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REVIEW

How does density of the inner mitochondrial membrane influence mitochondrial performance?

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Abstract

Our current understanding of variation in mitochondrial performance is incomplete. The production of ATP via oxidative phosphorylation is dependent, in part, on the structure of the inner mitochondrial membrane. Morphology of the inner membrane is crucial for the formation of the proton gradient across the inner membrane and, therefore, ATP synthesis. The inner mitochondrial membrane is dynamic, changing shape and surface area. These changes alter density (amount per volume) of the inner mitochondrial membrane within the confined space of the mitochondrion. Because the number of electron transport system proteins within the inner mitochondrial membrane changes with inner mitochondrial membrane area, a change in the amount of inner membrane alters the capacity for ATP production within the organelle. This review outlines the evidence that the association between ATP synthases, inner mitochondrial membrane density, and mitochondrial density (number of mitochondria per cell) impacts ATP production by mitochondria. Furthermore, we consider possible constraints on the capacity of mitochondria to produce ATP by increasing inner mitochondrial membrane density.

ATP synthase; cristae; intermembrane space; matrix; oxidative phosphorylation

INTRODUCTION

Mitochondria play a formative role in the evolution of complex life (1). Most energy in eukaryotic organisms stems from the use of adenosine triphosphate (ATP), which serves as the energy currency of the cell and is formed within mitochondria. The relative capacity of cells and tissues to produce ATP is dependent on the number of mitochondria within each cell and the capacity of the mitochondrial population to use oxygen and convert adenosine diphosphate (ADP) to ATP. This process, known as oxidative phosphorylation, is driven by chemiosmosis where the transfer of electrons by electron transport system (ETS) complexes facilitates the movement of ions across the semipermeable inner mitochondrial membrane (IMM). The movement of ions into the intermembrane space (IMS) forms a proton gradient (i.e., the proton motive force, pmf) that ultimately powers the phosphorylation of ADP at ATP synthase (2). This process is influenced by the availability of oxygen and electron-donating substrates, the enzymatic activity and the number of ETS complexes, and the morphology of mitochondria (e.g., Ref. 3).

To understand variation in mitochondrial function, investigators commonly measure mitochondrial density (number of mitochondria per cell) and coupling, relative number and relative enzymatic activity of complexes, relative abundance of proteins that regulate mitochondrial biogenesis and repair, relative abundance of markers that contribute to or directly indicate mitochondrial damage, among other measures (4–7). An increasing number of studies have also considered markers of mitochondrial fission and fusion, known as mitochondrial dynamics (e.g., Ref. 8). With a few notable exceptions (e.g., Refs. 9, 10), few studies have quantified the amount of IMM within mitochondria (hereafter referred to as "density" of the IMM) in an effort to understand variation among individual organisms.

Provided ETS complexes are embedded within the IMM (11), density of the IMM within a mitochondrion can influence the capacity of mitochondria to consume oxygen, pump protons into the IMS, and produce ATP. Changes to density of the IMM also directly impact mitochondrial morphology, including volume of the mitochondrial matrix, IMS, and the intracristal space (Fig. 1). In turn, these changes can influence mitochondrial performance. For the purposes of this review, mitochondrial performance is defined as function of the ETS, including electron transport, proton pumping, oxygen reduction, and ATP production. As such, an increasing number of studies have shown that IMM density can change in response to either an increase or a decrease in energetic demand or energetic strain on the mitochondrion (e.g., Refs. 9, 10, 12, 14). As the IMM expands, cristae formation appears to be initialized through the bending of the IMM by ATP synthase (15) and a change in phospholipid distribution (16), as described in *Formation of Mitochondrial Cristae*. This change in IMM density and number of cristae provides the space needed for

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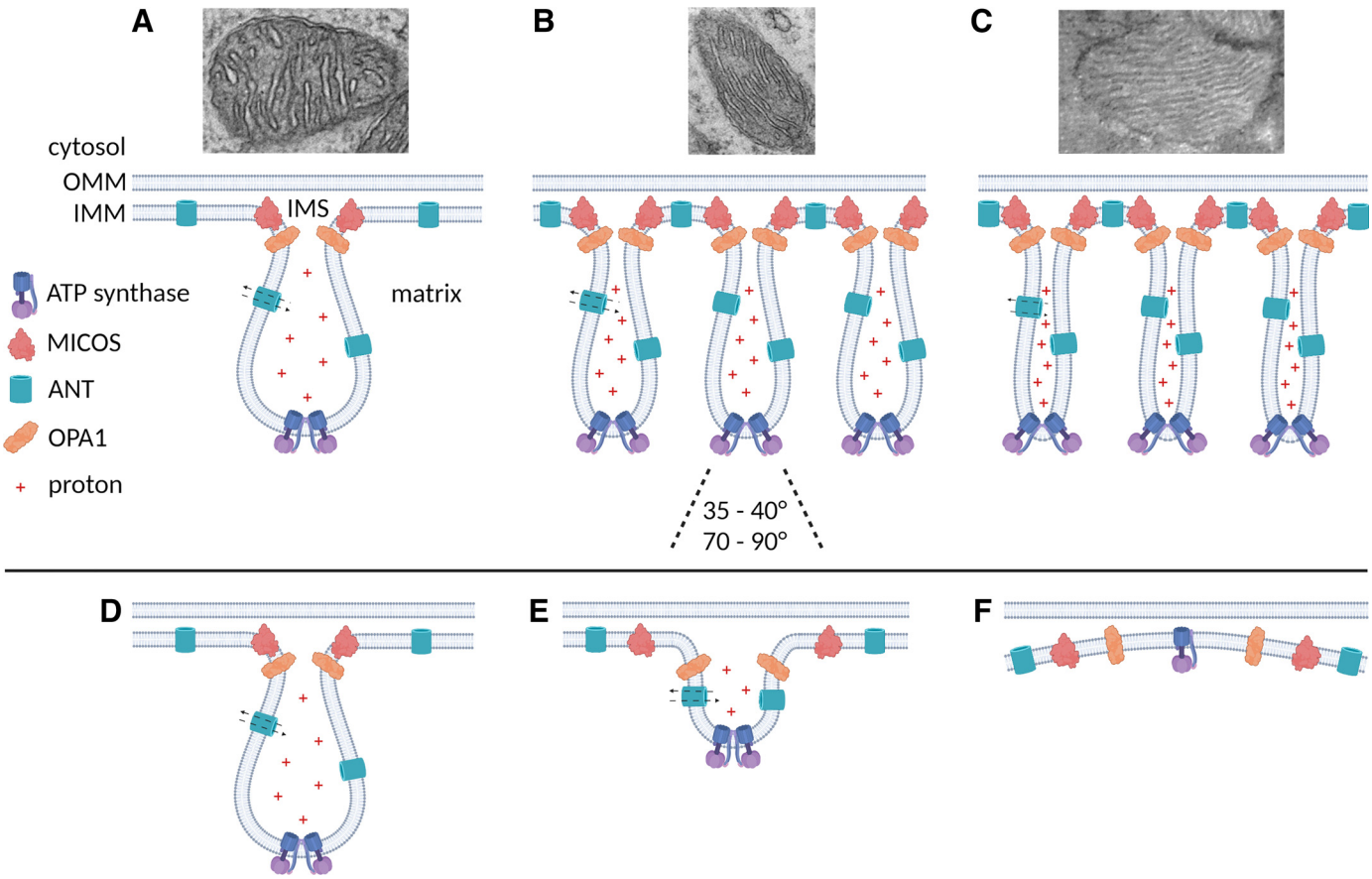

Figure 1. Illustration of how crista morphology changes with density of the inner mitochondrial membrane (IMM) under increased energetic demand (*A–C* and corresponding micrographs) and aging (*D–F*). *A–C* are predictions based on Heine et al. (12). Wide, distantly spaced cristae and/or a lower density of IMM in *A* may result in a lower concentration of protons (reduced proton motive force), altered crista junctions associated with OPA1 and MICOS (depending on size of the matrix), and fewer adenine nucleotide translocase (ANT) per unit volume within the mitochondrion. Here, fewer ANT exist within the mitochondrion (less IMM) to transport less ADP/ATP. Narrow, densely packed cristae in *B* may result in a greater concentration of protons (increased proton motive force), altered crista junctions (depending on size of the matrix), and more ANT per unit volume within the mitochondrion. *B* is predicted to occur as energetic demand increases. Here, more ANT exist within the mitochondrion (more IMM) to transport more ADP/ATP. Cristae that are too narrow at an exceedingly high density of IMM in *C* may result in decreased mitochondrial performance if the mitochondrion exceeds its optimal proton concentration and the production of reactive oxygen species increases significantly. Transition from *A* to *C* will fundamentally change the ratios of IMM to outer mitochondrial membrane, intermembrane space, and matrix, altering the ATP-producing capacity of the mitochondrion—provided the overall volume of the mitochondrion (i.e., enclosed within the outer membrane) remains constant. *D–F* is based on Daum et al. (13). *D* represents the standard morphology of cristae where ATP synthase are associated as dimers and crista junctions are intact. *E* represents the early stages of mitochondrial aging where crista junctions begin to disassemble, and ATP synthase complexes exist along shallow ridges as cristae recede. *F* represents complete vesiculation of the IMM as ATP synthase dissociate into monomers and the IMM becomes concave. Micrographs are of mitochondria within myocytes of *Tigriopus californicus* copepods (*n* = three copepods). The micrograph in *C* is from Heine et al. (12) Supplemental Material S3. Created with BioRender.com.

the cell to increase the number of ETS complexes present in the organelle, therefore forming a proton gradient within the new cristae. We propose that these changes in density of the IMM alter the capacity of mitochondria to produce ATP by changing the number of ETS complexes within the mitochondrion and altering the volume of IMS relative to volume of the mitochondrial matrix. Consequently, changes to density of the IMM can represent an adaptive response to changing energetic demand (17).

This review outlines support for the hypothesis that variation in density of the IMM influences performance of the ETS, and as a result, the capacity of mitochondria to produce ATP. We start by outlining the formation of mitochondrial cristae via ATP synthase dimers and phospholipid composition. We then describe the relationship between IMM density and capacity to build and maintain a pmf. Last, we address possible associations between density of the IMM and mitochondrial density. We conclude with suggestions for how we can better understand the roles of density of the IMM in mitochondrial energetic performance. We provide new hypotheses supported by existing evidence in the field and propose additional experiments to amend the gaps presented in the literature. Finally, the majority of ideas put forth focus primarily on a single cell type, myocytes, given most of the literature and resulting evidence stems from research on muscle cells. Literature not focused on myocytes is identified as such.

FORMATION OF MITOCHONDRIAL CRISTAE

The ability of mitochondria to produce ATP is directly dependent on the accumulation of protons within the intracristal space. This includes the volume of cristae, which can

influence the pmf, and how many cristae form within a single mitochondrion (i.e., density of IMM). Therefore, our understanding of variation in cristae morphology and density of the IMM is critical to understanding variation in mitochondrial function.

ATP synthase, or complex V, is best known for its role in phosphorylating ADP to ATP across the crista membrane, but it also plays a vital role in cristae formation. ATP synthases first form as singular proteins (monomers) within the IMM, which then combine with another ATP synthase to create a dimer. Because of long-range attractive forces, these dimers spontaneously associate to form rows that exist along the curved edges of cristae (15), forming larger structures made of four ATP synthases (tetramers). The degree at which these dimers are angled, bending the IMM to form cristae compartments, likely influences the number of cristae that can form within the confined space of the mitochondrion, and thus, the density of IMM (Fig. 1).

The angles formed by ATP synthase can directly impact the width and volume of IMS and, therefore, the concentration of protons within the intracristal space (18). The angle formed by ATP synthase dimers appears to vary by taxa, tissue type, and even within an individual organism over time (19–21). Both wide-angle dimers (70°–90°, shown in algal mitochondria) and small-angle dimers (35°–40°, shown in *Bos taurus* heart mitochondria) have been characterized (19, 22, 23). It is unclear whether these angles change in direct response to changes in energetic demand; however, wide-angle dimers bend the IMM more, leading to a more narrow IMS, which can affect pmf.

The formation of ATP synthase dimers and bending of the IMM are critical for the performance of the ETS and cellular function (Fig. 1). Reports of monomeric ATP synthase do not eliminate mitochondrial function altogether but are associated with lower mitochondrial membrane potential and organism growth in yeast (24). The formation of large- or small-angle dimers seems to depend on the tissue type, but the details of how the angle of ATP synthase dimers influences mitochondrial function requires further investigation. Although there are few molecular tools available to manipulate mitochondrial DNA products in vivo (but see Refs. 25 and 26), the effects of ATP synthase dimer angle on the energetic capacity of mitochondria can be deduced by comparing organisms and tissue types that vary significantly in energetic demand. Comparing organisms allows for identifying patterns of natural biological variation, which would be beneficial in understanding when a large- or small-angled dimer would provide energetic benefits to an organism. Laboratory techniques such as quantification of ATP synthase dimer angles using cryo-EM and measures of proton concentrations across the IMM using confocal microscopy can tell us the extent to which dimer angle is related to pmf and density of the IMM.

The distribution of phospholipids can also contribute to bending of the IMM. Cardiolipin (CL) is one of the main phospholipids involved in IMM morphology (27). Although not the most abundant phospholipid within the IMM, only making up ~18% of phospholipids, CL provides elasticity at key bending junctions in the IMM, allowing IMM to invaginate (28, 29). In particular, the loss of CL synthase, the enzyme that synthesizes CL, resulted in a reduction in the number of extended dimer rows, increased scattered dimer orientation, and mitochondrial dysfunction in skeletal muscle of *Drosophila* (30). Joubert and Puff (16) outline how CL remodeling in Barth syndrome (associated with mutations in the tafazzin gene) results in abnormal cristae organization and detriments to oxidative phosphorylation. In addition, Bashir et al. (31) showed that adults and adolescents with Barth syndrome have significantly lower ATP production via oxidative phosphorylation in calf muscle than individuals without Barth syndrome. This study also showed lower oxygen uptake during peak exercise ($\dot{V}O_{2peak}$) in both groups compared with control groups. As such, genetic mutations to the genes responsible for the expression of phospholipids lead to changes in the morphology of cristae and density of the IMM (32).

Another important observation regarding CL is that the phospholipid is also present in the plasma membrane of bacteria, where it is critical for ATP production (33). Given that CL is observed as a critical component in both bacterial and mitochondrial membranes, it is likely an important, conserved aspect of membrane structure and function. This evidence, in conjunction with mitochondrial dysfunction caused by reduced CL, lends weight to the idea that structure, and therefore density, of the IMM likely have critical roles in function of the ETS.

In addition to CL, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) also play critical roles in the morphology and function of cristae. For instance, PC promotes membrane fluidity and maintains a tubular morphology leading to the formation of planar bilayers of the inner and outer membranes. PE, however, is conical in shape and forms tension within the inner and outer membranes, influencing membrane fusion and protein movement (29). Because of its role in the formation of cristae, PE is found in larger quantities within the IMM than the outer membrane (29, 34). Although the ratio of phospholipids to proteins is lower within the IMM than the outer membrane (29), further research is needed to determine the extent to which this ratio changes with density of the IMM.

Notably, PC is found in a greater percentage on the matrix-facing side of the IMM bilayer, whereas PE and CL are both found on the crista lumen side of the IMM bilayer. It has been proposed that the biased distribution of these proteins within the IMM is to allow for stability to the curvature of the crista membrane (27). Further research is needed to understand the extent to which regulating these phospholipids impacts cristae morphology and overall mitochondrial function.

The MICOS (mitochondrial contact site and cristae organizing system) complex and OPA1 (optic atrophy 1) also play important roles in cristae organization and dynamics (Fig. 1). The MICOS complex is composed of seven subunits and serves to stabilize crista junctions and form contacts between the inner boundary membrane and outer membrane. It is also involved in the regulation of cristae biogenesis, fission, and fusion. See Anand et al. (35) for a recent, extensive review of the roles of the MICOS complex in cristae dynamics. OPA1 plays a critical role in cristae biogenesis and mitochondrial fusion (36). Variations in the short and long forms of the complex are proposed to influence the width of crista junctions. In addition, the disassembly of OPA1 leads to the release of cytochrome *c* through the widening of crista junctions and, ultimately, leads to apoptosis (35).

DENSITY OF THE IMM AND PROTON MOTIVE FORCE

The IMM is populated by complexes I–V of the ETS that contribute to the transfer of electrons from NADH and $FADH_2$ at complexes I and II, respectively, to complex IV where oxygen is reduced to water in the mitochondrial matrix. This transfer of electrons pumps protons across the IMM and into the IMS by complexes I, III, and IV. The protons pumped into the IMS flow down their electrochemical gradient and through ATP synthase to phosphorylate ADP to ATP (37). Aside from increasing the rate of respiration (38), mitochondria appear to upregulate or maintain their function by increasing density of the IMM (9, 10, 12, 39). By increasing density of the IMM, the mitochondrion may be able to support more ETS complexes within a given mitochondrial volume (i.e., newly formed crista membrane becomes populated by newly formed ETS complexes). Although such morphological changes have the potential to meet the increasing energetic demands of the cell (3), there are likely limits to how much IMM can exist within a mitochondrion without decreasing mitochondrial function/performance of the ETS.

Theoretically, a disproportionate change in IMM surface area relative to mitochondrial volume will impact volume of the IMS and volume of the matrix. A change in volume of the intracristal space is expected to alter the effort required to maintain the pmf and, thus, the capacity of the mitochondrion to produce ATP (see Refs. 40–42). The number of protons required to maintain the pmf will depend on *1*) proton consumption by ATP synthase, *2*) proton leak of the IMM, and *3*) volume of the intracristal space. Kilarski et al. (39) showed that in response to a 6-wk cold (5°C) acclimation, crucian carp (*Carassius carassius*) displayed a nearly twofold increase in IMM area relative to mitochondrial volume in muscle tissue. Because the oxygen content of water increases while diffusion rates across membranes and through solutions decrease at lower temperatures, it is hypothesized that the change in IMM density increases oxygen delivery when diffusion rates are low (39). We predict that this effect also allows mitochondria to concentrate protons within the IMS, reduce proton diffusion distance, and maintain oxidative phosphorylation. Furthermore, increases in IMM area may allow mitochondria to maintain the pmf if they become damaged due to increased oxidant exposure as described by Heine at al. (12).

In contrast, an exceedingly high density of IMM could have several negative impacts on mitochondrial performance, such as more diffusion bottlenecks and reduced flux of ATP synthase (43). When IMM density is exceedingly high, a high density of ETS complexes and limited IMS could result in the mitochondrion quickly exceeding its optimal proton concentration. When this occurs, ETS complexes can no longer efficiently move new protons into the IMS, and electrons can back up within ETS complexes I–III instead of being passed to oxygen as the terminal electron acceptor (40). This backup can allow electrons to escape the ETS and generate reactive oxygen species (ROS). Therefore, a very high density of IMM is predicted to be associated with low mitochondrial function and greater ROS production, and as a result, potentially an increase in oxidative damage. Alternatively, an increase in IMM density has been shown to occur alongside a decrease in ROS production when OPA1 is overexpressed and cristae are narrow (44). This may be the case in humans when OPA1 is lower in individuals with type 2 diabetes compared with athletes (45). Mitochondrial capacity could be reduced because a reduction in matrix volume may decrease the capacity of the mitochondrion to support the citric acid cycle which produces the electron-donating substrates necessary to power the ETS. To test these ideas, experiments can be performed which alter energetic demand while measuring mitochondrial behavior and morphology, along with mitochondrial or whole animal performance.

An additional change that may occur with an increase in density of the IMM may be an increase in the presence of adenine nucleotide translocase (ANT) within individual mitochondria, which is necessary for a proper functioning ETS. ANT exists within both the inner boundary membrane and crista membrane (43, 46, 47) and may increase when density of the IMM increases (Fig. 1, *A–C*). As reviewed by Mannella et al. (48), proapoptotic treatment alters inner membrane morphology and influences the organization of ANT. If more cristae form to meet increasing energetic demands of the organelle, that demand may be met by a greater influx of ADP and efflux of ATP, which can be managed by more ANT transporters within the inner boundary and crista membranes. Such an increase in transport may be difficult to implement (and decrease mitochondrial function) in mitochondria with fewer cristae when the demand for ATP is high but the surface area of IMM is insufficient to transport metabolites for oxidative phosphorylation.

As density of the IMM increases, the ratio of outer membrane to IMM should decrease if the size of the mitochondrion remains constant. As a result, there is likely a point where the transport of substrates into the mitochondrion becomes inefficient to support oxidative phosphorylation. Empirical evidence for a limit to the benefits of increased IMM density on respiratory performance comes from recent work by Heine et al. (12). Heine et al. showed that when *Tigriopus californicus* copepods are exposed to ultraviolet (UV) radiation (0.5 W/m^2), the metabolic rate of individual copepods was maintained at 3 h of UV irradiation but decreased after 6 h of exposure (0.13 ± 0.09 mmol O_2/L/15 s/mm body length for the control, 0.15 ± 0.06 at 3 h of exposure, 0.08 ± 0.05 at 6 h of exposure). Subsarcolemmal mitochondria within the copepod myocytes displayed an increased IMM density in both irradiation treatments (42.1 ± 3.76 intersects/μm^2 for the control, 69.8 ± 15.7 at 3 h of exposure, 81.9 ± 18.8 at 6 h of exposure). This suggests that more IMM may be a proximate response to increased oxidative stress from UV light but may ultimately fail to maintain metabolic rate at exceedingly high levels of UV radiation. Accordingly, increases in the density of the IMM can alter respiratory performance depending on the ratio of IMM to the aforementioned traits (Fig. 1, *A–C*).

ASSOCIATIONS BETWEEN CRISTAE DENSITY AND MITOCHONDRIAL DENSITY

Increased mitochondrial density may support some of the most energetically demanding aspects of animal performance, such as flight. Mathieu-Costello et al. (49), for example, found that relatively high mitochondrial densities per muscle fiber, upward of 39% of the fiber volume, are typical in

the supracoracoideus (the muscle supporting the upstroke) in hummingbirds (*Selaphorus rufus*). Furthermore, the location of mitochondria within cells also influences mitochondrial function. For example, mitochondria in the muscle of high-altitude deer mice localize to the region under the sarcolemma adjacent to capillaries. This behavior reduces the distance for oxygen diffusion (50) and potentially increases the ability of mitochondria to produce the pmf required to be distributed to the rest of the mitochondrial network (51, 52). Interestingly, recent work shows that increases in density of the IMM may accompany, or occur in place of, increases in mitochondrial density (e.g., Ref. 10).

Increasing density of the IMM may be an alternative means for mitochondria to meet increasing energetic demands in addition to increasing mitochondrial density. Nielsen et al. (10) found that while IMM density and mitochondrial density increased in human skeletal muscle following long-term endurance training but also, cristae density (R^2 = 0.58) in particular was a better predictor of $\dot{V}O_{2max}$ than mitochondrial density (R^2 = 0.41). Endurance-trained soccer players, for instance, had an average IMM density of 31.6 ± 1.8 $\mu m^2 \cdot \mu m^{-3}$ in the leg muscle (musculus vastus lateralis) compared with recreationally active men of the same age with an IMM density of 25.1 ± 1.1 $\mu m^2 \cdot \mu m^{-3}$. In addition, the muscle mitochondrial volume density of endurance-trained athletes was 110% higher than that of sedentary individuals. A similar observation was made with increases in both density of the IMM (see *Density of the IMM and Proton Motive Force* for estimates) and mitochondrial density in myocytes when *T. californicus* copepods were exposed to varying levels of UV radiation (see Ref. 13; 0.75 ± 0.43 mitochondria/μm^2 for the control, 2.13 ± 1.20 at 3 h of exposure, 1.89 ± 1.01 at 6 h of exposure). However, the effect sizes in this study were consistent between treatment groups for both mitochondrial density and density of the IMM in copepods. These results would be expected if mitochondrial density and morphology work in concert to influence metabolic rate. In addition to the work by Nielsen et al. (10) in humans, Suarez et al. (53) suggest that a greater IMM surface area in the skeletal muscle of hummingbirds could lead to a higher $\dot{V}O_{2max}$ compared with mammals, although these data are not directly correlated.

The aforementioned studies demonstrate how the function of the ETS can be influenced by numerous mechanisms including, but not limited to, increases in mitochondrial density and density of the IMM. However, other changes such as an increase in capillary density, the location of mitochondria near the cell sarcolemma (54), and the location of mitochondria near nuclei and capillary beds (52), likely play formative roles in both mitochondrial and cellular performance. Further work is needed to determine the extent to which associations may exist between mitochondrial density and density of the IMM to influence mitochondrial performance.

CONCLUSIONS AND FUTURE DIRECTIONS

The fields of physiological ecology and mitochondrial biology have made significant strides in understanding variation in whole animal performance (e.g., Ref. 55) and how mitochondrial morphology impacts mitochondrial performance (e.g., Ref. 3), respectively. By integrating theory and techniques common to these fields, we will gain a better understanding of the mechanisms responsible for the many fascinating and diverse adaptations to energetic challenges that have evolved among eukaryotes. Density and morphology of the IMM influence variation in ATP production within mitochondria; however, few studies have evaluated the role of change in IMM density and its relation to other mitochondrial traits under different energetic constraints. This review outlines our current understanding of these relationships as a crucial step toward understanding variation in mitochondrial performance.

In particular, we reviewed how mitochondrial performance is influenced by *1*) the relationship between density of the IMM and other morphological traits of the mitochondrion, *2*) bending of the IMM by ATP synthase and phospholipids, and *3*) possible relationships between density of the IMM and mitochondrial density. The aforementioned traits interact concurrently to influence oxygen consumption, ATP production, and ROS production within individual and populations of mitochondria. Last, few studies (e.g., Refs. 12, 43) have evaluated the limitations and possible detrimental effects of exceedingly high levels of IMM density. Such work shows that although increasing density of the IMM may be sufficient to influence function when mitochondria are compromised, or to increase oxygen consumption and/or ATP production under increased energetic demand, there is likely a point where exceedingly high levels of IMM density decrease mitochondrial performance due to a significant decrease in matrix volume and an increase in ROS production.

To further understand how density and morphology of the IMM can influence mitochondrial function and, therefore, whole animal performance, studies should include microscopy alongside measures of mitochondrial physiology and a relevant measure of organ or organism capacity. This approach may initially be most informative by investigating differences in mitochondrial phenotypes between extreme life history traits and in response to environmental change. For example, mitochondrial morphology and respiratory capacity can be measured in migrating and nonmigrating birds, in animals subject to extreme differences in temperature, and between protists and complex animals. Migrating birds are expected to maintain flight and, thus, sustain a high metabolic rate and mitochondrial respiratory performance for longer periods than nonmigrating birds (56), especially those that undergo nonstop, transoceanic migration. It is not clear what is different about the mitochondrial morphology and IMM structure of birds that must sustain high ATP production over long periods. As previously mentioned, changes to density and morphology of the IMM may play a significant role in metabolic plasticity among ectotherms; however, this field of inquiry is scarce. The respiration rates of crustaceans such as copepods increase significantly with temperature (e.g., Ref. 38), but the proportion of this increase that is due to increases in enzyme activity versus changes to morphology of the IMM is unknown. It is also prudent to understand how density and morphology of the IMM differ between exceedingly small eukaryotes such as protists and larger eukaryotes, including plants, animals, and fungi.

Understanding differences in mitochondrial morphology under different energetic challenges may begin to shed light on adaptive mechanisms that have evolved in different eukaryotes. Although we assume that morphological responses to energetic demand are under selection, the genetic basis of

variation in mitochondrial morphology is poorly understood. We predict that the energetic optima for each tissue will vary by taxa and, thus, the combination of mitochondrial and physiological solutions and reaction norms for each morphological variable will also vary, suggesting there will not be a common mechanism to maximize energetic capacity in all circumstances.

Perspectives and Significance

Future work may benefit from investigating variation in density and morphology of the IMM to understand variation in mitochondrial performance. Doing so can further our understanding of why we observe large amounts of variation in mitochondrial function between organisms, tissues, and life-history strategies (17). We hope this work encourages the integration of mitochondrial behavior and morphology with studies of whole animal performance in physiological ecology and evolutionary biology.

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Figure 1 was created from BioRender.com.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.B.H. conceived the review and wrote the original draft; K.B.H., H.A.P., and W.R.H. edited and revised the manuscript; K.B.H., H.A.P., and W.R.H. approved the final version of the manuscript.

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