal mitochondria preservation and inheritance.

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Box 1 – MtDNA in a shell

From the very first handful of bivalve mtDNA data, it appeared clear that the extent of genome rearrangements is uncommonly high and associated with higher evolutionary rates, and that the level of gene order variability was extremely high, even compared to other Mollusca (Gissi et al. 2008). Bivalve mtDNA variability involves rearrangements of all genes (from tRNAs to protein coding genes), and this fluidity is observable even at the subfamily level (e.g.: Brachidontinae (Lee et al. 2019); Psammobiidae (Sun et al. 2020)) and at the genus level (e.g.: *Laternula* (Williams et al. 2017); *Scapharca* (Li et al. 2020; Kong, Li, et al. 2020)). Genome size itself is also subject to wide deviations in this class: for instance, within the single order of Arcida (Pteriomorphia), mitochondrial genomes range from roughly 16 to 56 kb (as a result of multiple independent elongation/reduction events (Kong, Li, et al. 2020)), within Anomalodesmata (Euheterodonta) from 14 to 32 (possibly >40) kilobases (Williams et al. 2017), while *Dreissena polymorpha* (Euheterodonta) holds, with 67 kb, the metazoan record of single-molecule mtDNA length (McCartney et al. 2019). Most of these size variations are caused by shifts in non-coding region length, but also gene duplications are not uncommon in Bivalvia. Without considering the erratic nature of the tRNA content, the ribosomal gene *rrnS* has undergone two independent duplications in *Crassostrea* (Wu et al. 2010) and some Arcidae species (Kong, Li, et al. 2020), while the OXPHOS gene *cox2* has been duplicated at least three times. Until recently, *cox2* duplications were found exclusively in DUI species, one in the M-type mtDNA of *Musculista senhousia* (Passamonti et al. 2011), and one in the F-type of *Ruditapes philippinarum* (Ghiselli et al. 2013), and some sort of association to the peculiar mitochondrial inheritance mechanism was hypothesized (Capt et al. 2020). However, two copies of complete and divergent *cox2* have also been found within Arcida (Kong, Li, et al. 2020), in four species that are so far not considered DUI, therefore expanding the potentiality of such an event to the whole class. Up to now, what appears to be a DUI-specific common pattern for *cox2* is the elongation of the ORF: all three Unionidae families share a 3' elongation in the M-type *cox2* (up to a gene length of 1,380bp in *Hyridella menziensis* (Guerra et al. 2017)); *M. senhousia* and *R. philippinarum* have a 3' elongation in the duplicated copy of M-type and F-type *cox2*, respectively (Passamonti et al. 2011; Ghiselli et al. 2013); *Meretrix lamarckii* has a 100-amino acid insertion within the ORF (Bettinazzi et al. 2016); *Scrobicularia plana* and *Limecola balthica* have an M-type-specific insertion of >4.5 and >3.5 kb, respectively (Capt et al. 2020). These latter species have a tremendously big insertion (curiously similar, even in the position of the insertion, to what observed in *Campsomeris*, an ectoparasitoid Hymenoptera (Szafranski 2017)) and, while the *L. balthica* one splits the gene in

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3 two ORFs, the *S. plana* insertion is in-frame, that if translated would make it the longest COX2
4 protein in Metazoa. Other peculiar variations in the canonical mtDNA genes include the splitting
5 of *rrnL* in Ostreidae (Ren et al. 2010), the translocation of a portion of *nad5* in *Anodonta cygnea*
6 (a non-DUI Unionida (Chase et al. 2018)), and an intron-like ~600 bp insertion in the *cox1* of
7 *Cucullea labiata* (Arcoida (Feng et al. 2017)). Last but not least, multiple lineage-specific ORFans
8 have been found in DUI species (Breton et al. 2011; Ghiselli et al. 2013; Milani et al. 2013, 2014,
9 2015; Guerra et al. 2019; Ouimet et al. 2020). These ORFans are usually present in both F- and
10 M-type mtDNAs, and evidence of a protein product was found: polypeptides are usually localized
11 in mitochondria (*R. philippinarum* (Milani et al. 2014); *M. edulis* (Ouimet et al. 2020)), but have
12 been observed also on the nuclear envelope (*Venustaconcha ellipsiformis* (Breton et al. 2011))
13 or even inside it (*R. philippinarum* (Milani et al. 2014)). These proteins, that might have a viral
14 origin (Milani et al. 2013, 2014, 2015, 2016), were hypothesized to have a role in sex specification
15 and/or mitochondrial inheritance (i.e.: DUI maintenance (Milani et al. 2013, 2014, 2015, 2016;
16 Guerra et al. 2019)).
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Table 1 - Mostly investigated phenotypic traits related to mitochondrial function in bivalves (Section 4)

species/taxon	SMI/DUI	trait
Tolerance to fluctuating environmental factors		
<i>Mercenaria mercenaria</i>	SMI	(Ivanina et al. 2016)
<i>Argopecten irradians</i>	SMI	
<i>Crassostrea virginica</i>	SMI	(Sokolova 2004, 2018; Cherkasov et al. 2006, 2007, 2010; Ivanina et al. 2012)
<i>Crassostrea gigas</i>	SMI	(Sussarellu et al. 2013; Sokolova et al. 2019)
<i>Diplodon chilensis</i>	DUI?	(Yusseppone et al. 2018; Sokolova et al. 2019)
<i>Mya arenaria</i>	SMI	(Abele et al. 2002; Sokolova 2018)
<i>Laternula elliptica</i>	?	(Peck et al. 2002; Heise et al. 2003; Sokolova 2018)
<i>Mytilus edulis</i>	DUI	(Letendre et al. 2009; Müller et al. 2012; Sokolova et al. 2012)
<i>Dreissena bugensis</i>	?	
<i>Elliptio complanata</i>	DUI	(Hraoui et al. 2020)
Alternative oxidase and protection from oxidative stress		
<i>Crassostrea virginica</i>	SMI	(Liu and Guo 2017)
Bivalves		(Weaver 2019)
<i>Arctica islandica</i>	DUI	(Tschischka et al. 2000; Munro et al. 2013)
<i>Crassostrea gigas</i>	SMI	(Robertson et al. 2016)
Ageing and lifespan: membrane peroxidation and ROS generation		
<i>Arctica islandica</i>	DUI	
<i>Mya arenaria</i>	SMI?	
<i>Spisula solidissima</i>	SMI?	(Munro and Blier 2012)
<i>Mactromeris polynyma</i>	SMI?	
<i>Mercenaria mercenaria</i>	SMI?	
<i>Arctica islandica</i>	DUI	
<i>Mya arenaria</i>	SMI?	(Munro et al. 2013)
<i>Spisula solidissima</i>	SMI?	
<i>Arctica islandica</i>	DUI	(Rodríguez et al. 2019)
DUI vs SMI phenotypes		
<i>Arctica islandica</i>	DUI	
<i>Mytilus edulis</i>	DUI	
<i>Placopecten magellanicus</i>	SMI	(Bettinazzi et al. 2019)
<i>Mercenaria mercenaria</i>	SMI	
<i>Mytilus edulis</i>	DUI	
<i>Ruditapes philippinarum</i>	DUI	
<i>Mercenaria mercenaria</i>	SMI	(Bettinazzi et al. 2020)
<i>Nuttallia obscurata</i>	SMI	
<i>Placopecten magellanicus</i>	SMI	

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