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SYMPOSIUM

Mechanisms that Alter Capacity for Adenosine Triphosphate Production and Oxidative Phosphorylation: Insights from Avian Migration

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Synopsis Avian migration is among the most energetically demanding feats observed in animals. Studies evaluating the physiological underpinnings of migration have repeatedly shown that migratory birds display numerous adaptations that ultimately supply the flight muscle mitochondria with abundant fuel and oxygen during long-distance flights. To make use of this high input, the organs and mitochondria of migrants are predicted to display several traits that maximize their capacity to produce adenosine triphosphate (ATP). This review aims to introduce readers to several mechanisms by which organs and mitochondria can alter their capacity for oxidative phosphorylation and ATP production. The role of organ size, mitochondrial volume, substrate, and oxygen delivery to the electron transport system are discussed. A central theme of this review is the role of changes in electron chain complex activity, mitochondrial morphology and dynamics, and supercomplexes in allowing avian migrants and other taxa to alter the performance of the electron transport system with predictable shifts in demand. It is my hope that this review will serve as a springboard for future studies exploring the mechanisms that alter bioenergetic capacity across animal species.

Introduction

To migrate, birds must sustain an exceptionally high level of adenosine triphosphate (ATP) production over many hours while fasting (Jenni and Jenni-Eiermann 1998; McWilliams et al. 2004). To support flight under these conditions, long-distance migrants accumulate large fat depots prior to migration that support the demand for nutrients during migration, and they display several adaptations that maximize oxygen delivery to the mitochondria (Weber 2009). These adaptations are not observed in their non-migratory counterparts. The concept of **symmorphosis** suggests that the size or capacity of all parts of a physiological system must match to support the functional demand of the organism (Weibel et al. 1991). Thus, elevated oxygen and nutrient delivery to tissues, as observed in avian migrants, should be met with greater oxygen and nutritional **substrate** utilization and greater ATP production by the mitochondria. Thus, relative to non-migrants, migratory birds are predicted to display traits that increase their capacity to produce the ATP needed to sustain flight for extended periods of time. Given that mitochondria are responsible for at least 90% of the ATP used by animals to support essentially all metabolic processes (Pizzorno 2014), an evaluation of the performance the mitochondria vital to these movements will provide unparalleled insight into how some avian species, but not others, are able to accomplish these amazing energetic feats.

The evolution of the mitochondrion enabled multicellularity and the complexities of vertebrate animals and arthropods (Lane 2006). However, the diversity of life histories that emerged among complex animals necessitated not merely a large supply of energy but also a means to precisely regulate energy to match the needs of the individual organism (Ballard et al. 2007). To

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accommodate changes in energetic demand, mitochondria display **metabolic flexibility**. All eukaryotic organisms appear to have the capacity to alter the volume of mitochondria in cells (Devin and Rigoulet 2004) and the rate of **oxidative phosphorylation (OXPHOS)** (Box 1) (Devin and Rigoulet 2007). The rate of OXPHOS varies between a basal rate, which maintains the proton motive force while at rest, and a functional state, which is typically sufficient to support the cell's and organ's demand for ATP (Nicholls and Ferguson 2013). In addition, evidence is emerging to suggest that some organisms have evolved mechanisms that further optimize organ-specific ATP production by reversibly altering basal or maximum capacity for ATP production. These changes occur in response to training, stress, and predictable changes in season or life history (Staples 2014; Fiorenza et al. 2019; Stier et al. 2019; Rhodes et al. 2024).

Over the last decade, an increasing number of evolutionary and ecological physiologists have included measurements of mitochondrial performance in their studies (Salin et al. 2012; Pichaud et al. 2013; Staples 2014; Ivanina et al. 2016; Koch et al. 2021, among many others). Yet, other than leak and accumulated damage from reactive species, few researchers have delved into the mechanisms that support variation in the capacity of the ETS to perform OXPHOS. In general, measures of OXPHOS are relatively repeatable within individuals (Stier et al. 2019; Thoral et al. 2024). There is increasing evidence that rates of OXPHOS can be altered to match persistent or predicted changes in demand (Smith et al. 2018; Memme et al. 2021; Staples et al. 2022; Rhodes et al. 2024, among others).

The objective of this review is to provide a scaffold for evaluating how organisms alter their capacity for ATP production. While this manuscript is framed within the context of avian migration, where the capacity to migrate varies within and between closely related species, this manuscript was written to be used as a resource for researchers studying diverse taxa displaying an array of energetic adaptations. This review has likely overlooked known mechanisms for adjusting OXPHOS and future work will likely reveal new processes. Thus, readers should view this paper as a springboard rather than a guide for exploring the mechanisms responsible for variation in OXPHOS. This work starts with an overview of key processes that impact the capacity for ATP production upstream of the ETS, including substrate delivery, organ size, and mitochondrial volume, but emphasizes processes that regulate OXPHOS, including **mitochondrial complex enzymatic activity**, **mitochondrial morphology**, **mitochondrial dynamics**, and **supercomplex abundance** (Fig. 1).

Intra- and interspecific variation in the capacity to migrate

Across Aves, there is tremendous variation in the seasonal movements of birds and, hence, in the energetic demands of migratory movements (Rappole 2013). Individuals in many species of birds do not migrate. Among species where at least some individuals migrate, movements vary from a series of short flights that require little change in daily energy expenditure relative to the non-migratory period to the extreme movements across major oceans and from one side of the planet to the other (Battley et al. 2012). In numerous clades of birds, sister species and even subspecies vary in their use of migratory movements, suggesting that migration in birds is a highly labile trait. Several investigators have suggested that avian migration evolved numerous times at multiple taxonomic levels (Helbig 2003; Pulido 2007). Conversely, migration may have evolved fewer times but with frequent losses of migratory capacity (Zink 2011; Rolland et al. 2014; Gómez-Bahamón et al. 2020).

Approaches to studying the energetic adaptations that allow for migration have included both intraspecific and interspecific comparisons. Intraspecific comparisons allow researchers to characterize how a particular species supports migratory demands. In contrast, interspecific and even inter-subspecies comparisons of migratory and non-migratory birds will provide valuable insight not only into how birds differ but also into the processes modified across generations and whether those processes are associated with shifts in gene frequency or alternative mechanisms. Taken together, investigating not only what traits differ between migrants and non-migrants but also how those traits are regulated will be exceptionally valuable to understanding the source of such diversity.

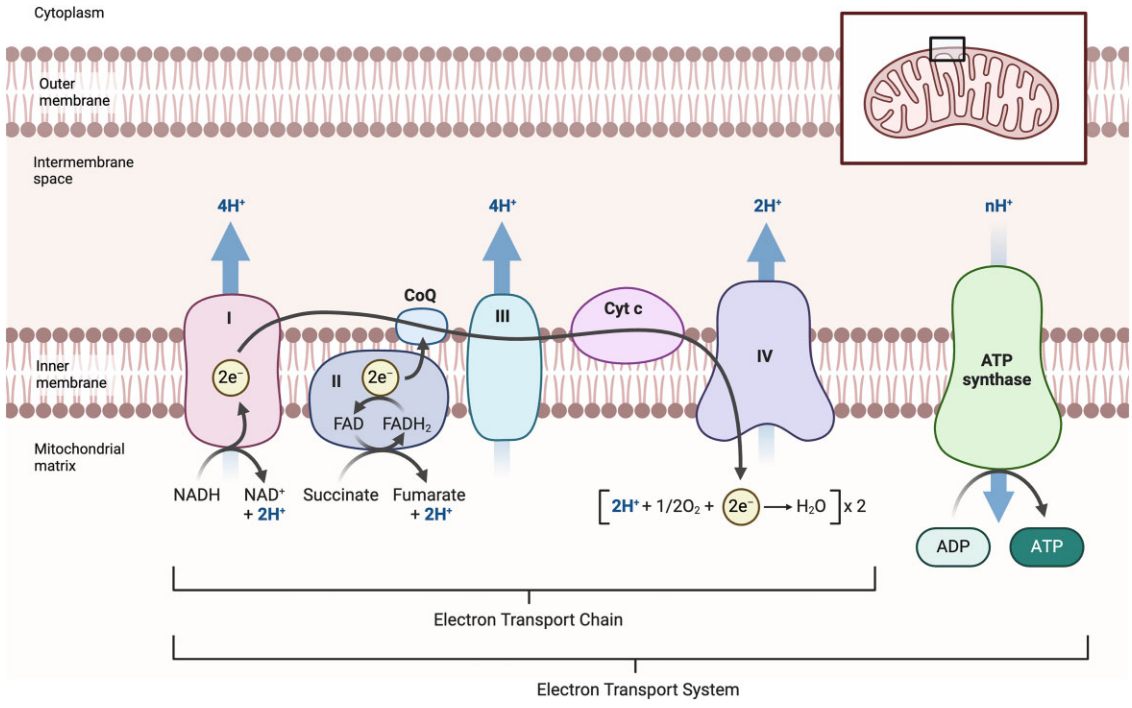
Migratory birds display a mix of fixed and flexible physiological traits that support the energetic demands of migration (McWilliams and Karasov 2001; Weber 2009). Compared with non-migrants, migrants persistently have greater concentrations of red blood cells and hemoglobin in their blood, indicating higher oxygen-carrying capacity (Yap et al. 2019; but see Minias 2019). They also have a higher resting metabolic rate (RMR) throughout the year than non-migrants (Jetz et al. 2008). Typically, individuals with a high RMR consume more ATP per unit of time, and elevated RMR can arise from several different variables, including higher organ mass, higher immune costs, and greater thermoregulatory demand, but it can also be associated with less efficient mitochondrial basal respiration (Speakman et al. 2004; Jetz et al. 2008; Burton et al. 2011). Traits that show within-individual flexibility in migrants include

Box 1: A primer on OXPHOS.

OXPHOS is the process that supports the conversion of ADP to ATP by the electron transport system (ETS), making ATP available as fuel for the organism. In eukaryotes, the process occurs within the mitochondria, where the ETS protein complexes sit within the inner mitochondrial membrane. The products of carbohydrate, lipids, and, in some cases, protein breakdown enter the citric acid cycle, where they are converted into molecules that donate electrons to complex I (NADH) or complex ll ($FADH_2$) of the ETS. The breakdown of each macronutrient provides NADH and $FADH_2$, but at different ratios of NADH:$FADH_2$. Thus, each macronutrient varies in its relative dependence on OXPHOS via complex I versus complex II, with the breakdown of all macronutrients supplying more higher energy NADH (which can support the production of 3 ATP per NADH molecule) than the lower energy $FADH_2$ (which can support the production of 2 ATP per $FADH_2$ molecule). Once taken up by complex I or II, electrons move between the complexes of the electron transport chain (ETC), where they are ultimately transferred with two hydrogen ions to an oxygen molecule to produce water.

The movement of electrons between complex I, III, and IV or II, III, and IV is coupled to the movement of protons from the mitochondrial matrix into the intermembrane space, creating a proton gradient. Complex V, the ATP synthase, has a channel that allows the proton ions to flow down their concentration gradient from the intermembrane space back into the matrix, where the protons support the addition of a phosphate ion to ADP to produce ATP (Stryer 1999; Cooper 2000). When a cell is at rest, the ATP synthase only allows enough protons through the ATP synthase channel to support the production of the ATP necessary to support housekeeping functions (Willis et al. 2016). With increased ATP demand, the ATP synthase is triggered to further open the proton channel, primarily via calcium ion signaling (De Marchi et al. 2014; Willis et al. 2016), allowing protons to move back into the matrix and supporting the synthesis of ATP. Thus, ATP production is fueled by demand for ATP and not by available substrate. Both the leak of electrons or protons from the ETS can reduce the efficiency and, thus, the rate of ATP production by the ETS. Proton leak can be passive through the inner membrane or active through uncoupling proteins or adenine nucleotide translocase (Jastroch et al. 2010). The leak of electrons can contribute to reactive species formation. Both proton and electron leaks are typically greatest when the availability of electron donors is high and the proton motive force is high, but the use of electrons to produce water and protons to support ATP production is low (Murphy 2009; Speakman and Garratt 2014; Willis et al. 2016).

For a more detailed description, I recommend the reader review the chapter on OXPHOS in college textbooks for cell biology, animal physiology, or biochemistry targeted to upper-level biology students. Then, consider reviewing more detailed references such as *Biochemical Adaptations* by Somero et al. (2016) and *Bioenergetics* textbooks by Nicholls and Ferguson (2013). I also found the hydraulic model described by Willis et al. (2016) helpful in my understanding of OXPHOS. Image created with BioRender.com.



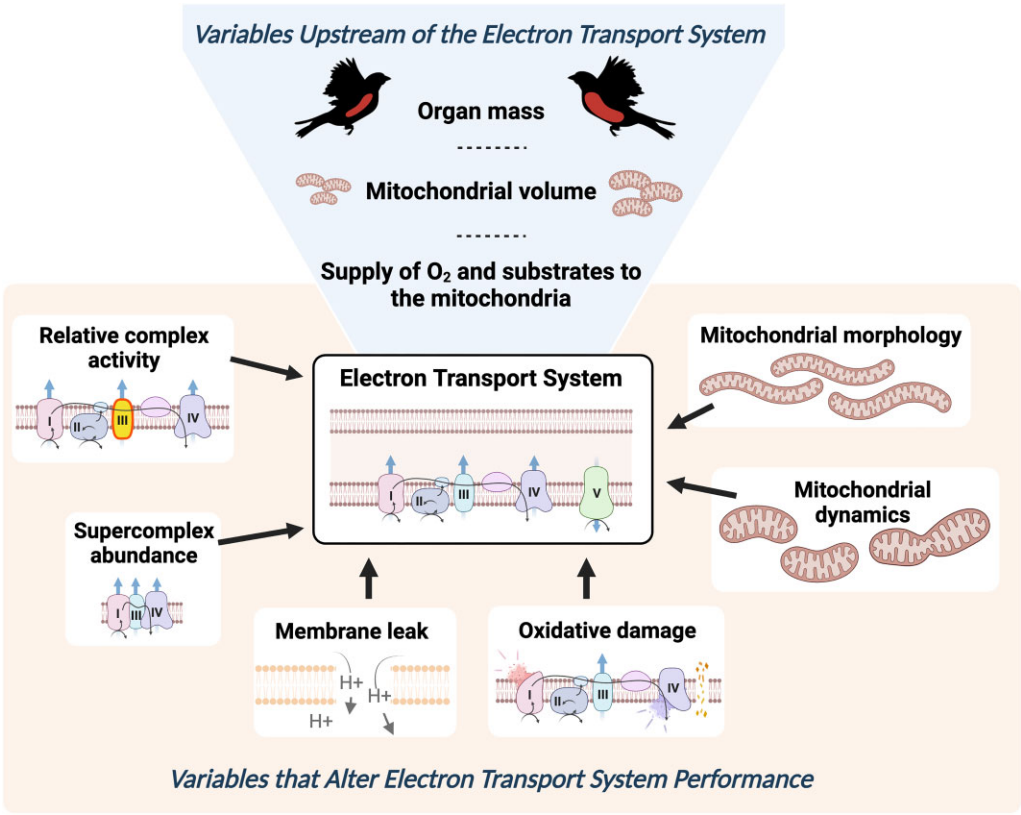


Fig. 1 Mechanisms contributing to variation in ATP production. Organ mass and mitochondrial volume determine how many ATP-producing units there are in the organ, and the supply of oxygen and mitochondrial substrates to the ETS can limit ATP production when supplied below the capacity of the ETS. Further, the capacity of the ETS itself can be up- or down-regulated by altering the enzymatic activity of the complexes, changing the proportion of ETS supercomplexes, altering inner membrane leak, changing mitochondrial morphology and dynamics, and with the accumulation of oxidative damage. Created with BioRender.com.

the size of the flight muscles (pectoralis and supracoracoideus) and gastrointestinal tract (Evans et al. 1992; Piersma and Gill 1998; Landys-Ciannelli et al. 2003). Flexibility in flight muscle size is associated with variable muscle fiber diameter and variable number of nuclei per volume of cytoplasm (i.e., myonuclear domain) (Evans et al. 1992; Vézina et al. 2021). Flexibility in the size and mass of the gastrointestinal track supports premigration hyperphagia and adipose deposition (Piersma and Gill 1998; Landys-Ciannelli et al. 2003). The relative mass of gastrointestinal organs declines during long-distance migration and may be rebuilt at stopover sites before declining again when migration continues (Dekinga et al. 2001). Birds may enhance their already high skeletal muscle mitochondrial volume in preparation for migration (Evans et al. 1992). An increase in the relative skeletal muscle size, **mitochondrial volume**, and **mitochondrial respiratory performance** is commonly observed with high-intensity training in humans (Fiorenza et al. 2019; Callahan et al. 2021). Changes in muscle size and mitochondrial volume are initiated in response to photoperiod in the days or weeks before the onset of migration in birds (DeMoranville et al. 2019; Vézina et al. 2021), before there is an apparent increase in time spent in flight. This suggests that changes in muscle size and mitochondrial volume occur in anticipation of high demand, not solely as a response to demand, as occurs with the initiation of a new training program in humans.

These data suggest that migratory birds have physiological and anatomical adaptations that support a high rate of oxygen and nutrient delivery to demanding tissues, such as the flight muscles. The avian lineage evolved several changes in cellular metabolism that allow the skeletal muscles of flying birds to function near their energetic capacity (i.e., VO_2max) (Guglielmo et al. 2002; McWilliams et al. 2004; Butler 2016). Birds support this high demand with a high density of mitochondria in their flight muscles and a high density of OXPHOS complexes per mitochondrion (Suarez et al. 1991; Rasmussen et al. 2004; Kuzmiak et al. 2012). Mammals fuel periods of prolonged exercise almost entirely with carbohydrates (respiratory quotient, RQ > 0.90; Brooks and Donovan 1983; O'Brien et al. 1993); in contrast, birds fuel flight primarily by oxidizing lipids (RQ = 0.72–0.73) (Rothe et al. 1987;

Kuzmiak-Glancy and Willis 2014). When metabolizing fatty acids, birds drive OXPHOS with fewer electron donors (NADH, $FADH_2$) in the matrix than observed in mammals using the same substrates; this lowers the number of electrons passing through the ETS, lowering the risk that electrons will escape. Reduced electron leak will result in the production of fewer reactive oxygen species and lower the potential for the accumulation of oxidative damage (Suarez et al. 1991; Barja et al. 1994; Rasmussen et al. 2004; Kuzmiak et al. 2012; Kuzmiak-Glancy and Willis 2014). Birds also use fat more efficiently than mammals, as indicated by a higher oxygen utilization capacity (**state 3 respiration**) when provided succinate or palmitoylcarnitine as an **OXPHOS substrate** (Kuzmiak et al. 2012). Whether these bioenergetic adaptations for flight are also sufficient to support long-distance migratory movements is unknown.

Variables altering ATP production upstream of OXPHOS

The metabolic rate of an individual is the sum of the oxygen utilized to support all metabolic processes in the body. Yet, changes in energy demand are often obscured from whole animal respiration measurements because organs interact and trade-off resources (Metcalfe et al. 2023). Thus, delving into the characteristics that impact the capacity of organs to produce ATP can provide valuable insight into how an organism supports the demands of a specific activity. In the context of avian migration, long-duration migratory movements rely on the concerted efforts of multiple organ systems. The lungs maximize oxygen uptake and the removal of carbon dioxide, the blood has high capacities in carrying oxygen and buffering carbon dioxide, and the digestive system atrophies, reducing maintenance demands, while adipose stores are efficiently mobilized to support demand (Weber 2009; Ivy and Guglielmo 2023). The synergy of these organ systems facilitates an increase in available oxygen and nutrients allocated to the flight muscles. Within this network, the organs that bear the highest energetic demands are the pectoralis, which supports the downstroke, and the supracoracoideus, which supports the upstroke. Hence, these muscles provide valuable targets for evaluating the bioenergetic demands of flight, though insights will also be gained from examining other organ systems (Jehl et al. 2015). For illustrative purposes, emphasis will be placed on the pectoralis.

The ability of the pectoralis to produce ATP is broadly dependent on its relative mass, the volume of mitochondria within the organ, and the relative rate of ATP production by the ETS within the mitochondria. Each of these variables and their supporting processes will determine the ATP output of the organ. Many organs change in mass relative to demand, undergoing hypertrophy, hyperplasia, and/or atrophy. A larger organ produces more ATP than a smaller organ if mitochondrial volume, morphology, and function are similar. Moreover, if organ mass is constant but mitochondrial volume is greater—whether associated with an increase in the number of cells with the same relative mitochondrial volume or maintenance of cell number alongside an increase in mitochondrial volume per cell—there will also be an increase in ATP production. Therefore, organ mass and mitochondrial volume are vital to understanding the capacity of an organ to produce ATP. In Gray Catbirds (*Dumetella carolinensis*), migration is associated with increased pectoralis mass but not mitochondrial volume (DeMoranville et al. 2019). In contrast, European Robins (*Erithacus rubecula*), Reed Warblers (*Acrocephalus seirpaceus*), and Common Blackbirds (*Turdus merula*) all display increased mitochondrial volume associated with migration (pectoralis mass was not given; Lundgren and Kiessling 1985). Evaluation of skeletal muscle phenotype is complicated by a mix of different skeletal muscle fiber types and mitochondrial populations that vary in function and capacity for ATP production (Lundgren and Kiessling 1988). As muscle fiber types are readily quantifiable and the respiratory performance of mitochondrial populations in the subsarcolemmal and intermyofibrillar space can be distinguished (Kavazis et al. 2009), it is worth considering differences in each population as has been characterized in other studies of bird skeletal muscle (Duchamp et al. 1991; Roussel et al. 2000; Teulier et al. 2016).

The capacity of mitochondria to produce ATP relies on a myriad of processes upstream of the ETS and processes that directly alter OXPHOS. While the demand for ATP drives the utilization of nutritional substrates (Trivedi et al. 2015; Willis et al. 2016; Boël et al. 2020), the ability of the body and cells to deliver substrates to the mitochondrial matrix and ETS can constrain ATP production. During migration, flight is primarily supported by the oxidation of fatty acids mobilized from adipose stores (but see Elowe et al. 2023 for evidence that protein catabolism can also be important). Supporting the utilization of fatty acids as fuel, birds in the migratory state upregulate fatty acid transport proteins that carry fatty acids into the cell, through the cytosol, and into the mitochondrion. These changes include a higher fatty acid translocase that carries fatty acids through extracellular and intracellular spaces, a higher fatty acid binding protein that carries fatty acids through the cytosol, and a higher carnitine palmitoyl transferase that prepares fatty acids for transport into the intermembrane space of the mitochondria

(Bonnefont et al. 2004; Zhang et al. 2015; Young et al. 2021; Rupert and Kolonin 2022; Agellon 2023). There is also an upregulation in the capacity for β-oxidation within the mitochondrial matrix, as indicated by greater β-hydroxylacyl CoA-dehydrogenase (Zhang et al. 2015; Young et al. 2021). β-oxidation catabolizes fatty acids for entry into the citric acid cycle and $\beta-$hydroxylacyl CoA-dehydrogenase is a key enzyme in that pathway. These processes will result in the production of more NADH and $FADH_2$ than found in non-migrants, which can be used to support OXPHOS via complex I and complex II (hereafter, complexes noted with C, i.e., Cl and Cll).

OXPHOS

Mitochondrial respiration measurements are typically completed in permeabilized cells, isolated mitochondria, or cultured cells (Brand and Nicholls 2011; Koch et al. 2021). While numerous variables can be measured in these contexts, many of which are specific to the type of instrument used (Zdrazilova et al. 2022; Walsh et al. 2023), two common measures of mitochondrial function are state 3 and state 4 respiration under *ex vivo* conditions. State 3 is a measure of the maximum respiratory performance of coupled mitochondria given unlimited substrate, oxygen, and adenosine diphosphate (ADP); state 4 respiration is a measure of basal respiratory performance when ADP has been depleted (Brand and Nicholls 2011). While some differences occur in the conditions under which state 4 measurements are completed (i.e., with or without the inhibition of the ATP synthase), state 4 is generally considered a measurement of leak respiration (Koch et al. 2021). Specifically, leak is associated with the passive or induced movements of protons from the intermembrane space back into the matrix. To compensate for the leak, the ETS must continuously use substrate and oxygen to maintain the proton gradient between the intermembrane space and the matrix and, thus, maintain a minimum proton motive force. Thus, state 4 respiration measures the amount of oxygen used to compensate for leak (Koch et al. 2021).

Only three published studies have evaluated the respiratory performance of mitochondria in migratory birds (Toews et al. 2014; Coulson et al. 2024; Rhodes et al. 2024). Toews et al. (2014) compared the respiratory performance of pectoralis mitochondria between Audubon's Warblers that carried the mitochondrial haplotype the migratory Myrtle Warbler (*Setophaga coronata*) or the mitochondrial haplotype non-migratory Black-fronted Warbler (*Setophaga nigrifrons*). They found that the pectoralis mitochondrial of individuals with the Myrtle Warbler hapotype displayed a higher acceptor control ratio, but not higher state 3 respiration, than individuals with the non-migratory Black-fronted Warbler haplotype. An important caveat in this study is that birds were collected during the breeding season, which is outside of the migratory period. The acceptor control ratio (ACR) is a ratio of state 3/state 2 respiration, with state 2 respiration being a measure of mitochondrial respiration after the addition of pyruvate and malate substrates but before the addition of ADP. ACR measures how efficiently substrate oxidation is coupled to ADP phosphorylation. This variation did not appear to be associated with variation in the enzymatic activity of complex l, which did not differ between groups (Toews et al. 2014). In contrast, Coulson et al. (2024) evaluated mitochondrial respiration in pectoralis mitochondria of Yellow-rumped Warblers (a.k.a. Myrtle Warblers) in a migratory versus non-migratory state and found that pectoralis mitochondria from warblers in the migratory condition displayed higher state 3 and state 4 respiration when provided palmitoylcarnitine substrate, suggesting that changes in mitochondrial respiratory function are necessary for migration.

In collaboration with the Hill and Kavazis labs, Rhodes and others in my lab evaluated the respiratory performance of pectoralis mitochondria in a migratory and non-migratory subspecies of White-crowned Sparrow (*Zonotrichia leucophrys gambelii* and *Z. l. nuttalli*, respectively). We evaluated mitochondrial function in birds from the migrant population during the spring as birds prepared for migration, during fall migration, and during the winter non-migratory period. We collected similar data from individuals from the non-migrant population during fall and winter but not spring so as not to include reproductively active birds in our study (Rhodes et al. 2024). We found that state 3 and state 4 respiration and the ratio between the two, the respiratory control ratio (RCR), of pectoralis mitochondria were upregulated just prior to and during the migration in the migrant. Interestingly, the upregulation of maximum mitochondrial respiratory capacity was observed regardless of the substrate used to stimulate OXPHOS, including (1) palmitoylcarnitine, as shown in Fig. 2, (2) a mix of pyruvate, malate, glutamate, and (3) succinate (Rhodes et al. 2024). RCR also followed the same pattern. If changes in leak were responsible for the pattern, RCR would have been similar between all groups, but it was not. Given that patterns of mitochondrial respiration were similar across substrates for state 3 and 4 respiration, we propose that the patterns observed are a product of a process that has global effects on the efficiency of oxygen utilization by the ETS. Thus, the upregulation of mitochondrial capacity must be associated with a mechanism that equally impacts oxygen utilization regardless of the path of the electrons through the

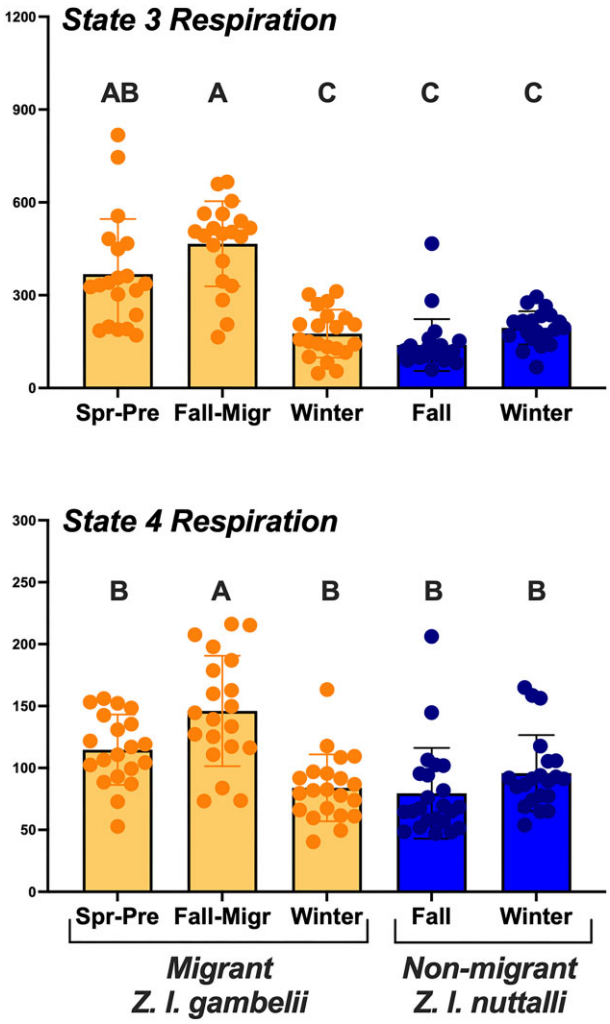


Fig. 2 Comparison of state 3 and state 4 respiration with palmitoylcarnitine substrate in isolated pectoralis mitochondria collected from migratory Gambel's (*Zonotrichia leucophyrs gambelii*) and non-migratory Nuttall's (*Z. l. nuttalli*) White-crowned Sparrows. Data presented includes samples collected during spring premigratory fattening (Spr-Pre), fall migration (Fall-Migr), and during winter (outside of the migratory period) for Gambel's individuals and during fall and winter for Nuttall's individuals. No animals were breeding during any of these periods. The letters above indicate the results of statistical comparisons between groups. If the letters are similar, the groups are statistically similar. If the letters are different, the groups are statistically different. Data adapted from Rhodes et al. (2024).

mitochondria. To deduce the adaptation responsible for this pattern, it is necessary to delve further into processes that regulate OXPHOS.

Mechanisms that alter the efficiency of OXPHOS

State 3 and state 4 respiration, and theoretically, the functionality of the mitochondria *in vivo*, can be modified by a variety of mechanisms, including the enzymatic activity of the ETS complexes, changes in mitochondrial morphology, fission and fusion dynamics, and the relative abundance of ETS complexes assembled into supermolecular structures called supercomplexes (Strohm and Daniels 2003; Mathers et al. 2017; Fiorenza et al. 2019; Hutchinson et al. 2022). The function of the mitochondrial complexes within the ETS can vary when there are non-synonymous differences in the mitochondrial or nuclear genes that code for proteins that make up each complex, with CI, CIII, CIV, and CV, including mitochondrial and nuclear proteins, and CII composed of only nuclear-encoded proteins (Hill 2019). For example, bar-headed geese (*Anser indicus)* that migrate at very high altitudes display lower heart CIV (cytochrome oxidase) activity than a lower-altitude migrant, the barnacle goose (*Branta leucopsis*). This difference appears to be associated with a single-point mutation in the COX3 subunit at a point that appears to be conserved across other vertebrate species (Scott et al. 2011). The functionality of the complexes also appears to be modifiable by post-translational modification (PTM) of one or more proteins within the ETS complexes. PTM can change the performance of proteins via phosphorylation, acetylation, or succinylation (among others) of specific amino acids (Hofer and Wenz 2014; Stram and Payne 2016). In thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*), a suppression of CIV activity in liver mitochondria during hibernation is due, at least in part, to a significant increase in phosphorylation of NADH-ubiquinone oxidoreductase, CI subunit. In contrast, the flavoprotein of CII is 4.6 times more phosphorylated between hibernation bouts when CII enzymatic activity is also elevated (Mathers and Staples 2019). To our knowledge, the PTM of mitochondrial or other proteins in the tissues of migrants has not been evaluated in any context. In our study of White-crowned Sparrows, we also measured the enzymatic performance of individual mitochondrial complexes. Surprisingly, none of the ETS complexes followed the pattern of up-and-down regulation that we observed for mitochondrial respiration. However, some differences did occur between groups (Rhodes et al. 2024). These observations suggest that a change in the function of individual complexes is unlikely to play a major role in the consistent change in respiratory capacity across respiratory states and substrates. Instead, alternative mechanism(s) must be responsible for much of the change in mitochondrial function that enables migration.

Aside from complex performance, multiple mechanisms have been proposed for how mitochondria alter the relative concentration of protons in the intermembrane space and the rate of electron movement through

Box 2: Glossary of terms displayed in bold throughout the text..

Bioenergetics is the study of the energy conversion processes across the inner mitochondrial membrane (Nicholls and Ferguson 2013).

Electron transport chain (ETC) vs. electron transport system (**ETS).** The ETC specifically references the electron-transporting enzymes contributing to OXPHOS in the mitochondria, including complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome c reductase), and complex IV (cytochrome c oxidase). The term ETS is used when referencing the ETC plus the ATP synthase (complex V).

Mitochondrial respiratory performance. The relative rate of oxygen utilization by the ETC. (I have intentionally used this term rather than more specific terminology in some instances because the conditions under which mitochondrial respiration is quantified often vary with the instrument used for quantifying mitochondrial respiration).

Mitochondrial volume is the volume that mitochondria occupy within the cell or organ. Because individual mitochondria vary in size, volume, and cristae surface area, it is important to consider the volume of mitochondria rather than their number. Physiologists commonly measure mitochondrial volume based on the relative abundance of citrate synthase. The amount of CS associated with each mitochondrion will vary with its crista surface area and, presumably, the number of ETS complexes within the organelle (Larsen et al. 2012; Heine et al. 2023).

OXPHOS is the primary source of ATP for eukaryotic cells. During OXPHOS, the ETC complexes couple the movement electrons with the production of a protein gradient that powers the phosphorylation of ADP to ATP by the ATP synthase, making ATP available as a source of fuel for the organism (Stryer 1999; Box 1).

OXPHOS substrate or **substrate**. In the context of OXPHOS, a substrate is an organic molecule used to induce NADH or $FADH_2$ production that donates electrons to the ETC. In vivo, ingested or stored macronutrients are catabolized to provide mitochondria with substrates to supply the citric acid cycle and the ETC. In vitro, researchers can use a variety of substrates. Pyruvate, malate, and glutamate are often supplied to mitochondria to support the production of NADH, and succinate is used to provide $FADH_2$. Palmitoylcarnitine is also often used in avian studies because of the high use of fatty acids as fuel by members of this taxonomic group. It is a fatty acid attached to a carrier protein that allows the fatty acid to be transported into the mitochondria, where the fatty acid must go through β-oxidation before being supplied to the TCA cycle, where the electron donors are produced (Stryer 1999; Kuzmiak et al. 2012).

Metabolic flexibility refers to phenotypic flexibility in metabolic processes, with phenotypic flexibility defined as rapidly reversible changes in phenotype involving multiple genotypes. In contrast, phenotypic plasticity is a signal genotype producing multiple phenotypes in response to an environmental variable. The former best represents changes in mitochondrial phenotype, which display rapidly reversible changes in mitochondrial respiratory performance and regulation by multiple genes from two distinct genomes, the nuclear and mitochondrial genomes (McWilliams and Karasov 2001; Piersma and Drent 2003).

Mitochondrial complex activity is a measurement of the catalytic rate of an individual ETC complex or, more specifically, the control that a complex exerts on the rate of respiration by ETC (Villani and Attardi 2007). These measurements are commonly completed for complex l, II, III, and lV but not V because measurements of l-IV can reliably completed on frozen tissue (Spinazzi et al. 2012)**,** but measurements of complex V activity require fresh tissue (Vives-Bauza et al. 2007).

Mitochondrial dynamics refers to measures of mitochondrial biogenesis (the production of new mitochondria), mitophagy (the disposal of damaged mitochondria), mitochondrial fission (the dividing of a mitochondrion into two daughter cells, often associated with removing a damaged portion of the organelle), and mitochondrial fusion (the union of two mitochondria) (Sebastián et al. 2017; Tanaka et al. 2020).

Mitochondrial morphology refers to the mitochondria's physical attributes, including the mitochondria's relative size, the density of the inner mitochondrial membrane, and the shape of the organelle and crista within it. Morphology can be influenced by fission and fusion dynamics (Heine and Hood 2020).

Mitochondrial supercomplexes. Countering the fluid/liquid-state model, which suggests that each of the ETS complexes moves independently through the inner membrane of the mitochondria, we now know that complexes I, III, and IV commonly exist in supermolecular structures called supercomplexes. These supercomplexes are thought to increase the efficiency of electron transfer between the complexes (Dudkina et al. 2010).

Reactive oxygen species (ROS) or **reactive species**. ROS are groups of highly reactive molecules that are generated by the reduction of an oxygen-containing molecule by a free electron. The leak of electrons from the ETS is commonly reactive with oxygen to form superoxide. Reactive species include all products of reduction by a free electron, including reactive oxygen species, reactive nitrogen species, reactive halogen species, and reactive sulfur species, and thus, the term reactive species is used to not exclude these molecules (Halliwell and Gutteridge 2015). Importantly, the production of ROS is the most important (but not necessarily exclusive) reactive species produced as a product of electron leak from the ETS (Murphy 2009), and thus, the production of other reactive species is often ignored.
State 3 respiration is the maximum performance of coupled mitochondria when substrate, oxygen, and ADP are not limiting.
State 4 respiration (i.e., **leak**) is a measure of basal respiratory performance of the mitochondria when ADP has been depleted and is generally considered a measurement of leak respiration (Koch et al. 2021). Specifically, (proton) leak is the passive or induced movements of protons from the intermembrane space back into the mitochondrial matrix. To compensate for the leak, the ETS must continuously use substrate and oxygen to maintain the proton gradient between the intermembrane space and the matrix and, thus, maintain a minimum proton motive force. Thus, state 4 respiration measures the amount of oxygen used to compensate for leak (Koch et al. 2021). Note that when the term leak is used, it is almost invariably used to describe proton leak, but electrons also leak from the ETS.
Symmorphosis suggests that the size or capacity of all parts of a physiological system must match to support the functional demands of the organism (Weibel et al. 1991).

the ETS to enable long-distance migration (Heine et al. 2023). These mechanisms include changes in mitochondrial morphology and associated mitochondrial dynamics. Mitochondria can adjust their shape and the density of the inner membrane, fuse and divide, move within the cell, and communicate with neighboring mitochondria (Heine and Hood 2020). An increase in inner mitochondrial membrane (IMM) density for the same volume of mitochondria will increase the surface area available for accumulating OXPHOS complexes and reduce the volume of the inner membrane space within each crista, thus increasing proton density and the relative concentration of NADH and $FADH_2$ in the matrix. These changes will increase the availability of NADH and $FADH_2$ as substrates for OXPHOS and protons for ATP synthase in the final step of ATP production (Heine et al. 2023). Mitochondrial volume in the pectoralis and supracoracoideus muscles in the Rufous hummingbird (*Selasphorus rufus*) is very high throughout the year, making up approximately 35% of muscle volume, with 40% of mitochondria found in the subsarcolemmal region adjacent to capillaries and the remaining interfibrillar region. The density of the IMM was exceptional and only matched by flying insects (Suarez et al. 1991). While Rufous Hummingbirds are migratory, they also use an exceptionally demanding form of flight, hovering. Data were not collected during migration, and, to our knowledge, no other studies have evaluated mitochondrial morphology associated with migration in birds. Yet, Rauhamaki et al (2014) found that the flight muscle mitochondria of the red admiral butterfly (*Vanessa atalanta*), a long-distance migrant, have greater cross-sectional area and denser cristae and that the mitochondria made up a greater percentage of sarcoplasmic volume than a butterfly that disperses shorter distances, the Glanville fritillary (*Melitaea cinxia*). Measures of morphology, including mitochondrial shape and intermitochondrial junctions, or electron-dense sites between neighboring mitochondria, should also be considered, given that such changes commonly occur when mitochondria have been exposed to oxidative stress and intermitochondrial junctions have been shown to increase associated with running in lab mice (Picard et al. 2013; Heine et al. 2021). While the functional significance of these changes in morphology remains an active area of investigation, a recent model suggests that more globular mitochondria generate more ATP than elongated mitochondria (Garcia et al. 2023).

Measures of mitochondrial dynamics typically include measures of mitochondrial biogenesis, mitophagy, and mitochondrial fission and fusion. Mitochondria biogenesis and mitophagy regulate the number and quality of mitochondria within the cell. Mitochondrial fission and fusion contribute to variation in mitochondrial morphology (as described above) and have the potential to alter the capacity for OXPHOS for a given volume of mitochondria. The detailed mechanisms that underlie each of these processes are reviewed elsewhere (Sebastián et al. 2017; Tanaka et al. 2020). Mitochondria regularly turnover within a cell, with half-lives varying from 9–24 days in rat liver, heart, and brain (Menzies and Gold 1971). An increase in mitochondrial biogenesis increases the rate of mitochondrial replication, which can increase mitochondrial volume within the cell (Popov 2020). Relative expression of peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α) is a

common marker of the rate of mitochondrial biogenesis (Liang and Ward 2006). The biogenesis rate responds to demand and has been shown to increase in skeletal muscle tissue with exercise (particularly intermyofibrillar mitochondria) and other organs in response to high demand, such as the liver during lactation (Hyatt et al. 2018) and in response to an oxidative event in the heart (Zhang et al. 2018). Mitophagy removes mitochondria from the cell; thus, it is essential for both maintaining mitochondrial volume in cells that are continuously producing new mitochondria and for the selective disposal of old and damaged mitochondria (Ma et al. 2020). During migration, the tissues of migrants have been shown to accumulate oxidative damage (Costantini et al. 2007; Jenni-Eiermann et al. 2014; Eikenaar et al. 2020). Given that mitochondrial reactive oxygen species production is highest at rest and low during exercise (Willis et al. 2016; Tanaka et al. 2020), the damaging reactive species in the skeletal muscle of migrants are likely of non-mitochondrial origin, associated with the action of oxidative enzymes such as NADPH oxidases and xanthine oxidases (Tanaka et al. 2020). Processes of mitophagy would be critical for removing this damage and maintaining mitochondrial performance during migration. An upregulation of PTEN-induced putative kinase protein 1 (PINK1) and E3 ubiquitin ligase Parkin (i.e., Parkin) is measured to quantify mitophagy (Ma et al. 2020).

Processes of mitochondrial fission and fusion also play important roles in maintaining mitochondrial quality within the cell, but they may also play a role in regulating OXPHOS. Both processes of mitochondrial fission and fusion appear to be continuously active, playing a role in maintaining the continuous replacement of mitochondria over time. Yet, each can also be altered in response to energetic demand and mitochondrial damage. The proteins mitofusion 1 (Mfn1), mitofusion 2 (Mfn2), and optic atrophy 1 (Opa1) play key roles in the coalescence of the outer (Mfn1, Mfn2) and inner membranes (Opa1) of the mitochondria when adjacent mitochondria fuse. Dynamin-related protein 1 (Drp1) and mitochondrial fission 1 (Fis1) cleave mitochondria and are upregulated with oxidative stress and disease. Fission appears to be the first step before removing damaged mitochondria via mitophagy and thus plays an important role in maintaining bioenergetic capacity (Wu et al. 2011). The response of skeletal muscle mitochondrial fission and fusion markers to exercise has been variable. Axelrod et al. (2019) showed that the skeletal muscle of humans participating in a regular exercise regime displayed Mfn1, Mfn2, and Opa1 levels that were similar to sedentary individuals but a reduction in Fis1 and Parkin that resulted in more elongated mitochondria. Fusion allows mitochondria to exchange mtDNA proteins, lipids, and metabolites. With nutrient depletion, elongated mitochondria exhibit increased cristae density, decreased cristae width, and increased dimerization and activation of the ATP synthase (Gomes et al. 2011; Pernas and Scorrano 2016). In contrast, Fiorenza et al. (2019) show that both mitochondrial fission (Drp1) and fusion (Mfn2) dynamics markers and OXPHOS capacity (state 3 for Cl and Cll substrates) were upregulated following regular high-interval training. Importantly, these effects were only observed at 40°C, temperatures that may be reached during the intense training, but not at 35°C, the typical temperature for skeletal muscle at rest. Such effects were not observed following a single bout of exercise in rats (Picard et al. 2013; Yoo et al. 2019). Further work is needed to characterize the relationship between mitochondrial morphology, fission and fusion markers, and OXPHOS. Given that long-distance migrants display long bouts of continuous flight with elevated oxidative stress, it is possible that both mitochondrial fission and fusion markers may be elevated. It would be interesting to explore the relationship between mitochondrial respiration and mitochondrial dynamics.

Another variable that has the potential to increase the efficiency of electron transfer within the ETS is mitochondrial supercomplexes. Mitochondrial complexes commonly assemble into supermolecular structures called supercomplexes. Supercomplexes form linkages between CI/CIII/CIV in the respirasome, as well as Cl/CIII and CIII/CIV (Novack et al. 2020). Close association between complexes is thought to increase the rate of electron transfer between the complexes, and both increase the efficiency of respiration and decrease the rate of formation of reactive oxygen species (Genova and Lenaz 2014; Huertas et al. 2017). In humans, the relative abundance of CIII and CIV in supercomplexes increases with exercise. Further, state 3 respiration strongly correlates with supercomplex abundance in skeletal muscle in humans (Greggio et al. 2017). In thirteen-lined ground squirrels, complex III is found in 3 configurations: associated with CI and CIV (i.e., the respirasome), CIV only, or with another CIII complex forming a dimer; it is associated with the respirasome or CIV approximately 50–75% of the time. Its associations shift with arousal from torpor, with the relative distribution CIII in brown adipose mitochondria associated with the respirasome being lower and CIV being higher after arousal from torpor than it was during torpor. This change may play a role in the release of mitochondria from a suppressed state during hibernation (Hutchinson et al. 2022). Whether changes in the relative association of ETS complexes with supercomplexes play a role in avian

migration is unknown but could be a fruitful avenue of investigation.

Identifying the mechanisms that underlie the lability of migration in birds

By crossing different populations of Blackcap warblers (*Sylvia atricapilla*) that varied in their migratory path, Berthold et al. (1992) unequivocally showed that the direction and distance of migration were heritable. Several investigators have searched for genes associated with variation in migratory behavior (Mueller et al. 2011; Delmore et al. 2016; Lundberg et al. 2017; Toews et al. 2019; Delmore et al. 2020, 2020). Despite the complexity of migration, it is noteworthy that in each of these studies, few genes were differentially expressed between migrants and non-migrants. Further, only one has identified a gene that may play a role in bioenergetic capacity or efficiency. Specifically, Toews et al. (2019) found distinct differences in vacuolar protein sorting 13A (VPS13A) between golden-winged and blue-winged warblers that vary in migratory distance, with the former moving from North America to South America and the latter flying from North America to Central America. While Toews et al. (2019) noted that VPS13A has roles in lysosomal degradation and lipid transfer to the ER and speculated that this gene could mitigate differences in oxidative damage between species, a more recent paper suggests that VPS13A also plays a role in mitochondrial morphology and mitophagy (Yeshaw et al. 2019).

Exercise regulates the PTM for numerous proteins that alter the efficiency of OXPHOS, particularly those associated with mitochondrial dynamics (Tanaka et al. 2020). Given their putative role in improving the ATP production capacity of skeletal muscle in response to exercise, PTMs are also likely to be common in proteins modifying OXPHOS with migration in birds. PTMs can reversibly or permanently modify the configuration, localization, and functional capacity of proteins following translation, contributing a greater diversity of protein phenotypes than there are genes encoding for them (Baer and Millar 2016). As described in the thirteen-lined ground squirrel above (Mathers and Staples 2019), such changes can be seasonal and may have dramatic impacts on the capacity to produce ATP. It will be interesting to see if PTM to proteins that modify ATP production correlate with the upregulation of OXPHOS that occurs prior to the onset of migration, as observed in White-crowned Sparrows. Given that PTMs are thought to represent a faster mechanism for the generation of phenotypic diversity than changes in the genome (Bradley 2022), PTMs could be important targets for up- and down-regulating bioenergetic capacity in migratory songbirds.

Conclusions

Further research into mitochondrial respiration coupled with the evaluation of variables that have the potential to up-or-down-regulate OXPHOS, such as mitochondrial complex activity, mitochondrial morphology and dynamics, and supercomplex abundance, will be valuable in deducing the mechanisms that underlie the capacity for and lability of avian migration. I hope this review will encourage others to consider quantifying OXPHOS and the mechanisms that underlie differences in OXPHOS performance when evaluating flexible variation in the energetic demands of organisms. Given the complexity of many of the physiological measurements described herein, this is an ideal topic for interdisciplinary collaboration, both in the completion of the physiological measurements described herein and evaluated the genomic basis for the patterns observed. Such comparisons will provide a more complete picture of how avian migrants and animals within other taxonomic groups support and evolve the fascinating array of energetic strategies that we observe today.

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Conflict of interest

The author declares no conflicts of interest.

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