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Permalink
https://escholarship.org/uc/item/9vg7705c

Journal
The Journals of Gerontology Series A, 76(3)

ISSN
1079-5006

Authors
Gonzalez-Armenta, Jenny L
Li, Ning
Lee, Rae-Ling
et al.

Publication Date
2021-02-25

DOI
10.1093/gerona/glaa299

Peer reviewed

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Journals of Gerontology: Biological Sciences
cite as: *J Gerontol A Biol Sci Med Sci*, 2021, Vol. 76, No. 3, 434–439
doi:10.1093/gerona/glaa299
Advance Access publication December 30, 2020

OXFORD

Research Report

Heterochronic Parabiosis: Old Blood Induces Changes in Mitochondrial Structure and Function of Young Mice

Jenny L. Gonzalez-Armenta, BS,[1,2,3] Ning Li, MD,[4] Rae-Ling Lee, BS,[2,3] Baisong Lu, PhD,[5] and Anthony J. A. Molina, PhD[6,*]

[1]Section on Molecular Medicine, Department of Internal Medicine, Wake Forest School of Medicine, Winston-Salem, North Carolina. [2]Section on Gerontology and Geriatrics, Department of Internal Medicine, Wake Forest School of Medicine, Winston-Salem, North Carolina. [3]J Paul Sticht Center for Healthy Aging and Alzheimer's Prevention, Wake Forest School of Medicine, Winston-Salem, North Carolina. [4]Department of Pediatric Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. [5]Wake Forest Institute for Regenerative Medicine, Wake Forest University Health Sciences, Winston-Salem, North Carolina. [6]Division of Geriatrics and Gerontology, Department of Medicine, University of California San Diego School of Medicine, La Jolla.

*Address correspondence to: Anthony J. A. Molina, Division of Geriatrics and Gerontology, Department of Medicine, University of California San Diego School of Medicine, 9500 Gilman Drive, MC 0665, La Jolla, CA 92093-0665. E-mail: ajmolina@health.ucsd.edu

Received: April 30, 2020; Editorial Decision Date: November 12, 2020

Decision Editor: Rozalyn M. Anderson, PhD, FGSA

Abstract

Heterochronic parabiosis models have been utilized to demonstrate the role of blood-borne circulating factors in systemic effects of aging. In previous studies, heterochronic parabiosis has shown positive effects across multiple tissues in old mice. More recently, a study demonstrated old blood had a more profound negative effect on muscle performance and neurogenesis of young mice. In this study, we used heterochronic parabiosis to test the hypothesis that circulating factors mediate mitochondrial bioenergetic decline, a well-established biological hallmark of aging. We examined mitochondrial morphology, expression of mitochondrial complexes, and mitochondrial respiration from skeletal muscle of mice connected as heterochronic pairs, as well as young and old isochronic controls. Our results indicate that young heterochronic mice had significantly lower total mitochondrial content and on average had significantly smaller mitochondria compared to young isochronic controls. Expression of complex IV followed a similar pattern: young heterochronic mice had a trend for lower expression compared to young isochronic controls. Additionally, respirometric analyses indicate that young heterochronic mice had significantly lower complex I, complex I + II, and maximal mitochondrial respiration and a trend for lower complex II-driven respiration compared to young isochronic controls. Interestingly, we did not observe significant improvements in old heterochronic mice compared to old isochronic controls, demonstrating the profound deleterious effects of circulating factors from old mice on mitochondrial structure and function. We also found no significant differences between the young and old heterochronic mice, demonstrating that circulating factors can be a driver of age-related differences in mitochondrial structure and function.

Keywords: Aging, Heterochronic parabiosis, Mitochondria, Mouse model

Mitochondrial bioenergetic decline is a hallmark of the aging process. Studies have reported reduced mitochondrial respiratory capacity in older adults, as indicated by reduced phosphocreatine recovery time and reduced mitochondrial respiration in skeletal muscle (1,2). Multiple studies have shown decreased mitochondrial enzyme activities in skeletal muscle, liver, and heart with increasing age, including: succinate dehydrogenase, citrate synthase, cytochrome c oxidase, and β-hydroxyacyl-CoA dehydrogenase (1,3,4). Reduced mitochondrial DNA copy number and increased number of mitochondrial DNA mutations in these tissues have been associated with age in both animal models and human subjects (4–7). Additionally, reduced mitochondrial content and alterations in mitochondrial dynamics are associated with aging (6). Altogether, these studies indicate that mitochondrial changes are systemic and

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therefore apparent in multiple tissues, including brain, heart, skeletal muscle, and liver (4,8). Yet, little is known about what may mediate the systemic nature of age-related bioenergetic decline.

Heterochronic parabiosis is a surgical technique that involves connecting 2 mice, in this case one old and one young, so that the circulatory systems of the mice become connected to study the systemic effects of aging. Using parabiosis, researchers have demonstrated that circulating factors in blood play a major role in many age-related processes that occur across a variety of tissues. Heterochronic parabiosis in mice has been reported to have positive effects on muscle, liver, brain, and other tissues for the older parabiont (9–14). Recently, it has been shown that old blood has a greater negative effect on young mice and resulted in decreased muscle performance and neurogenesis (10). While it is apparent that heterochronic parabiosis has the potential to modulate function at the cell and tissue level, the effects of circulating factors on mitochondrial bioenergetics have not been examined.

In this study, we used the heterochronic parabiosis model to test the hypothesis that circulating factors present in blood mediate age-related bioenergetic decline in skeletal muscle. We examined mitochondrial content, expression of mitochondrial electron transport chain complexes, and performed high-resolution respirometry of permeabilized skeletal muscle fibers in pairs of heterochronic and isochronic parabionts. To our knowledge, this study is the first to provide direct evidence that circulating factors alone can mediate age-related changes in mitochondrial structure and function.

Materials and Methods

Heterochronic parabiosis

C57BL/6 young (2 months) and old (18–20 months) female mice were connected as old isochronic controls, young isochronic controls, and heterochronic experimental pairs. Parabiosis surgeries were completed as previously described (15).

All muscle tissues were collected from the free-moving hindlimb opposite of the parabiosis surgery to eliminate any effects of partial immobilization potentially caused by the surgery. For western blot analysis, mice were connected for 35 days before experiments were performed. These mice included 10 old controls (5 pairs), 12 young controls (6 pairs) and 11 young and 11 old heterochronic mice (11 pairs). For high-resolution respirometry analysis, mice were connected for an average of 50 days (range: 40–53 days) before experiments were performed. These experiments included 2 old isochronic controls (1 pair), 4 young isochronic controls (2 pairs), and 6 young and 6 old heterochronic experimental mice (6 pairs). For electron microscopy, mice were connected for an average of 50 days (range: 40–53 days) before experiments were performed and included 2 old isochronic controls (1 pair), 2 young isochronic controls (1 pair), and 2 young and 2 old heterochronic experimental mice (2 pairs). All procedures and protocols were approved by the Wake Forest School of Medicine Animal Care and Use Committee.

Electron Microscopy for Mitochondrial Morphology

Muscle samples were obtained from a subset of mice and the deep or red *gastrocnemius* of 2 old isochronic controls, 2 young isochronic controls, and 4 heterochronic experimental mice were collected. The tissue was fixed with 2.5% glutaraldehyde in 0.1 M Millonig's phosphate buffer (pH 7.3) for a minimum of 1 hour. Subsequently, the samples were washed 3× in buffer and post-fixed with 1% osmium tetroxide in phosphate buffer for 1 hour. The samples were washed 3× in buffer, then dehydrated through a graded series of ethanol for 10 minutes each. For preparation of resin infiltration, the samples were incubated in propylene oxide 2× for 15 minutes each. Finally, the samples were gradually infiltrated with 1:1, 1:2, and pure solutions of Spurr's resin and allowed to cure in a 70 °C oven overnight; 90 nm longitudinal sections were obtained with a Reichert-Jung Ultracut E ultramicrotome, stained with lead citrate and uranyl acetate, and viewed with a FEI Tecnai Spirit TEM operating at 80 kV. Ten images from each sample were obtained with an AMT 2Vu CCD camera. Mitochondrial morphology parameters (total mitochondrial area, average mitochondrial area, and average mitochondrial length) were measured using ImageJ (National Institutes of Health).

Western Blotting

Skeletal muscle samples (*quadriceps femoris*) were obtained from 10 old isochronic controls, 12 young isochronic controls, and 22 heterochronic experimental mice. Whole tissue homogenate was loaded at a concentration of 20 µg total protein per well for separation by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked at room temperature for 1 hour in 5% nonfat dry milk in tris-buffered saline containing 0.1% Tween-20. Blots were probed overnight at 4 °C with primary antibodies (VDAC/Porin, Abcam, ab14734; GAPDH, Abcam, ab9484; Total OXPHOS Rodent WB Antibody Cocktail, Abcam, ab110413) and incubated with the appropriate HRP-conjugated anti-IgG antibody. Antibody-bound protein was detected by enhanced chemiluminescence and quantified by densitometry with ImageJ (National Institutes of Health). GAPDH was used as a loading control for whole tissue homogenate and VDAC/Porin was used to control for differences in mitochondrial content.

Preparation of Permeabilized Skeletal Muscle Fibers for Respirometry

Immediately after animals were euthanized, skeletal muscle tissue (*soleus*) was removed from 2 old isochronic controls, 4 young isochronic controls, and 12 heterochronic experimental mice. Skeletal muscle fibers for high-resolution respirometry were prepared as described previously (16). Briefly, the tissue was placed in ice-cold BIOPS (10 mM Ca-EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM potassium morpholineethanesulfonic acid, 0.5 mM DTT, 6.56 mM $MgCl_2$, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1). Any remaining tendon was removed and the tissue fibers were separated mechanically with sharp angular forceps under magnification, permeabilized with saponin (30 mg/mL) for 30 minutes on ice, and washed with buffer Z (105 mM K-MES, 30 mM KCl, 1 mM EGTA, 10 mM K_2HPO_4, 5 mM $MgCl_2 \cdot 6H_2O$, 0.5 mg/mL BSA, pH 7.4) for 15 minutes on ice before analysis.

High-Resolution Respirometry of Permeabilized Skeletal Muscle Fibers

Approximately 0.5–0.9 mg of tissue was added to each chamber and steady-state rate of respiration measurements were obtained after every substrate addition and expressed as picomoles per second per milligram of tissue. Each sample was run in duplicate. High-resolution oxygen flux measurements were measured in 2 mL buffer Z containing 20 mM creatine and 25 µM blebbistatin to inhibit contraction (17). This injection protocol was completed as follows: 2 mM malate, 4 mM ADP, 5 mM pyruvate, 10 mM glutamate, 10 mM succinate, 10 µM cytochrome c to test for mitochondrial membrane integrity, 2 additions of 0.25 µM FCCP followed by a

titration of 0.5 µM FCCP to obtain maximal ETS capacity, 0.5 µM rotenone, and 5 µM antimycin-A. Respiration measurements are summarized in Supplementary Table 1.

Statistical Analysis

For analysis of the electron microscopy and high-resolution respirometry data, normality was assessed by Shapiro–Wilk tests and homogeneity of variance was assessed by Levene's test before 1-way ANOVAs were performed. Significant ANOVA results were followed by a post hoc analysis to determine which groups were statistically different using Tukey's studentized range test. Significance between groups was defined as p <.05. All analysis was performed using SAS Enterprise Guide version 7.12 (SAS Institute Inc.).

Results

Old Blood Induces Changes in Skeletal Muscle Mitochondrial Morphology

To characterize the effects of heterochronic parabiosis on skeletal muscle mitochondrial structure, we examined overall mitochondrial morphology using electron microscopy. Representative images from each experimental group are shown in Figure 1A. We measured the total mitochondrial area (Figure 1B), average mitochondrial area (Figure 1C), and average length of mitochondria (Figure 1D) in each image field. Our results indicate that young heterochronic mice had significantly lower total mitochondrial area, and significantly lower average mitochondrial area compared to young isochronic controls. We did not observe any significant differences between young heterochronic mice and old heterochronic mice. Additionally, there was no significant improvement in old heterochronic mice compared to old isochronic controls.

Old Blood Induces Changes in Complex IV Expression in Skeletal Muscle

We performed western blots to examine the expression of proteins that are representative of mitochondrial electron transport system complexes in skeletal muscle (Figure 2). We found that VDAC/Porin expression was higher in the young isochronic controls compared to old isochronic controls. While not significant, these suggest differences in mitochondrial content. Therefore, we controlled for these differences in mitochondrial content mathematically when comparing differences in mitochondrial complex expression. Our results indicate that the expression of complex IV had a similar pattern as the electron microscopy data: young heterochronic mice had an overall trend for lower expression of complex IV than young isochronic controls, there was no difference between young and old heterochronic mice, and we did not observe a significant improvement in complex IV expression in old heterochronic mice when compared to old isochronic controls.

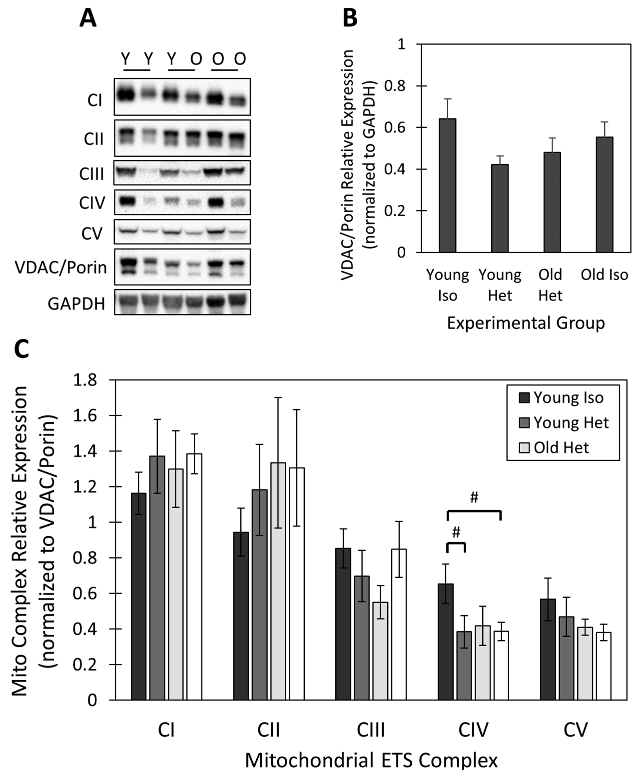

Figure 2. Western blot analysis of skeletal muscle from heterochronic parabiosis mice. (**A**) Representative western blots of mitochondrial complexes in skeletal muscle tissue homogenate images from young isochronic controls, young mice from heterochronic pairs, old mice from heterochronic pairs, and old isochronic controls. (**B**) Relative protein expression of VDAC/Porin in skeletal muscle tissue homogenate from young isochronic controls, young mice from heterochronic pairs, old mice from heterochronic pairs, and old isochronic controls. Data are presented as means ± SEM. (**C**) Relative protein expression of mitochondrial complexes in skeletal muscle tissue homogenate from young isochronic controls, young mice from heterochronic pairs, old mice from heterochronic pairs, and old isochronic controls. Data are presented as means ± SEM (#p < .10).

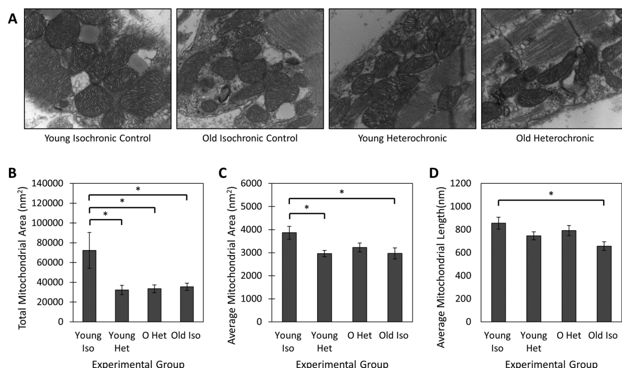

Figure 1. Electron microscopy of skeletal muscle from heterochronic parabiosis mice. (**A**) Representative electron microscopy images from young isochronic controls, young mice from heterochronic pairs, old mice from heterochronic pairs, and old isochronic controls. (**B**) Total mitochondrial area in electron microscopy field. Data are presented as means ± SEM (*p < .05). (**C**) Average mitochondrial area in electron microscopy field. Data are presented as means ± SEM (*p < .05). (**D**) Average mitochondrial length in electron microscopy field. Data are presented as means ± SEM (*p < .05).

Old Blood Induces Changes in Mitochondrial Respiration in Permeabilized Fibers

Young heterochronic mice exhibited lower oxygen flux across all respirometry measurements in permeabilized fibers when compared to young isochronic controls (Figure 3). Young heterochronic mice had statistically significantly lower mitochondrial respiration with complex I, complex I + II, and max ETS and a trend for lower complex II respiration when compared to young isochronic controls.

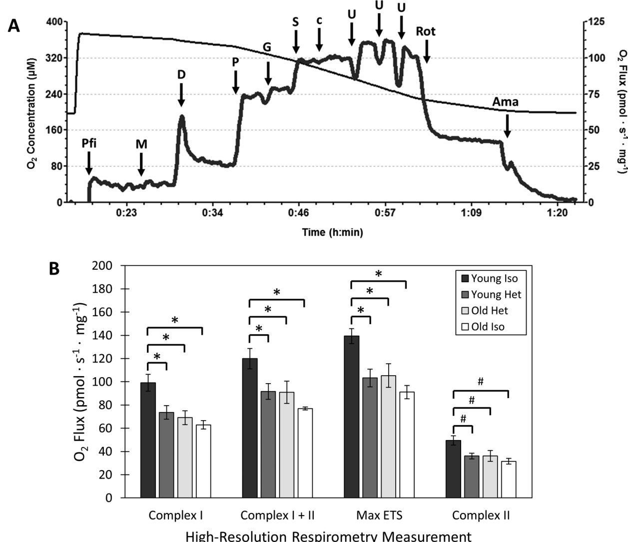

Figure 3. High-resolution respirometry of skeletal muscle from heterochronic parabiosis mice. (**A**) Representative trace of high-resolution respirometry of permeabilized *soleus* fibers with a SUIT protocol (Pfi = permeabilized fibers, M = malate, D = ADP, P = pyruvate, G = glutamate, S = succinate, c = cytochrome c, U = FCCP, Rot = rotenone, Ama = antimycin-A). (**B**) High-resolution respirometry of permeabilized skeletal muscle fibers. Data are presented as means ± SEM (* $p < .05$ and # $p < .10$).

We did not observe any significant differences in mitochondrial respiration with complex I, complex I + II, max ETS, and complex II between the young and old heterochronic mice groups. Again, we did observe significant improvements in mitochondrial respiration of skeletal muscle fibers from old heterochronic mice when compared to old isochronic controls.

Discussion

Our results from isochronic parabiosis controls are consistent with previous research that examined mitochondrial morphology, mitochondrial enzyme expression, and mitochondrial respiration across animals of different ages. We found reduced total mitochondrial area, reduced average mitochondrial area, and decreased mitochondrial length in the muscle of older animals compared to young animals. Others have reported similar differences in young and old, non-parabiosis, animals with regard to mitochondrial content reported by electron microscopy (6) and by surrogate measures such as citrate synthase activity (1,3). Moreover, older adults have been reported to have smaller, more fragmented mitochondria accompanied by reduced expression of markers of mitochondrial biogenesis and increased expression of markers of mitochondrial fission (18). We found reduced expression of complex IV in skeletal muscle of the old isochronic controls when compared to the young isochronic controls. Our results are consistent with the decrease in expression of complex IV in skeletal muscle in older individuals that have been reported previously (3,4). Other studies have also reported decreased expression of complex I as well as decreased activities of mitochondrial enzymes, such as: succinate dehydrogenase (complex II), citrate synthase, and cytochrome c oxidase (complex IV) with increasing age (3,4,18,19). We also observed decreased mitochondrial respiration in skeletal muscle from old isochronic controls when compared to young isochronic controls. This observation is consistent with previous studies that found decreased mitochondrial function as measured by both phosphocreatine recovery time and skeletal muscle mitochondrial respiration in older individuals (1,2,20–23). Overall, these studies indicate that the effects of age on mitochondrial structure and function are preserved in our isochronic parabiosis controls.

Our results provide striking evidence of the negative impact of circulating factors in old blood on mitochondrial structure and function. Young heterochronic mice had significantly lower total mitochondrial area, significantly lower average mitochondrial area, and a trend for lower average mitochondrial length in skeletal muscle when compared to young isochronic controls. We found a trend for reduced expression of complex IV in young heterochronic mice when compared to young isochronic controls. We also found significantly reduced mitochondrial respiration with complex I, complex I + II, and max ETS and a trend for lower complex II respiration in young heterochronic mice when compared to young isochronic controls. These mitochondrial alterations may underlie age-related differences in muscle function. Other labs have reported that young heterochronic mice exhibit decreased muscle performance, but no differences in muscle regeneration or fibrosis after muscle injury compared to young isochronic controls (10,24). Notably, lower skeletal muscle mitochondrial respiration has been associated with lower physical function as measured by muscle strength and gait speed (22). Taken together, these results suggest that altered mitochondrial structure and function is not only a major aspect of age-related decline in skeletal muscle, but that circulating factors can mediate these changes. Furthermore, circulating factors may play a role in the link between mitochondrial respiration and physical function.

We did not observe statistically significant increases in mitochondrial area or length, complex IV expression, or mitochondrial respiration in old heterochronic mice when compared to old isochronic controls. Other labs have reported that old heterochronic mice exhibit improved muscle regeneration and reduced fibrosis after muscle injury, but no differences in muscle performance when compared to old isochronic controls (10,24). Taken together, these results suggest that there is a greater negative impact of circulating factors in old blood on mitochondrial structure and function than can be restored by factors in young blood. Another potential explanation for this observation is that intrinsic properties of muscle from older animals may make these cells resistant to potential benefits of factors found in young blood. It should also be noted that there were a limited number of old isochronic controls and this may have limited our ability to detect differences between these groups.

It is notable that we did not observe any differences in mitochondrial morphology, complex IV expression, or mitochondrial respiration between young heterochronic or old heterochronic mice. When the circulating factors between young and old are mixed by heterochronic parabiosis, mitochondrial structure and function are similar between the parabiosed animals. Other labs have reported a similar responses in heterochronic mice with muscle regeneration and muscle performance (10,24). These results provide a striking demonstration that circulating factors can be a primary driver of mitochondrial structure and function. Completed studies have examined circulating factors as mediators of age-related differences. Some of these have also proposed specific proteins and metabolites that can impact mitochondrial function. For example, previous work from our lab has found that increased levels of IL-6 were associated with lower mitochondrial respiration in older adults (25). Additionally, other studies have also found that alterations in levels of IL-6 (26,27), BCAAs (28,29), and lipid metabolites (30,31) are associated with changes in mitochondrial function.

To our knowledge, this is the first study to examine the effects of age-associated circulating factors on mitochondrial structure and function using the heterochronic parabiosis model. While this study is limited in size, the results are striking and supported by the use of multiple complementary approaches to examine mitochondrial structure and function, including electron microscopy and high-resolution mitochondrial respirometric profiling to examine changes induced by circulating factors.

This study used multiple muscles for analyses: *gastrocnemius* for electron microscopy, *quadriceps femoris* for western blotting, and *soleus* for high-resolution respirometry. This approach ensured that adequate sample was available for each of our assays. Since we were primarily interested in mitochondrial changes, we chose muscles that were rich in type I muscle fibers that are rich in mitochondria and are highly oxidative (32). A benefit of this approach is that it gives a broader understanding of the mitochondrial changes across muscle tissues; however, there may be region specific effects that we are unable to appreciate. Future studies can include additional tissues for examination to determine if this is muscle-specific or is true across multiple tissue types. A potential limitation of this study is that the tissues were collected from 2 sets of parabiosis mice and there was variability in time that the animals were connected before analysis. A larger set of experiments would be needed in order to establish if there is a time course to the mitochondrial effects of heterochronic parabiosis. Moreover, this study only included female mice and therefore additional studies would be required to examine the effects of sex on these age-related changes in mitochondria. While equal numbers of parabiosis pairs were surgically created at the beginning of the study, attrition across groups led to a limited sample size, especially for the mitochondrial respiration controls and electron microscopy. This small sample size we were left with upon completion of the study may limit our ability to detect smaller changes. Additionally, the old isochronic controls in the mitochondrial respiration and electron microscopy experiments were from 2 animals from the same parabiosis pair; thus, these may not truly represent independent biological replicates. Outcomes that showed statistical trends for differences in this study can be further examined in future investigations.

The overall impact of this study stems from the finding that circulating factors can mediate alterations in mitochondrial structure and function associated with aging. Additionally, our results demonstrating that the heterochronic pairing of animals leads to similar mitochondrial structure and function between young and old parabionts suggest that circulating factors may be sufficient to mediate age-related bioenergetic differences in skeletal muscle metabolism. These results support a growing body of evidence that circulating factors are not only potential biomarkers of aging, but may underlie hallmarks of the aging process. These results will support future studies to identify circulating factors mediating age-related bioenergetic decline and their mechanisms of action.

Supplementary Material

Supplementary data are available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

Funding

This work was supported by National Institute of Health R56 AG057864 (PI: Molina) and by a training grant awarded to J.L.G.-A. (National Institute of Health T32 HL091797, PI: Parks).

Conflict of Interest

None declared.

Acknowledgments

The authors would like to thank Ken Grant and the Cellular Imaging Shared Resource at Wake Forest School of Medicine for their assistance with electron microscopy.

Author Contributions

J.L.G.-A. played a key role in the conceptualization and development of this study, performed all experiments, and played a lead role in data analysis and manuscript preparation. N.L. performed all parabiosis surgeries. R.-L.L. completed analysis of electron microscopy images. B.L. played a key role in the development of this study, provided the animal model, and supervised the parabiosis surgeries. A.J.A.M. provided oversight for the study and was responsible for its design and development, supervised the work performed by J.L.G.-A., provided all of the equipment and reagents for mitochondrial assessments, and worked closely with J.L.G.-A. on data analyses and manuscript preparation. All authors have read and approved the final manuscript.

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