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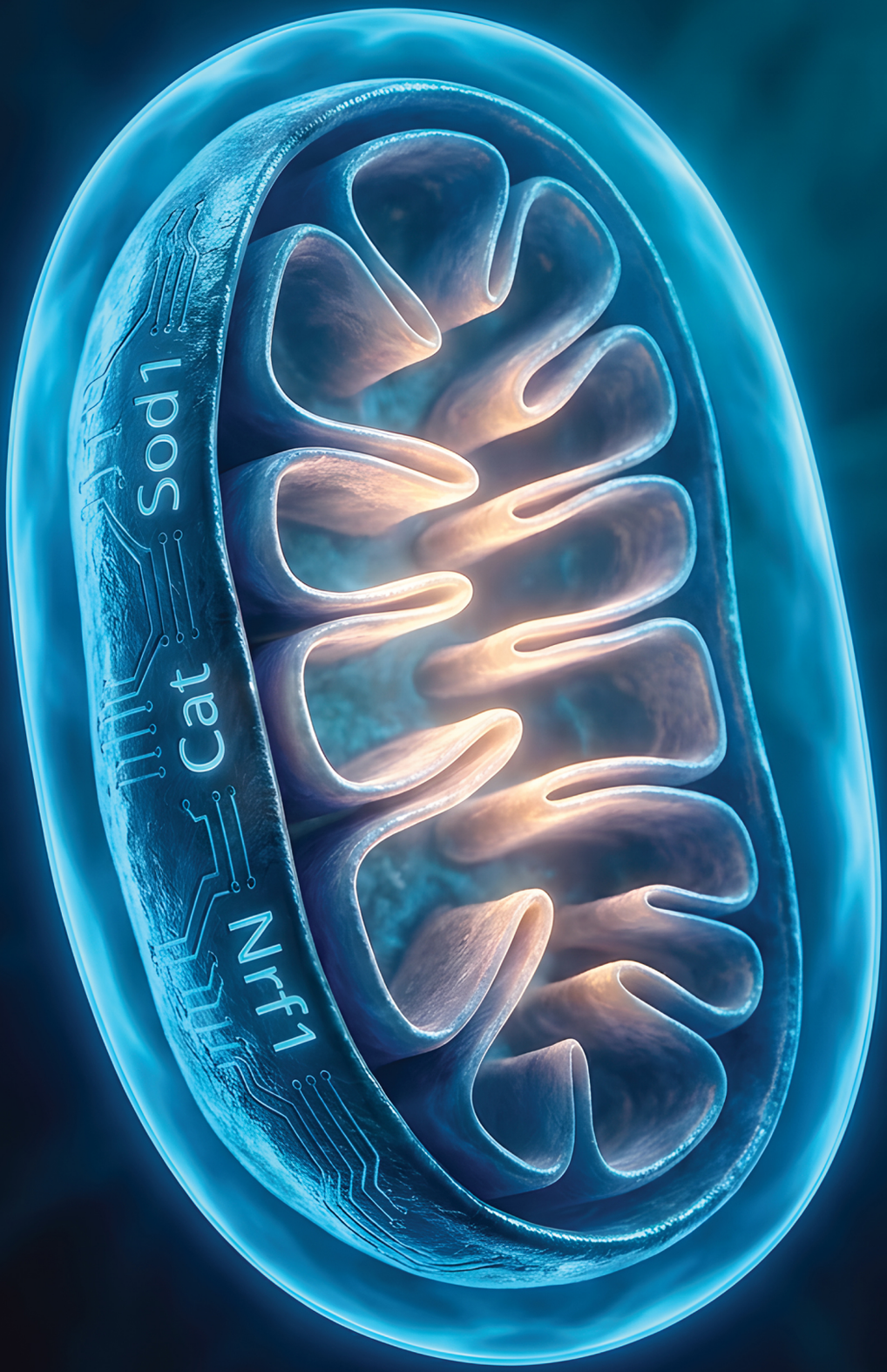

Spotlight

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RESEARCH ARTICLE

Environmental Effects on Physiological-Omics

Changes in liver and lung antioxidant and mitochondrial biogenesis gene expression in decompression sickness resistant rats

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Abstract

Susceptibility to decompression sickness (DCS) shows wide interindividual variability, the origins of which remain poorly understood. To better elucidate its mechanisms, we previously developed a rat strain with more than threefold higher resistance to DCS through selective breeding. In this study, we examined baseline expression of genes related to antioxidant defense and mitochondrial biogenesis in the lungs and liver of DCS-resistant and nonresistant Wistar rats. None of the animals were exposed to hyperbaric conditions, allowing us to focus on constitutive expression differences potentially underlying genetic resistance to DCS. Quantitative reverse transcription polymerase chain reaction was performed, and data were analyzed using two-way ANOVA to assess the effects of selection, sex, and their interaction. In the lungs, no significant selection effect was observed for genes involved in mitochondrial biogenesis (*Pgc1α*, *Nrf1*, and *Nrf2*) or antioxidant defense (*Sod1*, *Sod2*, *Gpx1*, and *Cat*). In contrast, in the liver, expression of both gene groups was significantly decreased in resistant rats ($P < 0.0083$, Bonferroni correction), except for *Pgc1α*. These lower hepatic expression levels align with the previously reported reduction in citrate synthase activity and lower basal oxygen consumption in the soleus muscle of resistant rats. Together, these findings suggest that hepatic metabolism and mitochondrial function may play key roles in DCS susceptibility, potentially through reduced mitochondrial activity and lower reactive oxygen species production in resistant animals.

NEW & NOTEWORTHY This study analyzed genes related to antioxidant defense and mitochondrial biogenesis in the lungs and liver of rats bred for resistance to decompression sickness (DCS). In resistant rats, no change was observed in lung gene expression, whereas both gene groups were downregulated in the liver ($P < 0.0083$, Bonferroni correction), except *Pgc1α*. These findings, along with previous ones, suggest that hepatic mitochondrial function and reduced reactive oxygen species production may contribute to DCS resistance.

decompression sickness; gene expression; mitochondrial biogenesis; reactive oxygen species; tissue specificity

INTRODUCTION

Decompression sickness (DCS) is characterized by a wide interindividual susceptibility, the origin and mechanisms of which remain quite unknown nowadays (1, 2). DCS risk has been claimed to depend on numerous physiological variables, including inflammation (3, 4), coagulation (5, 6), oxidative stress (7), and vascular dysfunction (7, 8), but little consensus has been reached. To better understand them, we have previously conducted a large-scale artificial selection program with Wistar rats, based on their resistance to DCS, and reported a threefold decrease in DCS occurrence (9). This selection program now provides us with a population with significantly increased spontaneous resistance to DCS. Previous investigations confirmed the involvement of these physiological functions by showing higher leukocytes count, lower coagulability, sensitivity to NO donor and arterial pressure in DCS resistant animals, but no changes in antioxidants enzymes activities (10, 11). In addition, we investigated mitochondrial function in saponin-permeabilized muscle fibers and observed lower basal oxygen consumption in the soleus muscle, whereas maximal consumption

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was unchanged (10). This decrease was accompanied by a decrease in citrate synthase (CS) activity in males, which might reflect a lower density of mitochondria in the soleus muscle, with perhaps a more efficient respiratory chain. Coherent with this altered mitochondrial function, DCS resistance was associated with modified metabolic pathways, as suggested by differences in the gut microbiota between DCS-resistant and nonresistant rats (12). Knowing that mitochondrial products can promote inflammation (13), as they can function as damage-associated molecular patterns (DAMPs), these observations led us to wonder whether changes in mitochondrial function could participate in resistance to DCS in our model.

To date, only a few studies have focused on mitochondrial function in the context of diving. They have mainly investigated the effects of diving on mitochondria, and none of them have focused on the possible role of mitochondria in the triggering of DCS. Results from in vitro studies showed that mitochondrial respiration and motility are altered after a simulated dive (14, 15). They also showed that mitochondrial dysfunction is independent of decompression and seems to imply pressure effects and reactive oxygen species (ROS) (16). Some work on eel also showed that mitochondrial function is improved in animals acclimatized to high hydrostatic pressure (17, 18). Finally, divers presenting neurological DCS symptoms seem to have more circulating mitochondrial DNA (mtDNA) than asymptomatic divers (19). Taken together, these results support the hypothesis that mitochondria could play a role in DCS.

Although the elevated pressure during the stay at the bottom potentially acts on all tissues, the vascular gas emboli formed during and after decompression are thought to act mainly on the lungs and liver (20). The lungs are the tissue where vascular bubbles formed during the decompression phase are filtered in the organism (21), and they are considered the primary organs possibly involved in DCS (22). Clopidogrel, a possible inhibitor of platelet activation and inflammation, has demonstrated an effect on reducing lung damages on DCS rats (23), and inhibition of NF-κB seems to prevent decompression-induced acute lung injury after unsafe fast ascent (24).

Although less studied than the lungs in relation to diving, several studies revealed that the liver is also injured by bubbles during decompression (20, 21, 25, 26), and we often observe bubbles in the liver of animals presenting DCS symptoms (27). An elevation of plasmatic markers of liver injury, including alanine transaminase, aspartate transaminase, and lactate dehydrogenase, was reported in mice after a rapid decompression (28). Some also observed damage in liver histology, with disorganized hepatocytes and a disordered lobular structure after rapid decompression (29). In addition, the liver is the place of the synthesis of many circulating proteins involved in coagulation and inflammation (30). DCS could be elicited by circulating factors (2, 31) and involves disseminated thrombosis (6) and inflammation (32). As the liver is extremely rich in mitochondria (33, 34), its dysfunction might lead the liver to trigger an inflammatory and/or coagulation response, leading to DCS.

Because it was shown previously that pressure-induced mitochondrial dysfunction involves the production of superoxide anions, the present study aimed at investigating in these two tissues (lung and liver), the mRNA levels of genes coding for antioxidant enzymes in both the mitochondria [superoxide dismutase 2 (*Sod2*) and glutathione peroxidase 1 (*Gpx1*)] and the cytosol [superoxide dismutase 1 (*Sod1*) and catalase (*Cat*)]. We additionally assessed the expression of *Nrf1*, *Nrf2*, and *Pgc1α*, which code for major signaling molecules related to mitochondrial biogenesis (35). NRF1 is also known for its association with small MAF (sMAF), which activates proteasome subunit genes in response to proteasome inhibitors (36), whereas NRF2 has anti-inflammatory, stem cell regulatory, or antiaging functions, and PGC1α can promote angiogenesis (37).

Our objective in this study is twofold: to gain a better understanding of two suspected initiation sites of decompression sickness (DCS) and to confirm the potential key role of mitochondria and antioxidant enzymes in its pathophysiology. To this end, we examined the steady-state expression of transcripts (rather than proteins) in rats selectively bred for resistance to DCS, but not themselves subjected to a simulated dive. Transcript-level analysis was chosen because it provides a sensitive measure of baseline gene regulation and allows early detection of molecular pathways potentially underlying DCS resistance, while avoiding the complexity and variability introduced by posttranslational protein regulation.

MATERIALS AND METHODS

Animals

This protocol complies with Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific research, and with Articles R214-87 to R214-137 of the French Rural Code and their subsequent modifications. It follows the replacement, reduction, and refinement (3Rs) rule and has been approved by the University of Brest ethics committee for animal experimentation [Autorisation de Projets utilisant des Animaux à des Fins Inventives et Scientifiques (APAFIS) authorization nos. 5250 and 10395-2017061909495511]. We have complied with all relevant ethical regulations for animal use. The rats used came from a selection protocol started with a batch of 52 males and 52 females of the Wistar strain from Janvier Labs (Le Genest-Saint-Isle, France). Received in our laboratory at the age of 6 wk, they were housed in the central animal facility of the university until the selection protocol, under controlled temperature (21 ± 1°C) and light conditions (12 h of light from 6:00 AM to 6:00 PM). The animals were fed ad libitum and were equipped with electronic chips for identifiers. The selection protocol is described in the article by Lautridou et al. (9). Control animals came from the same strain and from the same approved breeder, and they were housed in the same animal facility (under the same conditions) during the 2 wk preceding sampling. To avoid any bias linked to a persistent effect of diving on the expression of transcripts or miRs, the animals included in the study had never been exposed to any hyperbaric protocol before.

Four groups of six animals ($n = 6$) aged 18–19 wk were studied: six males and six females of the 7th generation (G7) of DCS-resistant rats, as well as six males and six females of

the nonresistant Wistar strain from which they came. The animals were anesthetized and analgesized before sampling with a cocktail of Ketamine 1000 (80 mg/kg) and Xylazine 2% (12 mg/kg) administered intraperitoneally. The resistant animals used in this procedure are supernumerary animals, and no animal has previously been subjected to hyperbaric exposure, so we can measure the stationary expression of targeted genes and not a response to a simulated dive. We then removed one lung and one liver lobe per animal, which we placed in cryotubes and in liquid nitrogen before storing them at −80°C. The animals were euthanized immediately after sample collection.

Extraction and Amplification

Extractions were made from liver and lung samples crushed in liquid nitrogen and then homogenized with Ultra-Turrax in the presence of NucleoZOL. After centrifugation, the nucleic acids contained in the aqueous phase were purified on NucleoSpin columns made for NucleoZOL (Macherey Nagel, Düren, Germany). After reverse transcription using the qScript cDNA synthesis kit (QuantaBio, VWR), the cDNAs were used to amplify, by quantitative polymerase chain reaction (qPCR), the genes of interest (*Sod1*, *Sod2*, *Cat*, *Gpx1*, *Pgc1α*, *Nrf1*, and *Nrf2*) using specific primers. All PCRs were carried out with an ABI Prism 7500 Fast from Applied Biosystems, and we have normalized expression levels with 18S RNA.

Statistics

The study was powered to detect approximately twofold differences in mean gene expression. The sample size was determined in accordance with the 3Rs principle, ensuring an ethically conservative use of animals while remaining consistent with established practice for qPCR studies in controlled rodent models. A total of four experimental groups were studied: resistant males (res males), resistant females (res females), nonresistant Wistar males (nonres males), and nonresistant Wistar females (nonres females), with six animals per group. This sample size ($n = 6$) was determined assuming an expected twofold difference in mean expression (Cohen's $d \approx 1.3$), a within-group coefficient of variation of 30%, $\alpha = 0.05$, and power $(1 - \beta) \approx 0.8$ for detecting main effects in a two-way ANOVA. This provided a balance between statistical sensitivity and ethical reduction of animal numbers. This design provides sufficient power to detect large, biologically meaningful baseline differences. Shapiro–Wilk and Bartlett tests were first applied to check normality and homogeneity of variances, respectively. When normality was confirmed, a two-way ANOVA (factors: selection and sex), followed by Tukey's post hoc tests, was used to compare the four group means (nonresistant Wistar males, nonresistant Wistar females, resistant males, resistant females). If normality was not confirmed, a Kruskal–Wallis nonparametric test followed by Mann–Whitney tests was applied. This allowed us to assess differences between groups, as well as the main effects of sex and selection, and their interaction. All data are expressed as means ± SE and were normalized to 18S rRNA expression levels. This approach controls the family-wise error rate (with Tukey's post hoc tests) across all group means and allows identification of statistical trends ($0.05 > P \geq 0.00833$) versus robust differences meeting the more stringent Bonferroni-adjusted threshold ($P < 0.00833$) applied across the seven genes analyzed per tissue. This dual reporting distinguishes biologically consistent tendencies from results that remain statistically robust after correction for multiple testing.

RESULTS

In the lung (Fig. 1), we observed a trend toward a main effect of selection, but not of sex, for the three transcription factors involved in mitochondrial biogenesis and antioxidant regulation: *Pgc1α* ($P = 0.0272$), *Nrf1* ($P = 0.0287$), and *Nrf2* ($P = 0.0179$, without family-wise error rate correction). However, these values do not reach the Bonferroni-adjusted significance threshold ($P < 0.00833$). Expression of all three genes tended to be higher in resistant rats than in their nonresistant Wistar counterparts, and no significant "selection × sex" interaction was detected, indicating similar patterns in both sexes.

Conversely, a main effect of sex was suggested for the antioxidant enzymes *Sod1* ($P = 0.0473$), *Sod2* ($P = 0.0176$), and *Gpx1* ($P = 0.0202$), but not for *Cat* ($P = 0.0854$). Expression of these genes was not significantly affected by selection, and again, no significant "selection × sex" interaction was observed.

In the liver (Fig. 2), a significant main effect of selection was observed for nearly all transcripts examined: *Sod1* ($P = 1.35 \times 10^{-6}$), *Sod2* ($P = 3.89 \times 10^{-5}$), *Cat* ($P = 1.07 \times 10^{-4}$), *Gpx1* ($P = 6.15 \times 10^{-5}$), *Nrf1* ($P = 0.0023$), and *Nrf2* ($P = 0.0023$), all of which were significantly underexpressed in resistant rats compared with Wistar controls. Only *Pgc1α* showed no difference between groups ($P = 0.734$).

A sex effect was also detected for *Cat* ($P = 0.0047$) and *Gpx1* ($P = 0.0073$), with higher expression in females than in males. No significant "selection × sex" interactions were found for any transcript.

DISCUSSION

In diving, studies have shown an increase in oxidative stress, as assessed by the higher plasmatic concentration of thiobarbituric acid reactive substances, proportional to decompression stress (7). This increase in oxidative stress is responsible for diving-induced vascular dysfunction (38), as well as inflammation mediated by microparticles (MPs) (39). Overall, diving and hyperbaric exposures seem to increase oxidative stress and consume antioxidant defenses, even if they remain within the normal range after the dive (40). A part of this oxidative stress can be prevented by the administration of antiplatelet agents (6).

Previous work from our group has established that hydrostatic pressure acts as a significant oxidative stressor. In a combined in vitro/in vivo approach, we demonstrated that pressure triggers superoxide production and that systemic antioxidant pretreatment in rats attenuated the consumption of plasma glutathione (a major antioxidant) but did not affect DCS outcome (41). In the context of natural resistance, we previously observed that resistant rats exhibit a lower basal mitochondrial oxygen consumption rate while maintaining a healthy respiratory control ratio (10). We interpret

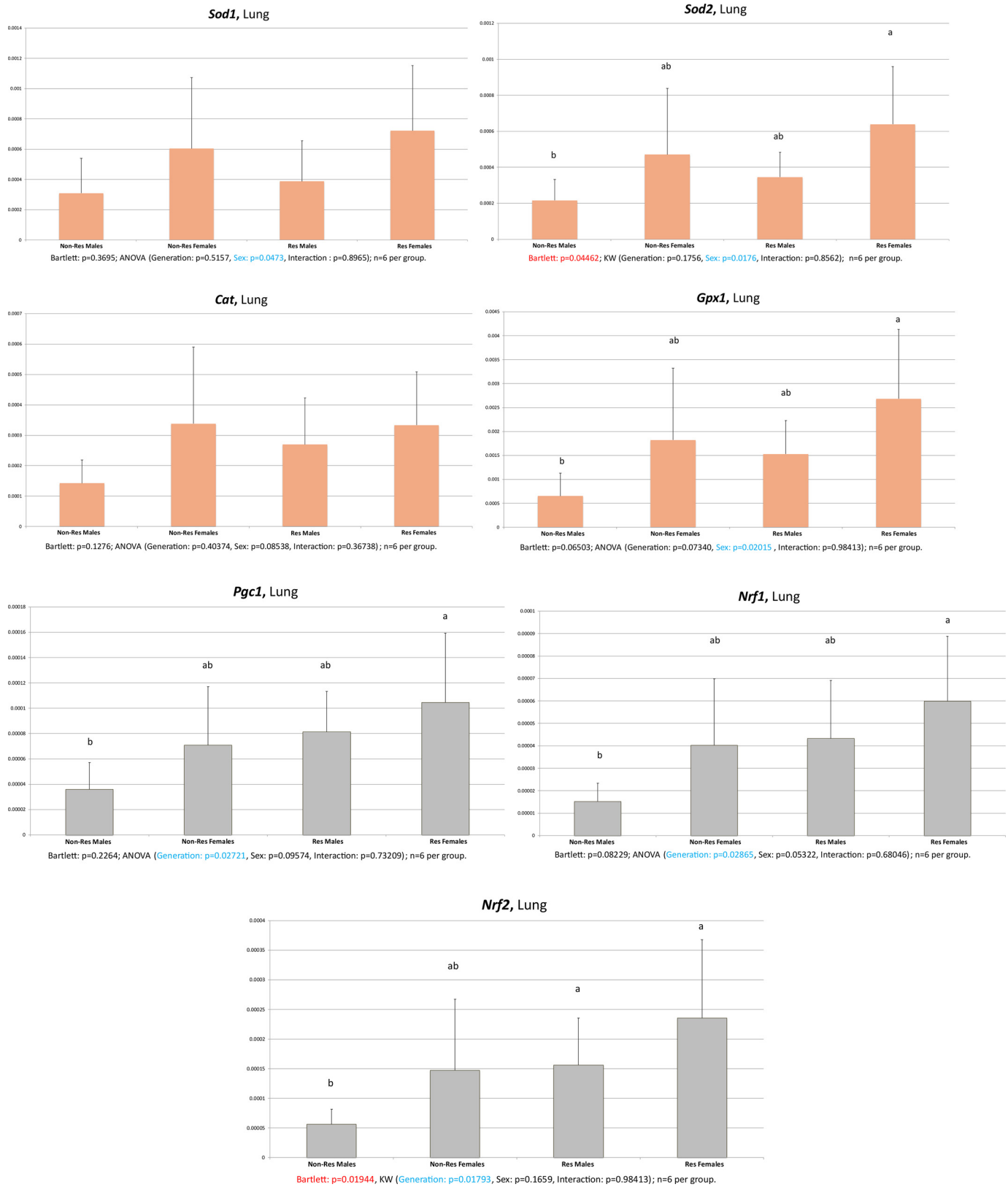
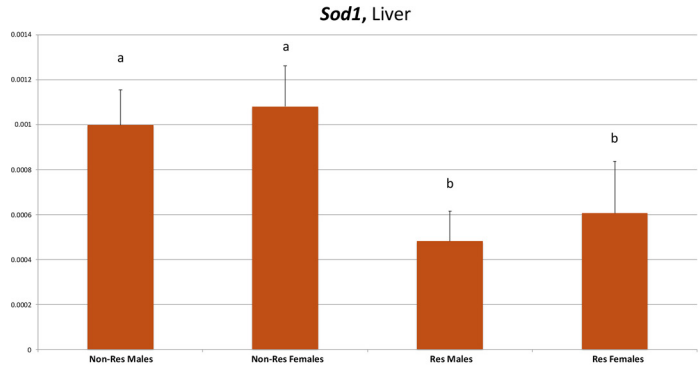
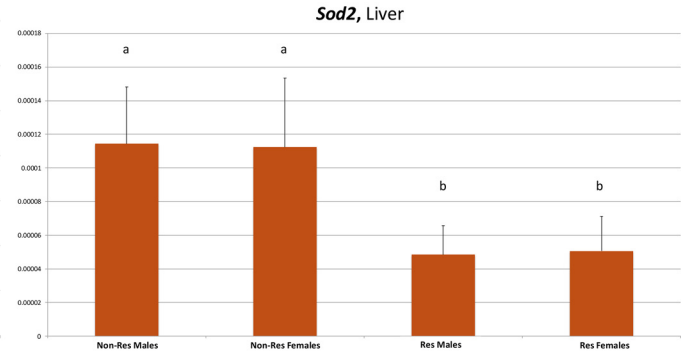


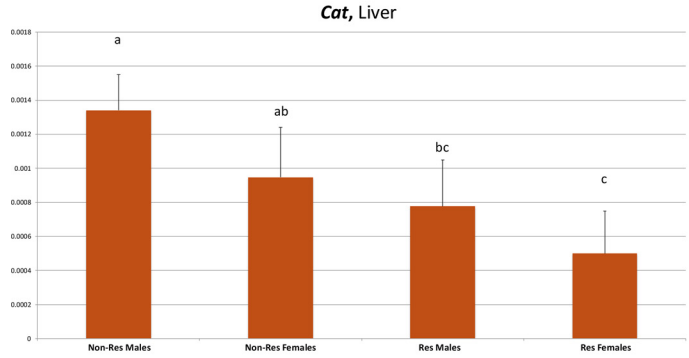
Figure 1. Lungs normalized mRNA expressions. Histogram of normalized lung expression levels. Significant effects of sex, selection, or their interaction are indicated in red. Trends ($0.05 > P \geq 0.00833$) identified in the Tukey post hoc tests are indicated in blue. Different letters denote statistically significant differences between experimental groups after post hoc correction. Four groups of six animals each ($n = 6$ per group) were analyzed: resistant males (res males), resistant females (res females), nonresistant Wistar males (nonres males), and nonresistant Wistar females (nonres females). All values are expressed as means ± SE and normalized to 18S rRNA expression.



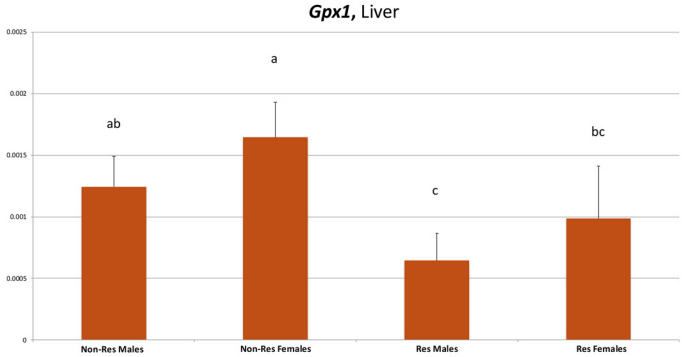
Bartlett: p=0.689; ANOVA (Generation: p=1.348e-06, Sex: p=0.1724, Interaction: p=0.7734); n=6 per group.



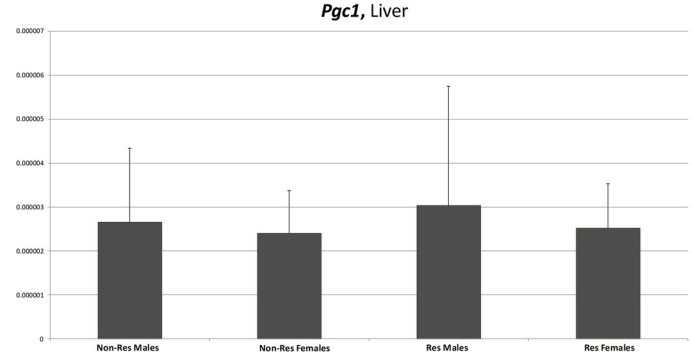
Bartlett: p=0.2259; ANOVA (Generation: p=3.894e-05, Sex: p=0.9969, Interaction: p=0.8636); n=6 per group.



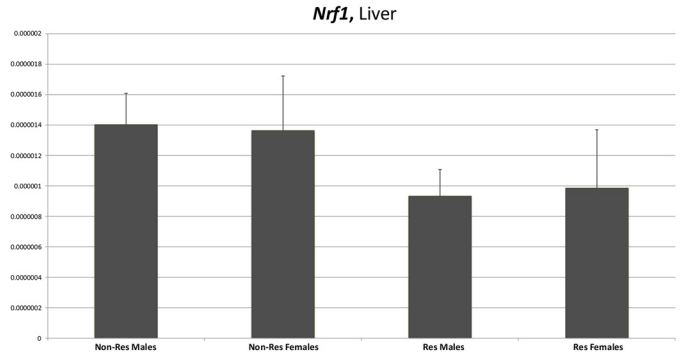
Bartlett: p=0.9081; ANOVA (Generation: p=0.0001072, Sex: p=0.0046759, Interaction: p=0.5858716); n=6 per group.



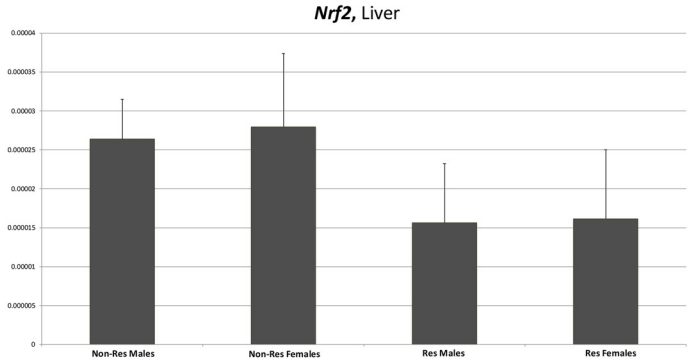
Bartlett: p=0.4839; ANOVA (Generation: p=6.15e-05, Sex: p=0.007303, Interaction: p=0.815503); n=6 per group.



Bartlett: p=0.08253; ANOVA (Generation: p=0.7336, Sex: p=0.6004, Interaction: p=0.8579); n=6 per group.



Bartlett: p=0.26; ANOVA (Generation: p=0.00232, Sex: p=0.979412, Interaction: p=0.705238); n=6 per group.



Bartlett: p=0.62; ANOVA (Generation: p=0.002277, Sex: p=0.746392, Interaction: p=0.868406); n=6 per group.

Figure 2. Liver normalized mRNA expressions. Histogram of normalized liver expression levels. Significant effects of sex, selection, or their interaction are indicated in red. Trends ($0.05 > P \geq 0.00833$) identified in the Tukey post hoc tests are indicated in blue. Different letters denote statistically significant differences between experimental groups after post hoc correction. Four groups of six animals each ($n = 6$ per group) were analyzed: resistant males (res males), resistant females (res females), nonresistant Wistar males (nonres males), and nonresistant Wistar females (nonres females). All values are expressed as means ± SE and normalized to 18S rRNA expression.

this not as mitochondrial dysfunction, but as a potential protective metabolic adaptation. This "metabolic idling" may limit baseline oxidant production, thereby priming systemic tissues, including the lung and liver, to better withstand the oxidative challenge of compression and decompression.

In this context, the differential expression of *Nrf1*, *Nrf2*, and *Pgc1α* observed here may represent adaptive regulation of antioxidant and mitochondrial biogenesis pathways aimed at maintaining redox homeostasis under conditions that predispose to oxidative injury. The decrease in hepatic expression of these genes in resistant rats could therefore reflect a protective metabolic adjustment, reducing mitochondrial activity and ROS generation in tissues more sensitive to hyperbaric stress.

However, these observations focus on a limited number of key gene expressions and do not provide direct evidence of increased mitochondrial adaptations. Therefore, further experiments involving controlled hyperbaric exposures and real-time assessment of mitochondrial ROS production, as well as mitochondrial mass or volume density, will be necessary to confirm this mechanistic link and to determine whether such transcriptional adjustments indeed confer protection against pressure-induced oxidative injury.

The importance of mitochondrial dysfunction is also supported by the increased amounts of circulating mtDNA in divers with neurological DCS symptoms, unlike endothelial cell DNA (19). Mitochondrial DNA (mtDNA) and mitochondrial reactive oxygen species are released in response to mitochondrial damage and act as danger-associated molecular patterns (DAMPs) (42). These contribute to the formation of the mitochondrial antiviral signaling protein complex and have been associated with the activation of the NLRP3 inflammasome. Such a pressure-induced activation of NLRP3 has been reported by Thom et al. (43). On the contrary, Sébert and Théron (17) reported that eel's oxidative phosphorylation is not altered by high hydrostatic pressures, unlike trout (which do not migrate under pressure), suggesting that resistance to elevated pressure partly relies on mitochondrial resistance. This hypothesis is also supported by our previous report of lower basal, but not maximal, mitochondrial oxygen consumption and CS activity in soleus muscles of resistant compared with Wistar males, which might reflect a decrease in the volume of mitochondria (10). However, as CS activity does not always scale linearly with mitochondrial number, this finding may also reflect functional remodeling or enhanced efficiency rather than a simple decrease in mitochondrial content. Taken together, these data suggest a key role of mitochondria in triggering inflammatory processes (13) and their potential involvement in DCS onset through the production of ROS.

We were first interested in the enzymatic activities of what constitutes the main line of antioxidant defenses (44): superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). Our present results seem to be coherent with the lack of differences in the maximal activities of the resistant animals in striated skeletal muscle (10), as we did not detect any changes in the amounts of mRNAs coding for *Sod1* and *Cat*, nor for *Sod2* and *Gpx1* in the lungs. Conversely, in the liver, they were all lower in resistant animals than in Wistar rats. Could this be explained by tissue-specific adaptations to varying levels of oxidative stress exposure? The lungs, being the first line of defense, are directly subjected to higher oxygen partial pressures from the breathing gas. If this initial barrier is effective at limiting oxidative exposure, then the liver, positioned further downstream in the gas exchange cascade, would primarily face metabolic oxidative stress. This stress would be comparatively lower if the metabolism of the resistant rats is reduced or if their mitochondria are more efficient.

Indeed, the mRNA levels of *Nrf1*, *Pgc1α*, and *Nrf2*, three key transcriptional regulators of mitochondrial biogenesis, tend to be increased in the lungs of rats selected for resistance to decompression sickness (DCS). In contrast, in the liver, the expression of these genes is decreased, except for *Pgc1α*, which remains unchanged.

According to several authors, including Fontecha-Barriuso et al. (45) and Scarpulla (35), *Pgc1α* acts upstream to activate *Nrf1* and *Nrf2* in the kidney, whereas Balestra et al. (46) reported a similar *Pgc1α-Nrf* regulatory mechanism in human peripheral blood mononuclear cells exposed to mild transient hyperoxia. Conversely, Ramachandran et al. (47) proposed that *Nrf1* can activate *Pgc1α* and *Nrf2*, suggesting that these transcription factors may operate within tissue-specific regulatory networks.

The lower hepatic expression of *Nrf1* and *Nrf2* observed here is consistent with a reduction in mitochondrial biogenesis at the liver level. This finding parallels the decrease in citrate synthase (CS) activity previously reported in the soleus muscles of resistant males. Whether this downregulation reflects a lower global metabolic demand or an enhancement of mitochondrial efficiency remains to be determined.

The lungs are the place of circulating bubble elimination in the body, so if we might think the bubbles play a role in triggering the inflammatory mechanisms involved in DCS, it possibly goes through circulating mediators activated at the pulmonary level, which might imply an adaptation more correlated to bubbles than to changes in gas partial pressures or absolute pressure. Moreover, the assumption that the liver encounters fewer bubbles than the lungs during diving is uncertain. The liver is a primary receiving site for iatrogenic venous air emboli originating from the inferior vena cava (20), and animal studies have reported abundant bubbles in the livers of rats exposed to rapid decompression (27). Since the liver is responsible for producing most circulating proteins, including those critical to coagulation and inflammation (48), it is worth considering whether hepatic bubble presence correlates more strongly with decompression sickness than overall venous gas embolism, which has a weak predictive value for DCS (2, 31). The present study does not allow us to determine whether the liver plays a central role in systemic DCS, since our animals were examined in a basal, nondiving state. Future studies involving controlled hyperbaric exposure will be necessary to explore this possibility. A large corpus of recent work has also demonstrated that microparticle (MP) release and DCS onset can be triggered not only by the presence of bubbles, but also by changes in gas partial pressures and absolute pressure (49–51). Therefore, we cannot exclude the possibility that the resistance observed in our selectively bred rats may stem from an improved cellular or systemic adaptation to these pressure

variations, such as a reduced oxidative or inflammatory response to pressure-induced microparticle release. In our previous work, we have also reported a significantly higher neutrophil-to-lymphocyte ratio and higher leukocyte counts in resistant animals, as well as a crossed miRNome/transcriptome analysis (52), which confirmed that resistance to DCS is fundamentally linked to innate immune signaling and inflammatory pathways. We have also found significant associations with single-nucleotide polymorphism in the *MyD88* and *NFKB1* genes, which are core components of the toll-like receptor (TLR) signaling pathway and might orchestrate the inflammatory response to decompression stress (53). These findings collectively suggest that although bubble presence remains a key suspect in DCS pathophysiology, the interplay between mechanical stress, gas exposure, and tissue-level sensitivity, especially in organs such as the lung and liver, may be equally critical. Future work should aim to decipher whether the resistant phenotype is more closely linked to a mitigated response to circulating bubbles or to an intrinsic resilience against the pressure-induced cellular activation known to initiate inflammatory cascades through MPs and other mediators.

Moreover, considering the established link between mitochondria, oxidative stress, and the initiation of inflammatory responses (54–56), our results align with findings by Blatteau et al. (19), who reported elevated levels of circulating mitochondrial DNA (mtDNA) in cases of neurological DCS; levels that were otherwise similar to those in nondiving controls in unaffected individuals. This suggests that when oxidative stress is well-regulated, mitochondria remain functional and are not excessively degraded, leading to stable circulating mtDNA levels. In contrast, in pathological conditions, such as neurological DCS, mitochondrial damage may exceed the cell's coping capacity, resulting in increased mitochondrial death and the release of mtDNA into the circulation. Based on this, we hypothesize that the mitochondria in our DCS-resistant rats may be more efficient, characterized by lower density and reduced basal oxygen consumption, which could contribute to decreased ROS production and enhanced cellular resilience to oxidative stress.

Limitations and Perspectives

The interpretation of mitochondrial status in the lung and liver was partly informed by previous findings in skeletal muscle (10) and endothelial cell models (41). Although DCS resistance appears to be a systemic phenotype, we recognize that metabolic and antioxidant responses can be tissue-specific. Future studies using direct organelle-specific measurements, such as high-resolution respirometry in liver and lung mitochondria, are required to confirm if the "metabolic idling" observed in muscle is indeed a cross-organ protective mechanism.

Our observations regarding mitochondrial biogenesis are based on the differential expression of a limited number of key genes. Although the regulation of *Pgc1α*, *Nrf1*, and *Nrf2* is a recognized signature of biogenesis, these transcriptomic changes do not provide direct evidence of increased mitochondrial mass or volume density. These findings should be viewed as hypothesis-generating, and further validation via protein expression (Western blot) or transmission electron microscopy is warranted.

Regarding mitochondrial biogenesis, we acknowledge that the differential expression of a limited number of genes (*Pgc1α*, *Nrf1*, and *Nrf2*) in the current study warrants a cautious interpretation. However, it is noteworthy that these findings align with our recent miRNome/transcriptome crossed-analysis (52), which identified significant modifications in pathways related to mitochondrial biogenesis and metabolic regulation in DCS-resistant phenotypes. This multiomics evidence suggests that the transcriptional "priming" of mitochondrial pathways is a robust feature of resistance. Nonetheless, as transcript levels do not always correlate with functional protein mass, these results should be viewed as hypothesis-generating. Further validation via transmission electron microscopy or mitochondrial protein quantification (e.g., PGC-1α or TOM20) is an essential next step.

In conclusion, although the present study identifies robust molecular markers of resistance, these markers represent a "snapshot" of the transcriptional landscape. Functional validation through direct physiological measurements in the target tissues remains a critical next step to fully elucidate their role in DCS resistance.

DATA AVAILABILITY

All data analyzed in this article are publicly available in figshare (https://doi.org/10.6084/m9.figshare.30011116). All the datasets are available through public repositories to support reproducibility, and the corresponding author can provide any details or materials upon reasonable request.

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GRANTS

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

E.D., E.G., F.G., and A.G. conceived and designed research; E.D., J.O., F.G., and A.G. performed experiments; E.D., J.O., E.G., F.G., and A.G. analyzed data; E.D., J.O., E.G., F.G., and A.G. interpreted results of experiments; E.D., F.G., and A.G. prepared figures; E.D., E.G., F.G., and A.G. drafted manuscript; E.D., J.O., E.G., F.G., and A.G. edited and revised manuscript; E.D., J.O., E.G., F.G., and A.G. approved final version of manuscript.

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