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RESEARCH ARTICLE

Physiological characteristics associated with increased resistance to decompression sickness in male and female rats

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Lautridou J, Dugrenot E, Amerand A, Guernec A, Pichavant-Rafini K, Goanvec C, Inizan M, Albacete G, Belhomme M, Galinat H, Lafère P, Balestra C, Moisan C, Buzzacott P, Guerrero F. Physiological characteristics associated with increased resistance to decompression sickness in male and female rats. *J Appl Physiol* 129: 612–625, 2020. First published July 23, 2020; doi:10.1152/japplphysiol.00324.2020.—Decompression sickness (DCS) is a complex and poorly understood systemic disease with wide interindividual resistance variability. We selectively bred rats with a threefold greater resistance to DCS than standard ones. To investigate possible physiological mechanisms underlying the resistance to DCS, including sex-related differences in these mechanisms, 15 males and 15 females resistant to DCS were compared with aged-matched standard Wistar males ($n = 15$) and females ($n = 15$). None of these individuals had been previously exposed to hyperbaric treatment. Comparison of the allelic frequencies of single nucleotide polymorphisms (SNPs) showed a difference of one SNP located on the X chromosome. Compared with nonresistant rats, the neutrophil-to-lymphocyte ratio and the plasmatic activity of coagulation factor X were significantly higher in DCS-resistant individuals regardless of their sex. The maximal relaxation elicited by sodium nitroprusside was lower in DCS-resistant individuals regardless of their sex. Males but not females resistant to DCS exhibited higher neutrophil and lymphocyte counts and higher prothrombin time but lower mitochondrial basal O_2 consumption and citrate synthase activity. Principal components analysis showed that two principal components discriminate the DCS-resistant males but not females from the nonresistant ones. These components were loaded with activated partial thromboplastin time, monocyte-to-lymphocyte ratio, prothrombin time, factor X, and fibrinogen for PC1 and red blood cells count and neutrophils count for PC2. In conclusion, the mechanisms that drive the resistance to DCS appear different between males and females; lower coagulation tendency and enhanced inflammatory response to decompression stress might be key for resistance in males. The involvement of these physiological adaptations in resistance to DCS must now be confirmed.

NEW & NOTEWORTHY By selective breeding of individuals resistant to decompression sickness (DCS) we previously obtained a rat model of inherited resistance to this pathology. Comparison of these individuals with nonresistant animals revealed differences in leukocyte counts, coagulation, and mitochondrial and vascular functions, but not resistance to oxidative stress. This study also reveals sex-related differences in the physiological changes associated with DCS resistance. A principal components analysis of our data allowed us to discriminate DCS-resistant males from standard ones, but not females. These differences represent possible mechanisms driving resistance to DCS. Although still far from the diver, this opens a pathway to future adaptation of personalized decompression procedures for "DCS-prone" individuals.

coagulation; decompression illness; inflammation; mitochondria; oxidative stress; prevention; SCUBA diving; vascular smooth muscle

INTRODUCTION

Decompression sickness (DCS) has a protean systemic pathology, occurring among people exposed to rapid drop in environmental pressure (scuba divers, airplane pilots, hyperbaric chamber workers, or astronauts). Among scuba divers, DCS consists of a wide range of symptoms, including skin rash, muscular and/or articular pain, and/or neurological impairments such as paresthesia, distal numbness, and paralysis; it may even result in death (48). There is evidence that wide interindividual variability exists for susceptibility to DCS. This so-called "probabilistic nature of decompression sickness" (3) has been well documented by experiments from animal models of DCS that provide many examples of this interindividual variability (9, 33). The risk of DCS correlates with the amount of circulating venous gas emboli (VGE) formed during and after decompression (16). However, a very high amount of detected VGE has been reported in a diver without any symptoms of decompression sickness (2), and, more generally, a large epidemiological study showed that similar amounts of VGE did not correlate strongly with similar susceptibility for DCS (12). This strongly suggests that other factors modulate the power of VGE to trigger DCS. DCS risk has been claimed to depend on numerous physiological variables, including inflammation (13, 47), coagulation (29, 30), oxidative stress (37), and vascular dysfunction (28, 37), but little consensus has been reached and the primary physiological variables that drive resistance to DCS remain to be specified.

We initiated a large-scale artificial selection program with Wistar rats, based on their resistance to DCS, and reported a

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threefold decrease of DCS occurrence (31). This selection program, which is still ongoing, provides us with a population with significantly increased resistance to DCS. Therefore, our objective is now to decipher the mechanisms of resistance to DCS by comparing these individuals with standard Wistar rats. In our preliminary study, we also observed that the gain in resistance was statistically significant as early as the second generation in females while this was only the case from the third generation in males, although the DCS resistance was no longer different in males than in females at the third generation. This led us to also investigate whether sex-related differences exist in the physiological characteristics associated with DCS resistance.

MATERIALS AND METHODS

Ethical approval. The protocol described in this study was conducted in accordance with the Directive 2010/63/EU of the European Parliament, of the Council on the protection of animals used for scientific purposes, and with the French national laws R214-87 to R214-137 of the Rural Code and subsequent modifications. It follows the 3Rs and was approved by the Ethics Committee of the Université de Bretagne Occidentale for Animal Experimentation (approval no. APAFIS#10395–2017061909495511).

Animals. The physiological modifications associated with the resistance to DCS were investigated in 165 rats from the sixth generation (G_6) of our selection protocol. Among them, 67 males and 68 females were submitted to a standardized hyperbaric protocol to assess DCS resistance. Biological analyses were performed on 30 other G_6 animals (15 males and 15 females). Because our aim was to identify the individual physiological characteristics associated with resistance to DCS independent of persistent physiological modifications induced by diving itself (15, 38), none of these individuals were previously exposed to hyperbaric treatment. They were compared with age-matched standard Wistar rats, i.e., the same as those we used for the founding stock, obtained from the same breeder (Janvier Laboratories, St. Genêts, France). We refer to these as G_0. The standard rats (G_0) were acclimated with the facility for 2 wk. The experimental setup is presented in Fig. 1. All animals were housed three per cage under controlled temperature (21 ± 1°C) and lighting (12 h of light per day, 0600–1800) at the university animal housing facility until the day of the hyperbaric treatment. They were fed standard rat chow and water ad libitum.

Hyperbaric protocol. At 11 wk old, the rats were placed in a 130-L steel hyperbaric chamber and compressed with air at a rate of 100 kPa/min up to 1,000 kPa absolute pressure (equivalent to 90 meters of sea water, msw). After 45 min at maximum pressure, decompression was conducted at a rate of 100 kPa/min with the stops (5 min at 200 kPa, 5 min at 160 kPa, and 10 min at 130 kPa). This hyperbaric protocol is known to trigger 63 ± 4% DCS (9). All simulated dives were performed in the morning (at 8:00, 10:00, and 12:00 AM) to avoid circadian differences because of the day-night cycle, and within 1 wk to avoid any difference in age (animals were 80 ± 1 days old at the time of the dive).

After decompression, animals were observed for 1 h for signs of DCS, especially respiratory distress, paralysis, convulsions, and death. This observation method has already been used in previous studies (33). To minimize the potential for animal suffering, a pain/distress scale was approved by the Animal Research Ethics Committee, and no rats displayed signs of distress at or above the level where early euthanasia was required. Except for DNA sampling for the TagSNP allele frequency shift analysis, no biological analyses were done on animals submitted to the hyperbaric protocol. Therefore, all biological investigations were performed on naïve animals, not subjected to the hyperbaric protocol before.

A control group consisted of the 52 male and 52 female standard Wistar rats that composed the founder population (G_1). These rats underwent the same diving protocol, were handled the same way, and were the same age on the day of their simulative dive.

TagSNP allele frequency shift. TagSNPs can be defined as a single nucleotide polymorphism (SNP) that will be used to represent a group of SNPs in a specific region of the genome. It allows for the identification of genetic variation associated to specific phenotypes without having to genotype each SNP of the chromosomal region. Because this technique is performed on DNA, the type of tissue used for extraction does not have an impact on the results. Liver tissue allows for an easy and repeatable DNA extraction process. Therefore, liver samples from 96 rats from the first and sixth generations were obtained after the hyperbaric protocol and stored at −80°C. For each individual, 25 mg of tissue were placed in a 96-deep-well plate. Samples were then shipped on dry ice to LGC Genomics Ltd., which performed a Kompetitive allele specific PCR genotyping analysis on 48 tagSNPs chosen from a list of Wistar-specific SNPs described by Nijman et al. (39). Because we previously hypothesized that the gain in resistance to DCS could be linked to a gene (or a major gene group) located on the X chromosome, each chromosome from 1 to 20 was assigned with one specific SNP, whereas the X chromosome was assigned with 28 SNPs.

Blood and tissue sampling. For biological analyses, rats (18–19 wk old) not previously exposed to hyperbaric treatment were anesthetized by intramuscular injection of ketamine (80 mg/kg) and xylazine (15 mg/kg). First, blood was sampled by intracardiac puncture and collected in 6.15×10^{-3} mol/L EDTA vacuum tubes for determination of blood count, or 0.109 mol/L trisodium citrate vacuum tubes for hemostasis tests.

Thereafter, the soleus muscle was finely minced (~5 mg/piece) for assessment of mitochondrial function and quantification of enzymatic activities.

Finally, the descending part of the thoracic aorta was carefully removed and immediately stored in iced-cold Krebs-Henseleit bicarbonate (KHB) solution previously gassed with 95% O_2-5% CO_2. The composition of the KHB solution was (in mM): 118.0 NaCl, 25.0 $NaHCO_3$, 1.18 KH_2PO_4, 4.74 KCl, 1.18 $MgSO_4$, 2.5 $CaCl_2$, and 10.0 glucose (pH = 7.4).

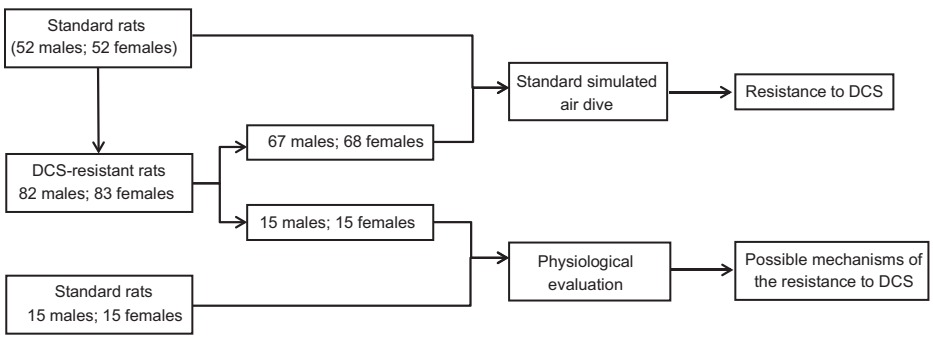

Fig. 1. Design of the experimental protocol.

Blood analysis. Hematocrit, hemoglobin, and absolute counts of erythrocytes, reticulocytes, platelets, monocytes, neutrophils, lymphocytes, eosinophils, and basophils were measured within 2 h on a Sysmex XE 5000 automat (Kobe, Japan). Neutrophil-to-lymphocyte ratio (NLR), monocyte-to-lymphocyte ratio (MLR), and platelet-to-lymphocyte ratio (PLR) were obtained by dividing neutrophil, platelet, and monocyte counts, respectively, by lymphocyte count.

For hemostasis tests, citrated tubes were gently turned upside down five times. The following three 15-min centrifugations were performed: one at 1,000 *g* and two at 3,000 *g* (centrifuge 2–16 K; Sigma) to obtain platelet-poor plasma. Plasma was frozen at −80°C until analysis. Measurements of factor II (FII), factor V (FV), factor X (FX), fibrinogen (Fib), prothrombin time (PT), and activated partial thromboplastin time (aPTT) were performed on thawed platelet-poor plasma on an automated analyzer (STA-R Evolution, Stago, Asnieres sur Seine, France). PT, aPTT times, and fibrinogen concentrations were measured with the following specific reagents: neoplastine Cl + 10, triniclot aPTT, and STA liquid Fib, respectively. FII, FV, and FX coagulation activities were determined using a PT-based clotting test incorporating FII-, FV-, or FX-deficient plasma (STA–Deficient; Diagnostica Stago). Coagulation factor activities are expressed as percentage of laboratory reference.

Vessel tension measurement. For the study of vascular smooth muscle (VSM) reactivity, aortas were carefully dissected to remove surrounding connective tissue and cut into rings of 2 mm length. Four rings from each vessel were then suspended in a jacketed 20-ml organ bath maintained at 37°C. The tension was measured isometrically using a force transducer (EMKA Technologies) and recorded digitally. Passive tension was maintained at 2 *g* throughout the experiment. Before each experiment was performed, the rings were allowed to equilibrate for a 45-min period and washed every 15 min. Thereafter, all rings were contracted with 80 mM KCl solution, obtained by isosmotic replacement of Na^+ by K^+ in KHB. Between trials, the rings were allowed to recover for 45 min and washed every 5 to 10 min. Thereafter, VSM contraction was directly stimulated again in two rings by 80 mM KCl solution before agonist-induced contraction was assessed by adding cumulative doses of phenylephrine (PE, 10^{-9} to10^{-4} M). In the other two rings, the sensitivity of VSM to the NO donor sodium nitroprusside was investigated by adding cumulative doses (10^{-9} to 10^{-4} M). All drugs were purchased from Sigma Chemicals. PE and sodium nitroprusside were prepared in distilled deionized water and frozen for later use.

Mitochondrial function. For permeabilized fiber preparation, immediately after sampling, a part of the soleus muscle fibers was placed at 4°C in *buffer A* (in mM: 10 EGTA, 20 taurine, 3 $MgCl_2$, 50 MOPS, 0.5 dithiothreitol, 15 phosphocreatine, and 5 ATP; at pH 7.4) containing saponin (100 μg/mL) to selectively destroy the integrity of the sarcolemma. After 20 min of incubation, the fibers were washed two times in *buffer A* for 10 min to completely remove the saponin and metabolites. After being rinsed, the permeabilized fibers were exposed (or not) to reactive oxygen species (ROS) before oxygen consumption measurement. For in vitro ROS exposure, a part of the permeabilized fibers was exposed to exogenous ROS by incubating them with $FeCl_2/H_2O_2$ according to the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{-}OH + {}^{\cdot}OH$). The permeabilized fibers were transferred to *buffer A* containing 1 mM $FeCl_2$ and 5 mM H_2O_2 for 30 min at 4°C. Other permeabilized fibers, which are regarded as the control, were incubated in *buffer A* without H_2O_2 or $FeCl_2$. After the incubation time, the fibers were washed two times for 5 min in *buffer A*. Measurements of oxygen consumption were then determined.

The oxygen consumption rate of control and ROS-exposed permeabilized fibers (~20 mg) was measured polarographically at 37°C, using a Strathkelvin 928 6-Channel Oxygen System and a Clark-type electrode in a closed thermostatted oxygraph cuvette equipped with magnetic stirring. The measurement was carried out in 2 ml of a respiratory buffer (in mM: 20 Tris, 150 KCl, 0.08 EDTA, 10 NaH_2PO_4, and 7.5 $MgCl_2$; pH 7.2). First, basal respiration ($\dot{V}_0$), defined as state 4 in mitochondria preparation, was assessed by adding the tricarboxylic acids cycle intermediates pyruvate/malate (12 mM/6 mM). Next, the maximal ADP-stimulated respiration ($\dot{V}_{max}$), defined as state 3 in mitochondria preparation, was measured by adding 5 mM ADP. The respiratory substrates (pyruvate/malate and ADP) were used at saturating concentrations. The oxygen consumption was expressed in nanomoles O_2 per minute per gram of wet weight. The respiratory control ratio (RCR) was calculated by the ratio $\dot{V}_{max}/\dot{V}_0$. Vulnerability to ROS was expressed in percentage of the oxygen consumption variation in exposed vs. nonexposed fibers.

Enzyme activities and protein content in soleus. Samples from frozen tissues were placed in an extraction buffer (75 mM Tris and 5 mM EDTA) at 4°C and pH 7.4 for homogenization with a Polytron homogenizer before centrifugation. After centrifugation of the resulting supernatant at 12,000 *g* for 10 min at 4°C, the activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) were determined at 25°C as was the protein content. SOD activity was measured at 480 nm using the method that inhibits the adrenaline-adrenochrome reaction. One unit of SOD is equal to the amount of sample needed to cause 50% inhibition compared with the control (100%). SOD activity was expressed in units per milligram protein. GPx activity was assessed at 340 nm using an indirect method. Briefly, it was determined from the decrease of NADPH induced by a coupled reaction with glutathione reductase. GPx activity was expressed in micromole NADPH oxidized per minute per milligram protein. CAT activity was determined at 240 nm through its ability to transform H_2O_2 into H_2O and O_2. The assay concentration of H_2O_2 was 10 mM in 75 mM Tris and 5 mM EDTA buffer at pH 7.4. CAT activity was expressed in $\mu mol\ H_2O_2 \cdot min^{-1} \cdot mg^{-1}$ protein. Citrate synthase activity (CS) was assessed at 412 nm using 5,5-dithio-bis-2-nitrobenzoic acid (DTNB). Samples from frozen tissues were placed in an extraction buffer (100 mM Tris) of pH 8.1 at 4°C for homogenization with a Polytron homogenizer. Measurement was performed on muscle homogenate at 37°C and expressed in nmol DTNB per minute per milligram protein. Protein content was measured by the colorimetric method (562 nm) using a BC Assay Protein Quantitation Kit (no. FT-40840A; Uptima-Interchim). The protein concentration was expressed in milligrams protein per gram tissue.

Statistics. All data were analyzed using Statistica software (v. 13; StatSoft France). The proportion of DCS outcomes between G_1 and G_6 in each sex was compared by χ^2 tests. For physiological parameters, a Shapiro-Wilk W test was first performed to assess normality. If the data were parametric, we proceeded with two-way ANOVA, with sex (male, female) and generation (G_0, G_6) as independent variables and the considered parameter as a dependent variable. When the ANOVA identified significant differences, a Fisher least-significant difference post hoc test was used to assess the differences. For nonparametrically distributed results, we used an ANOVA with a Kruskal–Wallis test on the following four groups: G_0 females (G_{0F}), G_0 males (G_{0M}), G_6 females (G_{6F}), and G_6 males (G_{6M}). Data were considered significant at $P < 0.05$ and reported either as means ± SE or as median (25th–75th percentiles) for the indicated samples.

Finally, a principal components analysis (PCA) was used to find a pattern in physiological variables. For determination of variables that represent each PC, a cut-off value of |0.5| is usually used for biological markers (56). Because of the low number of cases (15 animals per group) in our study, variables with a loading above the cut-off point |0.70| were considered to be the dominant variables in a component. We then performed regression analysis to explore the association of each PC with resistance to DCS. For these analyses, all variables were mean centered and standardized; missing values were replaced by the mean value of the group.

RESULTS

Resistance to DCS. At 11 wk old, body weight was 363 ± 4 and 235 ± 2 g in G_6 males and females, respectively. This was statistically significantly less than G_1 at the same age. Indeed, G_1 males and females weighted 426 ± 3 ($P < 0.001$) and 251 ± 2 ($P < 0.001$) g, respectively.

The prevalence of DCS was 28 and 20% in G_6 males and females, respectively, and was not different between the sexes [$\chi^2 = 1.09$, degrees of freedom (df) = 1, $P = 0.29$]. For both sexes, the prevalence of DCS was significantly lower than previously observed in G_1: 28 vs. 65% DCS for G_6 and G_1 males, respectively ($\chi^2 = 24.64$, df = 1, $P < 10^{-4}$), and 20 vs. 65% DCS for G_6 and G_1 females, respectively ($\chi^2 = 16.25$, df = 1, $P = 10^{-4}$).

Polymorphic SNPs. Among the 48 SNPs genotyped, only 18 were found polymorphic. The sequences and localization of these polymorphic SNPs are given in Table 1. Among these polymorphic SNPs, only the allelic frequency of the SNP 63759801–63759881, located on the X chromosome, was significantly different between G_1 and G_6 [χ^2 test, raw P value = 0.001, corrected P value < 0.0028 (Bonferroni correction)].

Complete blood count. At 18 to 19 wk old, body weight was 537 ± 13 vs. 451 ± 12 g ($P < 0.001$) in G_0 and G_6 males, respectively, and 313 ± 6 vs. 285 ± 5 g ($P = 0.005$) for G_0 and G_6 females, respectively. Data on blood parameters are presented in Table 2. Two-way ANOVA did not detect a significant effect of sex ($F = 0.300$, df = 1, $P = 0.587$) or generation ($F = 0.350$, df = 1, $P = 0.556$) on hematocrit, nor a sex times generation interaction ($F = 1.37$, df = 1, $P = 0.251$). The analysis did show that sex exerts a significant effect on reticulocyte count ($F = 6.780$, df = 1, $P = 0.014$) as well as on erythrocyte count ($F = 5.82$, df = 1, $P = 0.022$), but not on hemoglobin ($F = 1.09$, df = 1, $P = 0.305$). It also showed that reticulocyte count ($F = 6.603$, df = 1, $P = 0.016$), but not erythrocyte count ($F = 0.03$, df = 1, $P = 0.856$) or hemoglobin ($F = 0.18$, df = 1, $P = 674$), was significantly different in G_6 compared with G_0. Finally, there was no significant sex times generation interaction effect on erythrocyte count ($F = 2.72$, df = 1, $P = 0.110$), reticulocyte count ($F = 0.729$, df = 1, $P = 0.401$), or hemoglobin ($F = 1.64$, df = 1, $P = 0.210$), which suggests that reticulocyte but not erythrocyte count or hemoglobin was lower in both male and female DCS-resistant individuals than in standard Wistar.

Considering leukocytes, two-way ANOVA indicated a statistically significant effect of sex on both neutrophil ($F = 25.807$, df = 1, $P = 2.10^{-5}$) and lymphocyte ($F = 6.586$, df = 1, $P = 0.015$) counts while generation was significant for neutrophil ($F = 6.044$, df = 1, $P = 0.020$) but not lymphocyte ($F = 1.104$, df = 1, $P = 0.302$) count. However, significant sex times generation interactions were detected for both neutrophil ($F = 4.904$, df = 1, $P = 0.035$) and lymphocyte ($F = 4.662$, df = 1, $P = 0.039$) counts. Post hoc tests indicated that neutrophil count was significantly higher in G_6 than G_0 males but not females, such that it was not different between sexes for G_0 but was significantly higher in males than females for G_6. The same is true for lymphocyte count also. As a result, both NLR ($F = 6{,}548$, df = 1, $P = 0.016$) and MLR ($F = 6.116$, df = 1, $P = 0.019$) were significantly different between males and females, and generation exerted a major significant effect

Table 1. *Sequence and chromosomal localization of the polymorphic SNPs*

Chromosome	Localization	Sequence
9	28408403–28408483	ACCCCACCCCAACATTCTCCTTTAAAAACACTCCGAATCC[C/T]GCCTATGACTGTCATTTTCTATCCTGCTCATTTAGAAGTT
10	33090635–33090715	CTGAAAAATTTGCTATCAAAGACTTAATGACTATTTAAAA[T/C]CTCTTTCTATTCTTTGAGAGTTCGTATATGAGTATAATGT
11	20635967–20636047	TGACATAAGCATTGTATATACATTGTCAGCATTCACAGCA[T/C]CTTAAAATAATATATACAAAACAGAATCAATTTGAAAATA
12	24177615–24177695	GACTCCAGGCTGAAGCTGAGTCCTTATGGGAGATGGAGGA[T/C]GGCAGGGTCTGAGGCGCTGAGAGCCCCAGACACACTAGCC
15	21828053–21828133	ATCGGTGACGCGGCGTTGGAGAGGGTCTGCTGATAGTGAT[A/G]ACTGAGCCAAAAAGAAAGAGTAGTGTAGAGTGGACAAGGT
17	31376660–31376740	AATAATATGGTAGAGCCTAAATCATCATGGAGTTTATCTC[T/C]CACACTTTGTCATATATGAGTCTGACCAGAGCTCCCCAGT
18	16921137–16921217	TGCAACTACATCACACTGAGGTTCCTCACAGCCCTCACCT[A/G]GCTGCTGAGTCCTCGGTTATTGCTAGCTGCCTTCTGGGAG
X	63759801–63759881	AACCACATTGAGGTGTGATTTTGCAAATTCTCCATATCAC[A/G]ACCTTCACCCTTTGTCACCCAGTTCTCTAATCCAAGTTAC
X	37110132–37110212	ACTGCATGTTCCAGGTAAAATGTGTTTCTAGGTCTTGGTA[A/G]ATAACACAGAAGAATGAGAATTGACTAGAAGGAAGGGCTG
X	16143211–16143131	TGGGTATGGGGGCATGGGAGACACAGAAGGACAGAAAAAC[A/G]GAACAGCAGCCTAAGGTTCCTGTGTAATGTCAGTTCTTAA
X	105875099–105875019	TCCACAATGGAACATACACACTGTGGGTCTCAGCATAAGA[T/C]GTGGGTGGGCCTGGCCAGATAGCTGGAAGAAACAATACT
X	89753705–89753785	AGTGTATAATATGAGAAGCGTTCTCATTAGTAGTTATTATT[C/T]CATGACTATCAATGATAGAAAACCACTGACTTAAATTCTT
X	80329108–80329188	AGTTTGTGCATTAGTGTTAATCTTATGATCTTGTTTCTTC[T/C]GTAACAATATCATGGGTTTCAAAATTAAGAAACTAATAAT
X	130861798–130861878	TCCTTATACATCTTTGTGGACTGTAATAGCCTTTCTTAGG[G/A]AGACATTATTGTAAATCAAGCGCTAATTGCAGTCTACTTC
X	18038405–18038485	GGGCTCTTCTTACTGATTTATTCAATTAAAAGAGTATTTT[T/C]ATCTCAGGTTCTCTAATAAATGAAACATAACAATGTTTCT
X	86039984–86040064	CTCAACCTCTGAATTGAACTTCTACCAGGACTGTCCTACA[C/T]TCTGGAAACATATGTATCTTTTTTAGTAAGAAGAATGTGA
X	93189573–93189653	GAAAAGTCCAGAAGTTGCTGCATATTTTCTAAGCAATTAG[T/G]TGGTTCTGGCTGTGCCCTCTCTGGCTCTACTGTATAAATTA
X	134914274–134914354	TGTAACATTATAACACAGAAGAGACAATCTAATACAGCTG[T/C]AAAGTCAAGGGAAGTGATTCCTAGAAGAAAGGACACTTAA

SNPs, single nucleotide polymorphisms.

Table 2. *Blood count of DCS-resistant selected rats (G_6) and standard Wistar animals (G_0)*

	P Value			G_0		G_6	
	Sex	Generation	Sex × generation	Males	Females	Males	Females
Hematocrit, %#	0.587	0.556	0.251	46.26 ± 0.96	46.76 ± 1.03	46.73 ± 0.52	45.33 ± 0.77
White blood cells							
Neutrophils, giga/L#	2.10^{-5}	0.020	0.035	1.28 ± 0.18[b]	0.87 ± 0.16	1.95 ± 0.11[a,b]	0.90 ± 0.09[a]
Lymphocytes, giga/L#	0.015	0.302	0.039	8.39 ± 0.98[b]	7.96 ± 0.90	11.71 ± 1.10[a,b]	6.81 ± 0.77[a]
Monocytes, giga/L#	0.006	0.303	0.962	0.51 ± 0.11	0.28 ± 0.07	0.42 ± 0.05	0.20 ± 0.05
Eosinophils, giga/L*		0.333		0.025 [0.020, 0.035]	0.020 [0.010, 0.020]	0.020 [0.010, 0.100]	0.010 [0.010, 0.030]
Basophils, giga/L*		0.217		0.005 [0.000, 0.010]	0.005 [0.000, 0.010]	0.005 [0.000, 0.020]	0.000 [0.000, 0.000]
NLR#	0.016	0.035	0.820	0.14 ± 0.04	0.11 ± 0.02	0.17 ± 0.03	0.14 ± 0.04
MLR#	0.019	0.224	0.171	5.6 ± 3[a]	2.7 ± 1[a]	3.6 ± 1.3	2.8 ± 1.9
PLR*		0.343		72.1 [59.7, 79.3]	67.6 [65.6, 85.7]	55.6 [34.9, 1.3]	81.0 [66.9, 96.9]
Red blood cells							
Erythrocytes, tera/L#	0.022	0.856	0.110	8.44 ± 0.20	8.31 ± 0.13	8.73 ± 0.14	8.07 ± 0.14
Reticulocytes, tera/L#	0.014	0.016	0.400	0.33 ± 0.02	0.30 ± 0.01	0.30 ± 0.01	0.24 ± 0.01
Hemoglobin, g/dL#	0.305	0.674	0.210	15.10 ± 0.30	15.16 ± 0.32	15.34 ± 0.21	14.68 ± 0.28
Platelets, giga/L*		0.128		648 [502, 694]	575 [544, 643]	692 [638, 717]	594 [547, 694]
Coagulation parameters							
PT, %#	0.207	0.589	0.022	57.5 ± 3.7[a,b]	71.3 ± 5.5[a]	68.6 ± 2.2[b]	64.4 ± 3.1
aPTT, %#		0.204		0.90 [0.81, 0.99]	0.70 [0.53, 0.90]	0.54 [0.51, 0.55]	0.53 [0.49, 0.89]
Factor X, %	0.007	0.003	0.468	35.1 ± 2.5[a,b]	45.6 ± 3.6[a]	46.7 ± 2.8[b]	53.0 ± 2.5
Factor V, %		0.064		401 [313, 448]	455 [343, 607]	271 [223, 335]	256 [232, 490]
Factor II, %		0.318		39.0 [35.0, 47.0]	50.0 [40.0, 66.0]	42.0 [37.0, 44.0]	43.5 [39.0, 64.5]
Fibrinogen, g/L	5.10^{-4}	0.162	0.058	2.21 ± 0.11[b]	1.94 ± 0.21	2.69 ± 0.07[a,b]	1.86 ± 0.14[a]

DCS, decompression sickness; G_6, 6th generation; G_0, standard rats; NLR, neutrophil-to-lymphocyte ratio; MLR, monocyte-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; PT, prothrombin time; aPTT, activated partial thromboplastin time. #Variable was analyzed with two-way ANOVA test. In these cases, data are expressed as means ± SE. *Variable was analyzed with Kruskal-Wallis test, including 4 groups (G_0 males, G_6 males, G_0 females, and G_6 females). Data are expressed as medians [25th, 75th percentiles]. [a]Different from same generation, opposite sex. [b]Different from same sex other generation.

on NLR ($F = 4.866$, df = 1, $P = 0.035$) but not MLR ($F = 1.546$, df = 1, $P = 0.224$). Interestingly, the sex times generation interaction was no longer statistically significant for NLR ($F = 0.052$, df = 1, $P = 0.820$) or MLR ($F = 1.970$, df = 1, $P = 0.171$). Altogether, this suggests that, although NLR was higher in both males and females resistant to DCS, this occurred through increased neutrophil and lymphocyte counts in males but not females.

Because eosinophil and basophil counts were not Gaussian, Kruskal-Wallis tests were performed, which did not show any differences between groups [$H_{(3,\ N\ =\ 33)} = 3{,}402$, $P = 0.333$ and $H_{(3,\ N\ =\ 33)} = 4{,}438$, $P = 0.217$ for absolute eosinophil and basophil counts, respectively].

Platelet count was not normally distributed so data were analyzed with a Kruskal-Wallis test. No difference was observed for platelet count between G_0 and G_6 regardless of sex [$H_{(3,\ N\ =\ 33)} = 5.682$, $P = 0.128$].

Coagulation parameters. Values of coagulation parameters in the four groups are presented in Table 2. Two-way ANOVA showed that PT was not significantly altered by sex ($F = 1.679$, df = 1, $P = 0.207$) nor by generation ($F = 0.300$, df = 1, $P = 0.589$). However, the sex times generation interaction was statistically significant ($F = 5.919$, df = 1, $P = 0.022$). Indeed, PT was significantly lower in G_0 males than in either G_6 males or G_0 females, whereas no difference was found between G_0 and G_6 females. Conversely, a Kruskal-Wallis test failed to detect any statistically significant difference in aPTT between groups [$H_{(3,\ N\ =\ 28)} = 4{,}591$, $P = 0.204$]. Analysis of fibrinogen serum concentration indicated the significant effect of sex ($F = 15.742$, df = 1, $P = 0.572 \times 10^{-3}$) but not generation ($F = 2.078$, df = 1, $P = 0.162$). Although a tendency was detected for the sex times generation interaction ($F = 3.966$, df = 1, $P = 0.057$), suggesting that fibrinogen was higher in G_6 than G_0 males but not different in females, the difference did not reach statistical significance. FII was not different among groups [Kruskal-Wallis test: $H_{(3,\ N\ =\ 28)} = 3.516$, $P = 0.318$], nor was FV [Kruskal-Wallis test: $H_{(3,\ N\ =\ 28)} = 7.242$, $P = 0.064$]. Two-way ANOVA indicated the significant effects of sex ($F = 8.538$, df = 1, $P = 0.007$) and generation ($F = 10.799$, df = 1, $P = 0.003$), but not the sex times generation interaction ($F = 0.542$, df = 1, $P = 0.468$) on FX, indicating that this factor was higher in G_6 than in G_0 individuals. However, the difference was significant for males ($P = 0.008$) only, but not females ($P = 0.085$).

Antioxidant enzyme activity. Although protein content was significantly different between males and females ($F = 5.422$, df = 1, $P = 0.025$), it was not different between generations ($F = 0.084$, df = 1, $P = 0.773$), and there was no sex times generation interaction ($F = 0.952$, df = 1, $P = 0.335$). When reported per gram of protein, the SOD activity (Fig. 2*A*) was not significantly different between sexes ($F = 0.600$, df = 1, $P = 0.443$) or among generations ($F = 1.836$, df = 1, $P = 0.184$), and no interaction was detected ($F = 1.998$, df = 1, $P = 0.166$). Similarly, catalase activity (Fig. 2*B*) did not differ with sex ($F = 3.334$, df = 1, $P = 0.076$) or generation ($F = 0.310$, df = 1, $P = 0.580$), and there was no sex times generation interaction ($F = 0.102$, df = 1, $P = 0.751$). The same was true for GPx activity (Fig. 2*C*), which did not differ with sex ($F = 0.471$, df = 1, $P = 0.496$) or generation ($F = 1.553$, df = 1, $P = 0.220$), and there was no sex times generation interaction ($F = 1.128$, df = 1, $P = 0.295$). This indicates that the resistance to DCS was not associated with difference of the antioxidant capacity.

Mitochondrial function. The Shapiro-Wilk W test revealed that $\dot{V}_0$ and $\dot{V}_{max}$ were not normally distributed (Fig.

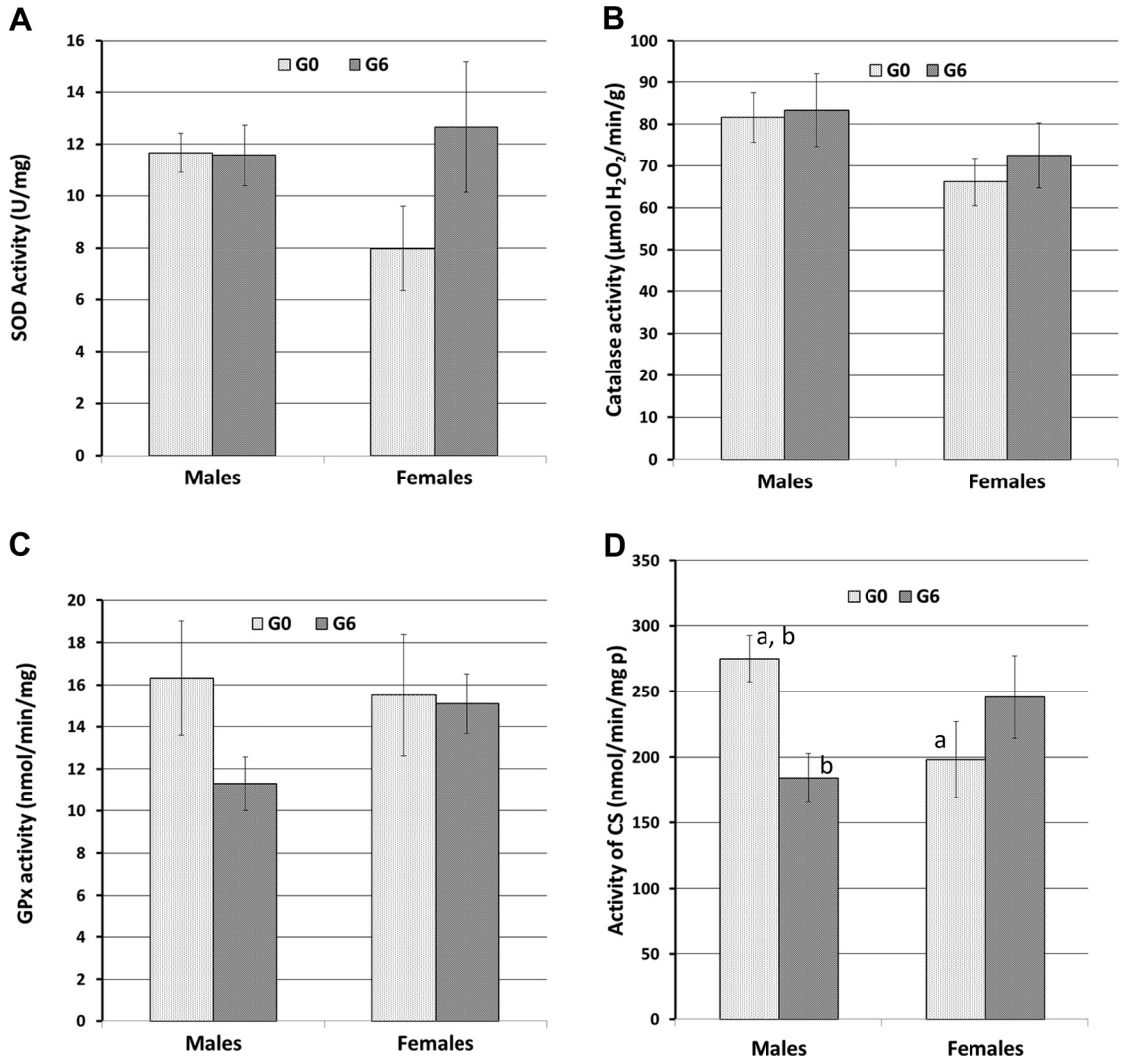

Fig. 2. Enzymatic activities in males and females resistant to decompression sickness [DCS, 6th generation (G_6)] and in their nonresistant [standard rats (G_0)] counterparts. Enzymatic activities of superoxide dismutase (SOD), catalase (Cat), glutathione peroxidase (GPx), and citrate synthase (CS) were measured in soleus muscle fibers. Data are expressed as means ± SE. [a]Different from same generation, opposite sex; [b]different from same sex other generation.

3). A Kruskal-Wallis test was therefore performed, which showed a statistically significant difference among groups for $\dot{V}_0$ [$H_{(3,\ N\ =\ 38)} = 13.438$, $P = 0.003$] but not $\dot{V}_{max}$ [$H_{(3,N\ =\ 38)} = 6.303$, $P = 0.097$]. $\dot{V}_{max}/\dot{V}_0$ ratios (RCR) did not differ [Kruskal-Wallis test: $H_{(3,\ N\ =\ 38)} = 4.292$, $P = 0.231$]. Multiple comparisons among groups showed a significantly lower $\dot{V}_0$ in G_{6M} than in G_{0M} [0.603 (0.563–0.624) vs. 0.816 (0.739–0.925) nmol $O_2 \cdot min^{-1} \cdot g^{-1}$, $P = 0.006$].

To assess whether the decrease of oxygen consumption by mitochondria could be due to decreased mitochondria content or activity, we also measured citrate synthase activity. Two-way ANOVA showed that sex ($F = 0.090$, df = 1, $P = 0.765$) and generation ($F = 0.728$, df = 1, $P = 0.399$) were not significantly associated, but that the sex times generation interaction ($F = 7.466$, df = 1, $P = 0.009$) was significant, indicating a change between G_0 and G_6, which differed according to sex (Fig. 2*D*). Indeed, citrate synthase (CS) activity was significantly higher in G_0 males than in either G_0 females or G_6 males. Meanwhile, it tended to be lower, but not significantly, in G_0 females than G_6 females.

ROS treatment decreased $\dot{V}_0$ and $\dot{V}_{max}$. However, two-way ANOVA showed that sex ($F = 0.142$, df = 1, $P = 0.708$) and generation ($F = 1.055$, df = 1, $P = 0.312$) factors did not exert a significant main effect on the decrease of $\dot{V}_0$ (Fig. 3*D*), nor did the sex times generation interaction ($F = 0.937$, df = 1, $P = 0.340$). The same was true for the decrease of $\dot{V}_{max}$ (Fig. 3*E*), which did not differ with sex ($F = 0.689$, df = 1, $P = 0.413$) or generation ($F = 0.006$, df = 1, $P = 0.937$), and there was no sex times generation interaction ($F = 0.765$, df = 1, $P = 0.389$).

Vessel reactivity. Two-way ANOVA indicated that the contractile response to 80 mM KCl depended on the sex of the animals ($F = 6.092$, df = 1, $P = 0.019$), but remained independent of the generation ($F = 1.476$, df = 1, $P = 0.233$). The sex times generation interaction was not significant ($F = 2.263$, df = 1, $P = 0.142$) (Fig. 4*A*).

PE induced a dose-dependent contraction of isolated rings in both sexes and generations (Fig. 4, *B* and *C*). The agonist-induced dose-response curves were compared by two-way ANOVA for repeated measures. We did not detect any signif-

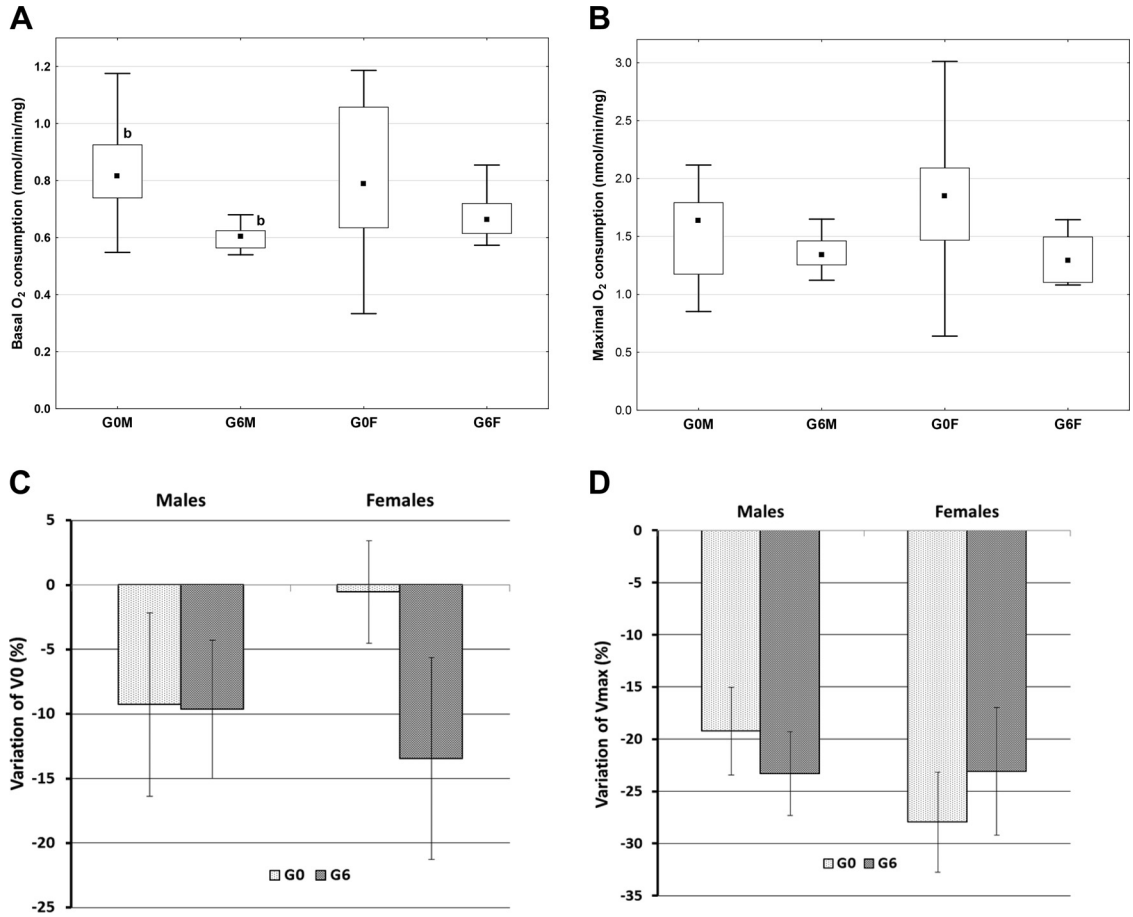

Fig. 3. Mitochondrial respiration of permeabilized soleus muscle fibers obtained from males and females resistant to decompression sickness [DCS, 6th generation (G_6)] and in their nonresistant [standard rats (G_0)] counterparts. *A* and *B*: basal and maximal oxygen consumption, respectively. Data are not normally distributed and are expressed as medians. Boxes indicate the 25th and 75th percentiles, error bars indicate the minimum and maximum. *C* and *D*: change in reactive oxygen species (ROS) treatment on basal ($\dot{V}_0$), maximal ($\dot{V}_{max}$) oxygen consumption, respectively. Data are expressed as means ± SE. [a]Different from same generation, opposite sex; [b]different from same sex other generation.

icant sex times PE interaction ($F = 0.546$, df = 1, $P = 0.772$), generation times PE interaction ($F = 0.518$, df = 1, $P = 0.793$), or sex times generation times PE interaction ($F = 0.633$, df = 1, $P = 0.703$).

To test NO-induced VSM relaxation, we compared sodium nitroprusside dose-response curves. The NO donor evoked a dose-dependent relaxation of isolated rings in both sexes and generations (Fig. 4, *D* and *E*). Two-way ANOVA for repeated measures showed that the generation times drug interaction was significant ($F = 4.567$, df = 1, $P = 6.61 \times 10^{-4}$). Conversely, the sex times drug interaction was not significant ($F = 0.906$, df = 1, $P = 0.478$) and neither was the sex times generation times drug interaction ($F = 0.937$, df = 1, $P = 0.458$). This suggests that dose-response curves to sodium nitroprusside differed between G_0 and G_6 in both males and females. Indeed, E_{max} was significantly influenced by generation ($F = 7.064$, df = 1, $P = 0.012$), but not by sex ($F = 0.295$, df = 1, $P = 0.590$) or the sex times generation interaction ($F = 0.954$, df = 1, $P = 0.336$) (Fig. 4*F*). Post hoc tests showed that maximal relaxation was lower in G_6 than G_0 for both sexes, although the difference was significant in females only. However, there were no significant effects associated with generation ($F = 0.029$, df = 1, $P = 0.865$), sex ($F = 2.759$, df = 1, $P = 0.107$) or sex times generation interaction ($F = 0.532$, df = 1, $P = 0.471$) on the EC_{50} with respect to drug (Fig. 4*G*).

Principal components analysis. In males, PCA showed that the first three PCs accounted for 56% of the variability (Table 3). PC1 is positively loaded by aPTT (0.868), MLR (0.830), $\dot{V}_0$ (0.786) and $\dot{V}_{max}$ (0.710), and negatively loaded by PT (−0.821), FX (−0.850), and Fib (−0.810). PC2 is negatively loaded by erythrocytes count (−0.735) and neutrophil count (−0.738). Although no parameters reached the threshold value of 0.7 on PC3, this component is mainly loaded positively by reticulocyte (0.633) and leukocyte (0.609) counts and negatively with the ROS-induced decrease of $\dot{V}_0$ (−0.669). A principal components regression analysis was performed to further explore the association of PCs with the resistance to DCS. Only PC1 and PC2 showed a significant relationship with resistance to DCS (Table 4). Indeed, Fig. 5*A* shows that these two components discriminate G_6 from G_0 males.

In females, the first three PCs accounted for 45% of the variability (Table 3). PC1 is loaded by leukocyte (0.906), monocyte (0.877), lymphocyte (0.821), neutrophil (0.756), and reticulocyte (0.723) counts. PC2 is negatively loaded by hematocrit (−0.831), hemoglobin (−0.812), and erythrocyte (−0.766) count while PC3 is negatively loaded by FII (−0.733) and FV (−0.714). However, in contrast to males,

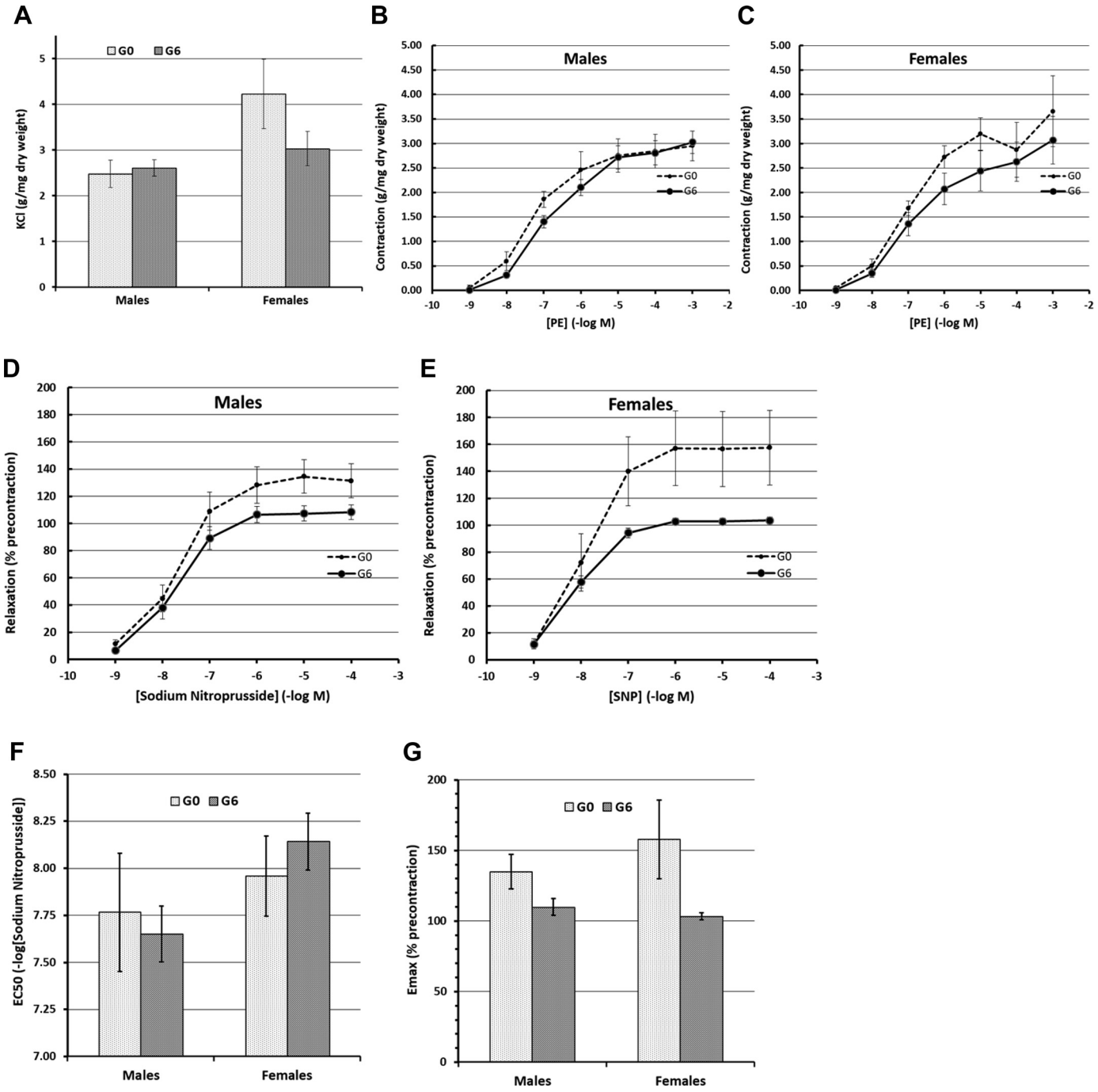

Fig. 4. Vasoreactivity of rings of thoracic aortas obtained from decompression sickness (DCS)-resistant [6th generation (G_6)] males and females and from their nonselected [standard rats (G_0)] counterparts. *A*: contraction induced by 80 mM of KCl. *B* and *C*: phenylephrine (PE)-induced contraction dose-response curves. *D* and *E*: sodium nitroprusside-induced relaxation dose-response curves. *F* and *G*: EC_{50} and E_{max} of sodium nitroprusside-induced relaxation dose-response curves. All data are expressed as means ± SE. Analysis did not detect any difference for KCl- or PE-induced contraction. Two way ANOVA for repeated measures showed a significant effect of generation but not sex ($P = 6.61 \times 10^{-4}$), and that E_{max} but not EC_{50} was decreased in G_6.

principal component regression analysis indicated that the resistance to DCS in females is not significantly related to any of these PCs (Table 4).

DISCUSSION

By selective breeding Wistar rats, we previously obtained animals with an up to three times higher resistance to DCS than their nonselected ascendants (31), independent of any diving acclimatization. Indeed, only 28% of the males and 20% of the females from the G_6 animals suffered DCS. This is statistically significantly lower than the 65% of DCS we previously observed in the founder population for animals aged the same age and submitted to the same hyperbaric protocol. Although we did not test animals from the G_0 group for their resistance to DCS, it is worth noting that the ratio of DCS among G_6 is also dramatically lower than the 63 ± 4% of DCS repeatedly ob-

Table 3. *Loadings of principal components 1 to 3*

	Males			Females		
	PC1 (22.70)	PC2 (17.43)	PC3 (16.12)	PC1 (18.42)	PC2 (13.81)	PC3 (12.79)
Hematocrit	0.386	−0.554	−0.005	−0.055	−0.831*	−0.274
Erythrocytes	0.277	−0.735*	0.012	0.088	−0.766*	−0.135
Reticulocytes	0.235	0.185	0.633	0.723*	−0.022	0.013
Hemoglobin	0.218	−0.534	0.116	−0.087	−0.812*	−0.334
White blood cells	−0.057	−0.522	0.609	0.906*	−0.151	−0.082
Neutrophils	−0.206	−0.738*	0.548	0.756*	0.298	0.059
Monocytes	0.652	−0.428	0.497	0.877*	0.126	0.069
Lymphocytes	−0.081	−0.459	0.584	0.821*	−0.177	−0.181
Eosinophils	0.122	−0.148	0.329	0.332	0.122	0.537
NLR	−0.209	−0.599	0.049	−0.034	0.483	0.274
MLR	0.830*	−0.264	0.225	0.599	0.457	−0.115
IG, %	0.490	−0.408	0.151	0.405	0.351	−0.162
Platelets	−0.282	−0.203	−0.064	0.262	0.137	−0.354
PLR	−0.219	0.233	−0.500	−0.581	0.277	−0.046
PT, %	−0.821*	−0.262	0.242	−0.087	0.057	−0.306
aPTT, %	0.868*	0.269	−0.110	0.148	−0.158	−0.123
FII, %	−0.605	0.213	0.480	0.043	0.377	−0.734*
FV, %	0.308	0.629	0.360	0.264	0.141	−0.714*
FX, %	−0.850*	−0.091	0.121	−0.012	0.690	−0.331
Fibrinogen, g/L	−0.810*	−0.350	0.216	0.136	−0.375	0.585
$\dot{V}_0$	0.786*	0.278	0.352	0.195	−0.073	−0.216
$\dot{V}_{max}$	0.710*	−0.219	−0.141	0.109	−0.266	−0.378
RCR	−0.063	−0.572	−0.520	0.057	−0.281	−0.204
$\dot{V}_0$, %	−0.115	−0.127	−0.669	0.486	−0.378	−0.054
$\dot{V}_{max}$, %	0.185	0.185	−0.440	0.325	0.159	0.484
KCl	0.242	−0.585	−0.561	0.352	−0.223	0.536
PE						
EC_{50}	0.060	−0.330	−0.389	−0.065	0.270	0.575
E_{max}	−0.044	−0.679	−0.526	0.371	−0.290	0.532
Sodium nitroprusside						
EC_{50}	−0.464	−0.018	−0.387	0.503	−0.046	−0.183
E_{max}	0.411	−0.105	−0.535	0.274	0.181	−0.209

PC, principal component; NLR, neutrophil-to-lymphocyte ratio; MLR, monocyte-to-lymphocyte ratio; IG, immunoglobulin; PLR, platelet-to-lymphocyte ratio; PT, prothrombin time; aPTT, activated partial thromboplastin time; FII, factor II; FV, factor V; FX, factor X; RCR, respiratory control ratio; $\dot{V}_0$, basal respiration; $\dot{V}_{max}$, maximal respiration; PE, phenylephrine. *Main loadings with cutoff >0.700 (absolute value).

tained in our laboratory with standard Wistar rats aged 11 wk old and submitted to the same hyperbaric protocol (9). Our aim therefore was to investigate the physiological differences of these "spontaneous" DCS-resistant Wistar rats with standard nonselected ones. Similarly to our previous report, both males and females in G_6 were significantly lighter than their G_1 counterparts while, in addition to age (which was precisely controlled in our protocol), body mass is a well-known risk factor of DCS in rats (9, 51). However, we previously demonstrated that generation accounted for the gain of resistance in both males and females, whereas the influence of body mass was significant for females but not males (31). Hence, factors other than body mass but related to the generation are also responsible for the difference in resistance to DCS between selected and nonselected rats. In this regard, evaluating whether our selection protocol increased the resistance to DCS through selection of the "good" genes and/or through regulation of gene expression by inherited epigenetic modifications (10) was not the main objective of the present study. These questions may be addressed in future works.

Table 4. *Principal component regression analysis showing the association between principal components and the resistance to DCS*

	Males		Females	
PC	OR (95% CI)	*P* value	OR (95% CI)	*P* value
PC1	1.27 [0.28, 5.67]	0.002	0.88 [0.32, 2.4]	0.058
PC2	1.61 [0.46, 5.68]	0.018	0.89 [0.34, 2.35]	0.138
PC3	1.45 [0.49, 4.27]	0.342	0.91 [0.36, 2.28]	0.534

Data are expressed as medians [25th, 75th percentiles]. DCS, decompression sickness; PC, principal component; OR, odds ratio; CI, confidence interval.

Physiological differences associated with resistance to DCS in both sexes. For decades, the mechanisms that drive resistance to DCS have been investigated mainly through preconditioning (20, 21, 24, 45, 52) or acclimatization (11, 15, 17, 38, 41), both strategies being efficient to increase resistance to DCS or decrease its severity. The novelty of our approach is to use animals that have not previously been exposed to hyperbaric conditions or other treatments, including preconditioning. The majority, if not all, of studies that investigated physiological mechanisms contributing to their efficacy for avoiding DCS were focused on inflammation (11, 15, 17). They showed that plasma levels of proinflammatory neutrophil gelatinase-associated lipocalcin and IL-8 are increased in divers, whereas the anti-inflammatory secretory leukocyte protease inhibitor is decreased by a 2-mo period of daily diving (17). Similarly, Eftedal et al. (15) reported an altered expression of genes associated with inflammation and innate immune responses in usual divers before a dive, with downregulation of genes

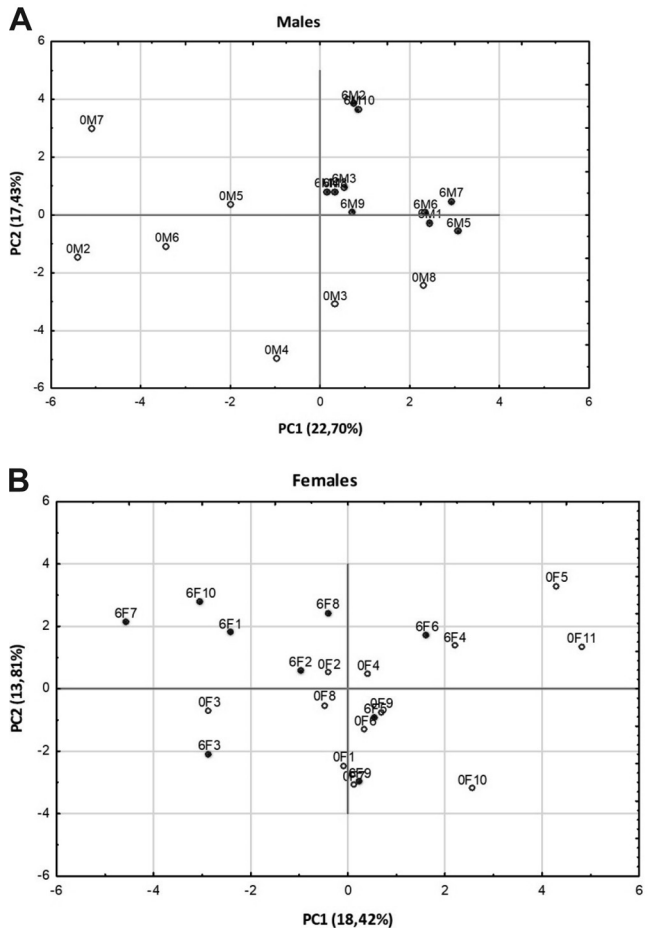

Fig. 5. Distribution of male (*A*) and female (*B*) decompression sickness (DCS)-resistant and standard non-DCS-resistant rats on the principal component plane. 0M and 0F, standard males and females, respectively; 6M and 6F, DCS-resistant males and females, respectively.

expressed by lymphocytes and upregulation of genes expressed by neutrophils, monocytes, and macrophages. The authors interpreted their observation as an adaptive response to enhance the cellular capacity for responses to repeated bouts of stress during diving. The higher NLR in our DCS-resistant rats might be coherent with the study by Ersson et al. (17) and could suggest the presence of a systemic inflammation in these animals. However, although NLR is an acknowledged marker of systemic inflammation (55), it is noteworthy that, during systemic inflammation, its increase is associated with lymphopenia and leukopenia (44), whereas we found that neither the neutrophil nor the lymphocyte counts were lower in DCS-resistant than standard individuals. It is unclear, therefore, whether lower-grade systemic inflammation is already present in our DCS-resistant individuals or whether the resistance to DCS is associated with an increased immune capacity that would enhance an acute proinflammatory response to diving and/or decompression-related stress, as suggested by Eftedal et al. (15).

Besides classically being considered a sterile systemic inflammatory disease, DCS also includes disseminated coagulation, most likely triggered by the presence of VGE. For single scuba dives, the activation of the coagulation cascade is well documented by the existence of a correlation between postdive decrease in platelet count and the severity of DCS (42) as well as by the beneficial effects of pretreatment with some, although not all, anticoagulant agents on the development of DCS (30). In addition, it was suggested that cross talk between platelets and leukocytes may be essential to the development of decompression sickness (29). To our knowledge, the possible involvement of changes in coagulation in the resistance to DCS has not previously been investigated. Surprisingly, males and females resistant to DCS both showed a higher FX activity. FX has a pivotal role within the coagulation cascade: it is activated either by the complex FVIIa-tissue factor in the extrinsic coagulation pathway or by FIXa-FVIIIa in the intrinsic coagulation pathway. In turn, activated FX promotes coagulation through generation of thrombin. Therefore, an increased coagulation tendency would have been expected in DCS-resistant rats from the higher activity of FX. However, this occurred neither in males nor in females resistant to DCS. Along with that, none of the other coagulation factors were different between generations. It is thus unlikely that the difference in FX associated with DCS resistance is related to the difference in coagulation function. Alternatively, it was reported that neutrophil can promote FX activation through the intravascular production of tissue factor (19, 26) and serine protease (35). Meanwhile, FX also stimulates the production of proinflammatory cytokines in various cell types, including leukocytes, endothelial cells, and vascular smooth muscle (4, 8, 18, 43). Further studies are needed to confirm whether the higher FX is related to its relationship with inflammation function rather than coagulation itself.

Neutrophil activation (46, 47) and perivascular adherence (34) associated with decompression sickness result from the action of circulating MPs released during diving through a mechanism that implies oxidative stress (53, 54). Increased oxidative stress has been repeatedly reported after diving. This increase correlates with decompression stress in a rat model of DCS (37) while ingestion of antioxidants prevents neutrophil activation (53) and vascular dysfunction (21, 40) associated with diving in humans. On the other hand, administration of antioxidants did not prevent DCS in rats (50). We did not detect any difference between DCS-resistant and nonresistant animals for the activities of antioxidant enzymes as well as for the effect of ROS on the mitochondrial oxygen consumption. In the present study, the activities of antioxidant enzymes were assessed at the whole cell level. Therefore, the possibility that changes might have occurred in particular cellular compartments cannot be ruled out. For example, in the study by Wang et al. (50), treatment with an antioxidant was shown to prevent cell death in cultured endothelial cells through inhibition of the production of superoxide anions in mitochondria, although without changing the occurrence of DCS in vivo. Nevertheless, our present results do not support the hypothesis that the increase in resistance to DCS was associated with an improved resistance to oxidative stress.

Large conductance arteries are altered after a single dive inducing DCS. This was shown in rat models of DCS by the impaired contractile response to phenylephrine but not to sodium nitroprusside (23, 36), although it was hypothesized that DCS did not act directly on vessel but, rather, through circulating factors. We are not aware of any data regarding a possible relationship between resistance to DCS and vascular function. The only study that compared the flow-mediated

dilation of the brachial artery between usual divers and nondivers did not find any difference (7). In our rat model, resistance to DCS was associated with decreased maximal vasorelaxation of VSM induced by NO, whereas the contraction elicited either by phenylephrine or by KCl was unchanged. KCl-induced contraction results from opening of L-type voltage-dependent Ca^{2+} channels. This substance is used to bypass intracellular second-messenger pathways as was the case for PE stimulation. From our results, none of these appear modified with increased resistance to DCS. Although many mechanisms can lead to blunted response of VSM to NO, it is unlikely that oxidative stress is involved in our resistant animals, as suggested by the lack of difference in the resistance to ROS between G_0 and G_6 animals and because ROS are known to increase the contractile response to PE as well (22). Alternatively, it was reported that factor X stimulates the production of NO in isolated rat aorta by activating protease-activated receptor-2 (27), which leads to chronic stimulation and, in turn, to desensitization of the soluble guanylyl cyclase (1, 6, 14). This results in an impairment of the production of cGMP in the VSM that, in turn, leads to blunted vasoreactivity to the NO donor as we observed in our DCS-resistant animals. Whether the lower reactivity of VSM to NO in rats resistant to DCS is linked to higher FX activity remains to be confirmed.

In G_6, both males and females showed lower reticulocyte count than their G_0 counterparts, whereas neither the blood concentration of hemoglobin nor the erythrocyte count differed between generations. Because reticulocytes account for only 3–4% of the erythrocyte population in blood, they probably play a minor role in rates of gaseous exchange, and, therefore, the substantially higher resistance to DCS we observed might not be associated with a large difference in oxygen transport. On the other hand, a decreased peripheral reticulocyte count can result either from a lower rate of release of reticulocytes in the peripheral blood or from a shorter maturation time in the peripheral blood. Which of these two mechanisms is responsible for the difference between DCS-resistant and standard animals needs further investigation.

Sex-related physiological differences associated with DCS resistance. We previously reported that the gain in resistance was statistically significant as early as the second generation in females while this was the case only from the third generation in males (31). This time shift in terms of resistance gain between males and females led us to hypothesize that the heritable determinants might be borne on the X chromosome. Even if modifications of other chromosomes cannot be excluded, the modification of allelic frequency on the X chromosome shown in this study supports our previous hypothesis. To investigate differences in the genome of these DCS-resistant rats is the focus of our actual studies. As stated above, the gain of resistance was not related only to generation for females but also to a decrease of body mass, which was not the case for males (31). Taken together, this suggests that the mechanisms that drive the resistance to DCS may not be exactly the same in males and females. This is also supported by the present results showing that males and females resistant to DCS exhibit higher NLR and FX than their nonresistant counterpart, albeit these differences were associated, respectively, to higher lymphocyte and neutrophil counts and lower PT in males but not females. Because we did not detect any difference between males and females for neutrophil and lymphocyte counts at G_0, the gain in resistance appeared associated with increased neutrophil and, to a lesser extent, lymphocyte counts in males. In females, however, these counts were not significantly modified. A similar observation was previously reported in mice by Desruelle et al. (13) who found that mice that did not suffer DCS had higher lymphocyte counts before a simulated air dive.

We also found that males but not females resistant to DCS had longer PT and lower mitochondrial basal oxygen consumption and content in skeletal muscles. Interestingly, for these two parameters, the difference of resistance to DCS between G_0 and G_6 was associated with the disappearance of a sex-related dimorphism. Indeed, the lower PT in male than female G_0 indicates a higher coagulation tendency in males than females that was no longer present at G_6. This was because of a longer PT in G_6 than G_0 males, whereas there was no difference between resistant and nonresistant females. Meanwhile, we did not detect any difference between generations or sexes for aPTT. It is, therefore, as if the gain in resistance was associated with lower coagulation tendency, which specifically results from modifications of the extrinsic coagulation pathway in males, whereas it did not change in females. Although in males only, it is tempting to state that a decreased coagulation tendency of the extrinsic pathway would result in lower disseminated coagulation in response to the presence of VGE. As already said above, this modification was not associated with differences between G_0 and G_6 in either the absolute platelet count, the platelet-to-lymphocyte ratio, or other coagulation factors, and despite increased FX. The mechanisms behind these changes are unknown but might include an increased inhibition of the coagulation. A similar pattern is also visible for mitochondrial function. In the present study, $\dot{V}_0$ was not different between sexes at G_0 despite a higher mitochondrial content in males than females, as assessed by CS activity. This could reflect the less efficient mitochondrial respiration already described in males than females (49). Males resistant to DCS exhibited lower $\dot{V}_0$ and citrate synthase activity than nonresistant ones, whereas no difference was detected between G_0 and G_6 females so that $\dot{V}_0$ and citrate synthase activity were no longer different between DCS-resistant males and females. This suggests, therefore, that resistance to DCS was associated with lower mitochondrial content and more efficient mitochondrial function in males. Whether the decreased mitochondrial content in males resistant to DCS is the cause of the lower $\dot{V}_0$ or an adaptation to lower metabolic demand at rest, as well as whether it occurs also in tissues other than skeletal muscle (and, more interestingly, nervous or pulmonary tissues), remains to be clarified.

That resistance to DCS does not rely on the same mechanisms in males and females was further confirmed by the principal components analysis (PCA). Nevertheless, in males, the first two PCs were significantly related to DCS resistance, and, as shown in Fig. 4*A*, they allow discriminating males resistant to DCS from standard ones. Among the variables that load these PCs above the threshold are coagulation parameters (aPTT, PT, and the concentration of fibrinogen), immune system parameters (MLR, neutrophil), and markers of mitochondrial function ($\dot{V}_0$ and $\dot{V}_{max}$). According to our hypothesis, FX might be more related to inflammation capacity than coagulation. This is very different from what we found in the females. Indeed, none of the PCs were significantly related to female resistance to DCS, and we were not able to discriminate

resistant from nonresistant females (which was not the case for males). Other parameters that are still unknown are therefore responsible for the higher resistance to DCS in females. However, the first three PCs explained not more than 56 and 45% of the total variance in males and females, respectively. This clearly indicates that, even in males, other factors not measured in our study also participate in resistance to DCS.

Limitations. In this study, we used standard Wistar rats obtained from an approved provider (Janvier Laboratories) as control rats. Hence, although our DCS-resistant animals are derived from a batch of animals of the same Wistar strain obtained from the same provider, the standard and resistant animals used for this study were not bred in the same conditions since their birth. Therefore, some difference in environmental conditions (including diet) might have influenced physiological parameters independent from the resistance to DCS. However, standard rats were kept for 2 wk before the experiments, which probably limited this potential bias. Furthermore, the hyperbaric protocol used by our group to assess resistance to DCS previously led to 63 ± 4% DCS, with a minimum of 50% and a maximum of 73%, despite large differences of time spent in our animal housing facility before the experimentations. The 20 and 28% DCS in our DCS-resistant groups are out of this range of variation. Additionally, during the selection process, animals from the fourth generation were not submitted to the hyperbaric protocol (because of technical issues) and were randomly bred. Interestingly, the following generation was less resistant than the third generation, implying that the selection protocol was indeed responsible for the gain of DCS resistance observed in the previous generations. Moreover, the resistance to DCS rose again in their progeny (i.e., the 6th generation), further confirming the impact of the selection protocol itself on the gain of DCS resistance. It is thus unlikely that our breeding conditions alone induced such a resistance. Another limitation comes from our approach, which compared physiological characteristics of two batches of animals that differed by their resistance to DCS. Although our results highlight various differences between these groups, we have not yet experimentally questioned the relationship between these physiological changes and resistance to DCS. It is therefore probable that not all of these differences are responsible for DCS resistance and that some may represent collateral modifications only. This is the subject of continued experimentation by our research group and others. Finally, a low polymorphism has been found during our SNP experiment probably because, even if the Wistar is an outbred strain, rats undergo a severe process of selection and inbreeding to produce its isogenic lineage and genetic standardization. We were not expecting such a low polymorphism, but our results show that G_1 still had sufficient polymorphism for the hyperbaric selection protocol. It is possible that a more genetically heterogeneous strain would have been better suited for this experiment, even if the conservation of genetic characteristics of a strain still requires standardization.

Conclusions. This study revealed differences in leukocyte counts, coagulation, and mitochondrial and vascular functions, but not resistance to oxidative stress between animals, which differed by their resistance to DCS. These differences represent possible mechanisms driving resistance to DCS. To our knowledge, it is also the first time that sex-related differences in these putative adaptations have been pointed out in the context of DCS resistance. Although merely speculative, the following working hypothesis could be suggested for future works. In this hypothesis, resistance to DCS would arise mainly from an enhanced immune capacity, documented by the higher NLR and FX, which would facilitate the response to stress related to diving conditions. In males, this mechanism would be further completed by higher leukocytes count, lower coagulation tendency of the extrinsic pathway in response to VGE, and improved mitochondrial function, whereas a decrease in body mass would be paramount in females. The importance of an enhanced inflammatory response for the resistance to DCS is also supported by a recent study that showed that administration of minocycline, a drug with immunomodulatory effects, increases the incidence of DCS in mice (13). A similar mechanism was previously documented in septic shock, in which early proinflammatory responses are needed for protection against advancement of this pathology (25). Together with the lower acute proinflammatory response capacity in females than males (32), this would also explain why females are considered at higher risk of DCS than males (9, 12) although they may form less VGE (5). In this hypothesis, the resistant individuals should exhibit a stronger and shorter inflammatory reaction induced by administration of LPS and/or bubbles at atmospheric pressure while drugs with immunomodulatory effects would increase their susceptibility to DCS. This remains to be confirmed.

Currently used decompression procedures based on calculated algorithms are presently considered to be relatively safe. Nevertheless, the fact that DCS still occurs even without violation of the algorithm recommendations (12) indicates that, for at least a proportion of the diver population, current algorithms are not conservative enough. It is now well recognized that the necessary improvement of these decompression algorithms, which are based on biophysical mechanistic computational models, will be possible by taking into account a diver's individual physiology. A principal components analysis of our data allowed us to discriminate resistant males from standard ones. Although still far from the diver, this opens a pathway to decompression management based on a precision medicine approach and future adaptation of personalized decompression procedures for "DCS-prone" divers.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

J.L., E.D., P.B., and F.G. conceived and designed research; J.L., E.D., A.A., A.G., K.P.-R., C.G., M.I., G.A., M.B., H.G., and F.G. performed experiments; J.L., E.D., A.A., A.G., K.P.-R., C.G., M.I., G.A., H.G., P.L., C.B., C.M., P.B., and F.G. analyzed data; J.L., E.D., A.A., K.P.-R., C.G., H.G., P.L., C.B., C.M., P.B., and F.G. interpreted results of experiments; J.L., E.D., and F.G. prepared figures; J.L., E.D., P.B., and F.G. drafted manuscript; J.L., E.D., C.M., P.B., and F.G. edited and revised manuscript; J.L., E.D., A.A., A.G., K.P.-R., C.G., M.I., G.A., M.B., H.G., P.L., C.B., C.M., P.B., and F.G. approved final version of manuscript.

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