Cytochrome c oxidase in Insects

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Flight dispersal represents a key aspect of the evolutionary and ecological success of insects, allowing escape from predators, mating, and colonization of new niches. The huge energy demand posed by flight activity is essentially met by oxidative phosphorylation (OXPHOS) in flight muscle mitochondria. In insects, mitochondrial ATP supply and oxidant production are regulated by several factors, including the energy demand exerted by changes in adenylate balance. Indeed, adenylate directly regulates OXPHOS by targeting both chemiosmotic ATP production and the activities of specific mitochondrial enzymes. In several organisms, cytochrome c oxidase (COX) is regulated at transcriptional, post-translational, and allosteric levels, impacting mitochondrial energy metabolism, and redox balance.

Keywords: metabolism ; bioenergetics ; respiration ; oxidative phosphorylation ; redox ; dispersal ; migration ; homeostasis ; kinetics ; allostery ; phosphomimetic

1. Introduction

1.1. Insect Flight Is Powered by Mitochondrial Metabolism

Although insects are the most diverse taxonomic group containing the largest number of species, studies dedicated to the understanding of insect's energy metabolism remain largely unexplored (≈1.8% of all original papers on mitochondria found in PubMed by November 2020). Insects possess several unique metabolic features that go beyond using them (mostly *Drosophila*) as a model organism to understand human diseases ^[1]. For example, mitochondrial transport and oxidation of dicarboxylates, such as succinate, are quite limited in insects ^{[2][3][4]}. Additionally, succinate oxidation in adult insects is loosely coupled to the generation of $\Delta \Psi_m$, resulting in low efficiency of mitochondrial ATP synthesis [5][6][2]. On the other hand, succinate oxidation is high in the midgut of tobacco hornworm larvae but severely reduced upon the commitment to the metamorphosis to pupae $[\underline{B}]$. In addition, the metabolism of mitochondrial Ca²⁺ in insects strikingly differs from mammals as very low Ca²⁺ uptake and stimulation of respiratory rates are observed ^{[6][9][10]}. The study of insect metabolism was critical in identifying and defining the biological significance of the glycerol phosphate shuttle [11][12] [13][14][15][16]. In this regard, insect mitochondria have a remarkably high capacity to oxidize G3P, which is only matched by mammalian brown adipose tissue [16][17]. Comparative analyses among distinct insect species revealed that G3P dehydrogenase (G3PDH) activity and G3P production are much higher than lactate dehydrogenase (LDH) activity and lactate levels during flight [13][14]. This strongly suggests that G3PDH activity plays a key role in re-oxidizing cytosolic NADH in insect flight muscles, which seems to allow intense contraction without acidification due to high lactate accumulation.

A key aspect is the substrate preference that insects use to support flight, which depends on several factors, including the type and duration of the flight activity, aging, and dietary strategies [18][19][20][21][22][23]. Regardless of the flight regimen, glucose, trehalose, diacylglycerol, and proline are the main substrates consumed to support insect's flight [18][24][25][26][27] [28][29][30][31][32][33][34][35]. While migratory insect species usually oxidize fatty acids in their long-range flights [35][36], hovering insects preferentially use proline and carbohydrates as main fuels [27][29]. A particular case is proline, which is used by different insect species not only as a fuel to support flight but also as a carbon source to stimulate pyruvate oxidation [27][28][29][30][31][32][33][34]. Indeed, proline acts as the main substrate to support flight activity in several insect species, including bumblebees and obligatory blood-feeding insect vectors of neglected tropical diseases [27][28][37]. Interestingly, the high rates of proline oxidation observed in blood-sucking insects seem to represent an important adaptation to blood-feeding habit associated with a protein-rich diet [27].

Insect flight muscles are remarkably unique in several aspects, from the morphological, physiological, and biochemical perspectives. For example, in *Drosophila* the structure of the tropomyosin binding protein troponin T (TnT) has a unique C-terminal extension of \approx 70 residues rich in glutamic acid and is essentially expressed in flight and leg muscles ^[38]. Importantly, TnT is essential for *Drosophila* motor activity, as climbing and flight abilities were strongly affected by genetic removal of TnT, possibly by acting as a mechanism for Ca²⁺ buffering ^[38]. From the morphological perspective, insect

flight muscles have remarkable architectural features suggesting that proximity of energy supplying (mitochondria) and demanding (myofibrils) sites would facilitate the flow of substrates to sustain such intense mechanical work during the flight. Structural hallmarks can also be extended at the organelle level, including the high mitochondrial abundance and density, which occupies $\approx 30-40\%$ of cell volume $\frac{[39][21][23][40]}{1}$. The densely packed cristae, resulting from the striking degree of inner membrane juxtaposition, also emerges as a remarkable feature among insect flight muscles $\frac{[21][41][40]}{1}$.

Hummingbird flight muscles have higher mitochondrial cristae densities as compared to insect flight muscle but have lower mitochondrial volume densities ^[40]. The metabolic consequence of such high tissue mitochondrial and cristae density is the extraordinary capacity of insect flight muscle to consume oxygen ^[22]. For example, in honeybees, respiratory rates relative to the mitochondrial volume are 2–3 times higher than observed in hummingbirds and 4–6 times higher than in mammals ^[40]. Thus, the enormous respiratory capacity of insect flight muscle is a consequence of their remarkable ability to produce ATP by means of OXPHOS.

1.2. Regulation of Mitochondrial Energy and Redox Metabolism in Flying Insects

To sustain the remarkable ATP turnover production rates, flying insects evolved two unique metabolic features: (i) many enzymes in flight muscle operate close to or at their V_{max} during maximal activity [42], and (*ii*) the cellular contents of some enzymes [E] are in larger excess compared to organisms with lower metabolic rates $\frac{[43]}{2}$. Indeed, reductions of \approx 50% in the activity of several metabolic enzymes caused no apparent effects on Drosophila wing beat frequencies, supporting the concept of a large excess of enzyme content in insect flight muscles [43][44]. However, the fine-tuning of respiratory rates is critical for the regulation of mitochondrial oxidant production and flight dispersal in insects [45][21][22][46]. For example, in the hawkmoth Manduca sexta, flight ability and oxidative damage increase in flight muscle proteins upon sugar-feeding [46]. The females of the major arbovirus vector Aedes aegypti represent an extreme example of the metabolic and physiological adaptations to the diet in a flying insect. Adult A. aegypti females usually acquire their nutrients from plant sap, which is rich in sugar and some amino acids, including proline $\frac{[47]}{2}$. However, to trigger the gonotrophic cycle and egg production, females need to feed on vertebrate blood (that is why A. aegypti is classified as facultative hematophagous insects). Vertebrate blood is rich in proteins and lipids but poor in sugars and strikingly differs from the usual mosquito diet on plant sap. Therefore, this exquisite food digestion and metabolism represent a substantial biochemical challenge for hematophagous organisms [48]. To overcome this, the metabolism of specific amino acids (including proline and tyrosine) represents an important adaptation for the blood-feeding habit [27](37)[49]. Mosquitoes have a high engorgement capacity, taking up close to three times their own weight on vertebrate blood per meal. It is thus remarkable their stupendous ability to fly even with such a high meal payload in their guts (imagine drinking a "smoothie" composed of \approx 7 L water, \approx 200 kg of meat, plus two tablespoons of sugar just before running a marathon!). The metabolic consequences of such a high protein, fat, and iron diet include the reduction of respiratory rates and mitochondrial oxidant production [21] and their flight activity [50]. The mitochondrial functional changes promoted by blood meal were a consequence of modulation of specific mechanisms, including the induction of mitochondrial fusion and specific reduction of COX activity ^[21]. These effects are fully reverted three days after a blood meal, indicating that blood-derived products act as signals to trigger mitochondrial functional and structural remodeling.

Energy metabolism is regulated by distinct factors and usually at multiple steps. In this regard, respiratory rates and mitochondrial ATP synthesis are classically regulated by three factors: (i) substrate supply, (ii) energy "wasting" by proton leak, and (iii) energy demand [51]. A classical model of metabolic regulation establishes that products of ATP hydrolysis, resulting from increased energy demand, bind to regulatory enzymes and activate catabolic pathways (the so-called "adenylate model of metabolic regulation"). Conversely, the flux of anabolic pathways is reduced by the same principle but on different enzyme targets. When energy demand ceases, decreased ATP utilization results in repression of catabolic pathways by ATP while activating anabolic pathways. The consequence would be a remarkable stability of cellular ATP levels (or energy homeostasis) even during massive energy demands such as during flight [52][53]. Mechanistically, two possibilities would explain how energy demand regulates respiratory rates and OXPHOS. The first one states that under high energy demand, ADP activates the F_1F_0 ATP synthase complex to produce ATP by using the energy from $\Delta\Psi_m$ through a chemiosmotic mechanism [54]. As the $\Delta \Psi_m$ magnitude directly affects the respiratory rates [54][55][56], the mechanism provided by the ATP/ADP ratio regulates respiratory rates in a $\Delta \Psi_m$ -dependent fashion $\frac{[54][55][56]}{1}$. The second possibility involves the allosteric regulation of several mitochondrial enzymes (including dehydrogenases and COX) by the ATP/ADP ratio independently of the $\Delta \Psi_m$ [32][57][58][59][60][61]. Interestingly, both possibilities were experimentally demonstrated in flying insects, providing supporting evidence for the regulation of respiratory rates and OXPHOS in this particular group of organisms [32][57][61].

Despite its simplicity, the "adenylate model of metabolic regulation" does not seem to be the major driver for the maintenance of ATP turnover rates ^{[43][62][63]}. For example, it is long known that during insect flight, respiratory rates rise several hundred-fold but, paradoxically, adenylate levels hardly change ^{[52][53]}. Additionally, the desert locusts

Schistocerca gregaria under anoxia show increased AMP, ADP, and Pi, but low ATP levels. This sharply contrasts with the postulate of the "adenylate model of metabolic regulation" since ATP turnover rates were not activated as it would be expected ^[62]. However, how would insect flight muscles increase respiratory rates without apparent changes in adenylate levels? A nice explanation is supported by experiments using *Drosophila* mutants to specific energy metabolism enzymes while assessing wing beat frequency as a proxy of flight capacity ^[43]. Reductions of up to ≈90% of most metabolic enzyme activities caused no apparent changes in wingbeat frequencies, strongly suggesting that insect flight muscle has an excess capacity of metabolic enzymes to sustain flight ^[43]. This observation fits with previous proposals suggesting that enzyme concentration [*E*] would be the strongest regulator of ATP turnover rates as seen in flying insects ^{[42][63][64]}. Indeed, hexokinase, phosphofructokinase, citrate synthase, and COX activities in honeybee flight muscle operate closer to V_{max} than in mammals ^[42]. Given that [*E*] is a key determinant for V_{max} and that activities of metabolic enzymes in insect flight muscle are much higher than in mammals ^[64], the remarkably high metabolic fluxes of insects during flight may be accomplished by having high [*E*] working closer to V_{max} than mammalian enzymes. Although metabolic fluxes seem to be mostly controlled by [*E*], this by no means excludes the regulatory roles of adenylates in fine-tuning other critical metabolic processes (such as the redox balance).

2. Cytochrome c Oxidase in Flying Insects

2.1. A Brief Historical Background

Our understanding of COX is historically linked to insects since the seminal works of Charles MacMunn and David Keilin, who firstly identified histo/myohematins and later cytochromes using insects as models of study ^{[65][66]}. Although MacMunn has found "cytochromes" in testes and gut of different insect species, Keilin observed that honeybees' thoracic muscles were "the best material for the study of the absorption spectrum of cytochrome" ^{[65][66]}. Keilin also noted that "among all organisms examined, the highest concentration of cytochrome is found in the thoracic muscles of flying insects", indicating the feasibility of insect flight muscle for cytochrome studies ^[66]. This postulate was later substantiated in fruit flies and blowflies when the so-called "sarcosomes" were finally defined as mitochondria, given their strong similarities in structural and biochemical properties ^[67]. Indeed, the high cytochromes content in insect flight muscle indicates their role in biological oxidations and energy transduction and correlates with the enormous energy demand that flight activity poses to this unique tissue ^{[67][68]}. Key observations also revealed the association of cytochromes during metamorphosis and development, as their levels sharply and specifically rise in flight muscle upon pupal–adult transition ^{[66][69][70]}. By using the common wax moth *Galleria mellonella* attached to glass slides, Keilin also established cytochromes as entities involved in redox reactions of respiration and metabolism ^[66]. Over the years, the function of cytochromes and COX to insects revealed its multiple facets to physiology and biology ^{[4][8][70][71][72]}.

2.2. Biological and Physiological Roles of COX to Flying Insects

Changes in several biological and physiological parameters, including shifts in dietary preferences, development of insecticide resistance, and aging were associated with altered COX function in flying insects [21][4][8][68][69][70][71][72][73][74][75] [76][77][78][79][80][81][82][83][84][85]. For example, in A. aegypti mosquitoes, a change from sucrose to blood diet transiently reduced flight muscle cytochromes $a + a_3$ content and COX activity ^[21]. These events are parallel to reductions in respiratory rates and hydrogen peroxide production, which are all reverted right upon blood being fully digested. Conceivably, reductions in flight muscle mitochondrial metabolism triggered by blood meal would have two physiological consequences for mosquitoes: (i) to spare nutrients from flight muscle to ovaries as a mechanism to support oogenesis, and (ii) to avoid the potential oxidative burst generated by the interaction of blood-derived products such as heme and iron with hydrogen peroxide produced by mitochondrial metabolism [75][76]. The first possibility fits within the broad concept of "flight-oogenesis syndrome", a physiological process by which some migrating insects alternate two energy-competing states: migration and reproduction [72], which is not the case for the mosquitoes. Alternatively, reductions in mitochondrial metabolism and oxidant production would represent a unique preventive antioxidant defense for mosquitoes (and other hematophagous organisms [21][75][76]) to avoid the potential toxicity of their unusual diet. In wood-fed Anoplophora glabripennis beetles, the expression of genes involved in protein metabolism and 13 subunits of COX was significantly higher than in artificial diet-fed insects [73]. This suggests that wood-feeding requires an increase of respiratory capacity in beetles, possibly to meet the energy demand posed by faster protein turnover. Associations between COX activity and insecticide resistance were also observed in flying insects. Strains of the housefly Musca domestica resistant to dichlorodiphenyltrichloroethane (DDT) exhibited higher COX activity compared to sensitive ones in a sex-independent manner ^[Z0]. In line with these observations, Sitophilus oryzae rice weevil and Blattella germanica cockroach strains resistant to insecticides have higher COX activity than nonexposed or susceptible individuals [78][79]. Regarding aging, specific reductions in COX function were observed in several flying insects along with their lifespan [4][80][81][82][83][84]. For example, selective downregulation of nuclear and mitochondrial COX subunits expression is associated with specific reductions in COX activity during *Drosophila* aging [4][82][83][84]. Interestingly, mitochondrial oxidant production is promoted

by inhibition of COX activity by using specific drugs in *Drosophila* and *Musca* ^{[4][81]}. On the other hand, *Drosophila* COX activity is reduced by redox imbalance, a pattern that is linked to specific degeneration of mitochondrial cristae ^{[84][85]}. Concerning the development, flight muscle mitochondria undergo important morphological changes, including the development of densely packed lamellar cristae as well as an increase in COX activity upon *Drosophila* emergence to adult stages ^[86]. Interestingly, COX activity and COXIV expression were essentially found at lamellar cristae, strengthening the concept that cristae are the true bioenergetic units responsible for mitochondrial energy transduction ^[87].

2.3. Regulation of COX Activity in Flying Insects

Evidence suggests that flight metabolism and dispersal potential are tightly linked to COX function. For example, longdistance migratory butterfly species have higher COX content and activity than short-distance fliers ^[88]. In this regard, the migratory butterfly *Vanessa atalanta* flight muscle mitochondrial area and cristae density were higher compared to the short-range butterfly *Melitaea cinxia* ^[88]. Remarkably, the relationship between dispersal potential and COX activity can also be observed within the same flying insect species. Recently established populations of *M. cinxia* butterflies have higher dispersal potential than old ones, a phenotype that is mirrored in COX activity ^[88]. This strongly indicates that COX represents a key metabolic mechanism for dispersal potential in flying insects.

Regarding the factors that regulate COX activity in flying insects, scarce information is available. Some of the known regulators of COX include oxygen availability, hormonal signaling, redox homeostasis, and adenylate balance $^{[61][89][90][91]}$ [$^{[92][93]}$]. For example, Tibetan highlander populations of migratory locusts, which are naturally exposed to low apO₂, preserve mitochondrial integrity, $\Delta\Psi_m$, COX activity, and turnover upon hypoxia exposure $^{[89]}$. On the other hand, lowlander populations exhibit considerable changes in mitochondrial functionality upon hypoxia $^{[89]}$. Remarkably, highlander populations also exhibited altered kinetics of COX, including higher affinity and V_{max} than lowlanders. This indicates that adaptations of flying insects to low apO₂ involve the regulation of COX by increasing its catalytic efficiency rather than its content $^{[89]}$. Metalation of COX cu_A sites is mediated by "syntheses of cytochrome *c* oxidase" (SCO) protein, which is absolutely required for COX assembly and function. In the eastern honeybee *Apis cerana*, COX activity requires SCO, whose expression is induced by different stress signals, including cold, transition metals, ultraviolet, and oxidant exposures ^[91].

Since COX activity is regulated by the adenylate balance in vertebrates ^{[59][60]}, an emerging question would be whether the flux of catabolic pathways can be controlled by allosteric regulation of COX activity in flying insects? Indeed, a direct demonstration of allosteric regulation of COX by adenylates in invertebrates was missing. Recently, our laboratory described for the first time that in insects, COX is allosterically regulated by adenylate balance, with direct consequences on mitochondrial metabolism ^[51]. We observed that ADP activates and ATP strongly inhibits respiratory rates sustained by G3P oxidation in *A. aegypti* flight muscle ^[61]. These effects were independent of substrate transport to mitochondria, TCA cycle enzyme activities, and $\Delta\Psi_m$. In addition, we observed that regulatory effects of adenylates were specific to COX activity and not to other ETS components. Indeed, ATP not only exerted powerful inhibitory effects on *A. aegypti* COX, reducing its activity by \approx 75% but also shifted the enzyme kinetics from a typical Michaelian hyperbolic-shaped curve to a sigmoidal one. This suggests that at least in *A. aegypti* mosquitoes, ATP promotes conformational changes in COX structure, possibly favoring the interaction of two complex monomers and resulting in positive cooperativity between the cyt *c* binding sites. Finally, the regulatory sites of adenylates on respiration seem to involve targets facing both the intermembrane space and matrix, as observed in vertebrates ^{[59][61][92][93]}. To our knowledge, this was the first evidence demonstrating allosteric regulation of COX by adenylates in invertebrates, underscoring this mechanism as a much broader and evolutionary conserved process of energy metabolism regulation than previously thought.

3. Phosphorylation as a Critical Mechanism of Post-Translational Modification and Regulation of COX

COX is a multimeric protein complex composed of three subunits encoded by the mitochondrial DNA (representing the catalytic enzyme core) and 11 subunits encoded by the nuclear genome in eukaryotes $^{[94][95]}$. Given that COX represents a key site of OXPHOS regulation $^{[96]}$, it is not surprising that in vertebrates it is under the control of several regulatory mechanisms at transcriptional, post-translational, and allosteric levels $^{[59][60][61][92][93][97][98][99][100][101][102][103][104][105][106]}$

Phosphorylation of specific Ser/Thr and Tyr residues in different COX subunits represents the best known posttranslational modifications of this enzyme complex. Notably, several lines of evidence support the notion that phosphorylation of specific subunits directly modulates COX function in vertebrates, essentially by sensitizing this enzyme complex to the allosteric effect of adenylates ^{[104][105][106][107][108][109][110][111][112][113][114][115][116]}. The first observation of COX phosphorylation identified a 17k Da protein as the major COX phosphoprotein in rat heart and liver mitochondria ^[104]. Subsequently, this 17k Da phosphoprotein was identified as the subunit IV of COX (COXIV) ^[104]. Mechanistically, cAMP-dependent protein kinase (PKA) phosphorylation of specific residues in different COX subunits renders the enzyme complex more prone to allosteric ATP inhibition, which is antagonized by dephosphorylation mediated by protein phosphatases ^{[106][107][111]}. PKA-dependent phosphorylation of COXII and COXV were also reported in bovine heart mitochondria, a step necessary for allosteric ATP-inhibition of COX activity ^[107]. Strengthening these observations, the presence of protein phosphatase 1 (or its activator, Ca²⁺) reverted the inhibitory effects of PKA-dependent phosphorylation of COX kinetics in a way that renders the enzyme sensitive to ATP inhibition ^[112]. In murine macrophages and rabbit hearts, hypoxic challenges induce PKA-dependent phosphorylation of subunits I, IV-1, and Vb ^{[113][114]}. Importantly, mitochondrial oxidants activate PKA during hypoxia ^[115] and phosphorylation of COX subunits reduced its activity, which in turn increased oxidant production ^[113].

COX phosphorylation can also be mediated by PKC ε , which associates with subunit IV in neonatal cardiac cells upon hypoxic preconditioning ^{[108][109][110]}. However, PKC ε -dependent phosphorylation increases COX activity, which contrasts with the functional outcome of PKA-mediated phosphorylation of COX ^{[106][107][108][109][110][116]}. This indicates that phosphorylation of specific residues confers unique functional outcomes for COX activity. COX is also regulated by proinflammatory cytokines such as tumor necrosis factor-alpha, which mediates Tyr phosphorylation of COXI, reducing its activity and compromising $\Delta \Psi_m$ and OXPHOS ^[116].

Although numerous phosphorylation sites on different COX subunits have been predicted over the years, only some of them were validated while new sites were found. For example, in a comprehensive tissue catalog phosphoproteome of 14 rat tissues identified eight phosphorylation sites in COXIV, two in COXVIc, and one in COXII [117]. A remarkable consequence of COX phosphorylation is the transition from the monomeric to the dimeric state of the enzyme complex [98]. Conversely, high cytosolic Ca²⁺ activates protein phosphatases which dephosphorylate COX and shifts the enzyme complex from the dimeric to the monomeric state [98][107][111]. The metabolic consequences of this transition include the blockage of the allosteric ATP inhibition of COX, reduced efficiency of electron transfer, increased $\Delta \Psi_m$ and mitochondrial oxidant production [98][107][111]. Since most COX structures solved by X-ray crystallography are in the dimeric form, this strongly indicates that reversible phosphorylation has a crucial role in determining the enzyme kinetics and activity. COX dimerization mediated by reversible phosphorylation might also explain the shift from hyperbolic Michaelian to sigmoidal allosteric kinetics induced by high ATP/ADP ratios and PKA-dependent phosphorylation, as cooperativity intrinsically requires at least an enzyme dimer to allow this regulation [98]. A beautiful example of regulation of COX activity identified a signaling cascade in which the TCA-derived carbon dioxide (CO₂) activates soluble adenylate cyclase (sAC) and the PKAdependent phosphorylation of COXI and COXIV-2 culminating with improved COX activity [118]. The consequences of COX phosphorylation through this signaling cascade are the activation of OXPHOS and reduction of mitochondrial oxidant production [118]. Likewise, the metabolic effects of the CO₂-sAC-cAMP-PKA signaling pathway were also observed in yeast, directly regulating COX activity and OXPHOS and underscoring the evolutionary conservation of this mechanism for metabolic regulation [119]. A further development identified COXIV-1 as a key target of PKA-dependent phosphorylation and regulation of COX activity by adenylates [120]. Specifically, Ser58 was the dominant site of COXIV-1 phosphorylation by PKA, and the exchange of this residue to a phosphomimetic aspartate increased COX activity while rendered the enzyme insensitive to adenylate regulation [120]. This observation contrasts with the experiments carried out in yeast COX5a, the mammalian orthologue of COXIV-1, where the substitution of Ser51 to Asp caused no apparent effects on COX activity and adenylate regulation [119]. However, changing Ser43 or Thr65 to Asp decreased COX activation by ADP while did not alter ATP inhibition of COX [119]. Therefore, maintaining a negatively charged environment provided by a phosphomimetic amino acid at critical phosphorylation sites seems to confer reduced COX sensitivity to allosteric regulation by adenylates, whether in yeasts or mammals [119][120].

It is long known that allosteric regulation of COX activity by adenylates plays a key role in controlling the electron flow through the ETS complexes [59][60][92][93][94][103]. Several lines of evidence demonstrate that adenylates act as specific and critical regulators of COX function in yeasts and vertebrates. Although most prokaryotes only code the core catalytic subunits of COX (subunits I-III), rendering bacterial COX activity insensitive to changes in ATP/ADP ratios [121], some prokaryote genomes code for additional COX subunits. For example, the cyanobacteria *Synechocystis* sp. codes for an additional proto-COXIV subunit that allowed adenylate regulation of COX activity [122]. The first observations on adenylate regulation demonstrated that in several vertebrate species, ATP binds to COX, reducing its affinity to cyt *c*, and ultimately decreasing its activity [123][124].

Interestingly, the specificity of nucleotide regulation of COX seems to be strong for adenylates as neither cytidine, guanosine, nor uridine-nucleotides promote conformational changes in the enzyme complex ^[92]. Subsequent research identified that adenylates regulate COX activity by binding to specific sites at subunits IV and VI ^{[59][60][92][93][94][103][120][125]}

^{[126][127][128]}. While the association of ATP/ADP to subunit IV takes place at both sides of the mitochondrial inner membrane ^{[60][93][120]}, subunit VI binds adenylate only at the matrix side ^{[125][127]}. The body of evidence accumulated clearly indicates that energy demand, through changes in ATP/ADP ratio, allosterically regulates COX activity and impacts respiratory rates.

To our knowledge, the only available evidence that adenylates regulate COX activity in insects was reported for the major arbovirus vector *A. aegypti* mosquitoes ^[61]. We observed that ATP/ADP controls mitochondrial G3P oxidation through specific and allosteric regulation of COX activity. Additionally, inhibition of the adenine nucleotide translocator decreases by \approx 50% the activating effects of ADP on uncoupled respiratory rates without affecting their apparent affinity. This strongly suggests the existence of two distinct sites by which adenylates regulate respiration: one at the matrix and the other at the intermembrane space ^[61]. Although the exact sites of adenylate binding in flying insects are yet to be determined, we postulate that subunits IV and VI are the most likely candidates to regulate COX activity.

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