

Exercise mitigates sleep-loss-induced changes in glucose tolerance, mitochondrial function, sarcoplasmic protein synthesis, and circadian rhythms.

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Running title: Exercise mitigates detrimental metabolic effects caused by sleep restriction

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1 **Abstract**

2 Sleep loss has emerged as a risk factor for the development of impaired glucose tolerance.
3 The mechanisms underpinning this observation are unknown; however, both
4 mitochondrial dysfunction and circadian misalignment have been proposed. Given that
5 exercise improves glucose tolerance, mitochondrial function, and alters circadian
6 rhythms, we investigated whether exercise may counteract the effects induced by
7 inadequate sleep. We report that sleeping 4 hours per night, for five nights, reduced
8 glucose tolerance, with novel observations of associated reductions in mitochondrial
9 function, sarcoplasmic protein synthesis, and measures of circadian rhythmicity;
10 however, incorporating three sessions of high-intensity interval exercise (HIIE) during
11 this period mitigates these effects. These data demonstrate, for the first time, a sleep loss-
12 induced concomitant reduction in a range of physiological processes linked to metabolic
13 function. These same effects are not observed when exercise is performed during a period
14 of inadequate sleep, supporting the use of HIIE as an intervention to mitigate the
15 detrimental physiological effects of sleep loss.

16 **Introduction**

17 The detrimental effects of sleep loss on glucose tolerance are now well-established, and
18 insufficient sleep is a risk factor for the development of type 2 diabetes (T2D) (1). In fact,
19 sleep loss is comparable with other more traditional risk factors that are associated with
20 the development of T2D, such as physical inactivity (1). Several studies have shown that
21 periods of sleep restriction, or reduced time in bed (TIB), typically with a sleep
22 opportunity of 4 to 5 h per night, cause significant reductions in a range of indices related
23 to glucose metabolism (2-5). The severity of this effect can be seen with only one night
24 of either sleep restriction (4 h TIB) or sleep deprivation (e.g., no sleep), which can reduce
25 insulin sensitivity (6-9). Despite these findings, there are limited data explaining the
26 physiological and molecular changes that underpin these effects. As a large proportion of
27 the population do not meet the current sleep recommendations (e.g., 7 to 9 h, per night)
28 (10, 11) and inadequate sleep is a consequence of many occupations (12, 13), gaining a
29 better understanding of these mechanisms may help to tailor specific interventions aimed
30 at counteracting the detrimental effects of sleep loss.

31

32 The physiological mechanisms that underpin the impairment of glucose tolerance
33 following sleep restriction are likely multifactorial. While not previously investigated in
34 the context of sleep loss, the development of insulin resistance has been associated with
35 a reduction in mitochondrial content and impaired mitochondrial respiratory function (14,
36 15). Furthermore, reductions in citrate synthase activity (a surrogate marker for
37 mitochondrial content (16)) and mitochondrial respiratory function have also been
38 reported in T2D patients, compared to obese non-diabetics, suggesting a link between
39 mitochondrial changes and the development of insulin resistance and T2D (14, 15).
40 Therefore, sleep-loss-induced reductions in glucose tolerance may, in part, be a
41 consequence of changes in mitochondrial content, function, or the processes that regulate
42 these properties - including mitochondrial dynamics and mitochondrial protein synthesis
43 (17). In support of this, 120 h of sleep deprivation was associated with a 24% reduction
44 in citrate synthase activity in human skeletal muscle (18). However, how these results
45 translate to the context of the sleep loss commonly experienced in society, such as
46 repeated nights of partial sleep loss, has not been determined and remains a critical gap
47 in the literature.

48

49 The detrimental effect of sleep loss on glucose metabolism may also be associated with
50 the misalignment of circadian rhythms (7, 8). One night of sleep deprivation (commonly
51 experienced by 20% of the world's population who perform shift-work) leads to a
52 reduction in glucose tolerance and concomitant alterations in the expression of skeletal
53 muscle clock genes (i.e., *Bmal1* and *Cry1* gene expression (7)) and the content of clock
54 proteins (i.e., BMAL1) (8), which are known to regulate circadian rhythms at a molecular
55 level (19). The functional significance of disrupting the molecular clock has been shown
56 in genetic mouse models (i.e., Clock mutant mice and the *Bmal1* KO mouse), which
57 display reduced glucose tolerance, mitochondrial respiratory function, and skeletal
58 muscle contractile function (20, 21). However, the effect of sleep restriction on markers
59 of circadian rhythmicity (e.g., skeletal muscle clock gene expression) and the potential
60 implications of such changes have not previously been examined.

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62 One approach to mitigating or ablating the impact of reduced sleep duration on glucose
63 tolerance is via exercise (17). Regular endurance exercise has been shown to exert
64 beneficial effects on glycaemic control via the activation of the insulin-independent
65 signalling pathway (22). High-intensity interval exercise (HIIE) is a time-efficient format
66 of endurance exercise, and is also a potent stimulus for the induction of mitochondrial
67 biogenesis (23), with increases in sarcoplasmic and mitochondrial protein synthesis, and
68 mitochondrial content and respiratory function, occurring concomitantly with
69 improvements in glucose tolerance (24-27). This raises the intriguing hypothesis that
70 exercise may also be useful to combat sleep-loss-induced impairments to glucose
71 tolerance, which are not necessarily reversed by a period of recovery sleep alone (28-30).
72 Furthermore, the same detrimental metabolic changes that occur in response to circadian
73 misalignment and altered expression of clock genes may also be ameliorated by
74 performing exercise (20, 31, 32). Consequently, HIIE may be able to mitigate the
75 detrimental effects of sleep loss on glucose metabolism, by increasing mitochondrial
76 content and function, and preventing changes in circadian rhythmicity (17).

77

78 Accordingly, the aim of this study was to investigate the effect of sleep restriction on
79 glucose tolerance, and to examine the underlying physiological alterations that might
80 contribute to these changes; specifically, by examining changes in mitochondrial content
81 and function, and circadian rhythmicity. Furthermore, we examined the role of exercise
82 as an intervention to mitigate the detrimental effects of sleep restriction. We hypothesised

83 that sleep restriction would reduce mitochondrial content and respiratory function, and
84 disrupt circadian rhythms, with a concomitant reduction in glucose tolerance, but that
85 performance of HIIE would ameliorate these effects.

86

87 **Results**

88 **Sleep Data**

89 To verify the efficacy of our sleep interventions, we measured the participants' total sleep
90 time (TST) via actigraphy (33). Mean nightly TST during the intervention was
91 significantly lower for the SR (sleep restriction) and SR+EX (sleep restriction and
92 exercise) groups compared to the NS (normal sleep) group ($P < 0.05$) (see Table 1). There
93 was no difference in nightly TST between the SR and SR+EX group. Polysomnography
94 (PSG), considered the gold standard assessment of sleep (34), was used to confirm
95 actigraphy TST data and to also assess sleep architecture on night 6 of the study ($n = 4$ per
96 group) (Supplementary Figure 1). Both the SR and SR+EX groups obtained significantly
97 less time in rapid eye movement (REM) sleep, non-rapid eye movement (NREM) stage
98 1 sleep, and NREM stage 2 sleep, compared to the NS group. Despite differences in TST
99 between NS and both the SR and SR+EX groups, there were no significant differences in
100 the absolute amount of sleep in the NREM stage 3 (N3) sleep between any of the groups
101 (N3 sleep \pm SD, NS = 72 ± 17 , SR = 75 ± 18 min, SR+EX = 71 ± 14 min, $P > 0.05$)
102 (Supplementary Table 1). Thus, as reported previously (2), N3 sleep was preserved
103 despite the reduced total sleep time.

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105 **Table 1. Actigraphy sleep analysis and step counts for each group.**

	NS	SR	SR+EX
Sleep duration baseline (min)	448 \pm 25	452 \pm 17	459 \pm 9
Sleep duration intervention (min)	449 \pm 22	230 \pm 5*#	235 \pm 5*#
Step count (habitual)	12260 \pm 3964	10965 \pm 2136	11831 \pm 919
Step count (intervention)	10652 \pm 2476	10033 \pm 1839	10953 \pm 2316

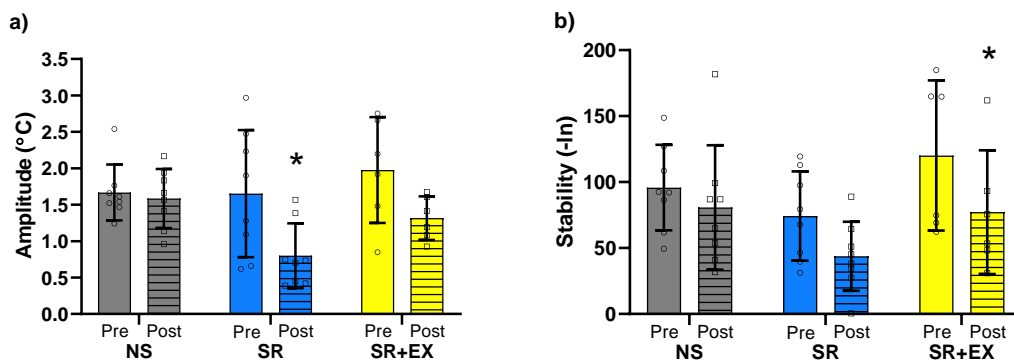
106 Values are mean \pm SD, Normal Sleep (NS), Sleep Restriction (SR) and Sleep Restriction
107 + Exercise (SR+EX), baseline (mean of first two nights of the study), intervention (mean
108 of 5 nights/days). * $P < 0.05$ compared to baseline within group, # $P < 0.05$ compared to
109 control (NS) during the intervention period.

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111 **Wrist skin temperature analysis**

112 Next we used peripheral wrist skin temperature, obtained over two 48-h periods, pre- and
113 post-intervention, to assess whether the significant reduction in TST in the SR and
114 SR+EX groups altered aspects of circadian rhythmicity, as previously suggested (7, 8).

115 There was a significant effect of time ($P=0.002$) for wrist skin temperature amplitude;
116 the skin temperature amplitude in the SR group was significantly lower from pre- to post-
117 intervention (mean amplitude change \pm SD $^{\circ}\text{C}$, 95% CI $^{\circ}\text{C}$, P value; $0.85 \pm 0.72^{\circ}\text{C}$, CI
118 $[0.20, 1.50^{\circ}\text{C}]$, $P=0.008$), indicating a decreased robustness of the temperature circadian
119 rhythm. There was no change in skin temperature amplitude in the NS ($0.08 \pm 0.67^{\circ}\text{C}$, CI
120 $[-0.57, 0.73^{\circ}\text{C}]$, $P=0.982$) or SR+EX ($0.66 \pm 0.65^{\circ}\text{C}$, CI $[-0.08, 1.43^{\circ}\text{C}]$, $P=0.091$) groups
121 (Figure 1). Furthermore, there was a significant effect of time ($P<0.001$) for wrist skin
122 temperature stability, with a significant reduction in the SR+EX group (53.2 ± 48.0 -ln,
123 CI $[13.7, 92.7$ -ln], $P=0.006$), but not the NS (15.1 ± 39.3 -ln, CI $[-21.9, 52.039$ -ln],
124 $P=0.658$) or SR (30.5 ± 30.9 -ln, CI $[-6.4, 67.5$ -ln], $P=0.126$) groups from pre- to post-
125 intervention.
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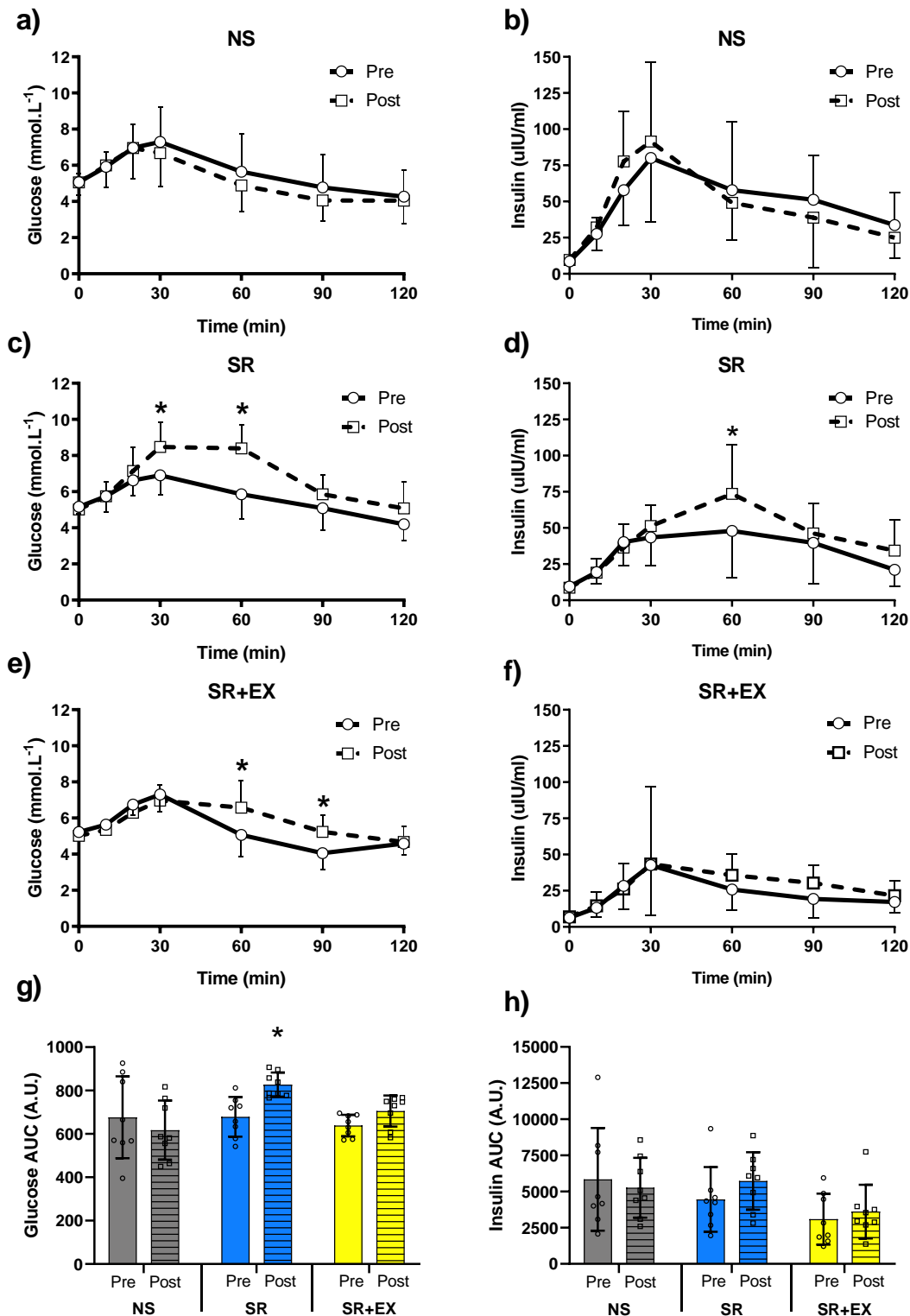


127 **Figure 1 - Measure of circadian peripheral skin temperature a) amplitude and b)**
128 **stability pre- and post-intervention.** Pre-intervention measurements are Day 2 and 3
129 (until 23:00 h) and the post-intervention measurements are Day 6 and Day 7. Normal
130 Sleep (NS, $n=8$), Sleep Restriction (SR, $n=8$) and Sleep Restriction and Exercise
131 (SR+EX, $n=6$). *Denotes significant within-group differences from pre- to post-
132 intervention ($P<0.05$).
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135 Glucose tolerance

136 As both sleep loss and disturbances to circadian rhythm have been associated with
137 impaired glucose tolerance, we examined plasma glucose and insulin concentrations in
138 response to an oral glucose tolerance test (OGTT), performed before and after the sleep
139 interventions. There were no significant differences between groups for pre-intervention
140 glucose ($P=0.771$) and insulin ($P=0.137$) area under the curve (AUC) values. Post-
141 intervention, there was a significant increase for total glucose AUC in the SR group (mean
142 change \pm SD, 95% CI, P value; 149 ± 115 A.U., CI $[54, 243$ A.U.], $P=0.002$) but not the
143 NS group (-59 ± 122 A.U., CI $[-36, 154$ A.U.], $P=0.356$) or the SR+EX group (67 ± 57 ,

144 CI [-162 to 28 A.U.], $P=0.239$). Although there was no interaction for changes in insulin
145 AUC for any group pre- to post-intervention ($P=0.085$), there was a 29% increase in
146 insulin AUC following the SR intervention (NS: -581 ± 1797 A.U., CI [-2037, 874 A.U.],
147 $P=0.933$; SR: 1275 ± 1787 A.U., CI [-180, 2731 A.U.], $P=0.100$ and SR+EX: $518 \pm$
148 1043 A.U., CI [-937, 1975 A.U.], $P>0.999$) (Figure 2 and Supplementary Tables 2 and
149 3).
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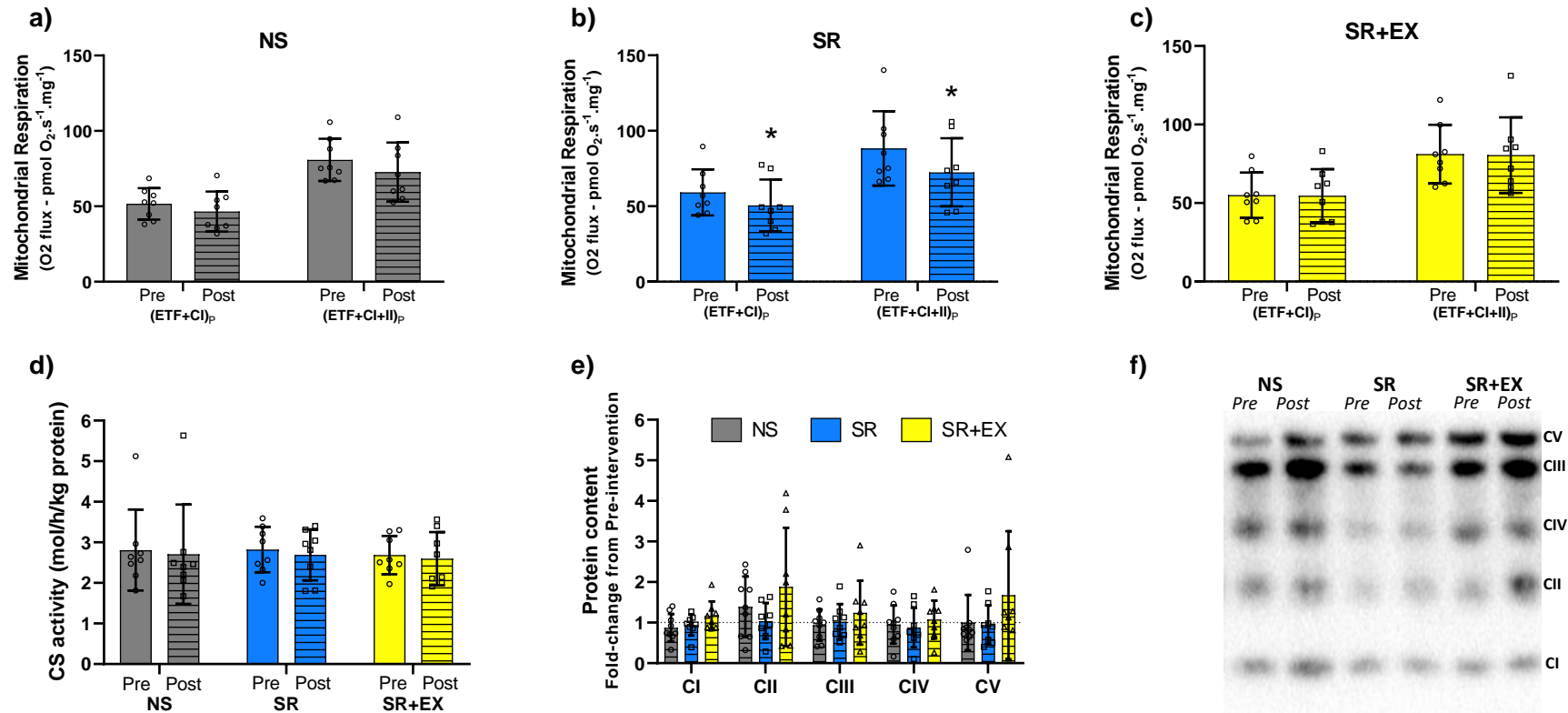
Figure 2 – Plasma glucose and insulin concentrations for pre- and post-intervention oral glucose tolerance tests (OGTT). Plasma glucose and insulin concentrations throughout the 120-minute OGTT in the (a, b) Normal Sleep (NS), (c, d) Sleep Restriction (SR), and (e, f) Sleep Restriction and Exercise (SR+EX) groups. g) Glucose and h) Insulin total area under the curve (AUC) during the OGTT. Values are mean \pm SD, individual data points are shown, * Denotes significant within-group differences from pre- to post-intervention ($P < 0.05$). $n = 8$ per group.

159 Mitochondrial content, function, and protein synthesis

160 As both insulin resistance and reduced glucose tolerance have been linked to changes in
161 mitochondrial characteristics, we investigated if skeletal muscle mitochondrial
162 respiratory function, content, and protein synthesis (via sarcoplasmic protein synthesis)
163 were influenced by the sleep loss and exercise interventions. There was a significant
164 interaction effect for maximal coupled mitochondrial respiration (ETF+CI+CII)_P
165 ($P=0.032$), which revealed a reduction from pre- to post-intervention (mean change \pm SD
166 pmol O₂.s⁻¹.mg⁻¹, 95% CI, P value) in the SR group (-15.9 ± 12.4 pmol O₂.s⁻¹.mg⁻¹, CI [-
167 25.6, -6.1 pmol O₂.s⁻¹.mg⁻¹], $P=0.001$) (~18% decrease and a coefficient of variation (CV)
168 of ~12%) (Figure 3a). This was not evident in the NS (8.1 ± 6.9 pmol O₂.s⁻¹.mg⁻¹, CI [-1.6
169 to 17.9 pmol O₂.s⁻¹.mg⁻¹], $P=0.122$) or SR+EX groups (0.6 ± 11.8 pmol O₂.s⁻¹.mg⁻¹, CI [-
170 9.1, 10.4 pmol O₂.s⁻¹.mg⁻¹], $P=0.997$) (Figure 3b and 3c). These results show for the first
171 time that sleep loss is associated with decreased mitochondrial respiratory function, which
172 was mitigated by exercise.

173

174 Mitochondrial content can be assessed via CS activity and the protein content of
175 mitochondrial complex subunits (16). There were no changes in whole-muscle CS
176 activity from pre- to post-intervention for any of the groups (interaction, $P=0.972$) (mean
177 difference, 95% CI, P value, NS 0.10 ± 0.45 mol/h/kg protein, CI [-0.24, 0.44 mol/h/kg
178 protein], $P>0.999$); (SR 0.13 ± 0.29 mol/h/kg protein, CI [-0.21, 0.47 mol/h/kg protein],
179 $P=0.992$) and (SR+EX $0.08, \pm 0.35$ mol/h/kg protein, CI [-0.26, 0.43 mol/h/kg protein],
180 $P>0.999$) (Figure 3d). As a further validation of changes in mitochondrial content, the
181 protein content for subunits of mitochondrial complexes were assessed via western
182 blotting. No significant interaction effects were observed for the protein content of
183 Complex 1 ($P=0.116$), Complex 2 ($P=0.649$), Complex 3 ($P=0.621$), Complex 4
184 ($P=0.718$) or Complex 5 ($P=0.158$) (Figure 3e), collectively indicating that the sleep and
185 exercise interventions did not affect mitochondrial content.



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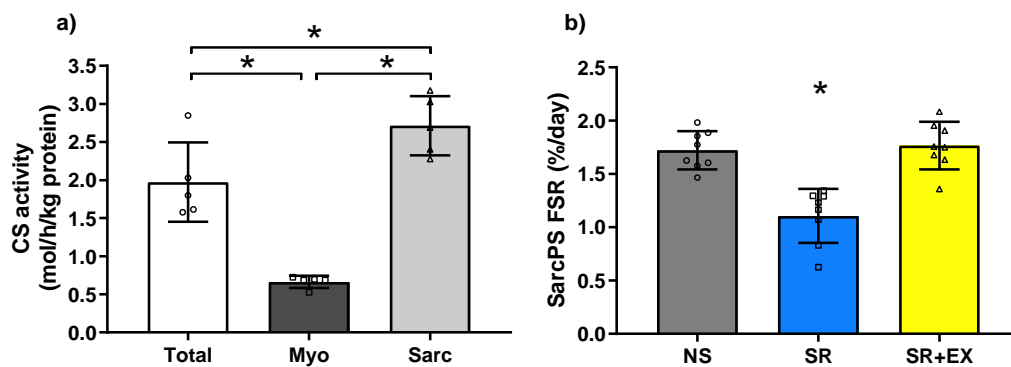
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Figure 3- Mitochondrial respiratory function and markers of mitochondrial content from pre-intervention compared to post-intervention.

Mitochondrial respiratory function in the (a) Normal Sleep (NS), (b) Sleep Restriction (SR) and (c) Sleep Restriction and Exercise (SR+EX) groups. (d) Citrate synthase activity and (e) fold-change of protein content for subunits of mitochondrial complexes (I – V) from pre- to post-intervention. Mitochondrial complex 1 (CI) - NDUFB8, Complex 2 (CII) - SDHB, Complex 3 (CIII) - Core protein 2 (UQCCRC2), Complex 4 (CIV) – MTCO, Complex 5 (CV) – ATP5A. (f) Representative image of protein content for mitochondrial complexes. Normal Sleep (NS), Sleep Restriction (SR) and Sleep Restriction + Exercise (SR+EX), (ETF+CI)_P – maximal coupled mitochondrial respiration through electron transfer flavoprotein (ETF) and CI; (ETF+CI+CII)_P – maximal coupled mitochondrial respiration through ETF, CI and CII; Pre – pre-intervention, Post – post-intervention. *n*=8 per group. * Denotes significant difference within group from pre- to post-intervention (*p*<0.05).

196 It has been argued that the best measure of mitochondrial biogenesis is mitochondrial
197 protein synthesis (MitoPS) (35). The sarcoplasm (skeletal muscle cytoplasm) is enriched
198 with mitochondria (see Figure 4a), and protein synthesis within this fraction
199 (sarcoplasmic protein synthesis - SarcPS) is likely reflective of MitoPS (24). Therefore
200 we assessed, for the first time, the effects of sleep restriction on SarcPS, which was
201 significantly lower in the SR group compared to both the NS group (between groups
202 difference FSR %/day \pm SD, 95% CI, P value, $-0.62 \pm 0.11\%$, CI $[-0.90, -0.33]$, $P < 0.001$)
203 and the SR+EX group ($-0.66 \pm 0.12\%$, CI $[-0.95, -0.37]$, $P < 0.001$); there was no
204 difference in SarcPS between the NS and SR+EX groups ($0.04 \pm 0.10\%$, CI $[-0.33, 0.24]$,
205 $P > 0.999$) (Figure 4b). These new data suggest that exercise can mitigate the lower rates
206 of SarcPS seen following sleep restriction alone.



207

208 **Figure 4 - Citrate synthase (CS) activity from fractionated skeletal muscle samples**
209 **and Sarcoplasmic protein synthesis (SarcPS) – a) CS activity of whole-muscle lysate**
210 **(Total), myofibrillar (Myo), and sarcoplasmic (Sarc) fractions were assessed from the**
211 **same muscle samples ($n=5$). b) Fractional synthetic rate (FSR) of SarcPS during the sleep**
212 **intervention. Data are mean \pm SD. Normal Sleep (NS), Sleep Restriction (SR), and Sleep**
213 **Restriction + Exercise (SR+EX), $n=8$ per group. *denotes significantly different from**
214 **other groups ($P < 0.05$).**

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216 **Glucose, circadian, and mitochondrial-related gene expression**

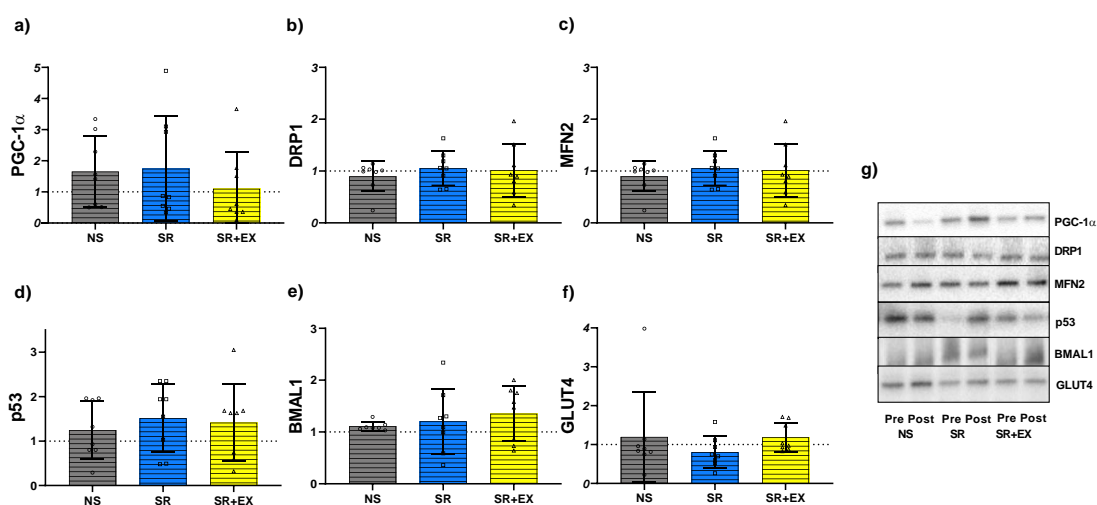
217 Given the changes observed for mitochondrial respiration, protein synthesis, circadian
218 rhythm (reflected by peripheral skin temperature), and glucose tolerance, we investigated
219 the expression of genes that regulate these processes (Table 2). In the SR group, there
220 was a significant reduction from pre- to post-intervention in the mRNA of *Mfn2* (mean
221 change \pm SD %, 95 % CI, P value; $-35 \pm 28\%$, CI $[-7, 79\%]$, $P=0.044$), *Bmal1* ($-29 \pm$
222 33% , CI $[-6, 64\%]$ $P=0.031$), and *Glut4* ($-38 \pm 33\%$, CI $[-13, 88\%]$, $P=0.020$), which
223 were not evident in the NS or SR+EX groups. There was also a decrease in *Tfam* and

224 β -*Had* mRNA expression with time; however, post-hoc analysis revealed no specific
225 group differences from pre- to post-intervention.

226

227 **Glucose, circadian, and mitochondrial-related protein content**

228 Following our discovery of reductions in the mRNA of some mitochondrial, circadian,
229 and glucose-related genes, we used the limited amount of remaining muscle biopsy tissue
230 to assess whether this leads to further changes at the protein level. There were no
231 significant interaction effects for PGC-1 α ($P=0.257$), DRP1 ($P=0.642$), MFN2
232 ($P=0.768$), p53 ($P=0.294$), BMAL1 ($P=0.778$), or GLUT4 ($P=0.466$) protein content,
233 from pre- to post-intervention (Figure 5).



234

235 **Figure 5 - Skeletal muscle mitochondrial, circadian, and glucose-related protein**
236 **content** a) PGC-1 α , b) DRP1, c) MFN2, d) p53, e) BMAL1, f) GLUT4, and g)
237 representative western blot images. Data are mean values \pm SD, normalised to pre-
238 pre-intervention values. Normal Sleep (NS), Sleep Restriction (SR) and Sleep Restriction and
239 Exercise (SR+EX), $n=8$ per group. * Denotes significant change from pre-intervention
240 ($P < 0.05$).

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Table 2 – Glucose, circadian, and mitochondrial-related skeletal muscle mRNA responses

Name	NS	SR	SR+EX	Interaction effect	Time effect
<i>Glucose metabolism related mRNA</i>					
<i>Glut4</i>	-14 ± 18	-38 ± 33*	16 ± 83	<i>P</i> =0.192	<i>P</i>=0.022
<i>Ampk1α</i>	-17 ± 30	-1 ± 52	-15 ± 24	<i>P</i> =0.952	<i>P</i> =0.060
<i>Pdk4</i>	0 ± 105	124 ± 286	60 ± 111	<i>P</i> =0.862	<i>P</i> =0.850
<i>β-Had</i>	-30 ± 42	-25 ± 39	-15 ± 42	<i>P</i> =0.872	<i>P</i>=0.011
<i>Circadian related mRNA</i>					
<i>Bmal1</i>	2 ± 22	-29 ± 33*	7 ± 51	<i>P</i> =0.154	<i>P</i>=0.041
<i>Clock</i>	6 ± 75	-7 ± 45	-14 ± 37	<i>P</i> =0.775	<i>P</i> =0.407
<i>Per1</i>	-7 ± 44	-28 ± 48	2 ± 71	<i>P</i> =0.527	<i>P</i> =0.123
<i>Per2</i>	-4 ± 32	-7 ± 51	-6 ± 48	<i>P</i> =0.719	<i>P</i> =0.410
<i>Cry1</i>	7 ± 45	12 ± 34	-10 ± 21	<i>P</i> =0.466	<i>P</i> =0.982
<i>Rev-Erb α</i>	-9 ± 38	-9 ± 47	-20 ± 42	<i>P</i> =0.583	<i>P</i> =0.130
<i>Mitochondrial related mRNA</i>					
<i>Pgc1α total</i>	-33 ± 51	-23 ± 49	14 ± 61	<i>P</i> =0.552	<i>P</i> =0.076
<i>p53</i>	48 ± 58	89 ± 100	6 ± 41	<i>P</i> =0.523	<i>P</i> =0.067
<i>Tfam</i>	-20 ± 33	-22 ± 41	-7 ± 59	<i>P</i> =0.816	<i>P</i>=0.035
<i>Nrf2</i>	-1 ± 34	-11 ± 33	-4 ± 43	<i>P</i> =0.777	<i>P</i> =0.179
<i>Dnm1l</i>	-14 ± 45	-3 ± 65	-4 ± 72	<i>P</i> =0.937	<i>P</i> =0.170
<i>Mfn1</i>	6 ± 39	-12 ± 47	-5 ± 53	<i>P</i> =0.414	<i>P</i> =0.524
<i>Mfn2</i>	18 ± 28	-35 ± 28*	2 ± 68	<i>P</i> =0.562	<i>P</i>=0.002
<i>Lc3b</i>	4 ± 50	-16 ± 40	-11 ± 55	<i>P</i> =0.486	<i>P</i> =0.177
<i>p62/SQSTM1</i>	39 ± 75	-1 ± 39	-8 ± 69	<i>P</i> =0.088	<i>P</i> =0.628
<i>Pink1</i>	-21 ± 34	-32 ± 45	2 ± 74	<i>P</i> =0.895	<i>P</i> =0.204
<i>Cox4</i>	-11 ± 22	-23 ± 28	-3 ± 65	<i>P</i> =0.537	<i>P</i> =0.088

258 Values are mean ± SD percent (%) changes from pre-intervention mRNA values. Normal
259 Sleep (NS, *n*=7), Sleep Restriction (SR, *n*=8), and Sleep Restriction and Exercise
260 (SR+EX, *n*=8). *Significantly different from pre-intervention value (*P*<0.05).

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265 **Discussion**

266 We discovered that in healthy young men, sleep restriction resulted in significantly
267 impaired glucose tolerance, with concomitant changes in circadian rhythmicity (skin
268 temperature amplitude and clock gene expression), skeletal muscle mitochondrial
269 respiratory function, and sarcoplasmic protein synthesis (SarcPS) – a proxy for
270 mitochondrial protein synthesis. However, performing three sessions of high-intensity
271 interval exercise (HIIE) during the sleep restriction intervention mitigated the
272 perturbations that were observed in the SR group. Mechanistically, there were also
273 differences between the SR and SR+EX groups in the expression and content of essential
274 glucose and mitochondrial-related regulatory markers. Our study provides novel insights
275 into the potential mechanisms underlying previously reported changes in glucose
276 tolerance with sleep loss and suggests exercise may be used as a therapeutic intervention
277 to attenuate such adverse effects.

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279 The sleep restriction protocol used in this study (consisting of 4 h TIB per night, for five
280 consecutive nights) resulted in a significant impairment in glucose tolerance.
281 Furthermore, the plasma insulin response during the OGTT for the SR group increased
282 by 29% from pre- to post-intervention. These results are consistent with previous studies
283 (3-5), including Rao et al. (2) who reported a 25% decrease in whole-body insulin
284 sensitivity (measured with a hyperinsulinaemic-euglycaemic clamp) following a similar
285 sleep restriction protocol (2). Therefore, the 22% increase in plasma glucose AUC
286 following sleep restriction observed in young, healthy men in this study solidifies the
287 evidence supporting the detrimental effect of even short periods of sleep restriction on
288 glucose tolerance.

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290 A novel aspect of this study was to examine the effect of three sessions of HIIE during
291 the period of sleep restriction as a means of mitigating sleep restriction-induced
292 reductions in glucose tolerance. In contrast to the SR group, adverse changes in glucose
293 tolerance were mitigated in the SR+EX group. While others have shown acute positive
294 (36) and protective (37) effects of exercise on sleep-loss-induced changes in glucose
295 tolerance, these studies assessed glucose tolerance either immediately after or within 24
296 h of exercise, raising the possibility these findings were confounded by the acute effects
297 of exercise on glycaemic control, which are known to persist for up to 48 h (22). In
298 contrast, we performed the OGTT 48 h post exercise, thus demonstrating for the first time

299 that performing HIIE during a period of sleep restriction prevents the negative effects to
300 glucose tolerance.

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302 The changes in glucose tolerance were mirrored by changes in skeletal muscle glucose
303 transporter 4 (*Glut4*) mRNA expression, whereby *Glut4* was lower in the SR group but
304 maintained in the SR+EX group. The maintenance of *Glut4* expression in the SR+EX
305 group is likely explained by the well-documented increase in expression commonly
306 observed with exercise (38). While there were no changes in GLUT4 protein content in
307 our study, a previous study that used streptozocin-induced diabetic rats demonstrates that
308 changes in skeletal muscle *Glut4* mRNA coincide with reductions in *in-vivo* glucose
309 uptake, but precede changes in GLUT4 protein content (39). Given that there was no
310 change in GLUT4 protein content in these previous studies, it may suggest that GLUT4
311 translocation may be impaired and supports previous research indicating impairments
312 within the insulin signalling pathway, following sleep restriction (40, 41).

313

314 Insulin resistance and reductions in glucose tolerance have previously been linked to
315 altered mitochondrial characteristics (14, 15); therefore, we also assessed changes in
316 skeletal muscle mitochondrial respiratory function. To our knowledge, ours is the first
317 report of a SR-induced reduction in skeletal muscle mitochondrial respiratory function in
318 humans. Previously, a study in humans investigating a single night of 4 h TIB reported a
319 decrease in insulin sensitivity with a concomitant increase in plasma acylcarnitines (9),
320 which was suggested to be indicative of both reduced fatty acid oxidation and impaired
321 mitochondrial function. The maintenance of mitochondrial respiratory function in the
322 SR+EX group is consistent with previous reports of the potency of HIIE for improving
323 mitochondrial respiratory function (26, 42); however, we admittedly do not have a
324 Normal Sleep and HIIE group that would allow us to ascertain whether HIIE enhanced
325 mitochondrial function. Nonetheless, our results do provide evidence supportive of a link
326 between reduced mitochondrial respiratory function and impaired glucose tolerance with
327 sleep restriction that is mitigated by the addition of HIIE.

328

329 Mitochondria are dynamic organelles and the production of new mitochondrial proteins
330 is an important determinant of their overall function (35). Mitochondrial protein synthesis
331 (MitoPS) is the measure that best reflects the process of mitochondrial biogenesis (23,
332 35). However, tissue availability for this project necessitated the use of sarcoplasmic

333 protein synthetic rate (e.g., SarcPS – Figure 4a) as a proxy for MitoPS. For the first time,
334 we report a lower rate of SarcPS in the SR group compared to both the NS and SR+EX
335 groups (Figure 4b). The significance of the lower rate of SarcPS in the SR group is
336 difficult to ascertain; however, we hypothesise it may underpin a reduced turnover of
337 mitochondrial proteins, with a subsequent impact on mitochondrial respiratory function
338 (35). Support for this notion comes from the observation that changes in MitoPS and
339 mitochondrial respiratory function have been shown to occur concomitantly (23, 43);
340 nonetheless, a direct link is yet to be established. Moreover, HIIE has consistently been
341 reported to increase both SarcPS and MitoPS (24, 44), and this likely accounts for the
342 higher rate of SarcPS in the SR+EX group compared to the SR group. Indeed, previous
343 reports indicate that SarcPS remains elevated for 48 h following endurance exercise (45),
344 and these increases are likely reflected in the integrative measure of protein synthesis
345 used in this study, which represents a summation of SarcPS throughout the entire
346 intervention.

347

348 Considering the changes observed to SarcPS, the effect of sleep restriction on markers of
349 mitochondrial content was also assessed. There were no changes to the protein content of
350 mitochondrial complex subunits or CS activity – both of which are valid markers of
351 mitochondrial content (16). While reduced CS activity has previously been reported
352 following sleep interventions, this was in response to extreme sleep deprivation (i.e., 120
353 h of continuous wakefulness) (18); thus, a direct comparison with our findings is difficult.
354 Despite no change in CS activity or protein content of mitochondrial complex subunits in
355 the SR+EX group, endurance exercise is well known to increase mitochondrial content
356 (25). Our results suggest changes in mitochondrial respiratory function and SarcPS
357 occurred independently of detectable changes in mitochondrial content. This dissociation
358 between changes in mitochondrial content and respiratory function has previously been
359 reported, and it has been suggested that these properties may be differentially regulated
360 (26, 43). One explanation for our observations may involve the processes regulating
361 mitochondrial dynamics/remodelling (i.e., fission and fusion), which can alter the
362 efficiency and oxidative capacity of the mitochondria, without necessarily altering
363 mitochondrial content (23). Therefore, we assessed genes and proteins known to regulate
364 mitochondrial remodelling. The reduced Mitofusin 2 (*Mfn2*) gene expression in this study
365 points to a potential link between altered mitochondrial morphology and the reduced
366 mitochondrial respiratory function and glucose tolerance observed in the SR group.

367 Considering exercise can also influence mitochondrial dynamics and mitophagy (46, 47),
368 and that *Mfn2* has previously been shown to be elevated in skeletal muscle 24 h post an
369 endurance exercise session (48), this may explain why *Mfn2* mRNA levels were
370 maintained in the SR+EX group.

371

372 As many of the molecular processes that regulate mitochondrial content, function, and
373 dynamics are regulated in a circadian manner (49, 50), we also examined aspects of
374 circadian rhythmicity using the robust measures of peripheral skin temperature (obtained
375 over 48 h, pre- and post-intervention) and analysis of molecular clock gene expression.
376 Peripheral skin temperature measures oscillate inversely to the rhythms of core body
377 temperature and therefore provides a physiological circadian output. We show that skin
378 temperature amplitude, a common measure of assessing the circadian nature of biological
379 processes, was significantly reduced in the SR group. Our data parallels that of Moller-
380 Levet et al. (51) who reported in human white blood cells, reduced amplitude of core
381 clock gene circadian expression following seven nights of sleep restriction (6 h TIB each
382 night). At the skeletal muscle level, we also show a significant reduction in *Bmal1* mRNA
383 from pre- to post-intervention in the SR group, but not in the NS or SR+EX groups. This
384 supports previous findings from Cedernaes et al. (7, 8) who reported a decrease in *Bmal1*
385 mRNA expression following 24 h of sleep deprivation. Moreover, reductions in *Glut4*
386 mRNA expression, similar to those we report in the SR group, have also been reported in
387 *Bmal1*^{-/-} mice (52), suggesting the reductions in *Bmal1* mRNA and changes to circadian
388 rhythm that were observed in the SR group may contribute to changes in *Glut4* mRNA.
389 These findings suggest that sleep restriction can disrupt the skeletal muscle circadian
390 clock, which has been associated with negative metabolic consequences, such as insulin
391 resistance and mitochondrial function (21, 52).

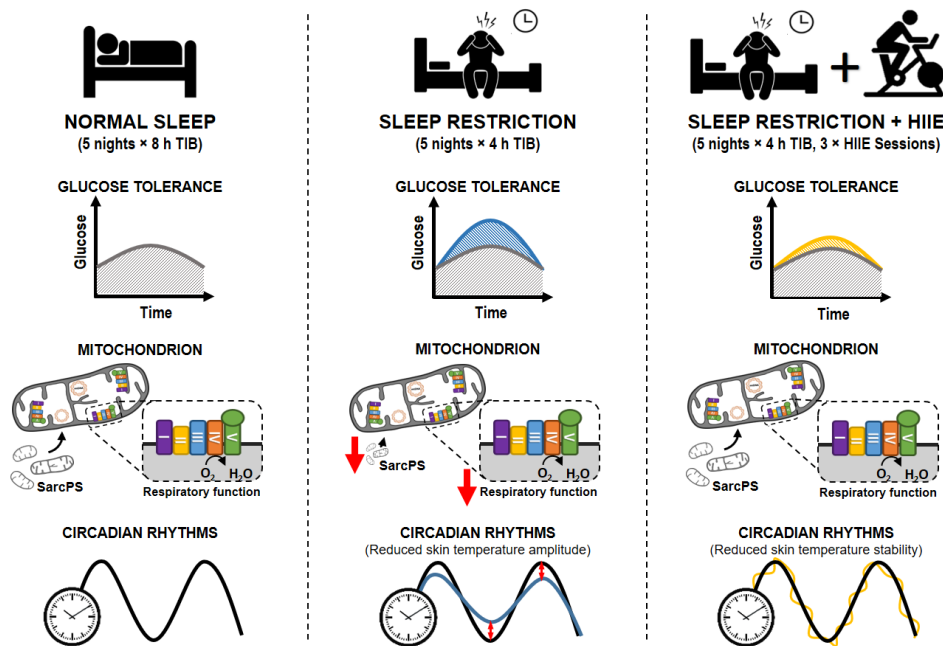
392

393 Exercise is considered a potent zeitgeber (i.e., circadian time cue) capable of altering
394 circadian rhythms (31, 53), therefore, we examined its effect on skin temperature and
395 clock gene expression. In this study, there was a reduction in skin temperature stability,
396 whilst skeletal muscle *Bmal1* mRNA expression was maintained in the SR+EX group,
397 but not the SR group. Exercise-like contractile activity in C2C12 myotubes induce time
398 of day related-phase shifts in *Bmal1* mRNA expression (53), which may help to explain
399 our findings. Nevertheless, further studies and additional muscle sampling time-points
400 are needed to clarify the effect of exercise performed at different times of the day in

401 humans, as well as changes in the underlying regulatory mechanisms (i.e., clock gene
402 expression). This will further optimise the implementation of exercise strategies to realign
403 changes in circadian rhythms induced by inadequate sleep.

404

405 In summary, we have provided the first direct evidence of a concomitant decrease in
406 mitochondrial respiratory function, SarcPS (of which MitoPS is a contributor), and
407 glucose tolerance, following sleep restriction in otherwise healthy young men. We
408 discovered alterations in mRNA expression of select genes involved in glucose uptake,
409 mitochondrial dynamics, and circadian rhythms (Figure 6). Collectively, these results
410 highlight a number of potential mechanisms by which sleep restriction may lead to
411 reductions in glucose tolerance. Importantly, HIIE mitigated these detrimental changes in
412 glucose tolerance and mitochondrial characteristics. While further research is still
413 required, these data provide a basis for the development of evidence-based health
414 guidelines and recommendations for those experiencing inadequate sleep, by highlighting
415 some of the underlying biological mechanisms that can be targeted by therapeutic
416 interventions such as exercise.



417

418 **Figure 6 – Summary of the effects of sleep restriction, with or without HIIE, on glucose**
419 **tolerance, mitochondrial characteristics, and circadian rhythms.** NS – Normal Sleep group,
420 SR – Sleep Restriction group, SR+EX – Sleep Restriction and Exercise group, HIIE – High-
421 intensity interval exercise, SarcPS – Sarcoplasmic protein synthesis, TIB – Time in bed.

422

423

424 **Study methodology**

425 **Ethics approval**

426 All procedures involved conform to the standards set by the latest revision of the
427 Declaration of Helsinki (except for registration in a database) and were approved by the
428 Victoria University Human Research Ethics Committee (HRE15-294).

429

430 **Participants**

431 Twenty-four healthy, recreationally active men, aged between 18 and 40 years of age,
432 volunteered to participate. Eligible participants 1) were not taking any medications, 2)
433 were not performing shift-work (within the previous three months), 3) had regular
434 sleeping habits (6 – 9 hours per night) and no previously diagnosed sleep disorders, 4)
435 had not travelled overseas in the previous two months, and 5) had a body mass index
436 between 19 and 30.

437

438 **Study overview**

439 Eligible participants attended the exercise physiology laboratory for baseline
440 anthropometric measurements (i.e., height and body mass), and aerobic fitness testing
441 (peak oxygen uptake [$\dot{V}O_{2\text{peak}}$] and peak aerobic power [\dot{W}_{Peak}]) that was performed to
442 volitional exhaustion on an electronically braked cycle ergometer (Excalibur, V2.0; Lode,
443 Groningen, Netherlands), using an incremental ramp protocol (30 W/minute). One week
444 prior to the intervention, a resting skeletal muscle biopsy was obtained to determine
445 baseline levels of deuterium oxide (D₂O) enrichment, and to assess basal mitochondrial
446 respiratory function. Following baseline testing, participants were matched for age, BMI,
447 habitual sleep duration, $\dot{V}O_{2\text{peak}}$, and mitochondrial respiratory function, and then
448 allocated to one of three experimental groups, in a counterbalanced order (Table 3).

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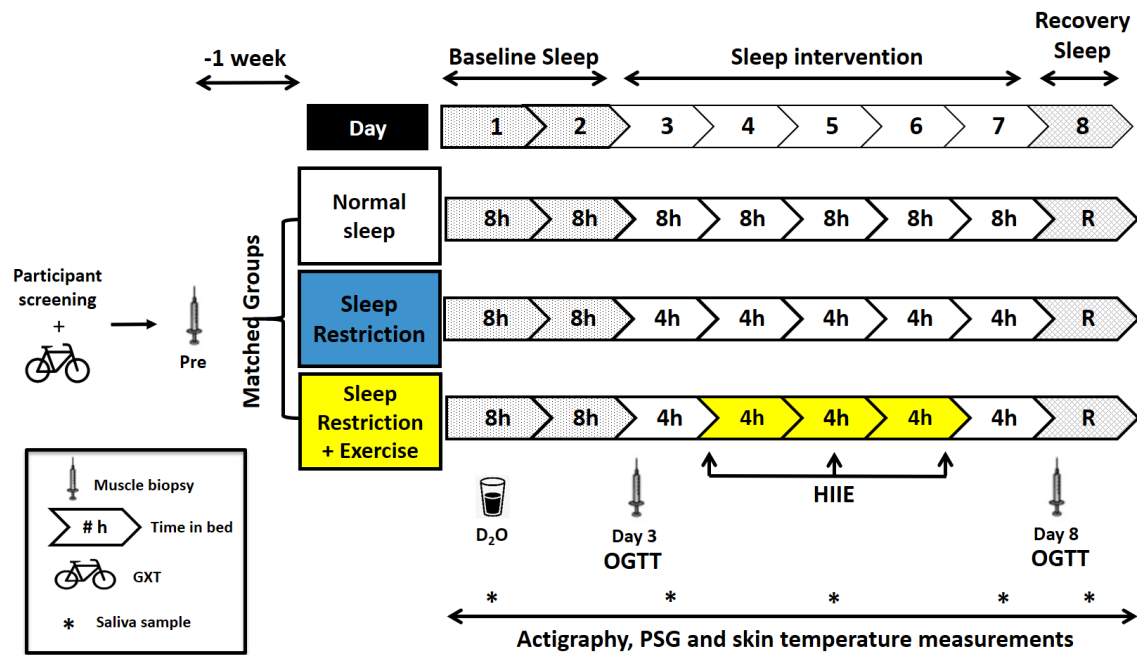
456 **Table 3.** Baseline characteristics of participants

	NS (n=8)	SR (n=8)	SR+EX (n=8)
Age (y)	24 ± 4	25 ± 5	24 ± 4
Height (cm)	177 ± 8	179 ± 6	179 ± 7
Mass (kg)	78.7 ± 13.3	74.5 ± 11.7	80.2 ± 9.5
BMI	25.2 ± 3.6	23.3 ± 3.0	24.6 ± 2.5
$\dot{V}O_{2\text{peak}}$ (mL.kg ⁻¹ .min ⁻¹)	43.7 ± 9.7	47.2 ± 6.7	48.0 ± 5.0
\dot{W}_{peak} (W)	319 ± 59	330 ± 44	362 ± 48
Habitual sleep duration (h:min)	7:37 ± 0:45	7:08 ± 0:44	7:17 ± 0:39
Mitochondrial respiration (ETF+CI+CII) _p (pmol.s ⁻¹ .mg tissue ⁻¹)	80.8 ± 17.7	88.3 ± 25.6	81.2 ± 19.7

457 Values are mean ± SD. There were no significant differences between the three groups
 458 for any of the baseline characteristics. NS – Normal sleep, SR – Sleep restriction, SR+EX
 459 – Sleep Restriction and Exercise, BMI – body mass index, \dot{W}_{peak} – peak power (W),
 460 (ETF+CI+CII)_p – maximal oxidative phosphorylation through electron transferring
 461 flavoprotein, complex 1 and complex 2 of the electron transport system (pre-study muscle
 462 biopsy).

463

464 The study consisted of an eight-night stay within a temperature-controlled sleep
 465 laboratory. All groups completed two initial nights of baseline sleep (8 h TIB from 23:00
 466 h to 07:00 h), followed by a five-night intervention period, during which the NS group
 467 spent 8 h TIB (23:00 h to 07:00 h), while both SR and SR+EX spent 4 h TIB per night
 468 (03:00 h to 07:00 h). Between 23:00 h and 03:00 h, lighting was dimmed to below 15 lux
 469 to reduce the effect of lighting on circadian rhythms (54). The SR+EX group also
 470 performed three exercise sessions during the intervention period on days 4, 5, and 6 at
 471 10:00 h. Following the intervention period, all groups completed a final night of *ad*
 472 *libitum* recovery sleep. Participants were monitored throughout the protocol and provided
 473 with a standardised diet consisting of fixed proportions (relative to body mass) of
 474 carbohydrates (4.5 g.kg⁻¹.d⁻¹), protein (1.5 g.kg⁻¹.d⁻¹) and fat (1 g.kg⁻¹.d⁻¹). All mealtimes
 475 (six throughout the day) were kept constant. An overview of the study protocol is shown
 476 in Figure 7.



477

478 **Figure 7.** Schematic representation of the study protocol. OGTT – oral glucose tolerance
 479 test, GXT – Graded exercise test, D₂O – deuterium oxide ingestion, HIIE – high-intensity
 480 interval exercise, R – *ad libitum* recovery sleep, PSG – polysomnography sleep
 481 monitoring, participant screening refers to medical questionnaires, exclusion criteria and,
 482 habitual sleep, and physical activity monitoring.

483

484

485 Experimental procedures

486 Sleep and physical activity monitoring

487 Sleep was assessed for one week prior to and then throughout the study using wrist-watch
 488 activity devices (Actiwatch 2, Philips Respironics, Murrysville, PA, USA) (33). Sleep
 489 architecture, duration, and quality were also determined via polysomnography (PSG)
 490 (Compumedics, AUS) on night 6 for a subset of participants from each condition ($n=12$;
 491 4 per group). Electrode placement for PSG monitoring was determined using the 10-20
 492 electrode placement system and scored in accordance to standard criteria (55). Habitual
 493 daily step counts were monitored using validated step-counting applications on the
 494 participants' personal mobile phone devices (i-Health app, Apple Inc., Cupertino, CA,
 495 USA; and Samsung Health, Samsung Electronics Co., Ltd., Suwon, South Korea) and
 496 these step counts were replicated throughout the study (Table 1) (data modified from
 497 (56)).

498

499

500

501 **High-intensity interval exercise (HIIE)**

502 The HIIE protocol was adapted from previous studies, which demonstrated improvements
503 in glucose tolerance (27, 57) and consisted of 10×60 -second intervals performed on a
504 cycle ergometer (Velotron, Racer-Mate, Seattle, WA, USA) at 90% of each participant's
505 \dot{W}_{peak} . Each interval was interspersed with 75 seconds of active recovery at 60 W. Each
506 session started with a 3-minute warm up at 60 W. The mean power per interval was 318
507 ± 53 W and the mean HR throughout the protocol was 156 ± 13 bpm.

508

509 **Wrist skin temperature measurements**

510 Wrist skin temperature was measured every 10 min (at a sensitivity of 0.0625°C) across
511 two 48 h periods (Pre-intervention - Days 2 and 3 (until 11 pm) and Post-intervention -
512 Days 6 and 7), using non-invasive temperature recording devices (iButtons, ThermoChron
513 iButton; Embedded Data Systems, Lawrenceburg, KY). This method has been shown to
514 be reliable and valid for evaluating temperature circadian rhythmicity, with peripheral
515 wrist skin temperature reported to have an inverse relationship to core body temperature
516 (58, 59). The data was analysed as previously described (59), for variations in temperature
517 amplitude and stability for each participant, using a modified version of the software
518 program JTK_CYCLE (60). Temperature amplitude is defined as the difference between
519 the maximum (or minimum) value of the trace and the mesor (which represents the mean
520 value of the data after smoothing with a cosine function) across a 24-h period (61);
521 changes in amplitude having previously been proposed to provide an indication of the
522 robustness of the rhythm (58, 62). The degree of phase homogeneity of a rhythm during
523 the period of data collection is considered a description of the 'stability' of the rhythm
524 (i.e., stability is considered high when the oscillatory pattern of the rhythm is nearly
525 identical from one day to the next, and considered low when there are large discrepancies
526 between oscillatory patterns from day-to-day) (58, 62). Data from eight participants in
527 both the NS and SR groups were included; however, due to technical issues, data from
528 the SR+EX group includes only six participants.

529

530 **Oral glucose tolerance testing (OGTT)**

531 To assess glucose tolerance, OGTT tests were performed on Day 3 (pre-intervention) and
532 Day 8 (post-intervention) at 08:00 h, following an overnight fast. Participants consumed
533 a 300 mL solution containing 75 g of glucose (Point of Care Diagnostics-Scientific, NSW,
534 Australia) and blood samples were collected after 0, 10, 20, 30, 60, 90, and 120 minutes.

535 Plasma glucose concentrations were measured on a glucose/lactate analyser (YSI, 2300
536 STAT plus, Yellow Spring, OH, USA). Plasma insulin concentrations were assessed
537 using an Insulin ELISA kit (ALPCO. 80-INSHU-E01.1, E10.1, Salem, NH, USA) and
538 run according to the manufacturer's instructions.

539

540 **Muscle biopsies**

541 One week prior to commencing the study, and on Day 3 and Day 8 of the intervention,
542 muscle biopsies were sampled from the *vastus lateralis* muscle using a suction-modified
543 Bergström needle, and under local anaesthesia of the skin and fascia (1% lidocaine). All
544 samples were collected at 10:00 h. Samples on Day 3 and 8 were collected post OGTT.
545 All samples were immediately frozen in liquid nitrogen and stored at -80°C, or set aside
546 for mitochondrial respirometry.

547

548 **High-resolution respirometry**

549 Immediately following the muscle biopsies, muscle fibres were placed in ice-cold biopsy
550 preserving solution (BioPS) and prepared as previously described (26). Mitochondrial
551 respiration was measured in triplicate (coefficient of variation [CV] = 12%) (from 2 to 4
552 mg wet weight of muscle fibers) in MiR05 at 37°C by using the high-resolution
553 Oxygraph-2k (Oroboros, Innsbruck, Austria). Oxygen concentration (in nanomoles per
554 milliliter) and flux (in picomoles per second per milligram) were recorded with DatLab
555 software (Oroboros). The following substrate–uncoupler–inhibitor titration (SUIT)
556 protocol was used to assess mitochondrial respiratory function; octanoyl-carnitine (0.2
557 mM) and malate (2 mM) for leak respiration (L) via electron transferring flavoprotein
558 (ETF), (ETF)L, ADP (5 mM, saturating concentration) was added for measurement of
559 oxidative phosphorylation capacity (P), (ETF)P. Pyruvate (5 mM) was then added for
560 measurement through Complex 1 (CI), (ETF+CI)P, followed by addition of succinate (10
561 mM) for measurement of oxidative phosphorylation capacity through complex 1 and 2
562 combined (ETF+CI+II)P. Cytochrome c (10 mM) was then added to test for outer
563 mitochondrial membrane integrity (an oxygen flux increase of < 15% from (ETF+CI+II)P
564 was considered acceptable). A series of stepwise carbonyl cyanide 4-(trifluoromethoxy)
565 phenylhydrazone (FCCP) titrations (0.75–1.5 mM), for the measurement of ETS capacity
566 (E) through (ETF+CI+II)E followed. Rotenone (0.5 mM), an inhibitor of CI, was then
567 added to determine E through CII (ETF+CII)E, whereas addition of antimycin A (2.5
568 mM), an inhibitor of CIII, allowed measurement of and correction for residual oxygen

569 consumption (ROX), which is indicative of non-mitochondrial oxygen consumption. As
570 there was no difference in mitochondrial respiration between ‘Pre’ and Day 3 resting
571 biopsies (mean (ETF+CI+II)P pmol O₂.s⁻¹.mg⁻¹ ± SD; Pre – 81.4 ± 21.6 pmol O₂.s⁻¹.mg⁻¹,
572 Day 3 - 80.6 ± 21.0 pmol O₂.s⁻¹.mg⁻¹, *P*=0.831), ‘Baseline’ mitochondrial respiration
573 was calculated as the mean from the ‘Pre’ study and Day 3 muscle biopsies.

574

575 **Assessment of sarcoplasmic protein synthesis (SarcPS)**

576 SarcPS was used as an indicator of mitochondrial protein synthesis (MitoPS), as
577 performed previously (24), due to the large amount of muscle needed to assess MitoPS
578 (e.g., 80 – 100 mg). In validating this approach, we measured CS activity (a validated
579 biomarker of mitochondrial content (16)) in whole-muscle lysate, as well as the
580 myofibrillar and sarcoplasmic fractions (Figure 4a). The whole-muscle CS activity was
581 significantly higher than the myofibrillar fraction (mean difference CS activity (mol/h/kg
582 protein) ± SD, 1.30 ± 0.48, CI [0.64, 1.97], *P*<0.001). The sarcoplasmic fraction CS
583 activity was also significantly higher than the myofibrillar fraction (2.05 ± 0.35, CI
584 [-2.71, -1.39], *P*<0.001) and whole-muscle fractions (0.74 ± 0.44, CI [-1.40, -0.07],
585 *P*=0.027) demonstrating that the sarcoplasmic fractions are enriched with mitochondria,
586 compared to both the myofibrillar fraction and whole-muscle sample.

587

588 To determine SarcPS, on Day 1 each participant ingested 150 mL of Deuterium Oxide
589 (D₂O) (70 atom %, Cambridge Isotope Laboratories) as previously described (63). Saliva
590 samples were collected prior to D₂O ingestion and then on Days 3, 5, 7, and 8 to determine
591 body water enrichment via cavity ring-down spectroscopy (Picarro L2130-I analyser,
592 Picarro, Santa Clara, CA). Total body water ²H enrichment was used as a surrogate for
593 plasma alanine ²H labelling, as previously described (63). The mean body water
594 enrichment (atom percent excess, APE) has been reported previously (56).

595

596 Frozen muscle samples (40 to 60 mg) were homogenised and prepared as previously
597 described (64). Cation exchange chromatography was then performed on the
598 sarcoplasmic samples using columns containing Dowex resin (Dowex 50wx8-200 ion
599 exchange resin, Sigma Aldrich) to extract free amino acids from the sarcoplasmic
600 fractions (64). The amino acid samples were then derivatised as their N-acetyl-n-propyl-
601 esters, as per previous protocols (65).

602

603 The $^2\text{H}/^1\text{H}$ ratio of the sarcoplasmic samples were determined using gas chromatography
604 pyrolysis isotope ratio mass spectrometry (GC-P-IMS) (Metabolic Solutions, Nashua,
605 NH, USA), to assess the incorporation of deuterium into protein-bound alanine. This was
606 used to assess the fractional synthetic rate (FSR) of sarcoplasmic proteins with the use of
607 the enrichment of body water, corrected for the mean number of deuterium moieties
608 incorporated per alanine (i.e., 3.7) as previously described (63), as the surrogate precursor
609 labelling between subsequent biopsies.

610

611 The following standard equation (63) was used to determine FSR:

612

$$613 \quad \text{FSR (\%/day)} = ((E_{t1} - E_{t0}) / (E_p \times \text{time})) \times 100$$

614

615 Where; FSR = fractional synthetic rate, E_{t1} = APE day 8, E_{t0} = APE day 3, E_p = average
616 saliva APE, time = time between biopsies, in days, and APE = atomic percentage excess.

617

618 **Preparation of whole-muscle lysates for western blots and CS activity assay**

619 Frozen muscle (10 to 20 mg) was homogenised as previously described (26) in an ice-
620 cold lysis buffer (1:20 w/v) containing 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1%
621 IGEPAL, deionised water and a protease/phosphatase inhibitor cocktail (Cell Signaling
622 Technology (CST), Danvers, MA, USA), adjusted to pH 7.4. Protein concentration was
623 determined in triplicate with a commercial colorimetric assay (Protein Assay kit-II; Bio-
624 Rad, Gladesville, NSW, Australia), against bovine serum albumin standards (BSA,
625 A9647; Sigma-Aldrich).

626

627 **Citrate synthase activity assay**

628 Citrate synthase (CS) activity was determined in triplicate on a 96-well microtiter plate
629 by adding 7.5 μL of a 4 mg/mL muscle homogenate (freeze thawed in liquid nitrogen
630 twice), 40 μL of 3 mM acetyl CoA, 25 μL of 1 mM 5,59-dithiobis(2-nitrobenzoic acid)
631 (DTNB), 165 μL of 100 mM Tris buffer (pH 8.3, kept at 30 °C). After addition of 15 μL
632 of 10 mM oxaloacetic acid, the plate was immediately placed in an xMark-Microplate
633 spectrophotometer (Bio-Rad) at 30°C, and after 30 s of linear agitation, absorbance at 412
634 nm was recorded every 15 s for 3 min. CS activity is reported as moles per hour per
635 kilogram of protein.

636

637 **Western blotting**

638 Muscle homogenate was diluted in 4X Laemmli buffer, and equal amounts of total protein
639 (15 or 20 µg) were loaded in different wells on Criterion™ 4-20% TGX Stain-Free™
640 Precast Gels (Bio-Rad, Australia). A stain-free system (Bio-Rad, Australia) was used as
641 a loading control, with protein expression normalised to total protein loaded per lane.
642 Each gel also contained four to six internal standards of varying dilutions, made from a
643 mixed homogenate of every sample in equal concentrations. These standards were used
644 to form a calibration curve, with density plotted against protein content. Protein
645 abundance was then calculated from the measured band intensity for each sample on the
646 gel, using the linear regression equation from the calibration curve.

647

648 Muscle lysates were separated via gel electrophoresis and transferred to PVDF
649 membranes. Membranes were then blocked in 5% non-fat dry milk diluted in Tris-
650 buffered saline with 0.1% Tween-20 (TBST) for 60 minutes. Membranes were then
651 incubated overnight at 4°C with the appropriate primary antibody, prepared at a 1:1000
652 dilution in TBST with 5% BSA and 0.02% sodium azide (unless stated otherwise). The
653 following primary antibodies were from Cell Signaling Technologies (CST) and include
654 PGC-1α (CST2178), DRP1 (CST5341), MFN2 (CST9482), p53 (CST2527), GLUT4
655 (CST2213). The following antibodies were obtained from Abcam; BMAL1 (AB93806),
656 Total OXPHOS (AB110413). The membranes were then incubated at room temperature
657 with the appropriate host species-specific secondary antibody for 60 min, before being
658 exposed to a chemiluminescence solution (Clarity™ Western ECL Substrate [Bio-Rad,
659 Hercules, CA, USA] or SuperSignal™ West Femto Maximum Sensitivity Substrate
660 [ThermoFisher, ThermoFischer Scientific, Wilmington, DE, USA]). Images were taken with
661 a ChemiDoc Imaging System fitted (Bio-Rad). Densitometry was performed with Image
662 Lab 5.0 software (Bio-Rad). Images are typically displayed with at least five bandwidths
663 above and below the band of interest.

664

665 **Real-time quantitative polymerase chain reaction (qPCR)**

666 RNA extraction with TRIzol (Life Technologies, 15596 026) from frozen muscle samples
667 (10 to 20 mg), quantification, and reverse transcription (iScript™ Reverse transcription
668 supermix, BioRad) were performed as previously described (66). Relative mRNA
669 expression was measured by qPCR (QuantStudio 7 Flex, Applied Biosystems, Foster

670 City, CA) using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Primers were
671 designed using Primer-BLAST to include all splice variants, and were purchased from
672 Sigma-Aldrich (Supplementary Table 4). The expression of each target gene was
673 normalised to the geometric mean of expression of the three most stably expressed
674 reference genes (as previously described) (66), and using the $2^{-\Delta\Delta C_t}$ method (where C_t is
675 the quantification cycle). Due to insufficient muscle sample collections for 1 participant,
676 only samples from 7 participants in the NS group were prepared for RT-PCR.

677

678 **Statistical analysis**

679 Statistical analyses were conducted using the statistical software package GraphPad
680 Prism (V7.03). Pre- to post-intervention changes in gene expression, protein content,
681 mitochondrial respiratory function, peripheral skin temperature, glucose tolerance, citrate
682 synthase activity, and plasma insulin concentrations were assessed for each group using
683 a mixed analysis of variance (ANOVA) with one between-subjects measure (group) and
684 a within-subjects measure (time). Significant effects of interaction (group \times time), time
685 (pre vs post), and group (NS vs SR vs SR+EX) are reported where effects are seen. Where
686 significant effects occurred, Bonferonni post-hoc testing was performed to locate the
687 differences. All statistical analyses of gene expression and protein content data were
688 conducted using raw values. Gene expression data in-text are reported as percent fold-
689 changes from pre-intervention values (mean % \pm SD) with 95% confidence intervals (CI)
690 from fold-change data (as a percentage), with the P value from the raw data reported.
691 Data in figures represent fold-changes from pre-intervention values, with individual
692 responses. A one-way ANOVA was used to assess differences between groups for the
693 mean actigraphy sleep data and SarcPS data. All data in text, figures and tables are
694 presented as mean \pm standard deviation (SD), and 95% confidence intervals with P values
695 ≤ 0.05 indicating statistical significance. Exact P values are presented, unless $P < 0.001$
696 or > 0.999 .

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872

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874

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876

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