

Invertebrates Mitochondrial Function and Energetic Challenges

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1. Introduction

The term “invertebrate” recalls all animal species lacking a backbone or a bony skeleton. Although “invertebrate” is not a scientific term that encloses a taxonomic rank, this group includes animal species represented by over 30 phyla and it includes the first animals that successfully inhabited the earth, an event that – according to the fossil evidence – dates back to around 600 million years ago. This group is composed of several different phyla, such as annelids, molluscs, sponges, cnidarians, echinoderms, and all species from the phylum Arthropoda – which is the largest among invertebrates and is comprised by insects, arachnids and crustaceans (nearly reaching 1,033,160 species).

Since they appeared for the first time during the Cambrian period, invertebrates have played an important ecological role since they are frequently the key constituents of many trophic chains and they occupy virtually every available ecosystem on Earth, being characterised by notable variations in temperature, oxygen concentrations, food availability and food quality. Also, many species occupy highly specific and important roles in nature as pollinators, parasites or vectors for parasitic diseases affecting human and animal health.

It is clear that the ability of invertebrates to inhabit almost every ecosystem – as well as the diverse array of morphological and behavioural strategies used to obtain nutrients from the environment – is an accurate reflection of the enormous ability of these organisms to solve their most basic energetic requirements. From blood-suckers such as mosquitoes, intestinal nematodes and leeches (hirudin), to small plankton marine feeders such as cnidarians and marine benthic bivalves, all species face changes in food availability throughout their life cycle which affect their energy stores and growth rates (Peck, 2002; Popova-Butler & Dean, 2009). A beautiful example of highly specific energy stores – crucial during invertebrates’ life cycle and important to human health – is that of the female mosquito (*Anopheles gambiae*), which usually feeds on sugar to gain energy to fly and to cope with metabolic

requirements; however, anautogenous mosquitoes require the energy resulting from blood digestion in order to produce eggs, and it is during blood sucking that *Plasmodium vivax* (the parasite from infected females) enters into the vertebrate host to produce Malaria, a major health problem around the world (Das et al., 2010).

Large energetic demands during external work are observed throughout the life of several invertebrate species, and a clear example may be found in insect flight, which is considered to be one of the most energetically demanding processes of animal locomotion (Harrison & Roberts, 2000). Besides this, being an aerobic process that requires a permanent oxygen supply and depends upon ATP cellular production, the high energetic cost of flying is related to the frequency of the flight muscles' contraction (Vishnudas & Vigoreaux, 2006). In vertebrate species, the existence of high-energetic molecules in the muscle (phosphocreatine) during its exercise has been well documented (Jubrias et al., 2001); however, in invertebrate species, the presence of phosphagen-kinases that catalyse the synthesis of these high-energetic phosphorylated molecules has not been widely distributed (Ellington & Hines, 1991). The insect flight muscle seems to lack such molecules, but some flying species are able to surpass such energy needs by the proximity of mitochondria to muscle myofibrils, thus facilitating the export of energy rich nucleotides – such as ATP – to myofibrils (Vishnudas & Vigoreaux, 2006).

Some other invertebrate phyla – such as crustaceans – are able to synthesise phosphagens differently from that of vertebrates, like phosphocreatine. Phosphoarginine – a phosphagen of L-arginine found in the tail muscle of shrimp and crabs as well as in the flight muscle of flying insects – is the chemical energy storage system of these tissues, and thus animals are able to rapidly produce ATP when it is required (Wegener, 1996; Kotlyar et al., 2000). The enzyme responsible for the synthesis of phosphoarginine from ATP and L-arginine in invertebrates is named 'arginine kinase' and it is also considered to be a major allergen protein for shrimp-allergic individuals (Garcia-Orozco et al., 2007).

Since energetics are considered to be a key factor in limiting organisms' adaptation to extreme temperatures, several invertebrate species inhabiting marine polar environments are known to show a remarkable plasticity as regards their cellular system. Such adaptations may include an increasing number of mitochondria per cell as the temperature decreases as well as differences in the mitochondrial characteristics relating to the species' lifestyle, from motile species to sedentary ones (Peck, 2002). Studies in the mitochondrial function of the eurythermal polychaete *Arenicola marina* have concluded that invertebrates inhabiting higher latitudes – and consequently exposed to cold temperatures – showed higher oxygen consumption, mitochondrial densities and mitochondrial capacities when compared with those organisms living at lower latitudes with higher temperatures (Sommer & Portner, 1999; Peck, 2002). This adaptation of cold-acclimatised organisms is thought to occur in order to equate the level of metabolic activity present at warmer temperatures.

Among other important environmental factors affecting the bioenergetic state of organisms, marine invertebrates face large daily fluctuations in the dissolved oxygen concentrations of water, as well as wide salinity changes between open ocean and coastal waters – where many species live at least during one specific stage of their life cycle – (Dall et al., 1990). Such variations can adversely affect some species whose physiological mechanisms usually do not allow them to cope with low oxygen levels (as oxyregulators) or to handle salinity changes (as osmoregulators). However, several species are able to swim or move from one place to other, searching for a suitable site to grow, reproduce and survive (Hochachka &

Somero, 2002; Abele et al., 2007). Nevertheless, other invertebrate species are highly adapted to live in extreme conditions such as those living in hypoxic or even anoxic environments, like the brine shrimp *Artemia franciscana* (Eads & Hand, 1999; 2003).

As has previously been stated, this chapter reviews the current state of knowledge of the mitochondrial function of invertebrate species. It asks two central questions: 1) How are invertebrates able to adapt to such diverse environmental conditions by using a common set of structures and mechanisms - their mitochondrial machinery - to fulfil their energy requirements along their entire life cycle? 2) Is it really important to understand the role of mitochondria in the life history of invertebrates? This chapter also includes original data on crustacean responses to the external factors affecting such mitochondrial functions as hypoxia, starvation and the energetically expensive molt cycle.

2. The highly conserved mitochondrial machinery of invertebrates: Same functions, different challenges

Following the endosymbiotic origin from primitive bacteria - at least 2 billion years ago - when atmospheric oxygen levels rose and subsequently remained relatively steady, mitochondria have experienced large changes among species, from α -proteobacteria to mammals. During the adaptation process of organisms to their new dynamic environment, some mitochondrial characteristics have remained highly conserved even among distantly related species, such as their rod shape - the overall structure including two phospholipid membranes - and, with some exceptions, their conserved characteristic genome content of 22 tRNAs, 2 rRNAs, and 13 genes encoding protein subunits of the enzymes from the oxidative phosphorylation system (OXPHOS) (Boore, 1999; Gray et al., 1999).

Besides mitochondrial encoded proteins, a significant fraction of the original mitochondrial genes have moved to the nucleus. Thus, in the mammalian mitochondria, approximately 76 subunits - which are part of the respiratory chain - are encoded by nuclear genes, and all of them must be imported into the mitochondria. The complete protein machinery involved in mtDNA replication, transcription and translation (including all of the ribosomal protein subunits) is encoded by nuclear genes (St. John et al., 2005; Falkenberg et al., 2005). Furthermore, several of these imported proteins are highly conserved among species, some of them accomplishing key roles as subunits alpha and beta of the ATP-synthase, which are part of the catalytic sites of the enzyme (Martinez-Cruz et al., 2011).

In addition to those key proteins that maintain a conserved function, hundreds of new proteins have been described among invertebrate species as being imported to mitochondria, each presumed to participate in at least one of the large number of metabolic pathways occurring in this organelle. However, its major conserved function allows mitochondrion to produce - from food assimilated compounds via oxidation - the proton motive force that drives ATP synthesis (Rich & Marechal, 2010). This complex process produces 95% of the cellular ATP that cells need for biosynthesis, transport and motility (Wilson et al., 1988; Dudkina et al., 2008; Diaz, 2010), and any significant change in the system could result in deleterious consequences for the whole cell metabolism and - consequently - reduce its efficiency or provoke its death (Mayevsky & Rogatsky, 2007).

Throughout the years (and mostly based in the study of human pathologies) researchers have found that mitochondria are involved in various critical functions - such as thermoregulation - in the synthesis of essential molecules - such as phospholipids and heme - in the programmed cell death or apoptosis of mediating multiple cellular signalling

pathways (Ryan & Hoogenraad, 2007). Mitochondria are also essential in the cholesterol metabolism and the detoxification of ammonia in the urea cycle. In addition, there is a close relationship between mitochondria and different cell types. It is well known that the number of mitochondria in individual cell types varies according to their function and energy requirements (St. John et al., 2005; Chen & Chan, 2009). Thus, highly energetic tissues as the flight muscle of flying insects and the midgut gland of crustaceans are known to contain a large number of mitochondria, just as occurs in the skeletal muscles of vertebrates during endurance training (Harrison & Roberts, 2000).

Mitochondria are known as dynamic organelles that cannot be made *de novo*, and instead they divide through a highly regulated process called mitochondrial fission, mediated by a defined set of new proteins recruited from the cytoplasm, which are added to pre-existing sub-compartments and protein complexes to a point whereby the organelle grows and divides (Ryan & Hoogenraad, 2007). Furthermore, mitochondria are now seen as a set of organelles that are able to migrate throughout the cell, to fuse and divide regulating mitochondrial function (Chen & Chan, 2009).

Recent findings have also confirmed the existence of dynamic mitochondrial supercomplexes - defined as the association of protein complexes distributed along the inner mitochondrial membrane - on mammals, plants, yeasts (*Yarrowia lipolytica*), and bacteria (Nübel et al., 2009; Wittig & Schägger, 2009; Dudkina et al., 2010). Complexes I, III and IV are able to associate in order to promote electron transport as single OXPHOS complexes or else as a supercomplex called respirasome (I + III₂ + IV₁₋₂) both of which can autonomously carry out respiration (Wittig et al., 2006). Furthermore, complex V - the mitochondrial F₁F₀ATP-synthase - is associated to form dimeric, trimeric and tetrameric organisations (Dudkina et al., 2008). Unfortunately, to our knowledge, there are no reports confirming the existence of these mitochondrial protein associations from invertebrate species.

A general description of the most recent advances covering mitochondrial enzymes participating in the electron transport chain and the OXPHOS, including some particular findings on the enzymes of some invertebrate species, is presented below:

2.1 Complex I, NADH: Ubiquinone-oxidoreductase (EC. 1.6.5.3)

Is an enzyme which provides the input to the respiratory chain by catalysing the transfer of two electrons from NADH from - glycolysis - to ubiquinone, and which utilises the free energy released in this redox reaction for the translocation of four protons across the membrane, from the matrix to the intermembrane space. The proton translocation from the mitochondrial matrix generates the proton-motive force required for ATP synthesis at the end of the respiratory chain during oxidative phosphorylation (Friedrich & Weiss, 1997; Dudkina et al., 2008). However, this proton-pumping enzyme is the largest, most complicated and least-well understood of the respiratory chain (Zickermann et al., 2008). Another unconventional function of complex I is the generation of reactive oxygen species (ROS) - such as the superoxide ion (O₂⁻) - and, even if it is not a strong oxidant, it is a precursor of most other ROS and, consequently, contributes significantly to cellular oxidative stress. In mammalian mitochondria, the superoxide production is predominantly produced by complex I (Turrens, 2003).

The scarce information available concerning mitochondrial complex I from invertebrates includes basic descriptive reports of the nucleotide sequences of the NADH subunits - most

of them from the mitochondrial genome-, their proteins, and an interesting study of site-directed mutagenesis aiming to understand the subunits' function in model insect species such as *Drosophila* spp. (Tovoinen et al., 2001; Sanz et al., 2010).

In addition, the existence of an alternative oxidase (AOX) in the animal mitochondria has been confirmed. Previously, this enzyme - which catalyses the O₂-dependent oxidation of ubiquinol, producing ubiquinone and H₂O - was thought to be limited to plants, some fungi and protists. The major difference between complex I and AOX is that the electron flow from ubiquinol to AOX is not coupled to the generation of a proton motive force, decreasing energy conservation in oxidative phosphorylation. The complementary DNA sequence that encodes AOX in invertebrate species from the phyla Porifera, Cnidaria, Nematoda, Anellida, Mollusca, and Echinodermata, has been characterised and it has been suggested that it may contribute on the acclimation of animals to stress conditions, mainly when the cytochrome pathway is inhibited (McDonald et al., 2009).

2.2 Complex II, Succinate: Ubiquinone- Oxidoreductase (EC 1.3.99.1)

Also called Succinate Dehydrogenase (SDH), is a functional member of the Krebs cycle and the aerobic respiratory chain, and it couples the oxidation of succinate to fumarate with the reduction of quinone to quinol (QH₂). Most probably, this enzyme presents the most striking differences among the mitochondrial complexes in the electron transport chain and OXPHOS (Rich & Marechal, 2010). It must be noticed that the oxidation of succinate to fumarate is the only Krebs reaction that takes place in the mitochondrial inner membrane itself; this reaction does not participate in proton translocation from one side to the other of the inner mitochondrial membrane. The energy carrier flavin adenine dinucleotide (FAD) forms a part of complex II, and succinate oxidation begins after the binding of succinate to the enzyme. This covalent binding of FAD to the enzyme increases the redox potential to a level that allows succinate oxidation (Rich & Marechal, 2010).

Contrary to the four human and yeast mitochondrial complexes, which include subunits that are encoded by the mitochondrial genome, the four subunits of SDH are encoded in the nuclear genome (SDH1 to SDH4; Figueroa et al., 2002).

Early studies of complex II (SDH) from invertebrates reported the isolation of mitochondrial fractions from the body muscles of the worm *Nereis virens* and from the tail muscle of the lobster *Homarus gammarus*, and reported high activity in both enzymes (Mattisson, 1965). Unfortunately, there is scarce new information available concerning complex II in invertebrates. However, the study of mitochondria from parasite species - used as animal models - can be considered a framework that has guided our knowledge in the understanding of such critical endogenous processes as aging, mitochondrial dysfunction and the role of the organelle in apoptosis (Grad et al., 2008; Wang & Youle, 2009). Thus, it has been suggested that mitochondria may influence the longevity of the nematode *Caenorhabditis elegans* through the rate of ROS production and by the stress-evoked signals that are known to act in a cell-non-autonomous manner during mitochondrial protein regulation (Durieux et al., 2011). Furthermore, *C. elegans* has been used as a model to investigate the mitochondrial mechanisms of human aging and tumourigenesis by studying the catalytic effects of mutation in the genes encoding the SDH iron-sulphur subunit. Promising results suggest that the SDH ubiquinone-binding site can become a source of superoxide and that the pathological consequences of SDH mutations can be diminished with antioxidants, such as ascorbate and N-acetyl-L-cysteine (Huang & Lemire, 2009).

2.3 Complex III, Ubiquinol: Cytochrome C Oxidoreductase or Cytochrome BC₁ (EC 1.10.2.2)

Is a multimeric enzyme complex involved in the transfer of electrons from ubiquinol to cytochrome C, and it is also coupled to electrons' transfer across the inner mitochondrial membrane. This bovine enzyme is formed by 10 nuclear encoded subunits, with only one encoded in the mtDNA (Xia et al., 1997). The catalytic mechanism of the enzyme includes the complex mechanism of the protonmotive Q-cycle that provides the additional efficiency of the energy conservation of the electrons transferred (Mitchell, 1976; Rich & Marechal, 2010).

In such species as mammals and yeasts it has been observed that as the rate of electron transfer is reduced, the enzyme may leak electrons to molecular oxygen, promoting the formation of the superoxide ion. This mitochondrial dysfunction has been widely studied, and its role in the O₂ sensing pathway has been investigated because the increasing production of reactive oxygen species (ROS) is the result of organisms in hypoxic/anoxic conditions (Guzy et al., 2007). New evidence suggests that ROS generated by the mitochondrial complex III are required for the hypoxic activation of transcription factors such as HIF (Hypoxia Inducible Factor); however, this topic will be more extensively discussed below.

The mitochondrial complex III from invertebrates has been poorly studied, but recent reports about these species confirm the importance of studying its basis and applications. An interesting example is the study about the control of Chagas disease, which severely affects the health of the human population in Latin America and which is caused by the protozoan parasite *Trypanosoma cruzi*. Genes et al. (2011) reported such bacteria species as *Serratia marcescens* biotype A1a, which is regularly found in the gut of the vector insect *Rhodnius prolixus*, and which demonstrates the trypanolytic activity conferred by prodigiosin. Prodigiosin is a potent bacterial tripyrrolic compound with various biological activities. This study suggests the abnormal mitochondrial function of *T. cruzi* since prodigiosin inhibits the mitochondrial complex III, affecting subsequent oxidative phosphorylation.

2.4 Complex IV, Cytochrome C oxidase (EC 1.9.3.1)

Is the terminal enzyme of the electron transport chain and it catalyses the reduction of molecular oxygen to water. The reduction of oxygen by this enzyme - which is responsible for biological energy conversion in mitochondria (Belevich et al., 2010) - is also linked to the translocation (pumping) of four protons across the membrane. This movement of electrons is subsequently coupled to ATP synthesis by the ATP-synthase (Khalimonchuk & Rödel, 2005). The cytochrome C oxidase (CO) has been described as one of the electron transport chain elements which is highly affected by changes in oxygen levels - since cytochrome C reduction is oxygen-dependent - and becomes more reduced when oxygen levels increase (Wilson et al., 1988).

The CO from eukaryotes consists of 11-13 subunits, depending on the species. It belongs to the family of heme-cooper enzymes, some of them suggested as hypoxia sensors. The enzyme is highly regulated by transcription factors, hormones, lipid membranes and the second messengers that control its activity (Ripamonti et al., 2006; Semenza, 2007; Fontanesi et al., 2008). As observed in other mitochondrial complexes, CO also includes mitochondrial encoded genes as subunits CO1, CO2, and CO3 which form the functional core of the enzyme; the rest are nuclear-encoded subunits and their functions - even in the most studied animal models - remain unclear, although they are assumed to participate in the

assembly, stability and regulation of the enzyme (Rich & Marechal, 2010). Moreover, CO is also regulated by the existence of various isoforms from each nuclear-encoded subunit which is known to be tissue- and specie-specific (e. g. CO5a and CO5b, CO6a, CO6b and CO6c, and CO7a, CO7b, CO7c, etc.; Diaz, 2010).

The CO genes' expression and the activity of the enzyme are known to be affected by external factors. In crustacean species, such as the grass shrimp *Palaemonetes pugio*, the gene expression of subunits CO1 and CO2 is positively or negatively regulated by low dissolved oxygen concentrations in water (Brouwer et al., 2008). References and further reading may be available for this article.

In insects, as with the sweet potato hornworm *Agrius convolvuli*, diapause - the delay in development in response to regularly and recurring periods of adverse environmental conditions - is induced by low temperatures. During this physiological state, the neurological activity, oxygen consumption rate and metabolic levels are low compared to undiapaused animals; and it has been found that the genetic expression of the CO1 subunit is down-regulated. When the organism terminates diapause, CO1 is up-regulated and the enzyme activity also increases (Uno et al., 2004). Other insect species, such as the cotton boll worm *Helicoverpa armigera*, show diverse responses during diapause: the levels of CO1 mRNA and enzyme activity are low, suggesting that the diapause state is different in each species (Yang et al., 2010).

In some species, CO participates in organism detoxification, as observed in the polychaetes *Hediste diversicolor* and *Marenzelleria viridis* which inhabit eutrophicated regions with low oxygen levels and high sulphide concentrations - where CO functions as an alternative pathway of oxidation - (Hahlbeck et al., 2000). In addition, when sulphide becomes hydrogen sulphide (HS) - a weak acid that occurs in marine and aquatic environments such as hydrothermal vents, mudflats and marshes - HS is known to reversibly inhibit CO activity, affecting the aerobic metabolism of certain species, such as the worm *Urechis caupo* (Julian et al., 1998).

2.5 Complex V, ATP synthase (EC 3.6.3.14)

Is a multimeric enzyme that transforms the kinetic energy of the protons' electrochemical gradient to synthesise the high energy phosphate molecule ATP. Nowadays, it is well-known that the enzyme can also hydrolyse ATP, functioning as an ATPase (Boyer, 1997; Tuena de Gomez-Poyou et al., 1999). This mitochondrial enzyme comprises a catalytic sector F_1 (composed by $\alpha_3\beta_3\gamma\delta\epsilon$ subunits), and a transmembrane hydrophobic sector F_0 (composed of at least three subunits: a , b_2 and c_{10-12}), both linked by a central and a peripheral stalk (Mueller et al., 2004). As in other mitochondrial complexes, this enzyme includes subunits encoded in both the nuclear and mitochondrial genomes, in a tightly coordinated process to assemble this multimeric complex (Itoi et al., 2003; Muhlia-Almazan et al., 2008).

During the oxidative phosphorylation process in mitochondria, the electron transport chain generates a proton gradient that is proposed to drive the rotation of F_0 , a central rotor located in the inner mitochondrial membrane. This rotation movement is believed to reverse the rotation of the F_1 nanomotor, inducing - via a conformational change - the sequential release of ATP from three identical catalytic sites followed by the sequential synthesis of newly formed ATP from $P_i + ADP$ at these sites (Cardol et al., 2005). Biochemical and structural studies of the F_1 sector from bovine enzymes have demonstrated that catalytic sites are integrated mainly by three β subunits that alternate with three α subunits. The

three catalytic sites formed by these three pairs of α/β subunits are grouped in segments forming a sphere, which is connected to the γ subunit which connects F_1 to F_0 (Lai-Zhang & Mueller, 2000).

Due to its complex structure and the dual role that the ATP synthase plays in cells, the current state of research concerning this mitochondrial enzyme is both abundant and relevant; however, for the majority of invertebrate taxa, the information regarding this enzyme appears to be almost non-existent, restricted to some insect species for the more studied models. Analyses of the mitochondrial transcriptome and proteome from these species – which have been exposed to different environmental conditions – have shown that the ATP-synthase subunits can be affected in their expression, and that specific subunits of this multimeric complex can also play additional roles in the mitochondrial function. These findings suggest that invertebrates are able to respond by changing their metabolism to maintain cell homeostasis.

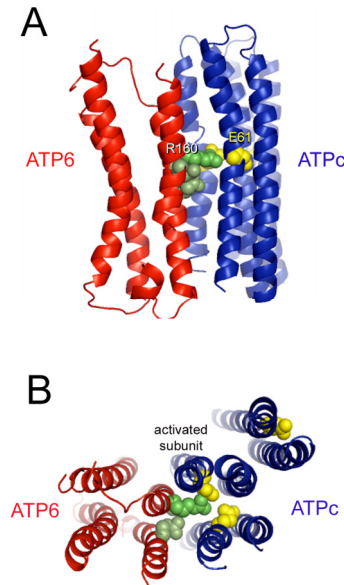
In the fruit fly *Drosophila melanogaster* and the California purple sea urchin *Strongylocentrotus purpuratus* the gene expression of the ATP-synthase subunit alpha (*atp α*) was measured at early developmental stages, and it was found that the amount of mRNA varies throughout development in both species. Contrary results showed that during the larval stage the nuclear and mitochondrially encoded ATP synthase genes appear to be temporally co-regulated in *Drosophila*, although in the sea urchin this development pattern was not observed (Talamillo et al., 1998). In 2005, Kidd et al. analysed null mutants of the ATP-synthase subunit ϵ in *Drosophila* spp., and a dramatic delay in the growth rate of the first instar larvae that finally died was reported. In addition, in fly embryos the ATP-synthase activity had a six-fold reduction.

Most likely, the first two studies concerning the ATP synthase of crustacean species were published in 2001. The authors characterised the enzymatic properties of F_1 and evaluated its sensitivity to specific inhibitors and modulators in the gills of the freshwater crayfish *Orconectes virilis*; they included, as an important contribution, the standardised methods for isolating mitochondria from crustacean tissues and some results about their enzyme stability at different temperatures and pH conditions (Li & Neufeld, 2001a, 2001b).

Recent reports on the most-studied shrimp species – *Litopenaeus vannamei* – have characterised and studied several mitochondrial and nuclear encoded subunits from tissues such as muscles, gills, pleopods and the midgut gland (Muhlia-Almazan et al., 2008; Martinez-Cruz et al., 2011). The complementary DNA sequences of the *atp6* subunit encoded in the mtDNA and the *atp9* (a nuclear encoded subunit) were characterised and their deduced proteins, as major components of the F_0 sector, were included in a molecular model which predicted that in the shrimp F_0F_1 ATP synthase the *atp9* oligomeric ring may contain 9-10 proteins (Figure 1; Muhlia-Almazan et al., 2008).

Over the last decade, the effects of a viral agent which provokes shrimp death have been deeply studied. The white spot syndrome virus (WSSV) is perhaps the most devastating shrimp disease, causing massive mortalities in global aquaculture systems (Sanchez-Paz, 2010). In 2006, Wang et al. analysed the gene expression profile of the fleshy prawn *Fenneropenaeus chinensis* in response to WSSV infection through cDNA microarrays. Genes including the ATP-synthase A chain and arginine kinase were found to be down-regulated during WSSV infection. Additional studies in other shrimp species, reported thirty additional genes which are involved in the antiviral process as part of the shrimp's defence system. One of the most interesting findings of these studies was that the interferon-like

protein (IntIP) – known as an antiviral factor – showed increased expression in virus-resistant shrimp (He et al., 2005). Later, Rosa & Barraco (2008) suggested that the shrimp interferon-like protein (IntIP) is rather a region of the insect mitochondrial b subunit of the ATP-synthase, due to the high identity between both proteins (60–73%). Recently, Liang et al. (2010) have suggested the ATP-synthase subunit β (*atp* β) – earlier called BP53 – as a protein involved in the WSSV binding to shrimp cells that may play an important role in the antiviral defence system of shrimp against WSSV.



A) Ribbon lateral view, and B) Ribbon front view of the subunit ATP6 complex with three ATP9 subunits. The predicted functional residues are marked in both subunits, R160 from ATP6, and E99 from ATP9. (Taken from Muhlia-Almazan et al., 2008).

Fig. 1. Molecular Model of the ATP9- ATP6 Subcomplex from the Shrimp *L. vannamei*.

Transcriptomes and proteomes have provided a lot of information, not only about the characteristics of specific sequences of nucleotides or amino acids, but also about the proteins' structure and function in invertebrate organisms under diverse environmental conditions (Clavero-Salas et al., 2007). Moreover, novel proteins have been reported as accessories to the mitochondrial protein complexes in invertebrates species, such as the ticks *Ornithodoros moubata* and *O. erraticus*, where six novel proteins similar to the ATP synthase subunit 6 (*atp6*) were identified in the salivary glands. These proteins are attractive targets for controlling ticks and tick-borne pathogens (Oleaga et al., 2007).

Actually, and based in the mitochondrial highly conserved function, generic models of the electron transport chain in mitochondria have been constructed using bioinformatic tools to predict how the rate of oxygen consumption through the system – and the redox states of some intermediates such as NAD/NADH, ubiquinone, and cytochromes – respond to physiological stimuli such varying oxygen levels and other rapid energy demands (Banaji, 2006).

Ultimately, it is remarkable that the mitochondrial function has remained in all animal species through its long and peculiar evolutionary history and under the influence of variable selective pressures. Moreover, structural and biochemical adaptations promoting highly effective mitochondrial functions have allowed organisms to inhabit unusual environments.

3. The Invertebrates mitochondrial genome

The study of the mitochondrial genome has provided enormous amounts of information from which it has become feasible to infer the origin of species by using comparative and evolutionary genomics (Jiang et al., 2009) in order to understand the ancient phylogenetic relationships among species, to comprehend population genetics (Boore et al., 1995; Boore, 1999), and to recognise the mechanisms coordinating the nuclear and mitochondrial genomes so as to synthesise a large number of functional proteins located in this organelle.

To date, the mtDNA of several invertebrates has been sequenced and characterised, including ascidians (Yokobori et al., 1999), echinoderms (Jacobs et al., 1988; Asakawa et al., 1995), insects (Clary & Wolstenholme, 1985), nematodes (Okimoto et al., 1992), molluscs (Yu & Li, 2011; Cheng et al., 2011), and various crustacean species such as shrimp and crabs (Staton et al., 1997; Shen et al., 2007; Peregrino-Uriarte et al., 2009). Several reports have shown that the mitochondrial genome of invertebrate species varies, and ranges between 12 and 20 kbp. This may be due to contrasting ecological habitats or it may be a response to different selective pressures (Table 1).

Phylum	Species	mtDNA size (bp)	GenBank Acc. No.	References
Porifera	<i>Plakinastrella sp.</i>	19,790	NC_010217	Lavrov et al., 2008
	<i>Negombata magnifica</i>	20,088	NC_010171	Belinky et al., 2008
	<i>Aphrocallistes vastus</i>	17,427	NC_010769	Rosengarten et al., 2008
Cnidaria	<i>Hydra oligactis</i>	16,314	NC_010214	Kayal & Lavrov, 2008
	<i>Aurelia aurita</i>	16,937	NC_008446	Shao et al., 2006
	<i>Fungiacyathus stephanus</i>	19,381	NC_015640	---
Platyhelminthes	<i>Symsagittifera roscoffensis</i>	14,803	NC_014578	Mwinyi et al., 2010
	<i>Clonorchis sinensis</i>	13,877	JF729304	Cai et al., 2011
	<i>Taenia taeniaeformis</i>	13,647	NC_014768	Liu et al., 2011
Rotifera	<i>Brachionus plicatilis</i>	12,672	NC_010484	Suga et al., 2008
Acanthocephala	<i>Leptorhynchoides thecatus</i>	13,888	NC_006892	Steinauer et al., 2005

Phylum	Species	mtDNA size (bp)	GenBank Acc. No.	References
Nematoda	<i>Caenorhabditis elegans</i>	13,794	NC_001328	Wolstenholme et al., 1994
	<i>Necator americanus</i>	13,605	AJ417719	Hu et al., 2002
Onychophora	<i>Oroperipatus sp.</i>	14,493	NC_015890	Segovia et al., 2011
Brachiopoda	<i>Laqueus rubellus</i>	14,017	AB035869	Noguchi et al., 2000
Echinodermata	<i>Acanthaster planci</i>	16,234	NC_007788	Yasuda et al., 2006
	<i>Strongylocentrotus purpuratus</i>	15,650	NC_001453	Qureshi & Jacobs, 1993
	<i>Cucumaria miniata</i>	17,538	NC_005929	Arndt & Smith, 1998
Mollusca	<i>Crassostrea gigas</i>	18,225	EU672831	Ren et al., 2010
	<i>Cepaea nemoralis</i>	14,100	NC_001816	Terrett et al., 1996
	<i>Octopus minor</i>	15,974	HQ638215	Cheng et al., 2011
Annelida	<i>Platynereis dumerilii</i>	15,619	AF178678	Boore & Brown, 2000
	<i>Lumbricus terrestris</i>	14,998	NC_001673	Boore & Brown, 1995
Arthropoda				
Subphylum Chelicerata	<i>Centruroides limpidus</i>	14,519	NC_006896	Davila et al., 2005
Subphylum Crustacea	<i>Litopenaeus vannamei</i>	15,989	DQ534543	Shen et al., 2007
Subphylum Myriapoda	<i>Scutigera coleoptrata</i>	14,922	NC_005870	Negrisoló et al., 2004
Subphylum Hexapoda	<i>Apis mellifera</i>	16,343	NC_001566	Crozier & Crozier, 1993

Table 1. Invertebrates' mitochondrial genome size of the species of different phyla.

Because of the wide variability of environmental conditions in which a large number of invertebrate species are distributed, several specific mtDNA-rearrangements have been found when compared with those observed in the mtDNA of mammals. Such novel arrangements include the mitogenome from the blue mussel *Mytilus edulis* (Hoffmann et al., 1992), and that of the fruit fly *Drosophila melanogaster* (Clary & Wolstenholme, 1985; Garesse, 1988) and the horseshoe crab *Limulus polyphemus* (Staton et al., 1997).

Also, some species - or groups of species - may lack some genes, such as nematodes whose mtDNA lacks a gene for ATP8 (Keddie et al., 1998), or cnidarians like the coral *Sarcophyton glaucum* which includes an unusual gene encoding an extra tRNA (Beaton et al., 1998). Moreover, major changes have been found in invertebrates' mtDNA, such as the mitochondrial genes of *Lumbricus terrestris*, which are all known to be encoded in the same strand and, unlike others, the genes coding A8 and A6 are separated by a long 2700 nucleotides fragment (Boore & Brown, 1995).

In 2006, the description of the mtDNA of the moon jellyfish (*Aurelia aurita*) was reported. It was surprising to find that mitochondria of this organism contain a linear genome, which became the first non-circular genome described in a Metazoan. Besides its linearity, its organisation involves two additional sequences of 324 and 969 nucleotides, the last (ORF969) encodes a putative family B-DNA polymerase, tentatively identified as *dnab*, which was previously only reported in algae mtDNAs (Shao et al., 2006). Subsequently, the linear mitogenome of Cnidarians of the genus *Hydra* was also reported, although it was found that it is fragmented as two linear mitochondrial “chromosomes” (mt1 and mt2) where all genes are unidirectionally-oriented (Voigt et al., 2008).

In addition, the invertebrate’s mitochondrial genetic code differs from the universal/standard genetic code, and it is suggested that this is species-specific since several studies have identified some changes in animal mitochondrial code, as shown by Table 2 (taken from Watanabe, 2010). As observed in this table, invertebrate mtDNAs are largely represented by different changeable codons - depending upon the species. This is the case for the AUA codon which usually codes Ile in the standard genetic code but in the mitochondria of some species of Nematoda, Mollusca, Platyhelminthes and Vertebrata it encodes a Met (Himeno et al., 1987; Bessho et al., 1992). Also, in several species, the start codon differs from the AUG but still codifies a methionine, and in most of the species the stop codon is an incomplete codon, such as UA or U (Watanabe, 2010).

Codon (Universal code)	AUA (Ile)	AAA (Lys)	AGA (Arg)	AGG (Arg)
Vertebrates (human, bovine, rat, mouse, chicken, frog)	Met	Lys	Term	Term
Prochordates (ascidian, asymmetron)	Met	Lys	Gly	Gly
Echinoderms (sea urchin, starfish)	Ile	Asn	Ser	Ser
Arthropods	Met	Lys	Ser	Ser
Most (shrimp, daphnia)	Met	Lys	Ser	Ser
Insect (<i>Drosophila</i>)	Met	Lys	Ser	-
Molluscs (squid, octopus, Liolophura, Mesogastropoda)	Met	Lys	Ser	Ser
Nematodes (nematodes, ascaris)	Met	Lys	Ser	Ser
Platyhelminthes	Met	Asn	Ser	Ser
Most (Echinostomida, Trematoda)	Ile	Asn	Ser	Ser
Rhabditophora (<i>Planaria</i>)	Ile	Lys	Arg	Arg
Coelenterates (jellyfish, coral, sea anemone, hydrozoa)	Ile	Lys	Arg	Arg

Table 2. The relationships between the genetic codes of animal mitochondria. Modified from: Watanabe, 2010. Bold letter: non-universal codon; Term: termination codon.

Although, to date, the mitochondrial genes expression mechanisms are not fully understood, and the evolutionary processes by which the mitogenome suffers a rearrangement are not clear. It is proposed that a new order in genes' arrangements must preserve or facilitate those signals or mechanisms required for the transcription and processing of RNAs to accomplish the mitochondrial function in animal species (Boore, 1999).

The mitochondrial DNA from animal cells is known to be easily affected, since it is not protected by DNA-binding proteins or histones such as nuclear DNA. Several studies have found that mtDNA can be affected by aging, hypoxia and random events of mutation or insertion/deletion (rates of mutation for mitochondrial genomes are known to be much higher than those in the nuclear DNA) that can produce increased oxidative stress and high levels of ROS in this organelle. Defective proteins which result from altered mtDNA molecules cause defective mitochondrial function, as an impaired respiratory chain and increased electron leaks so as to finally generate larger amounts of ROS (Wei et al., 1998).

Insects' mitogenomes are known to be affected at the transcriptional level by chemicals, since the mtDNA copy number has been shown to increase to meet the bioenergetic demands of the organism, as observed in the fly *D. melanogaster* when exposed to tetracycline. Treatment with this antibiotic causes an energetic deficiency, promoting an up-regulation of the mtDNA copy number (Moraes, 2001; Ballard & Melvin, 2007).

4. Invertebrate challenges and how marine species spend energy

In most animal species, high energy levels in their bodies reveal fast growth, adequate energy storage, effective reproduction strategies and viable descendants with characteristic short life spans; however, reduced energy levels in a biological system results in affected gene expression, low survival rates and reduced metabolic rates and, therefore, a need on the part of physiological mechanisms to slow the ageing rate until environmental conditions are enhanced and higher energy levels are again reached (Stuart & Brown, 2006). In their natural habitat, many invertebrate species must undergo endogenous physiological processes during their life cycle, such as molting, starvation, quiescence and metamorphosis, among others. Many of these processes imply high energetic expense, causing a low energy status that reduces their ability to reach the adult stage (Hochachka & Somero, 2002).

The role of metamorphosis - one of the most amazing physiological endogenous processes in nature - becomes strikingly important when considering the large number of animal species that undergo metamorphic changes. Frequently, the energetic balance of holometabolous insects during metamorphosis is negative, because there is no energy gain and species must face all these changes by using any energetic reserves previously stored (Nestel et al., 2003).

During their larval stages, insects - such as Lepidopterans - show fast growth rates, as observed in the tobacco worm larvae of *Manduca sexta* which increases its mass 10,000-fold in just 16 days at the final larval instar (Goodman et al., 1985). The midgut epithelium of this species is a highly aerobic tissue that digests and absorbs nutrients, and transports ions at high rates. During metamorphic changes, the midgut epithelium is programmed to die and the larval midgut should maintain structural and functional integrity until the pupal epithelium is formed. During this process, ATP synthesis and mitochondrial function must be obligatorily maintained. Thus, organisms resolve this by reducing mitochondrial

substrate oxidation, a clear indication that the electron transport chain may be a site of modulation during metamorphosis (Chamberlin, 2004).

Quiescence and estivation are also two responses that some species may display during unfavourable environmental conditions in which insufficient energy is available to grow and breed. These dormant states allow species to survive by reversibly down-regulating their metabolism to low levels for up to several years. Among invertebrates, many species show quiescent states at stress conditions, including nematodes, crustaceans such as the brine shrimp *Artemia franciscana* (Hand, 1998), the estivating pulmonate snail *Helix aspersa* (Pedler et al., 1996), and various insect species entering in diapause, such as *Helicoverpa armigera*. Studies have proposed that a coordination mechanism is required when animals enter into the dormant state so as to maintain cellular homeostasis by both energy-consuming and energy-producing pathways. During quiescence, *A. franciscana* can reduce its metabolism essentially to zero, this metabolic-rate suppression affects the mitochondrial respiratory capacity and the rates of ATP-consuming processes (Barger et al., 2003). In the embryos of *Artemia franciscana*, anoxia provokes the organism to enter into a quiescent state. During experimental gradual oxygen removal, various biochemical responses are observed, such as a pH decrease, the reduction of heat production and the depression of ATP levels. Also, genetic responses, such as the down-regulation of RNA transcription, are observed during quiescence (Hand, 1998).

Often, metabolic rates have been inversely related to the life span of mammals. Moreover, when mitochondrial respiration has been inhibited by RNAi techniques, the life span extends in *C. elegans* (Lee et al., 2003), and long-lived mutants of this nematode concomitantly show decreased metabolic rates (Stuart & Brown, 2006).

The process by which mitochondrial respiration affects or extends life span has been studied in several organisms, including yeasts, worms, flies and mice (Lee et al., 2010). Electron transport in mitochondria is the main producer of superoxide anion (O⁻), which in turn generates several types of reactive oxygen species (ROS), as has been mentioned (mitochondrial Complex III). In fact, according to various studies, ROS are not only undesirable toxic metabolites promoting organism oxidative stress, but they are also molecules that participate in the mitochondria-nucleus's signalling pathways (Storz, 2006). Emerging data on *C. elegans* suggests a new described pathway where superoxide serves as an intracellular messenger, whereby with increasing superoxide concentration a signal transduction pathway is triggered, resulting in changes in the pattern of gene expression of nuclear proteins and which finally results in an increased life span (Yang & Hekimi, 2010). However, different mechanisms have also been proposed as being implicated in the aging process, such as diet restriction, ubiquinone deficiency and the hypoxic response (Klimova & Chandel, 2008).

At this point, this chapter would not be complete if the energetic costs of flying for insect species were to be omitted. This activity is probably the most expensive process recorded in nature. It is by now a well-known and remarked-upon fact that the metabolic rate during insect flight increases over 50-100 fold above the resting rate (Ellington, 1985). Thus, it is clear that the flight muscle of insects is the model tissue that many researchers have adopted in order to understand mitochondrial function since it is capable of effectively producing and hydrolysing large amounts of ATP (Sherwood et al., 2005). Insect flight is a highly oxygen-dependent process, and the flight muscle metabolism is fully aerobic; thus, it has

been suggested that the amazing aerobic capabilities of insects are based on a highly efficient mode of oxygen delivery that includes their oxygen transport system in a well distributed system of tracheae and tracheoles (Wegener, 1996).

In addition, several studies have demonstrated that the function and energy needs of certain tissues are highly correlated with the number of mitochondria per cell (Robin & Wong, 1988). This agrees with the large quantities of mitochondria with pronounced cristae and large surface areas that are found in the flight muscle cells of the honey bee *Apis mellifera* (Suarez et al., 2000). To date, it is well-known that oxygen uptake rates in mitochondria cristae are much higher in the flying muscle of *A. mellifera* than that observed in mammals' mitochondria - this can explain the higher electron transport rates observed in such enzymes as cytochrome c oxidase, whose maximum catalytic capacity was recorded in this species during flight - (Suarez et al., 2000).

Besides the increase on the ATP hydrolysis rate during flight, other mitochondrial adaptations to the highly and continuous energy requirements of flying species have been reported, such as the remarkable dependence on the synthesis of energy-rich phosphate compounds like phosphoarginine. Phosphoarginine, as mentioned above, constitutes a usable pool of high energy phosphate (Hird, 1986) so as to maintain the high rate of ATP turnover in flying insects (Wegener, 1996).

In addition to the various metamorphic changes in their life, crustaceans undergo a frequent and cyclic process: molting. During the molt cycle, crustaceans are exposed to a temporary scarcity of food since they lack the ability to handle food until their new exoskeleton is synthesised. Several adaptive strategies have been recognised as being employed by these organisms so as to avoid the adverse effects of starvation, such as the storage of fuel compounds in their midgut gland (Sanchez-Paz et al., 2007), changes in locomotor activity (Hervant & Renault 2002), and a decrease in oxygen consumption (Morris et al., 2005). However, little attention has been paid to the bioenergetic consequences of starvation in shrimp; since the composition of food plays an important role in oxidative phosphorylation, the nutritional status of shrimp species, such as *Litopenaeus vannamei*, may affect its major bioenergetic functions.

In our lab, we have hypothesised that, due of its central role in the cell energy metabolism, the expression of genes encoding the different polypeptide subunits that compose ATP synthase during unpredictable episodes of food shortage may ultimately be modulated. Thus, we experimentally evaluate the effect of starvation in the gene expression of subunits *atp α* , *atp β* and *atp γ* in the shrimp midgut gland, during a period of short-term food deprivation (5 days). Our results (Figure 2) show that the mRNA amounts from subunits *atp α* and *atp β* which directly participate during ATP synthesis decreased as starvation time increased; however, no significant changes were observed in the mRNA amounts of *atp γ* , which forms the oligomeric ring from Fo in the shrimp ATP-synthase.

Sanchez-Paz et al., (2007) reported a gradual decrease of glycogen in the midgut gland of the white shrimp as starvation progressed. After a 24 h starvation period, the glycogen content dropped by about 50%, which correlates with an increase of the *atp γ* subunit after 24 h of starvation, suggesting that glycogen may be used as fuel to generate ATP and pyruvic acid. As glycogen stores become depleted, the organism must increasingly rely on fatty acid catabolism as a source for ATP synthesis. In general, starved shrimp showed a sharp decrease in their midgut gland lipidic constituents for up to 120 h (more noticeable in acylglycerides).

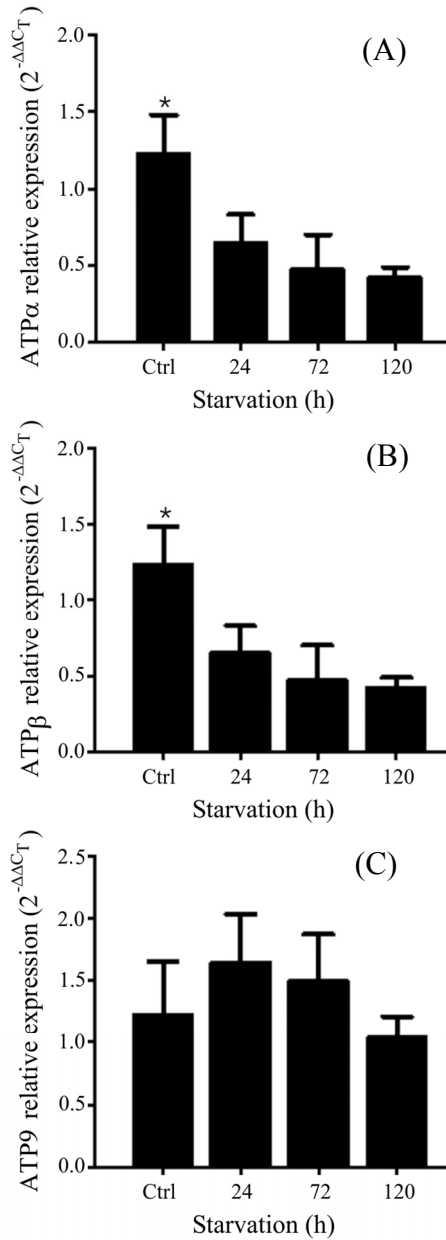


Fig. 2. The relative expression of A) ATP α , B) ATP β and C) ATP9 mRNA in the midgut gland of the white shrimp *Litopenaeus vannamei* in response to a short-term starvation period. Expression values are given based on normalisation to L8. The data is represented as the mean and standard deviation of triplicate determinations. (*) Statistical significance was considered at $P < 0.05$.

Various studies have shown that during starvation-induced lipolysis there is a decrease in the amount of ATP, which was accompanied by a fall in some subunits of the FoF₁-ATP synthase (Vendemiale et al., 2001). It is well-known that starvation tampers with cellular detoxification systems and may expose cells to oxidative injury (Di Simplicio et al., 1997; Vendemiale et al., 2001), leading to an impaired production of ATP and a reduced uptake of substrates for mitochondrial metabolism. The results from our study, together with results from previous studies, prompt us to suggest that shrimp are capable to satisfy their energy demands through a complex combination of mechanisms that enables them to survive the adverse effects of food scarcity.

Due to its density, viscosity (800 times more dense and 50 times more viscous than air) and low oxygen solubility, water - as a respiratory medium - imposes difficulties for aquatic breathers in obtaining the necessary supply of oxygen from their surrounding environment so as to keep breathing and bringing oxygen into their systems. This process becomes more complicated when considering additional parameters (such as temperature, salinity and depth) affecting the dissolved oxygen concentration of seawater, causing additional constraints on marine species' development (Sherwood et al., 2005). All the species inhabiting marine environments should face these dynamic environmental conditions, which in over the last few decades have been seriously affected by a wide variety of anthropogenic activities, such as industrial and agricultural runoffs (Wu, 2002).

Several studies have found that marine invertebrates may respond to stress conditions by changes at the transcriptional level. In crustacean species such as the crab *Eriocheir sinensis*, different gene expression profiles from gills were characterised during acclimation to high cadmium concentrations in water. Analyses have revealed over-expressed genes, such as disulphide isomerase, thioredoxin peroxidase and glutathione S-transferase. Under the same conditions, ATP synthase beta, alpha tubulin, arginine kinase, glyceraldehyde-3-phosphate dehydrogenase and malate dehydrogenase were down-regulated. The results demonstrated that acute and chronic exposure to waterborne cadmium induced a decreased abundance of the transcript-encoding enzymes involved in energy transfer; this suggests that chronic metal exposure induced an important metabolic reorganisation (Silvestre et al., 2006).

Some other species which face high cadmium concentrations are marine intertidal molluscs, such as oysters, which live in estuaries where fluctuating temperatures and levels of trace metals are known to directly affect mitochondrial function. Isolated mitochondria from the oyster *Crassostrea virginica* which were exposed to low cadmium concentrations (1 $\mu\text{mol}\cdot\text{L}^{-1}$) resulted in a progressive uncoupling that increased with the increasing dose of cadmium; this response agrees with that observed in mammals. However, unlike mammals, molluscs are ectotherms and the exposure to the combined effects of high temperatures and cadmium concentrations severely affected mitochondrial function since elevated temperatures increased the sensitivity of this organelle to cadmium and promoted an increase in the rate of ROS production (Sokolova, 2004). These results highlight the key role of temperature in the mitochondrial system of ectotherm species.

Most invertebrates are described as ectotherm species because their body temperatures vary with the environment. At very low temperatures, polar marine invertebrates were expected to show low metabolic rates, as previously observed in Antarctic fish; however, in 1999 Sommer & Portner found important intra-specific differences in the mitochondrial function of the polychaete *Arenicola marina* from the North Sea and the colder White Sea. Their results

concluded that invertebrate life is more costly at higher latitudes, where oxygen uptake, tissues mitochondrial densities and mitochondrial capacities were higher.

Remarkable abilities have been recorded in invertebrate species inhabiting extreme environments. The term “metabolic plasticity” perfectly describes such organisms as the intertidal periwinkle snail *Littorina littorea*, which has the ability to deal with very low temperatures and also to tolerate the changing environmental conditions imposed by the tidal cycle, implying continuous oxygen deprivation (Storey, 1993). Besides the biochemical and physiological mechanisms previously identified in this species, the over-expressed gene encoding a metallothionein (MT) was recently found during the exposure to low temperature and anoxic conditions of the tissues of *L. littorea*. Since thermogenesis is a process that requires high oxygen consumption and since it is also accompanied by a sharp rise in reactive oxygen species (ROS) generation, the authors describe the ability of MT to function as an antioxidant and as a reservoir of essential metals that contributes to survival under these conditions (English & Storey, 2003).

The deep sea hydrothermal vents are a different type of extreme environment where thermophilic species such as the Pompeii worm *Alvinella pompejana* inhabit. Shin et al. (2009) studied the structure and biochemical characteristics of the Cu,Zn-superoxide dismutase (SOD) of this species and found striking similarities between this enzyme and that of humans, but with an enhanced stability and catalysis – characteristics that may mean that this enzyme is potentially suitable for scientific and medical application. Other mitochondrial proteins have been proposed as a part of gene therapy for devastating human diseases by preventing the cell damage caused by oxidative stress. AOX – the mitochondrial alternative oxidase previously mentioned – is suggested to work in any cell, becoming chemically active only when it is required. AOX is provided to the cell by engineering a gene from a marine invertebrate snail *Ciona intestinalis*; this protein is under analysis as a therapeutic tool tested in mammalian disease models (Hakkaart et al., 2006).

5. How do invertebrates face hypoxia?

Hypoxia is probably one of the most studied factors affecting the central metabolic pathways of living organisms, including invertebrates. Aquatic species usually face hypoxic events in freshwater or marine environments as a daily cyclic routine in the shallow waters of lagoons, estuaries and mangroves during the dark hours, when plants and algae do not produce oxygen and organic matter is continuously oxidised (Dall et al., 1990). However, nowadays the frequency, abundance and severity of hypoxic events in coastal waters have increased due to anthropogenic activities resulting in deteriorating environments affecting marine organisms (Diaz, 2001). It is well known that hypoxia depresses the growth rate of marine animals, as it disturbs metabolic pathways and promotes the reallocation of energy resources (Wei et al., 2008; Wang et al., 2009).

Several studies have examined the physiological responses of invertebrate species to hypoxia, such as growth, stress resistance and even behaviour patterns in aquatic species able to vertically and horizontally migrate through the water column to reach more oxygenated zones (Eads & Hand, 2003; Burgents et al., 2005; Abe et al., 2007; Seibel, 2011). In fact, among invertebrates there are hypoxia-tolerant species, such as bivalve molluscs and annelids, with highly adapted structures and mechanisms to deal with hypoxia, and some others, such as crustaceans, whose tolerance to hypoxia depends on their habitat, food, and energy needs. Unfortunately, the responses to hypoxic conditions – at the molecular and

biochemical levels - of the mitochondrial proteins and enzymes that participate in the respiration process are still poorly studied for most invertebrate species.

The main physiological responses from invertebrates to hypoxia are somewhat similar to those from vertebrates since in the reduction or absence of oxygen, animal cells are not able to produce enough energy to survive. Such general responses are clearly a legacy of the evolutionary past from ancestral forms and they serve adaptive ends. In marine species, such as crustaceans and molluscs, reduced oxygen consumption and metabolic rates have been confirmed during hypoxia; in addition, glucose utilisation and lactate accumulation as indicators of a switch to anaerobic metabolism have been detected at low oxygen concentrations in water (Racotta et al., 2002; Martinez-Cruz, 2007; Soldatov et al., 2010). In the brine shrimp *A. Franciscana*, the intracellular pH falls at anoxia, heat production is reduced and ATP concentrations are also depressed to low levels (Hand, 1998; Eads & Hand, 2003).

A large amount of information is now available about the changes at the transcriptional level promoted by hypoxia in invertebrates, most of it concerning aquatic species. In our lab, we have evaluated the effects of hypoxia in the gene expression of F₀F₁ ATP synthase subunits, such as *atp9*, *atp6*, *atpα*, *atpβ*, *atpγ*, *atpδ*, and *atpε*, in different tissues of the white shrimp *L. vannamei*. Results show a general trend towards increase the amount of mRNA as oxygen concentrations decrease (Martinez-Cruz, 2007; Martinez-Cruz et al., 2011; Martinez-Cruz et al. in preparation). Also, significant changes in the amount of mRNA from the mitochondrial- and nuclear- encoded subunits of the ATP synthase were detected at different molt stages and tissues, according to the energy requirements of each stage and the specific requirements of the function of each tissue (Muhlia-Almazan et al., 2008). Chronic exposure to severe hypoxia (1.5 mg/mL during 7 days) also causes the increased transcription of mitochondrial-encoded genes, such as the 16S, CO1, and CO2 subunits from the cytochrome C oxidase in the grass shrimp *Palaemonetes pugio* (Brouwer et al., 2008). To date, microarray technologies have revealed a set of genes that are up- and down-regulated in *P. pugio* during chronic, acute and moderate hypoxia; the results revealed that various genes encoding mitochondrial proteins were affected (Li & Brouwer, 2009).

In the absence of oxygen, animal cells activate transcription factors - such as the well-studied vertebrates hypoxia-inducible factor (HIF) - which has been reported in invertebrates from worms to flies (Semenza, 2007). When activated, HIF leads the organism to exhibit metabolic adaptation to hypoxia by regulating the genetic expression of some proteins and enzymes involved in central biological processes such as glycolysis, erythropoiesis, breathing and angiogenesis so as to maintain cell homeostasis (Klimova & Chandel, 2008). In the shrimp *P. pugio*, a homolog protein to HIF- α called gsHIF was found in this hypoxia-tolerant species. It includes all the conserved domains of vertebrates' HIF proteins, and an additional polypeptide sequence of 130 residues that has not been found in databases, and its participation in the functional properties of the protein has not yet been determined (Li & Brouwer, 2009). In the white shrimp *L. vannamei*, HIF-1 is a heterodimer formed by two subunits: HIF-1 β , which is constitutively expressed in shrimp cells and HIF-1 α , which is differentially expressed in hypoxic conditions. HIF-1 is suggested in crustaceans to be the master regulator that senses decreased oxygen availability and transmits signals promoting the physiological responses mentioned above (Soñanez-Organis et al., 2009). Additional functions have been attributed to HIF in coral species, such as *Acropora millepora*, where the diel cycle in the central metabolism appear to be governed by the circadian clock and regulated by the HIF system operating in parallel (Levy et al., 2011).

As a part of the HIF-regulated metabolic responses to hypoxia in invertebrates, the activities of specific enzymes – most of them part of the central metabolism – are known to increase. In bivalves such as *Anadara inaequivalvis*, the increased activities of enzymes – such as malate and lactate dehydrogenases – were detected at hypoxia (Soldatov et al., 2010). Also, increases in the catalase and GST activities during anoxia in the estuarine crab *Chasmagnathus granulata* have been observed. It has been suggested that such responses may be a strategy to prepare the organisms for oxidative stress in an effort to protect tissues against oxidative damage during re-oxygenation. An important decrease in SOD activity (which occurred after aerobic recuperation) was also detected; and it could have been caused by the accumulation of hydrogen peroxide production during re-oxygenation (de Oliveira et al., 2005).

At normoxia, the small levels of ROS produced by the metabolism in normal animal mitochondria come from carrying electrons along the mitochondrial complexes I, II, and III (Turrens, 2003). However, when oxygen levels are reduced, the presence of the final electron acceptor in the mitochondrial respiratory chain fails, producing a reduction in the rate of electron transport and a decrease in oxygen consumption. Under these conditions, the membrane potential increases as does ROS production (Guerrero-Castillo et al., 2011).

It has been reported that in invertebrate species considered to be hypoxia-tolerant, the absolute rate of H₂O₂ production is at least an order of magnitude less per mg of mitochondrial protein than that measured on mammalian species (Abele & Puntarulo, 2004). However, some other species which are not tolerant to hypoxia tend to produce higher levels of ROS at low oxygen levels; thus, it is suggested that they display alternate pathways in order to maintain the mitochondrial respiratory rate and avoid an over-production of ROS (Guerrero-Castillo et al., 2011).

Nowadays, the alternative mechanism of proton sinks has been evidenced in invertebrates since uncoupling proteins (UCPs) have been identified in these species (Abele et al., 2007). Such proteins have been involved in various functions, including thermoregulation, body composition, antioxidant defence and apoptosis. UCPs are thought to dissipate the proton gradient across the inner mitochondrial membrane and may help in controlling ROS production (Yu et al., 2000).

In *Drosophila*, an UCP5 protein over-expressed in a heterologous system has shown to have similar functional abilities to an uncoupling protein (Fridell et al., 2004), while in the marine eastern oyster, *Crassostrea virginica*, UCP5 is represented by two transcript forms: UCP5S (small) and UCP5L (large). However, their function has not been determined since its gene expression is not affected by hypoxia, cadmium exposure or different temperatures (Kern et al., 2009). In addition, a novel protein (UCP6) in invertebrates is considered to be an ancestral form of the vertebrates UCP1, UCP2, and UCP3 (Sokolova & Sokolov, 2005).

In mammals, it is known that less-severe hypoxia induces protective mechanisms. This phenomenon – called hypoxic preconditioning (HP) – appears in two forms: immediate preconditioning (which occurs only a few minutes after a sub-lethal hypoxic episode and declines after 4 h) and delayed preconditioning (which requires gene expression changes and takes place 12 to 24 h later and can last for days) (Dirnagl et al., 2009). In the nematode *C. elegans*, the delayed form of HP has been found to induce unfolded protein response pathways – at this point, misfolded proteins serve as early hypoxic sensors that trigger signalling pathways to induce a hypoxia protective response (Mao & Crowder, 2010).

6. The role of mitochondria in invertebrate programmed cell death (Apoptosis)

Besides the various functions just described, mitochondria also acts as the arsenal of the cell. Numerous and complex processes, still poorly understood, can trigger the release of mitochondrial components into the cytoplasm and subsequently induce cellular apoptosis of the organelle (Hengarter, 2000). It is not our intent here to provide exhaustive coverage of all the issues relating to apoptosis in great detail, but rather to give the reader a basic description of the process – to highlight its importance and to show the challenges that those interested in this topic will face.

As has been mentioned, studies in invertebrate biology are paramount to an understanding of biodiversity and to the search for potential uses for their metabolic capabilities and products for biotechnologies. Besides, comparative sciences may facilitate the use of invertebrate models in understanding the biology and pathology of farmed animals and humans. This is due – in spite of differences in the biochemical, physiological, and cellular characteristics that make invertebrates and vertebrates so obviously different – to the fact that most parts of such grades of their biology have remained similar in both groups through their evolution. For example, invertebrate cells – whether wounded by harsh environments or by the expression of abnormal proteins – die as do vertebrate cells, indicating that the powerful advantages of invertebrate molecular genetics might be successfully used for testing specific hypotheses about human diseases, for the discovery of drugs and for non-biased screens for suppressors and enhancers of maladies (Driscoll & Gerstbrein, 2003). The same criteria apply for all cellular functioning, as for apoptosis.

Apoptosis (from the Greek: “falling off”) – or programmed and regulated cell death and elimination – is a pivotal process in embryogenesis, the orderly elimination of wounded or infected cells, and the maintenance of tissue homeostasis. The process is so important that it is estimated that on a daily basis the human body must get rid of approximately 10^{10} cells. Through apoptosis, cells die quietly in a controlled, regulated fashion; while in another forms of cell death – such as in necrosis – a series of uncontrolled events occur leading to serious and irreversible damage. Given the proper conditions, apoptosis destroys the cell swiftly and neatly. In contrast, necrosis causes the rupture of the cell, releasing its content into the surrounding tissue. Tampering with apoptosis may result in devastating health problems, such as cancers, immune diseases, neurodegenerative disorders and the proliferation of viruses. Apoptosis is executed by a variety of membrane, organelle, cytoplasmic and nucleus signalling, and initiator and effector molecules, including a subfamily of cysteine proteases known as caspases (Jiang & Wang, 2004).

In mammals, the active role of mitochondria in apoptosis induction has been well-established. In invertebrate models of apoptosis, such as the fly *Drosophila melanogaster* and the worm *C. elegans*, the role that mitochondria play during apoptosis and, in particular, during apoptosis initiation is less clear (Rolland & Conradt, 2006). While key regulators of apoptosis in *Drosophila* and *C. elegans* have been found in association with mitochondria, the significance of these associations has not been rigorously tested.

The regulated destruction of a cell is a basic process in Metazoa, as multicellular animals are obligated to remove damaged or harmful cells. During apoptosis, cells die in an orderly, regulated sequence of molecular, biochemical, and cellular processes. According to the endosymbiotic theory, the origin of apoptosis is currently regarded as the result of molecular interactions in which some components of a signal transduction pathway affects

other pathways through interaction of some initiator and effector proteins. Accordingly, apoptosis could have arisen simultaneously with – and as a by-product of – endosymbiosis (Kroemer, 1997). However, it has also been proposed that apoptosis may be the result of the acquisition of the aerobic metabolism by early eukaryotes (Frade & Michaelidis, 1997).

Apoptosis is a unique phenomenon of tissue kinetics as it can be said that life is critically controlled by the operational centre of cell, the nucleus. Instead, death is a process controlled by the power house of the cell, the mitochondria. Thus, even cells lacking nucleus commit apoptosis. In general, the two-step membrane depolarisation and free radical release taking place in the mitochondria may trigger apoptosis. This in fact is not so peculiar if we understand that mitochondria were once free-living bacteria which did not need an external gene control for achieving their functions. Once each came into symbiosis forming a eukaryotic cell, it retained some capacity to operate partially independently.

There are several major apoptotic pathways, but the most well-known and studied are the extrinsic and the intrinsic pathways, which respond to different environmental and cellular challenges in vertebrates. The intrinsic pathway is also called the mitochondrial pathway because of the involvement of mitochondria. There are mitochondrial proteins that induce this process (proapoptotic) and others that limit cell death (antiapoptotic). Both proteins interact so as to cooperate and govern the cell's fate. Also, the origin of the activation signals of apoptosis taking place on the mitochondria is a clue molecule, cytochrome C (Cyt C), which is released from the mitochondria to form the apoptosome complex. The intrinsic pathway – with some differences – is a mostly conserved pathway among metazoans (for a comprehensive review look at Wang & Youle, 2009). Cyt C is a key component of the apoptosome complex for activating the initiator caspase-9 after its release from mitochondria. Under non-apoptotic conditions, Cyt C is kept inside the respiratory chain. Against some cellular challenges, like the alteration of the DNA in the mitochondria or the nucleus, Cyt C is released from its membrane, crossing the external membrane and initiating the formation of the apoptosome complex. In essence, mitochondrial proteins – like Cyt C and caspases – are not hired guns and during non-apoptotic conditions they are responsible for various basic mitochondrial roles for normal cell functioning. The compartmentalisation of such mitochondrial proteins isolates them from interacting with partners or targets, a mechanism to prevent the unwanted activation of apoptosis in normal cells. Only after their appropriate release into the cytoplasm do such proteins play the role of triggers to initiate the cell's suicide.

The classical invertebrate model organisms for the study of apoptosis are *C. elegans* and *Drosophila*. In spite of the fact that the regulators of apoptosis have been found in such model organisms, the involvement of mitochondria in apoptosis is not conclusive. So far, no irrefutable evidence of the release of Cyt C from the intermembrane space has been found. Also, the involvement of Cyt C in the apoptosome formation in *Drosophila* is controversial, and some evidence suggests that Cyt C is not necessary (Rolland & Conradt, 2006).

The current evidence indicates that the whole process of apoptosis -including the involved proteins and the regulation mechanisms- in crustaceans is far more diverse than has been assumed from the studies with model organisms. Recent studies have shown that several proteins in the apoptotic network are quite conserved between mammals and arthropods; however, it is clear that the integration of such homologous proteins in the physiology and pathophysiology of crustaceans needs further experimental assessment. Some unresolved questions regarding this topic are: how does the regulation of the process occur? Is

crustacean apoptosis transcriptionally regulated, as in *Drosophila* (RHG 'killer' proteins)? Or is it controlled by pro- and anti-apoptotic Bcl-2 family proteins, as in vertebrates? The issues that should be investigated in the short-term are whether the calcium-induced opening of the mitochondrion permeability transition pore (MPTP), commonly found on vertebrate species, also occurs in crustaceans. Furthermore, the study of the differences in the regulation of the intrinsic pathway of crustacean apoptosis will lead to an understanding of their adaptation to challenging environments; this is because marine organisms have to deal with seasonal as well as circadian changes in environmental variables. Some examples are UV radiation, temperature and dissolved oxygen, and even some biological stresses such as toxins that may vary over time. But this is not all: other variables that may inhibit apoptosis must be considered. "Characterisation of the players, pathways, and their significance in the core machinery of crustacean apoptosis is revealing new insights for the field of cell death" (Menze et al., 2010).

Apoptosis is a key host response to viral infection. Viruses that can modulate a host's apoptotic responses are likely to gain important opportunities for transmission. Here, we review recent studies that demonstrate that the particles of Invertebrate Iridescent Virus6 (IIV-6) (Iridoviridae, genus Iridovirus), or an IIV-6 virion protein extract, are capable of inducing apoptosis in lepidopteran and coleopteran cells, at concentrations 1000-fold lower than that required to shut-off the host's macromolecular synthesis (Williams et al., 2009). Throughout the process of pathogen-host coevolution, viruses have developed a battery of distinct strategies to overcome the biochemical and immunological defences of the host. Thus, viruses have acquired the capacity to subvert host cell apoptosis, control inflammatory responses, and evade immune reactions. Since the elimination of infected cells via programmed cell death is one of the oldest defence mechanisms against infection, disabling host cell apoptosis might represent an almost obligatory step in the viral life cycle. Conversely, viruses may take advantage of stimulating apoptosis, either to kill uninfected cells from the immune system or else to induce the breakdown of infected cells, thereby favouring viral dissemination (Galluzzi et al., 2008).

7. Conclusion and future perspectives

As stated by Van der Giezen in 2009 "over the last 5-10 years, it has become apparent that the organelle known as the mitochondrion is a much more fluid entity than generally believed," so "why should mitochondrion be the same in all eukaryotes while other cellular structures show such great evolutionary malleability?"

It is our belief that since natural selection has given invertebrates the opportunity to evolve in quick steps, a large window is opening in the field of mitochondrial research among these species, giving an outstanding opportunity to researchers to contribute to an increase in knowledge, not only because there is scarce information, but also because many species have shown special and unique characteristics that need to be explained.

At this point, the information reviewed clearly shows that invertebrates display remarkable physiological capabilities, including highly specialised mechanisms for adjusting mitochondrial functions to solve their energetic demands under the stressful conditions they usually face. These species also include within their systems ancient and novel molecules and structures acting to reach an adaptive state, from the increasing number of mitochondria per cell to the highly complex function of the HIF system.

It is also remarkable that the number of invertebrate species considered as potential models in the study of mitochondrial function has increased. New data on marine invertebrates, such as molluscs and crustaceans and non-*Drosophila* species, are emerging. Since there is still an immense lack of knowledge about invertebrates, important efforts in new animal models should focus on i) the description of mitochondrial systems in species inhabiting extreme environments, ii) the recognition and understanding of the causes and effects of mitochondrial disorders, and iii) the development of unsolved phylogenetic relationships among species and phyla. This may also open important opportunities for new biotechnological applications to better face the effects of global changes such as warming, hypoxic conditions and chronic stressors that specifically affect the central metabolic pathways in such species.

If the regulation of apoptosis in crustaceans is as varied as their diversity as a species, or at least their Families, then the potential for discovering novel biomolecules is immense. Such molecules may find uses in biotechnologies across diverse industries, including pharmacology. We endorse the hypothesis that an advanced knowledge in apoptosis will provide some clues about how crustaceans deal with viral infections and enable the proposal of feasible strategies to protect farmed crustaceans.

8. References

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