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Article

Sex Specific Differences in Response to Calorie Restriction in Skeletal Muscle of Young Rats

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Abstract: Calorie restriction (CR), defined as a reduction of the total calorie intake of 30% to 60% without malnutrition, is the only nutritional strategy that has proven to extend lifespan, prevent or delay the onset of age-associated diseases, and delay the functional decline in a wide range of species. However, little is known about the effects of CR when started early in life. We sought to analyze the effects of CR in the skeletal muscle of young Wistar rats. For this, 3-month-old male and female rats were subjected to 40% CR or fed *ad libitum* for 3 months. Gastrocnemius muscles were used to extract RNA and total protein. Western blot and RT-qPCR were performed to evaluate the expression of key markers/pathways modulated by CR and affected by aging. CR decreased body and skeletal muscle weight in both sexes. No differences were found in most senescence, antioxidant, and nutrient sensing pathways analyzed. However, we found a sexual dimorphism in markers of oxidative stress, inflammation, apoptosis, and mitochondrial function in response to CR. Our data show that young female rats treated with CR exhibit similar expression patterns of key genes/pathways associated with healthy aging when compared to old animals treated with CR, while in male rats these effects are reduced. Additional studies are needed to understand how early or later life CR exerts positive effects on health- and lifespan.

Keywords: calorie restriction; aging; mitochondria; inflammation; autophagy; senescence

1. Introduction

Calorie restriction (CR), defined as a reduction of the total calorie intake of 30% to 60% without malnutrition, has been shown to extend lifespan across a wide range of species (1, 2). Furthermore, CR has been indicated as a robust strategy to delay the functional decline associated with aging and to prevent the onset of age-related diseases (1, 3). Several conserved signaling pathways are modulated by CR, including the mechanistic target of rapamycin (mTOR) pathway, the insulin-like growth factor (IGF)-insulin pathway, the AMP-activated protein kinase (AMPK) pathway, the nuclear factor erythroid 2-related factor 2 (NFE2L2/NRF2) pathway, or via the modulation of sirtuins (3, 4).

CR is thought to exert a protective role on muscle aging, as it preserves muscle mass and function (5). Although some reports showed no differences in mitochondrial biogenesis, mitochondrial function was found to be preserved in skeletal muscle of animals under CR (6, 7). Furthermore, CR has been shown to improve proteostasis in skeletal muscle, as it modulates protein degradation to prevent the accumulation of oxidative-damaged proteins (8, 9). The attenuation of oxidative stress damage has also been reported in rat

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cardiac muscle, as well as activation of autophagy with CR (10). In addition to a reduction in oxidative stress, inflammation levels have also been found reduced in the skeletal muscles of animals under CR (9, 11).

However, little is known about the effects of CR when started early in life. Some studies have reported no differences in mitochondrial function or antioxidant enzymatic activities (12), in oxidative metabolism (13), or in mTOR signaling and protein degradation (14) in young rats under CR. Therefore, the aim of this study was to analyze the effects of CR in the skeletal muscle of young Wistar rats. For this, male and female rats aged 3 months were subjected to 40% CR or fed *ad libitum* for 3 months. Following treatment, gastrocnemius muscles were used to determine the levels of markers of mitochondrial function, antioxidant, autophagy, nutrient-sensing, inflammation, and senescence pathways.

2. Materials and Methods

2.1. Animals and diets

All experiments were performed according to general guidelines for animal care approved by the institutional ethics committee and EU regulations (2010/63/UE). Female and male Wistar rats aged 3 months were purchased from Charles River Laboratories (Barcelona, Spain) and housed at 22 °C and 65 ± 3% humidity with a 12-hour light/dark cycle. Animals were divided into four experimental groups with different diets for 12 weeks. One group of females (n=8) and one group of males (n=3) were fed *ad libitum* with a pelleted standard diet (A04, Panlab, Barcelona). Another group of females (n=8) and of males (n=4) were subjected to 40% CR. Food for CR treated-rats was adjusted weekly to compare it with that of the control group and correct for growth requirements.

At 24 weeks of age, rats were sacrificed by decapitation and tissues were collected, weighted, and immediately frozen in liquid nitrogen and stored at -80 °C until analysis. Serum glucose and triglycerides were measured using and Accutrend® GCT-meter (Roche Diagnostics, Switzerland).

2.2. Total protein extraction

Gastrocnemius muscles (50 mg) were placed in a chilled mortar and pulverized with a pestle using liquid nitrogen. RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 1 mM EDTA) with protease and phosphatase inhibitors (Halt™ protease and phosphatase inhibitor Single-use cocktail, EDTA-free, 100X, ThermoFisher Scientific, #78443) was added (10% w/v). Samples were sonicated on ice at 40% amplitude for 10 s three times (VibraCell 75185). Then, samples were centrifuged at 600 *x*g for 10 min at 4 °C to remove any debris. Supernatants were recovered and protein content was quantified using the bicinchoninic acid (BCA) protein assay kit (Pierce, Germany).

2.3 Western Blot analysis

For each blot, 40 µg of total proteins were separated on SDS-PAGE gels and electrotransferred to 0.22 µm nitrocellulose membranes using the Transblot Turbo transfer system (Bio-Rad). Ponceau S staining was used as a loading control. Membranes were then blocked with 5% non-fat powdered milk in TBS with 0.05% Tween-20 for 1h. Antibodies against proteins analyzed are listed in Supplemental Table 1. After overnight incubation of primary antibodies, secondary antibodies conjugated with horseradish peroxidase were incubated for 1 h and protein bands were detected with Immun-Star™ WesternC™ Chemiluminiscent Kit (Bio-Rad) Western. The chemiluminiscence signal was acquired with a Chemidoc XRS densitometer (Bio-Rad) and results were analyzed with Quantity One Software (Bio-Rad).

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2.4. RT-qPCR

Gastrocnemius muscles (25 mg) were pulverized in liquid nitrogen as previously described and 1 mL of Tri Reagent® (Sigma-Aldrich) was added to each sample. RNA was isolated following manufacturer's instructions and quantified using a BioSpec-nano spectrophotometer (Shimadzu Biotech, Kyoto, Japan).

Total RNA (1 µg) was reverse transcribed to cDNA at 37 °C for 50 min with 200 U M-Mlv reverse transcriptase in a 20 µL volume of reaction mixture containing 1X First-Strand Buffer, 2.5 µM random hexamers, 500 µM each dNTP, 20 U RNAse inhibitor, and 10 mM DTT. Samples were diluted 1/10 and stored at -20 °C until use.

PCR reactions were performed using SYBR Green chemistry on a LightCycler® 480 System. Target genes, primers, and annealing temperatures are specified in Supplemental Table 2. The reaction mixture contained 7.5 µL SYBR TB Green® Premix Ex Taq™ (RR420A, Takara) with 0.5 µM of forward and reverse primers) and 2.5 µL of cDNA. The amplification program included a denaturation step at 95 °C, 5 min, followed by 45 cycles with a denaturation step (10 s, 95 °C), an annealing step (10 s, temperature depending on primers, see Supplemental Table 2), and an elongation step (12 s, 72 °C). A negative control without cDNA was run in each assay. Ct values obtained were analyzed, considering the efficiency of each reaction, using the GenEx Standard Software (Multi-DAnalises, Sweden). Two housekeeping genes, *Rpl32* and *Tbp*, were used according to the Normfinder algorithm.

2.5. String analysis

Protein-protein interactions (PPIs) were analyzed using the STRING database (v.11.5; www.string-db.org) by querying genes that were differentially expressed and proteins obtained in the results. The network edges represent either the evidence of the PPI or the confidence. All active interaction sources were selected, and the minimum required interaction score was set at medium confidence (0.400).

2.6. Statistical analysis

Results are presented as mean ± SEM unless otherwise specified. Differences between CR and control groups were assessed by Student's t-test, and interaction effects between sexes were generated by two-way ANOVA and post-hoc Student's t-test. A p value < 0.05 was considered statistically significant. To test potential associations between the measured parameters, a Principal Component Analysis (PCA) was computed. Statistical analyses were performed using R Studio version 3.5.2 of the R programming language (R Project for Statistical Computing; R Foundation, Vienna, Austria).

3. Results

3.1. CR reduced body and tissue weights

Body mass, tissue weights, and serum levels of glucose and triglycerides of control and CR male and female rats are shown in Table 1. Body weight was significantly decreased in animals subjected to CR compared to control animals. Both sexes exhibited similar weight loss, -27.1% in females, and -31.3% in males. Treatment with CR also reduced the weight of adipose tissue (i.e., inguinal, lumbar, mesenteric, and ovarian/epididymal adipose tissues) and the liver (-62.2% and -31.3% in females, and -67.0% and -33.2% in males, respectively). Skeletal muscle weight was lower in female rats compared to their male counterparts and CR reduced muscle weight similarly in both sexes. However, the muscle somatic index (MSI), obtained by adjusting muscle skeletal weight to body mass, was increased in both male and female CR rats, suggesting that this tissue is preserved under CR conditions in young animals. In fact, total protein content of skeletal muscle tissue remained unchanged with diet (Figure 1A). Finally, serum glucose was unaffected by diet, while triglycerides were reduced by 50% only in male rats subjected to CR. Principal component analysis (PCA) showed a clear separation between female and male rats treated with CR (Figure 1B).

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Table 1. Body and tissue weights, glucose and triglycerides levels in control and restricted rats. Two-way ANOVA was performed to assess for significance. *Abbreviations*: S, sex differences; D, diet differences; SxD, interactive effect between sex and diet; NS, non-significant. * Statistical differences between calorie restricted and control groups.

	Female			Male			
	Ad libitum	Restricted	*p-value*	Ad libitum	Restricted	*p-value*	*Statistics*
Body mass (g)	264 ± 6	192 ± 5*	<.001	484 ± 13	322 ± 8*	0.001	S, D, SxD
Liver weight (g)	8.21 ± 0.24	5.64 ± 0.17*	<.001	14.7 ± 0.5	9.77 ± 0.29*	0.005	S, D, SxD
Gastrocnemius weight (g)	3.09 ± 0.08	2.52 ± 0.05*	<.001	5.21 ± 0.18	4.26 ± 0.12*	0.021	S, D
WAT weight (g)	12.7 ± 1.5	4.81 ± 0.5*	<.001	30.4 ± 2.4	11.1 ± 0.8*	<.001	S, D, SxD
Muscle Somatic Index x100 (A.u.)	11.7 ± 0.2	13.1 ± 0.3*	<.001	11.2 ± 0.1	12.9 ± 0.2*	0.001	D
Glucose (mg/dL)	153 ± 14	142 ± 7	0.222	176 ± 7	161 ± 9	0.272	NS
Triglycerides (mg/dL)	173 ± 22	182 ± 10	0.183	330 ± 52	179 ± 17	0.024	NS

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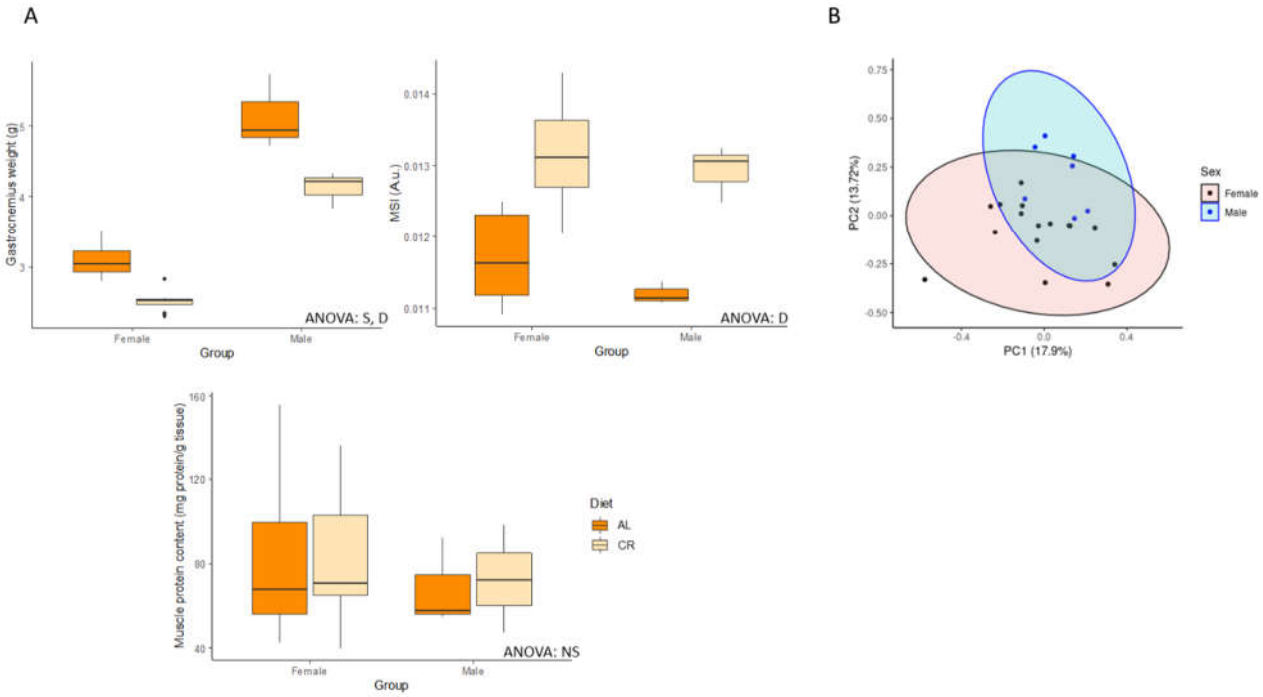

Figure 1. Effect of calorie restriction on skeletal muscle. (a) Boxplots showing gastrocnemius weight, muscle somatic index (MSI), and muscle protein content in females and males rats under *ad libitum* or calorie restriction diets. (b) Principal component analysis (PCA) plot performed on all data including weights, mRNA and protein expression, except for the mitochondrial complexes. Two-way ANOVA was performed to assess for significance. *Abbreviations*: S, sex differences; D, diet differences; N, non-significant.

3.2. CR effects on mitochondrial function and dynamics

To evaluate the effects of CR on mitochondrial bioenergetics, protein levels of OXPHOS complexes were analyzed by Western Blot (Table 2, Figure 2). Complex I and V showed no differences with CR, while complexes II, III, and V were increased in CR female rats. In male rats, only complex III showed a decrease with CR. However, Complex IV/Complex V ratio remained unvaried with CR in both sexes. Mitochondrial dynamics was also evaluated via the analysis of mitochondrial fission-related proteins GTPase dynamin-related protein 1 (DRP1) and mitochondrial fission 1 protein (FIS1) levels were also analyzed. Female rats did not show any differences in the levels of these proteins with diet, while male rats subjected to CR showed a significant decrease in both DRP1 and FIS1 expression levels (Figure 3).

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Table 2. Protein levels of OXPHOS complexes and mitochondrial fission proteins analyzed by Western Blot. Two-way ANOVA was performed to assess for significance. *Abbreviations*: S, sex differences; D, diet differences; N, non-significant. * Statistical differences between calorie restricted and control groups.

	Female			Male			
	Ad libitum	Restricted	*p-value*	Ad libitum	Restricted	*p-value*	*Statistics*
Complex I subunuit NDUFB8 (A.u.)	1.00 ± 0.13	1.06 ± 0.05	0.35	0.59 ± 0.28	0.93 ± 0.19	0.17	NS
Complex II 30 kDa subunit (A.u.)	1.00 ± 0.17	1.44 ± 0.17	0.04*	0.99 ± 0.40	0.94 ± 0.13	0.45	NS
Complex III, Core protein 2 (A.u.)	1.00 ± 0.26	1.64 ± 0.20	0.04*	2.13 ± 0.03	1.02 ± 0.26	0.02*	SxD
Complex IV subunit I (A.u.)	1.00 ± 0.37	2.11 ± 0.65	0.08	1.74 ± 1.01	2.10 ± 0.48	0.37	NS
Complex V alpha subunit (A.u.)	1.00 ± 0.10	1.25 ± 0.09	0.04*	1.15 ± 0.38	1.07 ± 0.12	0.41	NS
Complex IV/Complex V ratio	1.76 ± 0.59	3.30 ± 1.16	0.14	1.97 ± 1.02	3.32 ± 0.85	0.19	NS
DRP1 (A.u.)	1.00 ± 0.09	1.23 ± 0.18	0.14	1.69 ± 0.37	0.56 ± 0.03	0.01*	D, SxD
FIS1 (A.u.)	1.00 ± 0.18	1.17 ± 0.20	0.26	1.61 ± 0.40	0.79 ± 0.13	0.02*	NS

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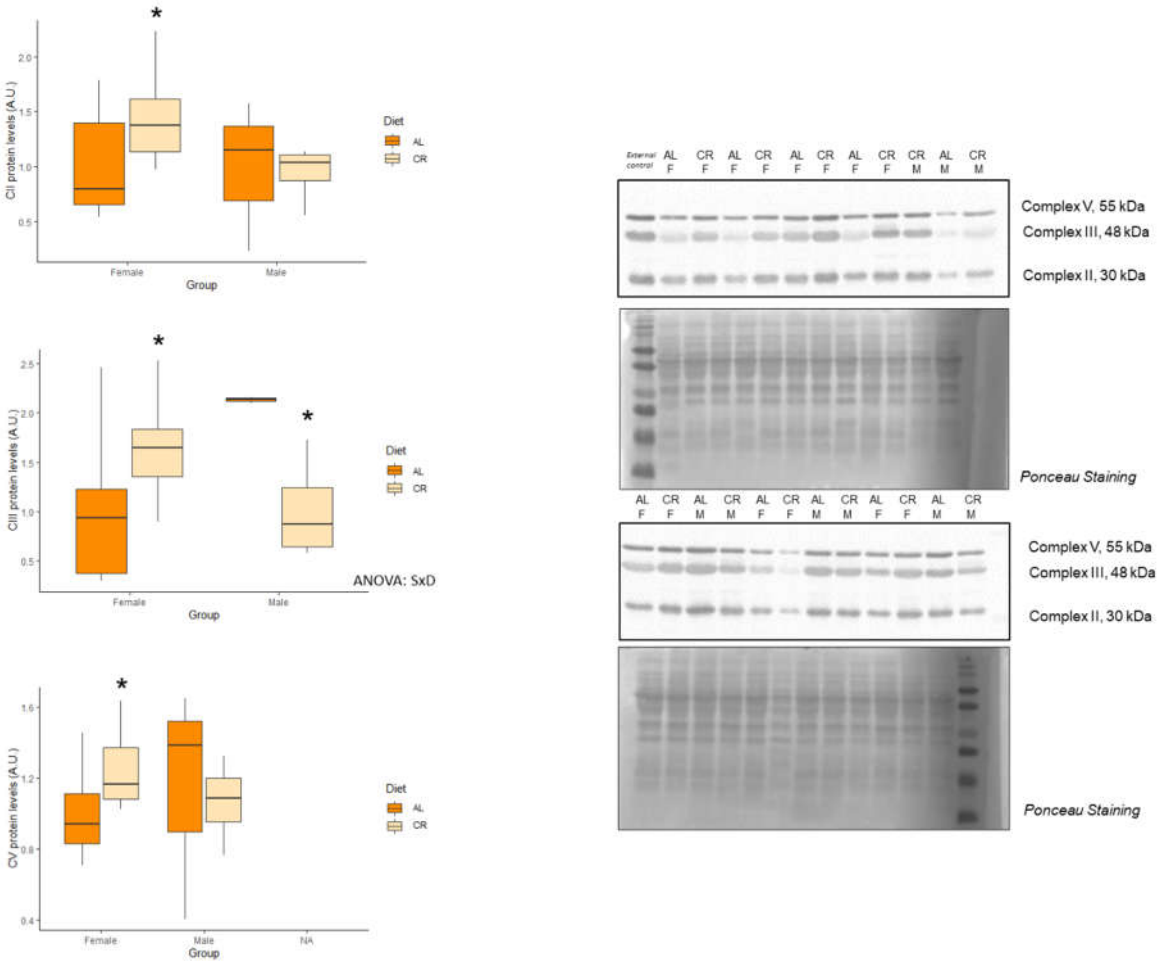

Figure 2. Effects of calorie restriction on oxidative phosphorylation (OXPHOS) complexes. Boxplots showing the protein levels of Complex II, Complex III, and Complex V and representative bands of the western blot. Two-way ANOVA was performed to assess for significance. *Abbreviations*: SxD, interactive effect between sex and diet; D, diet differences; NS, non-significant. * Significant difference between calorie-restricted and control group.

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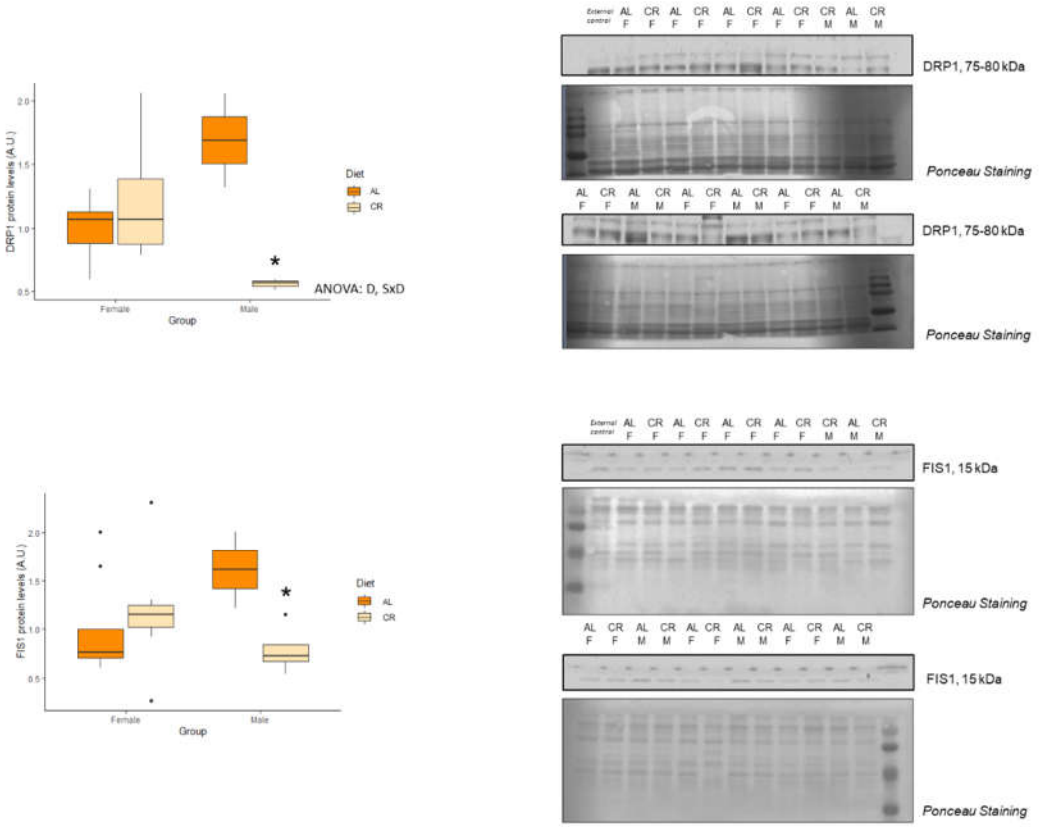

Figure 3. Effects of calorie restriction on proteins related to mitochondrial dynamics. Boxplots showing the protein levels of DRP1 and FIS1, and representative bands of the western blot. Two-way ANOVA was performed to assess for significance. *Abbreviations*: SxD, interactive effect between sex and diet; D, diet differences; NS, non-significant. * Significant difference between calorie-restricted and control group.

3.3. Antioxidant proteins

Table 3 shows the mRNA levels and Table 4 the protein levels of several antioxidant proteins. No differences were found in mRNA expression of the analyzed genes, and there was only an increase in the level of SIRT3 protein (Figure 4) in female rats subjected to CR. However, no changes were observed in the acetylated levels of SOD2, a known target of SIRT3. On the other hand, NRF2 expression levels, a main transcription factor involved in antioxidant pathways were different between males and females (Figure 4).

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Table 3. mRNA expression levels of antioxidant genes. Two-way ANOVA was performed to assess for significance. NS: non-significant. *Sod1*: superoxide dismutase 1; *Sod2*: superoxide dismutase 2; *sirt3*: sirtuin 3; *Nfe2l2*: nuclear factor erythroid 2-related factor 2; *Foxo3*: forkhead box O3.

	Female		Male		
	Restricted vs Ad libitum (Fold-change)	*p-value*	Restricted vs Ad libitum (Fold-change)	*p-value*	*Statistics*
Sod1	-0.15 ± 0.28	0.26	0.25 ± 0.22	0.35	NS
Sod2	-0.48 ± 0.32	0.15	0.30 ± 0.19	0.20	NS
Sirt3	-0.21 ± 0.13	0.15	-0.16 ± 0.04	0.17	NS
Nfe2l2	0.19 ± 0.11	0.18	-0.10 ± 0.17	0.40	NS
Foxo3	0.10 ± 0.21	0.38	0.27 ± 0.08	0.05	NS

Table 4. Protein levels of antioxidant enzymes and factors analyzed by Western Blot. Two-way ANOVA was performed to assess for significance. *Abbreviations*: S, sex differences; NS, non-significant.

	Female			Male			
	Ad libitum	Restricted	*p-value*	Ad libitum	Restricted	*p-value*	*Statistics*
SOD 2 (A.u.)	1.00 ± 0.29	0.96 ± 0.20	0.46	1.44 ± 0.68	1.13 ± 0.59	0.38	NS
Acetylated SOD2 (A.u.)	1.00 ± 0.18	1.33 ± 0.20	0.12	1.43 ± 0.21	1.22 ± 0.21	0.28	NS
Acetylated SOD2/Total SOD2 ratio	9.3 ± 1.0	11 ± 1.1	0.11	9.4 ± 3.2	12 ± 3.4	0.31	NS
SIRT3 (A.u.)	1.00 ± 0.19	2.46 ± 0.42	0.01*	1.33 ± 0.08	1.60 ± 0.34	0.31	NS
NRF2 (A.u.)	1.00 ± 0.10	0.93 ± 0.07	0.27	0.48 ± 0.27	0.90 ± 0.15	0.10	S
FOXO3A (A.u.)	1.00 ± 0.36	0.93 ± 0.38	0.45	0.43 ± 0.27	0.72 ± 0.22	0.23	NS

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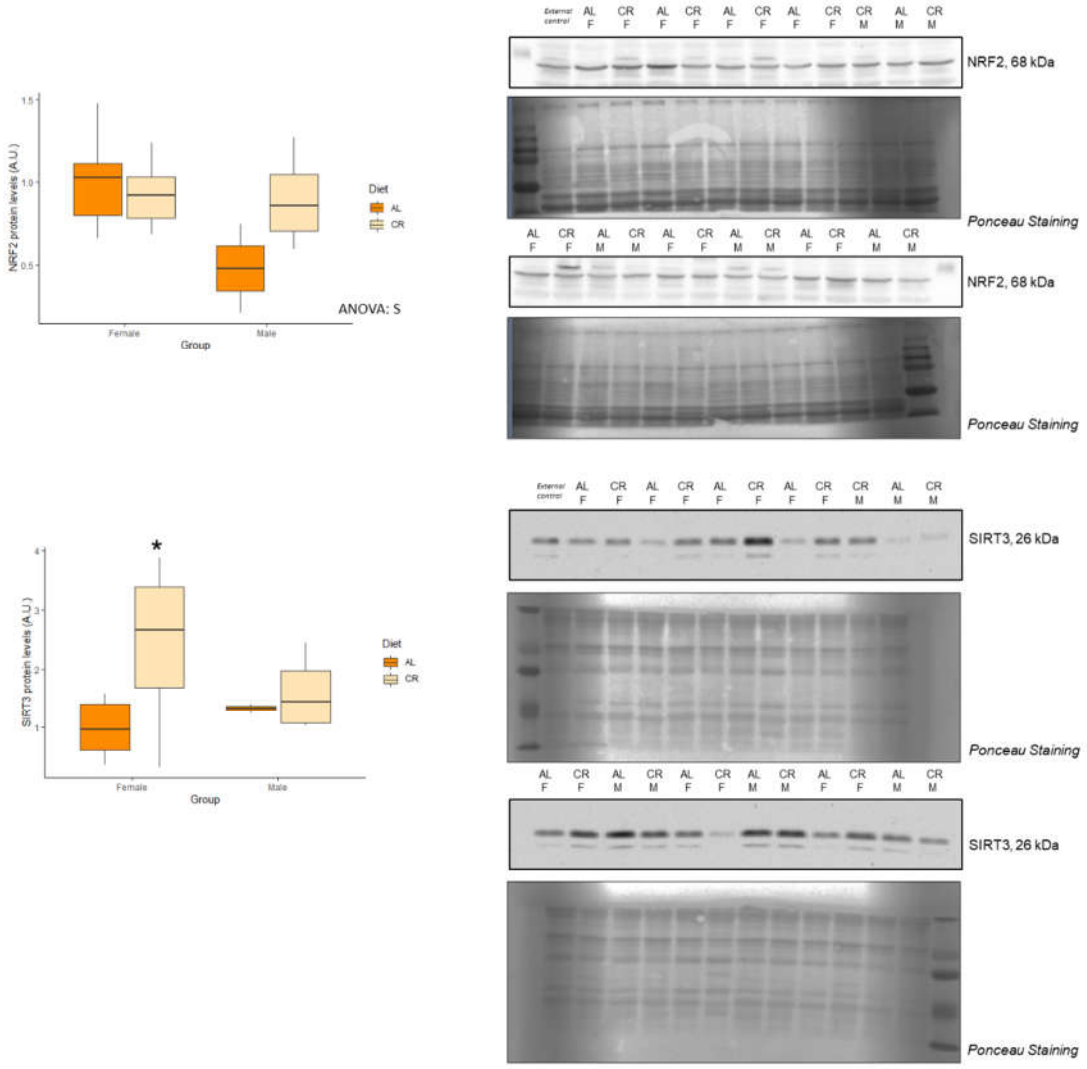

Figure 4. Protein levels of NRF2 and SIRT3 antioxidant factors. Boxplot showing the protein levels of NRF2 and SIRT3, and representative bands of the western blot. Two-way ANOVA was performed to assess for significance. *Abbreviations*: S, sex differences. * Significant difference between calorie-restricted and control group.

3.4. Autophagy and apoptosis

MRNA levels of *Map1lc3a* and *Sqstm1* (Table 5), and protein levels of LC3 and Caspase 3 (Table 6) were measured as makers of autophagy and apoptosis. No differences were observed in *Map1lc3a* and *Sqstm1* gene expression, and no changes were found in LC3A/B protein levels or the LC3-II/LC3-I ratio. However, interactive effects between sex and diet were found for caspase 3 in that caspase 3 expression levels in restricted males were lower (Figure 5).

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Table 5. mRNA expression levels of autophagy markers. Two-way ANOVA was performed to assess for significance. Abbreviations: NS, non-significant; *Map1lc3a*, microtubule-associated protein 1 light chain 3 alpha; *Sqstm1*: sequestosome 1.

	Female		Male		
	Restricted vs Ad libitum (Fold-change)	*p-value*	Restricted vs Ad libitum (Fold-change)	*p-value*	*Statistics*
Map1lc3a	0.89 ± 0.25	0.13	0.34 ± 0.23	0.15	NS
Sqstm1	-0.07 ± 0.13	0.36	0.26 ± 0.08	0.06	NS

Table 6. Protein levels of LC3-I and LC3-II, and caspase 3 analyzed by Western Blot. Two-way ANOVA was performed to assess for significance. Abbreviations: S, sex differences; D, diet differences; SxD, interactive effect between sex and diet; NS, non-significant.

	Female			Male			
	Ad libitum	Restricted	*p-value*	Ad libitum	Restricted	*p-value*	*Statistics*
LC3 I (A.u.)	1.00 ± 0.13	1.19 ± 0.12	0.16	0.59 ± 0.10	0.82 ± 0.08	0.07	NS
LC3 II (A.u.)	1.00 ± 0.16	1.35 ± 0.18	0.08	0.85 ± 0.20	1.09 ± 0.20	0.22	NS
LC3-II/LC3-I ratio	0.34 ± 0.03	0.4 ± 0.05	0.14	0.49 ± 0.05	0.44 ± 0.04	0.24	NS
Caspase 3 (A.u.)	1.00 ± 0.15	1.00 ± 0.09	0.49	1.11 ± 0.30	0.34 ± 0.11	0.07	S,D,SxD

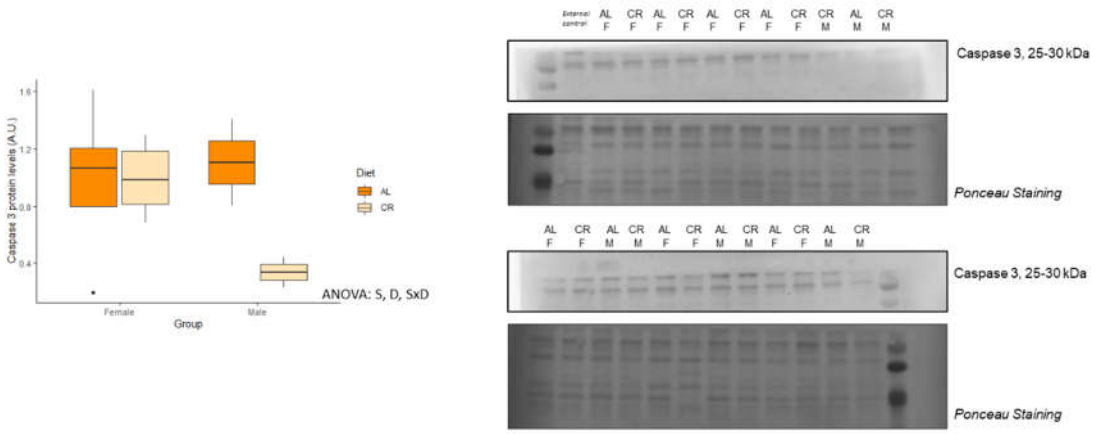

Figure 5. Protein levels of caspase 3. Boxplot showing the protein levels of Caspase 3 and representative bands of the western blot. Two-way ANOVA was performed to assess for significance. Abbreviations: S, sex differences; D, diet differences; SxD indicates interactive effect between sex and diet.

3.5. Nutrient-sensing pathways

Table 7 shows the mRNA levels of several key genes related to nutrient-sensing pathways. Interestingly, there were no differences between groups/sex in expression levels of *Pik3ca*, *Akt1*, *Gsk3b*, and *Mtor*. Only the expression of *Hif1a* showed an interactive effect

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between sex and diet and this effect was significantly reduced in male rats subjected to CR (Figure 6A).

Table 7. mRNA expression levels of autophagy markers. Two-way ANOVA was performed to assess for significance. S indicates sex effect; SxD indicates interactive effect between sex and diet. NS: non-significant; * Significant differences between calorie-restricted and control groups. *Pik3ca*: phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; *Akt1*: AKT serine/threonine kinase 1; *Gsk3b*: glycogen synthase kinase 3 beta; *Mtor*: mechanistic target of rapamycin kinase; *Hif1a*: hypoxia inducible factor 1 subunit alpha.

	Female		Male		
	Restricted vs Ad libitum (Fold-change)	*p-value*	Restricted vs Ad libitum (Fold-change)	*p-value*	*Statistics*
Pik3ca	0.41 ± 0.21	0.08	0.01 ± 0.22	0.49	NS
Akt1	0.44 ± 0.42	0.22	-0.69 ± 0.43	0.12	NS
Gsk3b	0.10 ± 0.24	0.36	-0.23 ± 0.13	0.16	NS
Mtor	0.79 ± 0.57	0.15	0.04 ± 0.29	0.46	NS
Hif1a	-0.05 ± 0.25	0.45	-0.49 ± 0.06	<0.01*	S, SxD

As seen in Table 8, protein levels of IRb and IRS were not influenced by CR. Similarly, AKT, LKB1, and LDH were unaffected by diet. Interestingly, only levels of ERRα were increased in rats treated with CR (Figure 6B). Both AMPK and GSK showed a tendency (p=0.07 and p=0.05, respectively) to decrease in females subjected to CR. Finally, IDH2 was lower in only in males subjected to CR.

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Table 8. Protein levels of several nutrient-sensing pathways markers analyzed by Western Blot. Two-way ANOVA was performed to assess for significance. D indicates diet differences. NS: non-significant. All data were normalized to ad libitum females.

	Female			Male			
	Ad libitum	Restricted	*p-value*	Ad libitum	Restricted	*p-value*	*Statistics*
IRb (A.u.)	1.00 ± 0.27	1.22 ± 0.20	0.26	0.74 ± 0.36	0.63 ± 0.09	0.38	NS
IRS1 (A.u.)	1.00 ± 0.39	0.53 ± 0.10	0.16	0.64 ± 0.29	0.79 ± 0.18	0.33	NS
AMPK (A.u.)	1.00 ± 0.12	0.76 ± 0.08	0.07	1.03 ± 0.13	0.56 ± 0.34	0.19	NS
LKB1 (A.u.)	1.00 ± 0.14	0.79 ± 0.11	0.14	1.07 ± 0.06	0.90 ± 0.33	0.37	NS
AKT (A.u.)	1.00 ± 0.15	0.90 ± 0.08	0.28	1.10 ± 0.21	1.01 ± 0.19	0.28	NS
GSK (A.u.)	1.00 ± 0.24	0.51 ± 0.15	0.05	0.36 ± 0.09	0.50 ± 0.02	0.10	NS
ERRA (A.u.)	1.00 ± 0.26	1.21 ± 0.21	0.27	0.75 ± 0.31	1.17 ± 0.36	0.21	D
LDHA (A.u.)	1.00 ± 0.16	0.75 ± 0.10	0.10	0.77 ± 0.01	0.49 ± 0.12	0.10	NS
IDH2 (A.u.)	1.00 ± 0.25	0.79 ± 0.12	0.23	0.25 ± 0.03	0.61 ± 0.11	0.04*	NS

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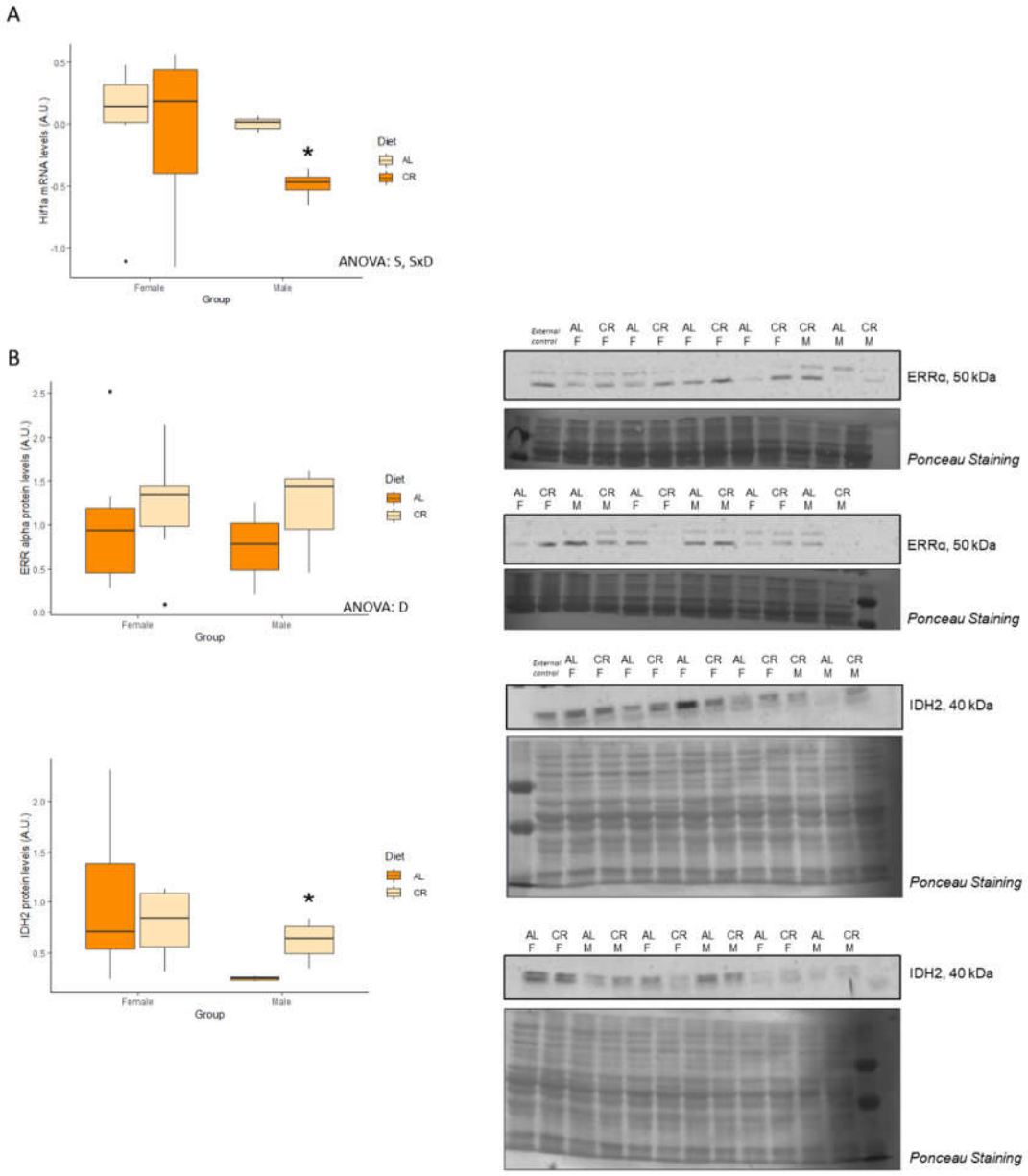

Figure 6. Levels of nutrient-sensing pathways markers. **(a)** Boxplot showing the mRNA levels of *Hif1a*. **(b)** Boxplot showing the protein levels of ERR*α* and IDH2, and representative bands of the western blot. Two-way ANOVA was performed to assess for significance. SxD indicates interactive effect between sex and diet, D indicates diet differences. * Significant difference between calorie-restricted and control group.

3.6. Inflammation and senescence

Levels of mediators of inflammation and senescence are shown in Tables 9 to 11. No changes were observed in mRNA levels of TNF and TGFb, while IL1b (Figure 7A) expression increased only in males under CR, while IL1R protein levels remained unvaried by diet. IL6 protein levels increased with CR only but this effect was only observed in females. On the other hand, *NF-kB* mRNA expression did not change with CR, but interestingly protein levels of NF-kB (Figure 7B) increased with CR in females. IkB protein levels (Figure 7B) decreased with CR but this effect was noted only in males. We did not find any changes in *Tp53*, *Cdkn2a*, and *Sirt6* mRNA levels, and only a tendency (p=0.07) of increased expression of *Cdkn1a* in male rats subjected to CR. P53 protein levels were also unaffected by diet or sex.

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Table 9. mRNA expression levels of inflammatory markers. Two-way ANOVA was performed to assess for significance. NS: non-significant Rela: RELA proto-oncogen, nuclear factor kappa B subunit; Il1b: interleukin 1 beta; Tgfb1: transforming growth factor beta 1; Tnf: tumor necrosis factor.

	Female		Male		
	Restricted vs Ad libitum (Fold-change)	*p-value*	Restricted vs Ad libitum (Fold-change)	*p-value*	*Statistics*
Rela	0.20 ± 0.12	0.25	0.05 ± 0.09	0.25	NS
Il1b	-1.20 ± 0.50	0.16	0.24 ± 0.28	0.04*	NS
Tgfb1	0.49 ± 0.35	0.15	-0.63 ± 0.69	0.26	NS
Tnf	-0.17 ± 0.54	0.39	-0.23 ± 0.51	0.36	NS

Table 10. Protein levels of several inflammation-related pathways markers analyzed by Western Blot. Two-way ANOVA was performed to assess for significance. NS: non-significant. All data were normalized to ad libitum females.

	Female			Male			
	Ad libitum	Restricted	*p-value*	Ad libitum	Restricted	*p-value*	*Statistics*
IL1R (A.u.)	1.00 ± 0.16	1.13 ± 0.20	0.30	0.52 ± 0.04	0.89 ± 0.28	0.17	NS
IL6 (A.u.)	1.00 ± 0.10	0.67 ± 0.08	0.01*	0.66 ± 0.12	0.87 ± 0.29	0.29	NS
NF-κB (A.u.)	1.00 ± 0.13	1.42 ± 0.20	0.04*	0.55 ± 0.19	0.37 ± 0.01	0.15	NS
IκB (A.u.)	1.00 ± 0.16	0.97 ± 0.13	0.45	1.09 ± 0.02	0.68 ± 0.12	0.04*	NS

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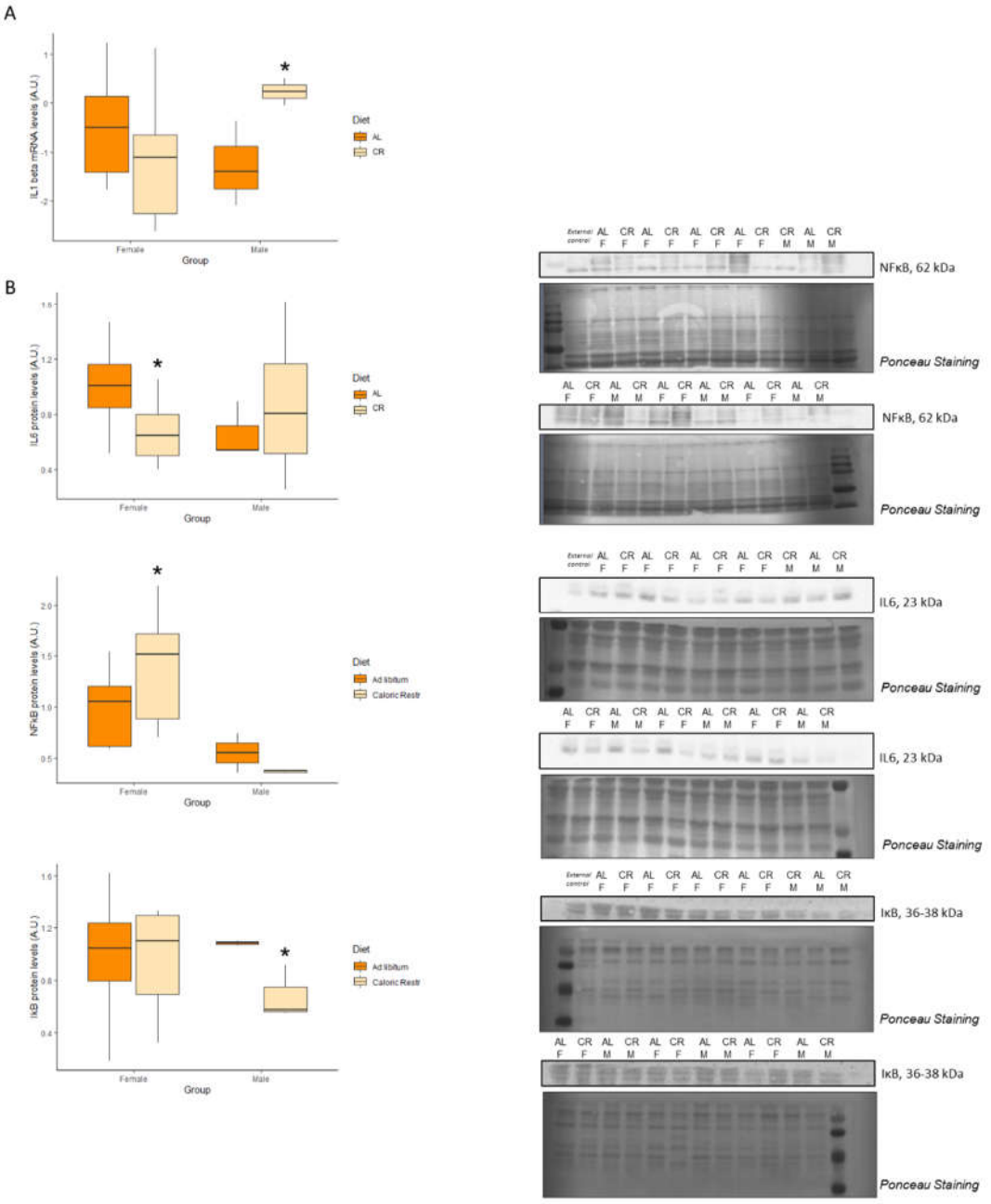

Figure 7. Levels of inflammation markers. **(a)** Boxplot showing the mRNA levels of *IL1B*. **(b)** Boxplot showing the protein levels of IL6, NFκB, and IκB, and representative bands of the western blot. * Significant difference between calorie-restricted and control group.

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Table 11. mRNA expression levels of senescence markers. Two-way ANOVA was performed to assess for significance. NS: non-significant. *Cdkn1a*: cyclin-dependent kinase inhibitor 1A; *Cdkn2a*: cyclin-dependent kinase inhibitor 2A; *Tp53*: tumor protein p53; *Sirt6*: sirtuin 6.

	Female		Male		
	Restricted vs Ad libitum (Fold-change)	*p-value*	Restricted vs Ad libitum (Fold-change)	*p-value*	*Statistics*
Cdkn1a	0.14 ± 0.26	0.43	1.28 ± 0.37	0.07	NS
Cdkn2a	1.11 ± 0.62	0.09	-0.50 ± 0.44	0.21	NS
Tp53	0.74 ± 0.54	0.12	-0.56 ± 0.38	0.17	NS
Sirt6	0.78 ± 0.07	0.20	-0.43 ± 0.28	0.14	NS

3.7. Analysis of protein-protein interaction

Finally, we analyzed possible protein-protein interactions using the STRING database. Figure 8A shows the main interactions and the interaction type found by querying the genes and proteins that were significantly affected by CR. The PPI enrichment p-value for this network was 5.05×10^{-11}, with 24 edges. Specific networks for females and males were also constructed. In Figure 8B and 8C, the network with the differentially expressed genes and proteins for females and males are shown, respectively. The thickness of the edges represents the strength of data supporting each PPI. The PPI enrichment values for these networks are 0.0103 for females and 0.000826 for males.

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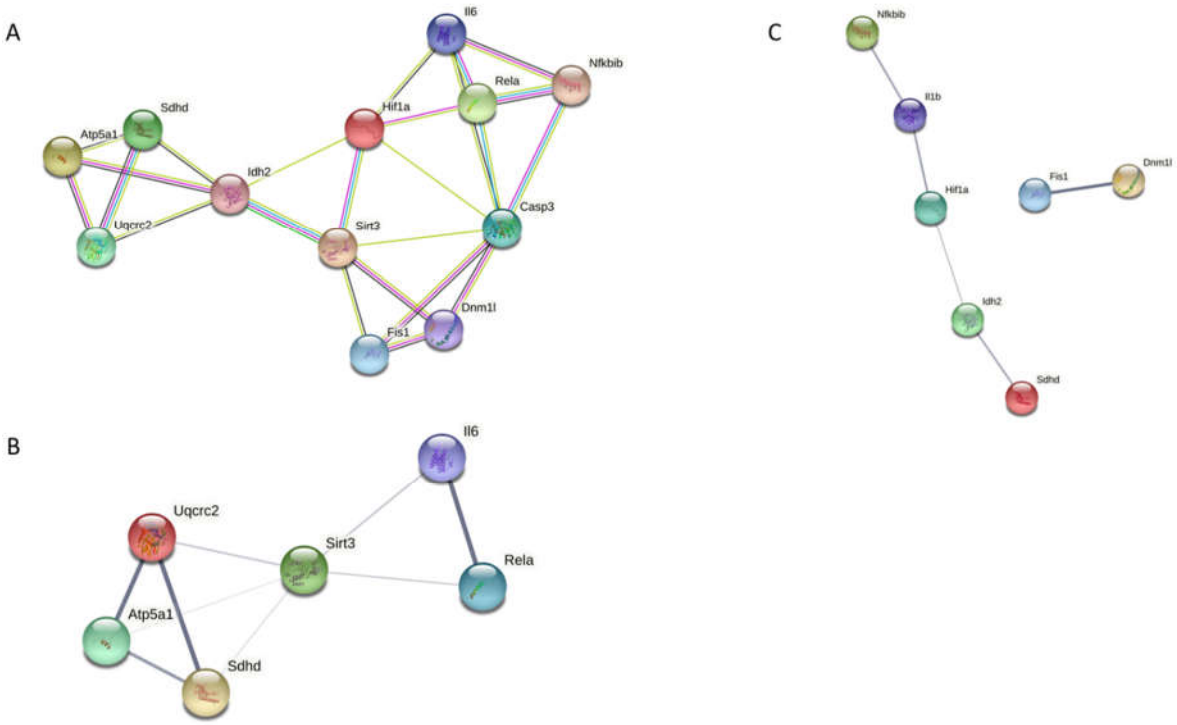

Figure 8. PPIs networks. (A) Network showing PPIs among genes and proteins differentially expressed in both sexes. (B) Network showing PPIs among genes and proteins differentially expressed in females. Thickness of edges represents the strength or confidence of data. (C) Network showing PPIs among genes and proteins differentially expressed in males. Thickness of edges represents the strength or confidence of data.

4. Discussion

In this study, we analyzed the influence of 40% CR on markers of skeletal muscle function in 3-months old Wistar rats. A general decrease in body, liver, adipose, and skeletal muscle weights in rats treated with CR was observed as expected for CR treatment. We also measured the expression of some of the age-related markers listed among the hallmarks of aging and related to mitochondrial biology, antioxidant activity, autophagy and apoptosis, nutrient-sensing pathways, inflammation, and senescence. Interestingly, we found significant differences among dietary treatment and sex in only some mitochondrial-related proteins and in a few markers of antioxidant, apoptosis, inflammatory and nutrient-sensing pathways.

CR reduced body weight, liver weight, fat mass, as well as gastrocnemius muscle weight, which is in line with what has been previously reported (12, 13, 15, 16). However, MSI, an index of sarcopenia (17, 18), and the protein content of skeletal muscle, were increased with CR in both males and females, which is in agreement with previous observations in the tibialis cranialis and the soleus muscles from rats under to energy restriction (17). Furthermore, Colom et al. (12) also reported conserved MSI and protein content in gastrocnemius muscles in male rats, and even an increase in protein content in female rats. Conversely, Miller et al. (19) showed that the synthesis of mitochondrial protein rate was similar in skeletal muscle of rats, regardless of age and CR treatment. These results indicate that skeletal muscle function might be preserved by CR in young male and female rats.

Mitochondrial dysfunction, such as alterations in the OXPHOS complexes and in mitochondrial dynamics, has been indicated as a key hallmark of aging [2, 20]. In skeletal muscle, CR has shown to preserve mitochondrial function by increasing OXPHOS activities or mitochondrial respiration (7, 12). In the present study, we observed a slight increase in the protein level of several subunits of the oxidative phosphorylation complexes in

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females under CR, although the complex IV/complex V ratio remained unvaried, thus indicating a preserved mitochondrial oxidative capacity. On the contrary, there was only a decrease in the complex III subunit in male rats subjected to CR. Previous studies conducted in young male rats showed no changes in OXPHOS levels or function with CR (6, 21), although Wang et al. (22) suggested that the effect of CR on mitochondrial function might be fiber-type dependent. On the other hand, Gutiérrez-Casado et al. (23) reported a decrease in complex III in the skeletal muscle of mice subjected to 18 months of CR. Furthermore, we also observed a decrease in both DRP1 and FIS1 fission-related proteins in male rats treated with CR. Mitochondrial fission is thought to be induced in aged skeletal muscle, and long-term CR has been shown to reduce the levels of FIS1 and DRP1 (18, 24). These results suggest that fission is attenuated by CR, presumably to stimulate mitochondrial fusion or to avoid mitochondrial degradation (25). Interestingly, in the current study, we did not observe any changes in key autophagy markers, which seem to be induced in older animals subjected to CR (23).

Oxidative stress is closely related to mitochondrial dysfunction and also plays a role in aging (25). CR has been reported to reduce oxidative damage in several tissues and animal models (7, 10, 24, 26). In our study, SIRT3 was increased in females under CR, although other antioxidant proteins were unvaried. This limited effect could be explained because animals were too young to 'consider' oxidative stress a determinant factor. In fact, previous studies have shown that the enzymatic activity of some antioxidant proteins are preserved in young skeletal muscle and these antioxidant defenses were higher in females than in males (12). This result is consistent with our findings of increased levels of NRF2 in females.

Interestingly, we found that protein levels of caspase 3 was decreased in male rats under CR and was unvaried in females. Caspase 3 has been reported to be involved in muscle differentiation and the promotion of myogenesis (27, 28). In fact, this protein regulates multiple pathways involved in skeletal muscle growth. Caspase 3 is responsible for the cleavage of Pax7, a transcription factor regulating self-renewal of satellite cells (29), the stem cells of skeletal muscle. Thus, the decrease in caspase 3 would indicate a preservation of stem cell niche in males. On the other hand, caspase 3 also regulates p21 expression, which results in cell cycle arrest and the initiation of the cell's differentiation program (29). In this regard, we only observed a slight trend of increased *Cdkn1a* mRNA expression levels in males. The function of skeletal muscle was preserved in both males and females, as discussed previously based on the MSI.

Very few changes were observed in genes and proteins related to nutrient-sensing pathways, which is in agreement with other animal studies, suggesting that the benefits of CR are limited in younger animals whose muscle function is adequate (13, 14). *Hif1a* expression was significantly reduced in males under CR. HIF1, an hypoxia-inducible factor, mediate transcriptional responses to hypoxia and has been shown to increase with aging, and CR is able to attenuate this effect (30). On the other hand, in the current study, ERRα was increased with CR, perhaps due to its involvement in the regulation of mitochondrial function and cellular metabolism (31).

In our study, contradictory results were also found when analyzing the effect of sex and diet on inflammatory markers. Male rats under CR showed increased expression of *IL1b* and decreased levels of IκB, a negative regulator of the transcription factor NF-κB (30), which may suggest an increase in inflammation due to CR when started at a younger age. Conversely, there was a decrease in IL6 levels and an increase in NF-κB in females. Previous reports have shown that CR decreases key inflammatory mediators in several tissues, including skeletal muscle (11, 30, 32, 33). It should be noted that most of our results are indirect measures of inflammation. For instance, in the current study, only an increase in the *IL1b* mRNA expression was observed, which does not necessarily translate into higher protein levels of this cytokine. Furthermore, no downstream effects of IκB and NF-κB were observed. Both IL6 levels, a key inflammatory marker, and the decrease in response to CR observed in the current study are in agreement with results from previous studies [11,33].

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Finally, we were not able to detect any changes in markers of senescence. As mentioned before, this may indicate an attenuated effect or a ceiling effect of CR in young animals, as age-related senescence is not yet a determinant factor in these animals.

This study has limitations. First, the sample size was small and therefore there may be limited statistical power to detect differences in gene or protein expression. Second, the treatment length was only of 3 months and therefore the identification of biological pathways affected by CR starting early in life, and how these pathways are related to lifespan, cannot be determined. Finally, our findings are exploratory and therefore it is not possible to make causal conclusions about the effects of early-onset CR on muscle health. Despite these limitations, our study provides convincing evidence that CR started early in life shows a sexual dimorphism effect in skeletal muscle.

5. Conclusions

In summary, our results suggest a sexual dimorphism in markers of oxidative stress, inflammation, apoptosis, and mitochondrial function in response to CR in young rats. Furthermore, our data show that young female rats under calorie restriction exhibit similar expression patterns of key hallmarks of muscle aging to those observed in older animals under CR, which are related to pro-longevity effects. Interestingly, these effects are reduced in younger male rats under CR . A 'ceiling effect' for CR may be present in younger animals, although no adverse effects were reported in skeletal muscle. Future studies are needed to understand whether and how early or late CR may exert positive effects on healthspan and lifespan.

Supplementary Materials: Supplemental Table 1. Antibodies used for Western Blot. Supplemental Table 2. Primers and conditions used for RT-qPCR.

Author Contributions: Designed the research, conceptualization: M.T-M., M.G.-F., P.R., J.O.; Conducted the analyses: M.T-M., M.G.-F., C.N.; Wrote the manuscript: M.T-M., M.G-F., P.R., J.O., D.W., A.P.; Critically reviewed manuscript: D.W., A.S-P, A.P.; Had primary responsibility for the final content: M.T.M., M.G.F., P.R., J.O.; All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Animal experiments were performed in accordance with general guidelines approved by our institutional ethics committee and the EU rules (86/609/EEC)

Data Availability Statement: The data used to support the findings of this study are available from the corresponding author upon request

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