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RESEARCH ARTICLE

High fatty acid oxidation capacity and phosphorylation control despite elevated leak and reduced respiratory capacity in northern elephant seal muscle mitochondria

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ABSTRACT

Northern elephant seals (*Mirounga angustirostris*) are extreme, hypoxia-adapted endotherms that rely largely on aerobic metabolism during extended breath-hold dives in near-freezing water temperatures. While many aspects of their physiology have been characterized to account for these remarkable feats, the contribution of adaptations in the aerobic powerhouses of muscle cells, the mitochondria, are unknown. In the present study, the ontogeny and comparative physiology of elephant seal muscle mitochondrial respiratory function was investigated under a variety of substrate conditions and respiratory states. Intact mitochondrial networks were studied by high-resolution respirometry in saponin-permeabilized fiber bundles obtained from primary swimming muscles of pup, juvenile and adult seals, and compared with fibers from adult human vastus lateralis. Results indicate that seal muscle maintains a high capacity for fatty acid oxidation despite a progressive decrease in total respiratory capacity as animals mature from pups to adults. This is explained by a progressive increase in phosphorylation control and fatty acid utilization over pyruvate in adult seals compared with humans and seal pups. Interestingly, despite higher indices of oxidative phosphorylation efficiency, juvenile and adult seals also exhibit a ~50% greater capacity for respiratory 'leak' compared with humans and seal pups. The ontogeny of this phenotype suggests it is an adaptation of muscle to the prolonged breath-hold exercise and highly variable ambient temperatures experienced by mature elephant seals. These studies highlight the remarkable plasticity of mammalian mitochondria to meet the demands for both efficient ATP production and endothermy in a cold, oxygen-limited environment.

KEY WORDS: Mitochondria, Adaptive thermogenesis, Diving mammals, Hypoxia adaptations

INTRODUCTION

Muscle mitochondria are functionally dynamic organelles capable of responding to bioenergetic demands by regulating the capacity and efficiency of oxidative phosphorylation and selectivity of metabolic substrate utilization. In addition, incomplete coupling of mitochondrial electron transfer to phosphorylation of ADP results in oxygen consumption in the absence of ATP production, reflecting a dissipation of mitochondrial membrane potential independent of proton flux through the ATP synthase. In endotherms, this respiratory 'leak' is thought to contribute significantly to metabolic heat production at the expense of phosphorylation efficiency (van den Berg et al., 2011). However, the extent to which modulation of these processes contributes to phenotypic adjustments of mammalian muscle at the organismal and evolutionary levels remains largely speculative because of a paucity of studies evaluating mitochondrial physiology in species uniquely adapted to extreme bioenergetic stress and environmental conditions.

Diving mammals exhibit the remarkable capacity for extended periods of exercise (foraging) under apneic conditions at a variety of depths and water temperatures. Prominent among these impressive feats is that of the northern elephant seal (NES) [*Mirounga angustirostris* (Gill 1866)], which can dive for up to 2 h without resurfacing for air, reaching depths of up to 2000 m in water temperatures near 2°C (Robinson et al., 2012). Remarkably, NES are thought to rely almost exclusively on aerobic metabolism of lipids to meet energy demands during dives, despite the steady decline in oxygen (O_2) availability (Le Boeuf et al., 1988; Hindell et al., 1992; Le Boeuf et al., 1996; Meir et al., 2009). This is possible because of a number of physiological adjustments that maintain a supply of substrate and O_2 to muscle mitochondria in the absence of exogenous O_2 supply (Meir et al., 2009; Davis, 2014) and high biomechanical efficiency ('gliding') to reduce energy demands during dives (Williams et al., 2000). Paradoxically, muscle mitochondrial content in diving mammals, assessed by electron microscopy and marker enzyme content in Weddell seals, actually decreases as individuals reach maturity, despite evidence for improvements in aerobic exercise capacity to sustain longer and deeper dives in adulthood (Kanatous et al., 2008). The mechanism of this response and the potential involvement of adaptive changes in mitochondrial respiratory function in the diving mammal phenotype are entirely unknown.

The purpose of this investigation was to evaluate the ontogeny and comparative physiology of mitochondrial respiration in NES skeletal muscle. High-resolution respirometry methods were employed under a variety of substrate conditions and respiratory states to examine differences in respiratory capacity, leak, substrate utilization and phosphorylation control between adult NES and human muscle mitochondria, and adaptations that occur as NES reach maturity. This study is the first to comprehensively investigate muscle mitochondrial respiratory function in a diving mammal, and

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reveal novel adaptive changes that complement known aspects of the NES phenotype, and highlight the remarkable plasticity of mitochondrial respiratory function in response to bioenergetic and thermoregulatory stress.

RESULTS

Comparison of adult human and NES muscle mitochondrial function

Mitochondrial respiratory function was determined in permeabilized muscle fiber bundles obtained from the m. longissimus dorsi of adult male NES (N=7) and vastus lateralis of adult male humans (aged 26±3 years; N=4) by high-resolution respirometry. Two substrate-uncoupler-inhibitor titration protocols were run in duplicate to evaluate mass-specific O_2 flux in response to sequential (additive) administration of various respiratory substrates, inhibitors of Complex I [rotenone (Rot)] and/or ATP synthase [oligomycin (Omy)], or the uncoupling protonophore carbonylcyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP). These protocols provide a comprehensive evaluation of muscle respiratory capacity and substrate control during oxidative phosphorylation (OXPHOS; P state), as well as the extent of non-phosphorylating respiratory 'leak' (LEAK; L state) and the enzymatic capacity of the electron transfer system (ETS; E state). A detailed description of the respiration protocols and associated respiratory states generated by the sequential titration of each constituent are provided in Table 1.

Protocol 1

Protocol 1 examined various respiratory states of muscle mitochondria from humans (N=4) and adult NES (N=7) in the presence of fatty acid substrate [palmitoylcarnitine + malate (PalM)]. Fig. 1A illustrates the tissue mass-specific O_2 fluxes from human and NES muscle following the sequential addition of each protocol constituent corresponding to the respiratory states described in Table 1. Across all respiratory states, NES muscle exhibited O_2 fluxes that were 29–46% lower than those obtained from human vastus lateralis, indicating a lower respiratory capacity of NES versus human muscle per milligram of tissue. The greatest relative difference in flux between humans and seals was during LEAK respiration following the addition of fatty acid (PalM) in the absence of adenylates (FAO_L), which was ~46% lower in NES than in human muscle (P=0.04). As expected, adding a saturating concentration of ADP increased respiratory flux 5- to 6-fold in both species, reflecting the maximal OXPHOS capacity using fatty acid oxidation (FAO) as the source of electrons for the respiratory system (FAO_P). The relative difference in flux between humans and seals decreased to 29% in the FAO_P state, which corresponded to a significantly greater index of fatty acid OXPHOS coupling control ($FAO_{P–L/P}$) in seals versus humans (P<0.05; Fig. 1B). This indicates a greater control of FAO capacity by ADP (the phosphorylation system) in NES than in human muscle.

Upon adding pyruvate, respiratory flux increased by 82% in humans and 50% in seals, demonstrating an expected additive effect of pyruvate oxidation on OXPHOS capacity in the presence of fatty acids in both species. The greater relative stimulation of respiration by pyruvate in humans restored the relative difference in O_2 flux between species to ~40% (P<0.001), indicating a much greater responsiveness of human mitochondria to pyruvate. This equates to a 16% greater contribution of FAO to OXPHOS in NES versus human mitochondria when both pyruvate and fatty acids are provided at saturating concentrations (FAO_P/PM_P, P=0.04; Fig. 1C). Subsequent addition of glutamate increased respiratory flux to similar relative extents in both species, indicating a comparable limitation of maximal respiratory flux through Complex I by pyruvate and lipid oxidation pathways in both human and seal muscle (Gnaiger, 2009).

Respiratory responses to the addition of cytochrome *c* (Cyt *c*) were negligible in both human and seal muscle fibers, confirming the preservation of mitochondrial outer membrane integrity in the

Table 1. High-resolution respirometry protocols and associated respiratory flux states assessed in mitochondrial respiration experiments

Protocol constituents (listed in order of titration)	Abbreviation	Respiratory flux state; explanation
Protocol 1		
Palmitoylcarnitine + malate (0.2 mmol l^{-1} + 1 mmol l^{-1})	PalM	FAO_L; LEAK respiration in the presence of high fatty acid concentration, but no ADP
ADP (5 mmol l^{-1})	ADP	FAO_P; Fatty acid OXPHOS capacity, limited by electron flux through ETF
Pyruvate (5 mmol l^{-1})	Pyr	PM_P; Lipid + pyruvate OXPHOS capacity; limited by PM oxidation capacity
Glutamate (10 mmol l^{-1})	Glut	CI_P (also PGM_P); Complex I-supported OXPHOS capacity (+ fatty acids)
Cytochrome *c* (10 µmol l^{-1})	Cyt *c*	Test of outer mitochondrial membrane integrity (fully intact mitochondria give minimal response)
Carbonylcyanide *p*-trifluoromethoxy-phenylhydrazone (1 µmol l^{-1} followed by 0.5 µmol l^{-1} titrations)	FCCP	CI_E (also PGM_E); Maximal non-coupled Complex I-supported ETS capacity
Protocol 2		
Pyruvate + malate (5 mmol l^{-1} + 1 mmol l^{-1})	PyrM	PM_L; LEAK respiration in the presence of high pyruvate concentration, but no ADP
ADP (5 mmol l^{-1})	ADP	PM_P; Pyruvate OXPHOS capacity, limited by pyruvate oxidation capacity
Glutamate (10 mmol l^{-1})	Glut	CI_P (also PGM_P); Complex I-supported OXPHOS capacity (without fatty acids)
Succinate (10 mmol l^{-1})	Succ	$CI+II_P$; Complex I+II-supported OXPHOS capacity; limited by ADP phosphorylation capacity ($\Delta\Psi_{mt}$)
Cytochrome *c* (10 µmol l^{-1})	Cyt *c*	Test of outer mitochondrial membrane integrity (fully intact mitochondria give minimal response)
Rotenone (0.5 µmol l^{-1})	Rot	CII_P; Complex II-supported OXPHOS capacity
Oligomycin A (0.5 µmol l^{-1})	Omy	CII_L; Complex II-supported LEAK respiration in the presence of high adenylate concentrations

Protocol constituents are listed in the order they are added in the respiration experiment, generating the cumulative respiratory states described in the right column. See Results and Pesta and Gnaiger (Pesta and Gnaiger, 2012) for additional explanation and interpretation of respiratory states. ETF, electron transferring flavoprotein; ETS, electron transfer system; PGM, pyruvate + glutamate + malate; PM, pyruvate + malate; OXPHOS, oxidative phosphorylation; CI/II, respiratory Complexes I/II; $\Delta\Psi_{mt}$, mitochondrial membrane potential.

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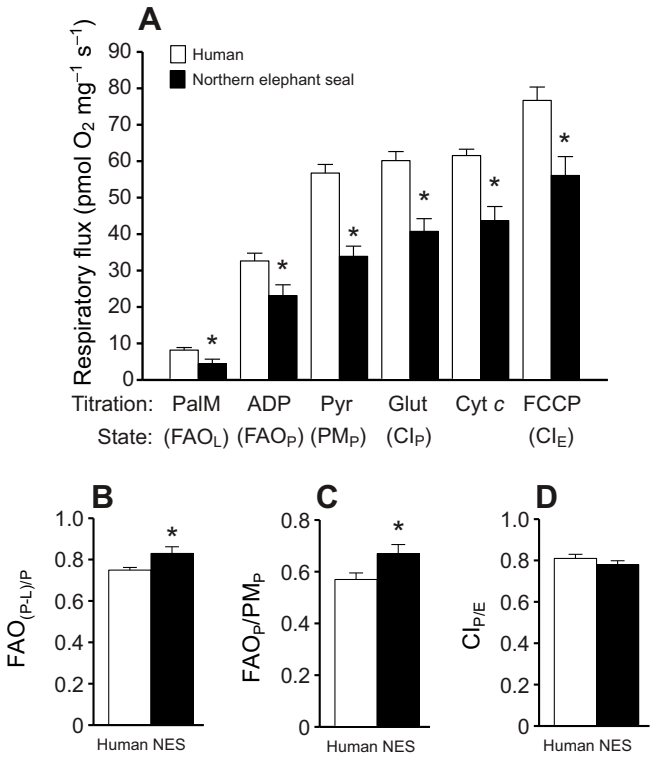

Fig. 1. Respirometry data from adult human and northern elephant seal muscle mitochondria in the presence of fatty acids in Protocol 1. (A) Mass-specific respiratory flux of permeabilized muscle fibers from humans (N=4) and seals (NES; N=7) during each of the respiratory states examined in Protocol 1 (see Results and Table 1 for details and abbreviations). (B) Fatty acid oxidative phosphorylation (OXPHOS) coupling control factor, calculated as (P–L)/P using the fatty acid oxidation (FAO) OXPHOS capacity (FAO_P) and preceding LEAK respiration without ADP (FAO_L) in A (an index of OXPHOS coupling efficiency). (C) Relative contribution of FAO to combined FAO+PM-supported OXPHOS capacity. (D) Combined fatty acid + Complex I OXPHOS flux control ratio, calculated as the OXPHOS capacity (CI_P) divided by subsequent electron transfer system (ETS) capacity (CI_E) in the presence of fatty acids. Data are means ± s.e.m. *P<0.05 versus human.

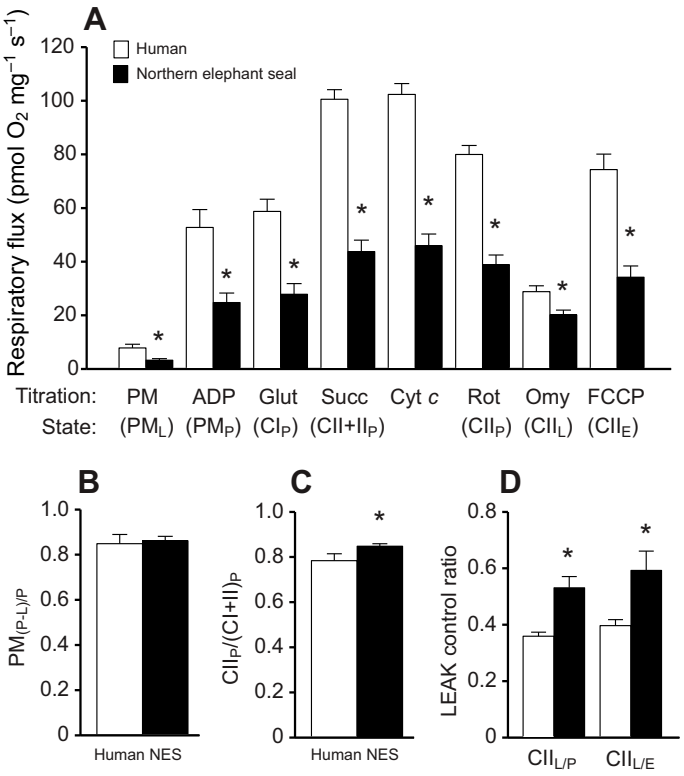

Fig. 2. Respirometry data from adult human and northern elephant seal muscle mitochondria in the presence of Complex I and Complex II substrates by Protocol 2. (A) Mass-specific respiratory flux of permeabilized muscle fibers from human (N=4) and seals (NES; N=7) during each of the respiratory states examined in Protocol 2 (see Results and Table 1 for details and abbreviations). (B) OXPHOS coupling control factor for pyruvate oxidation, calculated as (P–L)/P using the pyruvate+malate OXPHOS capacity (PM_P) and preceding LEAK respiration without ADP (PM_L) in A. (C) Complex II-linked OXPHOS flux control ratio [$CII_P/(CI+II)_P$], approximating the relative contribution of Complex II to the combined CI+II OXPHOS capacity. (D) Complex II-linked LEAK control ratios, calculated as the CII-linked LEAK respiration in the presence of oligomycin (CII_L) divided by the preceding uninhibited OXPHOS flux ($CII_{L/P}$) or subsequent non-coupled ETS capacity ($CII_{L/E}$) in A. Data are means ± s.e.m. *P<0.05 versus human.

samples used in our studies. Subsequent addition of the protonophore FCCP stimulates the maximal non-coupled ETS capacity (CI_E), which increased flux ~27% in both human and seal muscle fibers, demonstrating similar restraint of OXPHOS capacity by the phosphorylation system in both species. Expressing the maximal Complex I-supported OXPHOS rate (CI_P) relative to the fully non-coupled ETS capacity (CI_E) reveals the extent to which maximal OXPHOS utilizes the full enzymatic capacity of the ETS ($CI_{P/E}$, Fig. 1D). This was nearly identical in seals and humans, indicating that both species operate at ~80% of ETS capacity during CI_P in the presence of fatty acids.

Protocol 2

Protocol 2 provides assessments of respiratory LEAK and OXPHOS capacities using pyruvate + malate alone (PM_L and PM_P), maximal Complex I-linked OXPHOS following addition of glutamate (CI_P), combined Complex I- and Complex II-linked OXPHOS after the addition of succinate ($CI+II_P$), and the Complex II OXPHOS capacity after the addition of Rot (CII_P). Respiratory LEAK capacity was also assessed following CII_P by the addition of Omy, generating the CII-linked Omy-induced LEAK in the presence of high adenylates (CII_L). Similar to data from Protocol 1, NES muscle exhibited mass-specific O_2 fluxes that were 30–58% lower than those from human muscle in the presence of Complex 1 and Complex II substrates (Fig. 2A). Relative differences in OXPHOS capacities were generally consistent (52–58%) and highly significant (P<0.001) under all substrate conditions, confirming the lower respiratory capacity of NES compared with human muscle indicated by Protocol 1. Pyruvate + malate OXPHOS coupling control ($PM_{P–L/P}$; Fig. 2B) was nearly identical in seals and humans, indicating similar control of pyruvate oxidation capacity by the phosphorylation system. The addition of glutamate increased OXPHOS flux to similar extents in both species, as seen in Protocol 1. As expected, flux increased further and to similar extents in both species upon adding succinate, supplying additional electrons to the ETS through Complex II, thereby fully reconstituting the supply of reducing equivalents from the tricarboxylic acid (TCA) cycle to the ETS ($CI+II_P$) (Gnaiger, 2009; Pesta and Gnaiger, 2012). As in Protocol 1, responses to the subsequent addition of Cyt *c* were negligible in both human and seal muscle fibers, indicating structurally sound mitochondria in our experiments.

The Complex I inhibitor Rot was added next, generating the maximal Complex II-linked OXPHOS capacity (CII_P). Expression of this rate as a percent of the preceding Complex I+II OXPHOS

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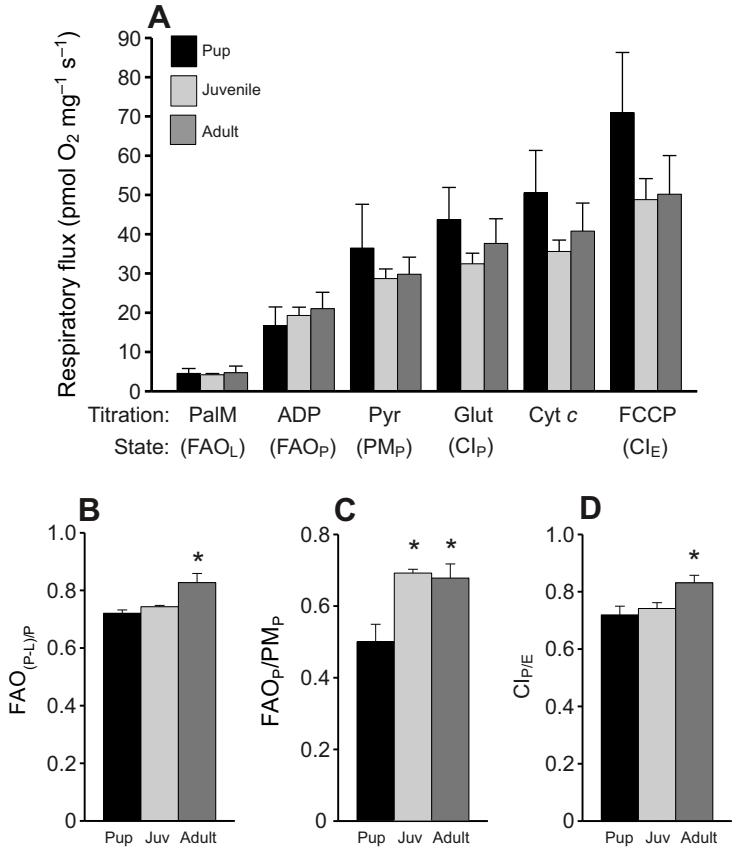

Fig. 3. Ontogeny of mitochondrial respiratory function in northern elephant seal muscle assessed in the presence of fatty acids by Protocol 1. (A) Mass-specific respiratory flux of permeabilized muscle fibers from elephant seal pups (*N*=4), juveniles (*N*=6) and adults (*N*=7) for each of the respiratory states examined in Protocol 1 (see Results and Table 1 for details and abbreviations). (B) Fatty acid OXPHOS coupling control factor, calculated as (P–L)/P using the FAO OXPHOS capacity (FAO_P) and preceding LEAK respiration without ADP (FAO_L) in A (an index of OXPHOS coupling efficiency). (C) The relative contribution of FAO to combined FAO+PM-supported OXPHOS capacity. (D) The combined fatty acid + Complex I OXPHOS flux control ratio, calculated as OXPHOS capacity (CI_P) divided by the subsequent ETS capacity (CI_E) in the presence of fatty acids. Data are means ± s.e.m. *P<0.05 versus pups.

capacity [$CII_P/(CI+II)_P$] suggests a slightly greater relative capacity of Complex II in seals versus humans (P=0.02; Fig. 2C). Subsequent addition of Omy blocks proton flux through ATP synthase, thereby revealing the maximal CII-linked respiratory LEAK in the presence of high concentrations of adenylates (CII_L) (Pesta and Gnaiger, 2012). The addition of Omy inhibited flux in human fibers by 64%, indicating that respiratory LEAK represents ~36% of CII-linked OXPHOS capacity ($CII_{L/P}$). Interestingly, the inhibitory effect of Omy was only 46% in seals, which equates to a 50% higher relative LEAK in seals versus humans in the presence of adenylates (P<0.001; Fig. 2D). Subsequent addition of FCCP releases the restraint of respiration by the high mitochondrial membrane potential ($\Delta\Psi_{mt}$), generating the CII-linked ETS capacity, which restored respiration to near the previous OXPHOS capacity.

Ontogeny of NES muscle mitochondrial function

To evaluate the effect of ontogeny on the mitochondrial phenotype of NES, we compared mitochondrial respiration data from adult male NES (*N*=7) with those obtained from male weaned pups (*N*=4) and juveniles (*N*=6) using the same high-resolution respirometry protocols (Figs 3, 4).

Protocol 1

When comparing mass-specific O_2 flux across the three stages of development, no statistically significant differences were found for any of the respiratory states assessed in Protocol 1 (Fig. 3A), indicating generally similar muscle mitochondrial functional capacity in the presence of saturating concentrations of fatty acid. However, trends for progressively lower LEAK and higher OXPHOS capacities as animals develop from pups to adulthood result in a significantly greater degree of fatty acid OXPHOS coupling ($FAO_{P–L/P}$), indicating a progressively greater control of FAO by ADP as animals develop from pups to diving adults (P<0.01; Fig. 3B). The addition of pyruvate tended to increase flux to a greater extent in pups compared with juveniles and adults, which equated to a much higher relative contribution of fatty acid versus pyruvate oxidation to OXPHOS flux in the juveniles and adults versus pups (P<0.01; Fig. 3C). The calculated $CI_{P/E}$ was significantly higher in adults compared with juveniles and pups (P<0.05; Fig. 3D), indicating a greater utilization of ETS capacity during maximal OXPHOS in adults versus juveniles and pups. Respiratory responses to the addition of Cyt *c* were <10% in all samples used in our analyses, confirming the preservation of mitochondrial outer membrane integrity.

Protocol 2

In contrast to data obtained in the presence of fatty acid in Protocol 1, mass-specific O_2 fluxes were significantly lower in adult seals compared with both pups and juveniles under all respiratory states evaluated in Protocol 2 using Complex I and Complex II substrates in the absence of fatty acid (Fig. 4A). No significant differences were seen in PM OXPHOS coupling control ($PM_{P–L/P}$), indicating similar control of pyruvate oxidation by ADP throughout ontogeny (Fig. 4B). Differences among groups became more apparent with the sequential addition of glutamate and succinate, eliciting a strong trend for a progressive reduction in muscle OXPHOS capacity as seals develop from pups to adulthood. Group differences in CII_P were congruent with the preceding Complex I+II capacities, which equated to nearly identical $CII_P/(CI+II)_P$ flux control ratios, indicating a similar contribution of CII to total OXPHOS capacity throughout ontogeny (Fig. 4C). However, CII-linked LEAK normalized to either the OXPHOS or ETS capacities was markedly

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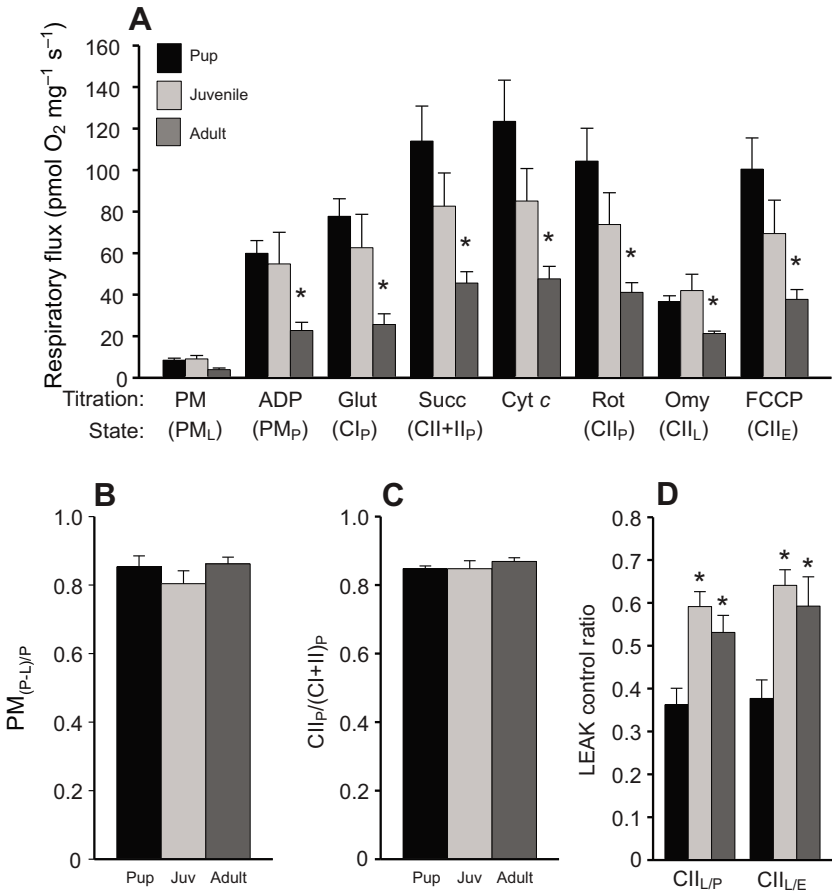

Fig. 4. Ontogeny of mitochondrial respiratory function in northern elephant seal muscle assessed in the presence of Complex I and Complex II substrates by Protocol 2. (A) Mass-specific respiratory flux of permeabilized muscle fibers from seal pups (N=4), juveniles (N=6) and adults (N=7) for each of the respiratory states examined in Protocol 2 (see Results and Table 1 for details and abbreviations). (B) OXPHOS coupling control factor for pyruvate oxidation, calculated as (P–L)/P using the pyruvate+malate OXPHOS rate (PM_P) and preceding LEAK without ADP (PM_L) in A. (C) Complex II-linked OXPHOS flux control ratio [$CII_P/(CI+II)_P$], approximating the relative contribution of Complex II to the combined CI+II OXPHOS capacity. (D) Complex II-linked LEAK control ratios, calculated as the CII-linked LEAK respiration in the presence of oligomycin (CII_L) divided by the preceding uninhibited OXPHOS flux ($CII_{L/P}$) or subsequent non-coupled ETS capacity ($CII_{L/E}$) in A. Data are means ± s.e.m. *P<0.05 versus pups.

higher in juveniles and adults compared with pups (P<0.01; Fig. 4D), with no significant difference between juveniles and adults. As in Protocol 1, respiratory responses to the addition of Cyt *c* were negligible, confirming the integrity of the samples used in our analyses.

DISCUSSION

NES possess the remarkable ability to rely largely upon aerobic metabolism for up to 2 h of apnea while foraging in cold water at depths of up to 2000 m (Robinson et al., 2012). Several biochemical, physiological and biomechanical adaptations are known to facilitate these impressive feats by enhancing muscle O_2 storage, diffusion and metabolic efficiency (for a review, see Davis, 2014), but the present study is the first to comprehensively evaluate muscle mitochondrial respiratory function in a diving mammal. Our studies indicate that adult NES muscle maintains a high capacity for fatty acid oxidation and enhanced OXPHOS coupling control despite a lower mass-specific respiratory capacity compared with humans and seal pups naïve to diving. The ontogeny of this phenotype indicates that it is an adaptive response to the diving lifestyle rather than an intrinsic property of NES muscle mitochondria. Interestingly, adult and juvenile seal mitochondria also exhibit a ~50% greater capacity for respiratory LEAK in the presence of high substrate and adenylate concentrations, which also appears to be an adaptive response to the diving lifestyle. To our knowledge, this is the first report of seemingly paradoxical elevations in both OXPHOS control and respiratory LEAK in mammalian muscle mitochondria, highlighting the plasticity of mitochondrial respiratory function to meet the unique bioenergetic and thermoregulatory demands of the diving mammal phenotype.

Tissue mass-specific OXPHOS capacities from adult NES muscle were 25–58% lower than those obtained from human vastus lateralis depending on the substrates used. This is consistent with a lower respiratory capacity of adult NES versus human muscle per unit mass, as would be predicted by the well-established inverse relationship between muscle mitochondrial volume density and body mass across mammalian species (Mathieu et al., 1981; Dobson and Headrick, 1995). However, the highly variable relative differences in respiratory flux between seals and humans highlights the importance of considering substrate utilization and multiple respiratory states when evaluating adaptive changes in muscle aerobic capacity. Previous studies indicate that diving mammals rely almost exclusively on lipid metabolism for muscle ATP production (Davis, 1983; Davis et al., 1991; Davis et al., 1993; Kanatous et al., 2008; Trumble et al., 2010; Trumble and Kanatous, 2012; Houser et al., 2013; Crocker et al., 2014). Consequently, it was not surprising that compared with humans, adult NES mitochondria exhibited a greater capacity to oxidize lipid versus carbohydrate substrates (Fig. 1C). Moreover, OXPHOS capacity of NES muscle with palmitoylcarnitine + malate (FAO_P) was 75% of fluxes seen in human muscle, whereas CI+CII-linked OXPHOS capacity supported by maximal convergent electron input from the TCA cycle was only 43% of that seen in human muscle. Taken together, these findings confirm long-standing evidence for a preferential reliance upon fatty acid metabolism in carnivorous marine mammals at the level of locomotor muscle mitochondria. Fatty-acid-supported respiratory flux in mitochondria is limited by electron delivery through the electron transferring flavoprotein, which obtains reducing equivalents from flavin adenine nucleotide in the acyl-CoA dehydrogenase reaction of the beta-oxidation cycle (Pesta and

Gnaiger, 2012). Therefore, NES mitochondria likely maintain comparatively high lipid OXPHOS capacity by a selective increase in the transport, oxidation and/or delivery of reducing equivalents derived from fatty acids to the respiratory system. Notably, myoglobin has been reported to facilitate myocellular transport of fatty acids under oxygenated conditions (Shih et al., 2014) and is far more abundant in NES versus human muscle *in vivo* (Gros et al., 2010; Hassrick et al., 2010). The vast majority of myoglobin is leached from fibers during the preparation and permeabilization procedure to avoid confounding effects on O_2 transport. However, we cannot rule out potentially additive effects of myoglobin on NES muscle fatty acid oxidation capacity *in vivo*, nor a potential contribution of trace levels that might remain in specialized cellular compartments, such as bound to mitochondria (Yamada et al., 2013), following fiber permeabilization in our experiments.

A previous study in Weddell seals demonstrated a paradoxical decrease in muscle mitochondrial volume density as seals reached maturity, despite evidence for improved aerobic exercise capacity to sustain longer and deeper dives in adulthood (Kanatous et al., 2008). Consistent with this finding, we observed a progressive decrease in tissue mass specific OXPHOS capacity as NES developed from pups to adults in the present study (Fig. 4A). However, despite this apparent decline in overall muscle respiratory capacity, mass-specific rates of FAO-linked OXPHOS capacity tended to increase in juveniles and adults, along with highly significant increases in OXPHOS control (Fig. 3B). Improvements in the fatty acid OXPHOS coupling control factor ($FAO_{P–L/P}$) resulted primarily from increased OXPHOS capacity rather than decreases in respiratory LEAK, suggesting an improvement in the control and/or capacity of the ADP phosphorylation system on respiratory flux in the presence of fatty acids. Consistent with this interpretation, a significantly higher CI-linked OXPHOS capacity was observed in adult NES compared with pups and juveniles when expressed as a percent of maximal (non-coupled) ETS capacity ($CI_{P/E}$) in Protocol 1 (Fig. 3D). Taken together, these findings suggest that NES mitochondria become more effective at generating ATP from fatty acids as they mature, a process that overcomes a decline in total mass-specific respiratory capacity as body mass increases, ultimately improving muscle aerobic capacity for longer and deeper dives in adulthood. The molecular basis of this adaptation will require further investigation, but might involve a selective increase in content or coupling of phosphorylation system components [e.g. ATP synthase and the adenine nucleotide translocase (ANT)] in conjunction with an upregulation of fatty acid oxidation enzymes including the electron transferring flavoprotein in muscle mitochondria.

Enhanced OXPHOS ‘efficiency’ is typically defined by reduced rates of LEAK respiration, improved indices of phosphorylation control and/or a decrease in the amount of O_2 required to phosphorylate a given amount of ADP during classic State 3 respiration (the ADP/O ratio) (Gnaiger et al., 2000; Jacobs et al., 2012; Pesta and Gnaiger, 2012). Accurate assessment of ADP/O is difficult in permeabilized muscle fibers because of the presence of residual ATPase; therefore, calculation of OXPHOS coupling control factors that account for changes in LEAK relative to OXPHOS capacities under common substrate conditions [i.e. (P–L)/P] represent sensitive expressions of OXPHOS coupling efficiency in permeabilized fibers (Gnaiger, 2009; Pesta and Gnaiger, 2012). Such factors range from zero in a fully non-coupled state (where L=P) to a maximally coupled 1.0 (where L=0). As discussed above, OXPHOS coupling control in the presence of PalM or PM was similar or higher in adult seals compared with humans and seal pups in this study. However, despite this, CII-linked LEAK respiration normalized to OXPHOS and ETS capacity was nearly 50% higher in juvenile and adult seals compared with pups and humans (Fig. 2D, Fig. 4D). This seemingly paradoxical finding is perhaps the most intriguing aspect of the mature NES mitochondrial phenotype, a discussion of which highlights both the plasticity of mitochondrial respiratory function in response to bioenergetic and/or thermal stress and the key role these adaptations may play in the development of the diving mammal phenotype.

In both CI- and CII-linked respiratory LEAK states examined herein, mitochondrial membrane potential is high as a result of saturating concentrations of respiratory substrates in the absence of proton flux through the ATP synthase (Pesta and Gnaiger, 2012). Therefore, respiration results from dissipation of the inner membrane chemiosmotic gradient due to proton leak, slip and/or cation cycling across the inner mitochondrial membrane. A primary mechanism of respiratory LEAK in skeletal muscle is thought to be increased proton conductance by uncoupling proteins (UCPs) and ANT (Parker et al., 2008a), both of which may be activated by fatty acids, reactive oxygen species (ROS) or membrane lipid peroxidation products (Parker et al., 2008b; Jastroch et al., 2010). Given the demonstrated preference of mature NES mitochondria for fatty acid (versus pyruvate) compared with pups and humans (Fig. 1C. Fig. 3C), and the known reliance of NESs on lipids for energy production (Houser et al., 2013; Crocker et al., 2014), activation of UCPs or the ANT by fatty acids might be expected to drive respiratory leak in the juvenile and adult seals. However, LEAK in presence of PalM was not increased with ontogeny; rather, it decreased relative to OXPHOS capacities, resulting in higher indices of fatty acid OXPHOS coupling in mature seals compared with pups and humans. Moreover, NES pups subsist exclusively on milk from lactating cows, which is even richer in lipid than the diet of foraging juvenile and adult seals (Crocker et al., 2014; Fowler et al., 2014). This argues strongly against a specific role for fatty acids in inducing the enhanced respiratory LEAK seen in mature NES muscle mitochondria. Indeed, elevated CII-linked LEAK in the juveniles and adults was observed in the absence of fatty acids, suggesting the involvement of Complex II, a higher rate electron delivery to ETS and/or the presence of high adenylate concentrations in this phenomenon.

Interestingly, non-shivering thermogenesis in cold-acclimatized ducklings (*Cairina moschata*) is associated with an upregulation of avian UCP and increased CII-linked Omy-induced LEAK respiration in skeletal muscle mitochondria, despite no change in the CII-linked coupling control or the efficiency of ATP production (Teulier et al., 2010). The striking similarity of these findings to the respirometry data in the present study suggests that cold acclimatization may play a role in the enhanced LEAK that develops in juvenile and adult NES, perhaps via UCP activation. A thermogenic role of mitochondrial uncoupling in seal locomotor muscle was proposed by Grav and Blix almost 35 years ago (Grav and Blix, 1979); however, the extent to which mature NES experience cold stress during dives given their thick insulating blubber layer and large body size is unclear. Arterial and venous blood temperatures in free-diving juvenile NES were highly variable, decreased with dive duration and were as low as 30°C in the longest dives (Meir and Ponganis, 2010). Enhanced muscle thermogenesis may support temperature regulation in perfused muscle, particularly during periods of low swimming effort (Ponganis et al., 1993). Notably, enhanced proton conductance by both UCP and ANT was linked to adaptive thermogenesis in skeletal muscle of cold-adapted penguins, but by different mechanisms of activation of UCPs (by ROS) and ANT (by fatty acids) (Talbot et

al., 2004). Mitochondrial ROS release was not investigated in the present study, but it is plausible that conditions encountered by NES during deep foraging dives promote a physiologic ROS release that facilitates adaptive increases in respiratory LEAK capacity of muscle mitochondria under conditions of high mitochondrial membrane potential.

Mitochondrial ROS generation is driven primarily by the reduced state of the respiratory complexes and the P_{O_2} of the local environment (Korshunov et al., 1997; Barja, 2007), both of which are near maximal in the CII-linked LEAK state. Indeed, CII substrates (succinate + Rot) in combination with Omy are routinely employed to assess maximal ROS emission from isolated mitochondria (Starkov, 2010). While CII-linked LEAK is not a physiological respiratory state, it reflects the concomitantly high reducing pressure (high NADH/NAD+) and low phosphorylation pressure (high ATP/ADP) that occurs in skeletal muscle mitochondria with chronic overnutrition (e.g. a high-fat diet) combined with a sedentary lifestyle. In humans and rodents, this drives a persistent elevation in mitochondrial ROS that induces UCP3 expression and respiratory uncoupling, but ultimately leads to excessive oxidative stress and the development of muscle insulin resistance (Hesselink et al., 2003; Fisher-Wellman and Neufer, 2012). Conversely, a similar oversupply of fatty-acid-derived reducing equivalents unmatched by ATP demand occurs transiently immediately following acute aerobic exercise, leading to brief periods of mitochondrial ROS release (Anderson et al., 2007). This 'physiological' ROS release triggers UCP3 activity/expression and induces respiratory uncoupling, which limits subsequent ROS emission during fatty-acid-supported respiration and enhances fatty acid OXPHOS capacity in muscle mitochondria (Pilegaard et al., 2000; Anderson et al., 2007; Fernstrom et al., 2007).

During development, weaned pups are known to upregulate expression of UCP2 in muscle (Martinez et al., 2013). While its cellular role is less defined, UCP2 may play a similar role in enhancing fatty acid metabolism and reducing ROS (Brand and Esteves, 2005). During deep foraging dives, NES engage in brief bouts of stroking 'exercise' to acquire prey, separated by longer periods of 'gliding' that reduce energy costs and facilitate longer and deeper dives (Davis et al., 2001; Aoki et al., 2011). Both activities are supported almost entirely by aerobic metabolism of fatty acids, made possible by high cellular levels of lipid and oxygen (Snyder, 1983; Ponganis et al., 1993; Guyton et al., 1995; Noren et al., 2001; Kanatous et al., 2002; Williams et al., 2004; Trumble et al., 2010). As noted above, such conditions might favor enhanced mitochondrial ROS release, leading to cellular oxidative stress unless balanced by adaptive increases in uncoupling and/or antioxidant defenses. Interestingly, muscles of diving mammals, including NES, have been shown to possess a greater capacity for ROS generation than terrestrial mammals *in vitro* without elevations of oxidative stress markers *in vivo* (Wilhelm Filho et al., 2002; Vázquez-Medina et al., 2006; Vázquez-Medina et al., 2010). This is has been attributed to a parallel upregulation of antioxidant defenses (Murphy and Hochachka, 1981; Wilhelm Filho et al., 2002; Zenteno-Savín et al., 2002; Vázquez-Medina et al., 2006; Vázquez-Medina et al., 2010), perhaps complemented by increased levels of myoglobin in seal muscle [known to possess oxidant scavenging properties (Flögel et al., 2004; Garry and Mammen, 2007)]. These adapations may at least partially represent hormetic responses to repeated bouts of ROS release and hypoxia associated with the semi-terrestrial diving mammal lifestyle (Zenteno-Savín et al., 2002; Vázquez-Medina et al., 2011). Based on the evidence above, mitochondrial ROS generation might play a similarly adaptive role by increasing respiratory LEAK through activation of UCPs and perhaps the ANT, thereby limiting excessive ROS production and contributing to non-shivering thermogenesis during prolonged cold water dives.

In summary, we provide the first comprehensive comparative and ontogenetic evaluation of muscle mitochondrial respiratory function in a diving mammal. Our results demonstrate maintenance of fatty acid oxidation capacity and improved OXPHOS control in NES muscle despite a reduced overall mass-specific respiratory capacity that develops as animals mature from pups to adults. A seemingly paradoxical increase in respiratory LEAK under conditions of high membrane potential, substrate and adenylate concentrations may serve to increase muscle thermogenesis and reduce generation of ROS during deep foraging dives. Future studies investigating the molecular underpinnings of this remarkable NES mitochondrial phenotype may reveal additional insights into how mitochondrial plasticity contributes to diving mammal physiology, with potential relevance to the study of oxidative stress and metabolic disorders in humans.

MATERIALS AND METHODS

Animal subjects

All NES (*Mirounga angustirostris*) sampling was conducted under National Marine Fisheries Service Marine Mammal Permit no. 786-1463, and all procedures were approved by the Colorado State University Institutional Animal Care and Use Committee (protocol no. 11-3085A). Seven adult male NES were sampled post-breeding season, May–July, six juvenile male NES were sampled mid-molt, April–May, and four pups were sampled early in the post-weaning fast (pup weaning dates established when their mothers departed for sea); all sampling occurred at Año Neuvo State Reserve, CA. Animals were captured as previously described (Boaz et al., 2012); briefly, an initial dose of Telazol was administered via intramuscular injection and immobilization was maintained through subsequent ketamine injections into the extradural vein. Muscle biopsy sampling was performed using a local anesthetic and samples were taken from the mid-belly of the muscle using an established procedure (described below). Following the biopsy procedure, animals were monitored until full voluntary locomotion was regained.

Human subjects

Human subjects (N=4) were recreationally active males aged 26±2 years (mean ± s.e.m.) without a history of regular tobacco use or medications (body mass=93.4±2.5 kg). Assessments of body mass index (28.9±1.2 kg m^{-2}), body composition (26.5±1.5% fat; assessed by dual-energy X-ray absorptiometry using a DXA-IQ; Lunar Radiation Corp., Madison, WI, USA) and peak oxygen consumption (41.2±0.8 ml kg^{-1} min^{-1}) assessed during a graded exercise test using a Parvo Medics Metabolic Cart, were typical of healthy sedentary young adults. Muscle biopsy sampling was performed by a trained technician using an established procedure (described below) that conformed to the standards set by the Declaration of Helsinki of 1975, as revised in 1983, and was approved by the Institutional Review Board at Colorado State University. The nature, purpose and risks of the procedure were explained to each subject before written informed consent was obtained.

Skeletal muscle sampling and handling

Human subjects

Upon completion of health screening, research participants reported to the laboratory following a 12 h fast and 24 h abstention from vigorous exercise to provide skeletal muscle samples. The medial vastus lateralis muscle was sampled using the Bergström technique under local anesthesia (1% lidocaine s.c.) and immediately placed into ice-cold biopsy preservation medium (BIOPS) containing (in mmol l^{-1}) 10 Ca^{2+}-EGTA, 20 imidazole, 50 potassium-4-morpholinoethanesulfonic acid, 0.5 dithiothreitol, 6.56 $MgCl_2$, 5.77 ATP and 15 phosphocreatine at pH 7.1 until processing for mitochondrial respiration experiments.

Elephant seals

Following sterilization of the biopsy area with betadine and subsequent subcutaneous administration of local anesthesia (1% lidocaine), a small incision was made and muscle biopsies were taken. Three muscle samples, weighing approximately 50 mg each, were collected from the primary swimming muscle, m. longissimus dorsi, using a 6 mm biopsy cannula (Depuy, Warsaw, IN, USA). Biopsies were immediately placed into ice-cold BIOPS until processing for mitochondrial respiration experiments. Because of location logistics, samples in BIOPS were shipped on ice to Colorado State University in Fort Collins, CO, for mitochondrial respiratory analysis within 36 h. Mitochondrial integrity was confirmed in all samples by examining respiratory responses to 10 µmol l^{-1} Cyt *c*, which should be negligible in mitochondria with fully intact outer membranes. Any samples showing a >10% increase in respiratory flux under near-maximal OXPHOS states were excluded from the study.

Preparation of permeabilized muscle fibers

Structurally sound fiber bundles were selected from biopsies maintained in ice-cold BIOPS, and mechanically separated, removing any visible adipose and connective tissue using fine forceps under a dissecting microscope. Teased fiber bundles (2–6 mg) were then transferred to BIOPS containing 50 µg ml^{-1} saponin for 20 min of permeabilization of the sarcolemma while leaving the mitochondria and intracellular structures intact (Gnaiger, 2009), followed by 3×10 min washes in ice-cold MiR06 respiration buffer containing (in mmol l^{-1}) 0.5 EGTA, 3 $MgCl_2$, 60 K-lactobionate, 20 taurine, 10 KH_2PO_4, 20 HEPES and 110 sucrose, with 1 g l^{-1} fatty-acid free BSA and 2800 U ml^{-1} catalase. Permeabilized fibers were carefully blotted on Whatman filter paper for 2–3 s to remove excess buffer, weighed and immediately placed in the Oxygraph chamber containing MiR06 at 37°C for stabilization prior to respiration experiments described below.

Mitochondrial respiration

Mitochondrial respiratory function was determined in permeabilized muscle fiber bundles obtained from the medial longissimus dorsi (seals) and vastus lateralis (humans) by high-resolution respirometry using an Oxygraph-2k high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria). Oxygen flux was monitored in real-time by resolving changes in the negative time derivative of the chamber oxygen concentration signal following standardized instrumental and chemical background calibrations using Datlab software (Oroboros Instruments). All respirometry data were collected at 37°C in a hyperoxygenated environment (275–400 µmol l^{-1}) to avoid potential limitations in oxygen diffusion in permeabilized fiber bundles (Gnaiger, 2009; Pesta and Gnaiger, 2012). A detailed description of the respiration protocols and associated respiratory states generated by the sequential titration of each constituent are provided in Table 1. Any fiber preparations exhibiting a greater than 10% increase in flux in response to Cyt *c* were excluded from analyses. Selected flux control ratios were calculated for determination of respiratory coupling/leak, substrate control and the relative contribution of fatty acid versus pyruvate as described in the Results and Discussion.

Statistical analyses

All data are presented as group means ± s.e.m., with the number of samples per group noted in the Results and figure legends. Data from respiration studies comparing adult seals and humans (Figs 1, 2) were compared by independent-sample *t*-tests. Data from seal pups, juveniles and adults (Figs 3, 4) were compared by one-way ANOVA, with *post hoc* Tukey's tests to reveal individual group differences. The level of statistical significance was set at $P<0.05$ for all analyses.

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Competing interests

The authors declare no competing financial interests.

Author contributions

Conception and design of the study: A.J.C., E.G. and S.B.K. Execution of the experiments: A.J.C., C.H.L., A.S., A.N., A.K., J.W.B., R.L.S., C.B., D.P.C., D.E.C. and S.B.K. Interpretation of the findings: A.J.C., A.S., E.G., D.E.C. and S.B.K. Drafting and revising the manuscript: A.J.C., A.S., E.G., D.E.C. and S.B.K.

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