The effect of acute sleep deprivation on skeletal muscle protein synthesis and the hormonal environment

Short title: Sleep deprivation and muscle protein metabolism

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Summary:

Inadequate sleep has profound, negative consequences on human tissues including skeletal muscle. Poor muscle health is associated with a series of chronic and metabolic conditions that are 15-30% more prevalent in individuals who chronically experience short and/or poor-quality sleep. Animal models suggest that inadequate sleep may directly impair muscle protein metabolism, which is critical to maintain muscle mass and function. This study aimed at providing the first proof-of-concept that total sleep deprivation decreases muscle protein synthesis in humans. To this end, thirteen young males and females experienced one night of total sleep deprivation or slept normally at home. Anabolic and catabolic hormonal profiles, muscle fractional synthesis rate and markers of muscle protein metabolism were assessed the following day. Cortisol release was higher, and there was a trend for testosterone and muscle fractional synthesis rate to be lower in the sleep-deprived than in the control condition. Markers of muscle protein degradation did not change. Exploratory analyses of the male and female cohorts revealed that the trend for sleep deprivation-induced decrease in fractional synthesis rate was significant and specific to the male cohort, and may be driven by testosterone. In conclusion, one night of total sleep deprivation disrupts the balance of anabolic and catabolic hormones and induces a trend towards a decrease in muscle protein synthesis. These results suggest a potentially direct relationship between inadequate sleep and poor muscle health in humans that may be sex-specific.

Keywords: muscle protein metabolism, fractional synthesis rate, anabolism, testosterone.

Introduction

Inadequate sleep (e.g., short sleep duration, poor quality sleep) is linked with a range of negative physiological and psychological outcomes (Kecklund & Axelsson, 2016). Beyond rapidly impeding simple and complex cognitive functions, inadequate sleep impairs whole-body homeostasis, leading to undesirable physiological consequences in the short- and longer-term (Reutrakul & Van Cauter, 2018). Most metabolic tissues including liver, adipose tissue and skeletal muscle are at risk of developing sleep-associated adverse outcomes.

Skeletal muscle is a primary regulator of human metabolism. It acts as a major storage site for energy substrates and accounts for a large proportion of whole body energy expenditure at rest and during exercise (Hargreaves & Spriet, 2018). Inadequate sleep has the potential to profoundly affect muscle homeostasis. Chronic disruption of the body's circadian rhythm, such as that induced by inadequate sleep, significantly alters the regulation of numerous genes and their associated phenotype within skeletal muscle (Harfmann, Schroder, & Esser, 2015). Inadequate sleep also affects substrate metabolism in the muscle. Even relatively short periods of sleep restriction (less than a week) can compromise glucose metabolism, reduce insulin sensitivity and impair muscle function (Bescos et al., 2018; Buxton et al., 2010).

Importantly, skeletal muscle is made up of 80% of proteins and maintaining optimal muscle protein metabolism is critical for muscle health. Situations where muscle protein synthesis rates chronically lags protein degradation rates can result in an accelerated loss of muscle mass. Low muscle mass is both a hallmark and a precursor to a range of chronic health conditions including neuromuscular diseases, sarcopenia and frailty, obesity and type II diabetes (Russell, 2010). Population-based studies report that the risk of developing these conditions is 15-30% higher in individuals who chronically experience short and/or poor

quality sleep (Kowall et al., 2016; Lucassen et al., 2017; Wu, Zhai, & Zhang, 2014). To this end, a growing body of evidence suggests that inadequate sleep may directly affect muscle protein metabolism (Aisbett, Condo, Zacharewicz, & Lamon, 2017; Monico-Neto et al., 2013).

Rodent studies first demonstrated a possible causal link between inadequate sleep and disrupted muscle protein metabolism. Specifically, rats subjected to 96 h of sleep deprivation (specifically paradoxical sleep deprivation, i.e., where rapid eye movement sleep is restricted) experienced a decrease in muscle mass (Dattilo et al., 2012) and muscle fibre cross-sectional area (de Sa Souza et al., 2016). In this rodent model, sleep deprivation attenuated markers of the protein synthesis pathways and increased muscle proteolytic activity (de Sa Souza et al., 2016); effects that were partially restored following a 96-h recovery period. These findings were paralleled by a recent human study reporting a catabolic gene signature in skeletal muscle following one night of total sleep deprivation (Cedernaes et al., 2018).

Factors that regulate skeletal muscle protein metabolism at the molecular level are influenced by mechanical (muscle contraction), nutritional (dietary protein intake) and hormonal inputs (Russell, 2010). Testosterone and IGF-1 positively regulate muscle protein anabolism by promoting muscle protein synthesis (Sheffield-Moore et al., 1999; Urban et al., 1995), while repressing genes that activate muscle protein degradation (Zhao et al., 2008). In contrast, cortisol drives catabolism by activating key muscle protein degradation pathways (Kayali, Young, & Goodman, 1987). Observational evidence suggest that inadequate sleep alters anabolic (Leproult & Van Cauter, 2011; Reynolds et al., 2012) and catabolic (Cedernaes et al., 2018; Dattilo et al., 2020) hormone secretion patterns in humans, providing a possible mechanism for impaired muscle protein metabolism. However, human studies looking into the mechanisms possibly linking inadequate sleep to clinically observable changes in body

composition, muscle function and metabolism are lacking. This proof-of-concept study sought to determine if a 30-h sleep deprivation period promotes a catabolic hormonal environment that, in turn, may compromise muscle protein synthesis and markers of muscle metabolism in young, healthy male and female participants.

Methods

Participants

Thirteen young (18-35 years old), healthy males and females gave their informed consent to participate in this randomized, crossover-designed study. Participants were excluded if they had a history of recent transmeridian travel (i.e., no travel across multiple time zones in the previous four weeks), shiftwork, frequent napping, or had a diagnosed sleep disorder. Participants were required to have habitual bed (2200–0000) and wake (0600–0800) times that were broadly consistent with the experimental protocol. Effects of female reproductive hormone fluctuations were minimised by testing female participants during the same phase of the menstrual cycle, and avoiding the follicular phase. A detailed account of the strategy for female volunteer recruitment and testing have been comprehensively described elsewhere (Knowles et al., 2019). The study was approved by the Deakin University Human Research Ethics Committee (2016-028) and conducted in accordance to *The Declaration of Helsinki* (1964) and its later amendments. Participants' characteristics are summarized in Table 1.

Table 1. Participants' characteristics

Sex	Age	Weight (kg)	Height (cm)	BMI ($kg \cdot m^{-2}$)
Male (N=7)	22 ± 1.8	71.6 ± 11.3	173.5 ± 9.0	22.6 ± 4.1

Female (N=6)	20 ± 1.3	60.1 ± 10.3	170.5 ± 5.1	20.7 ± 3.2

Mean ± SD; BMI = Body Mass Index

Pre-study procedure

Participants were instructed to maintain their habitual sleep behaviour prior to entering the study. Participants wore an actigraph (Actical MiniMitter/Respironics, Bend, OR) on their non-dominant wrist, and completed a sleep diary for one week prior to the study. The sleep diary was used to corroborate the actigraphy data and minimise possibility of incorrectly scoring periods of sedentary wakefulness as sleep. These data were also used to ensure participants were not sleep restricted prior to trial participation and did not have detectable sleep problems.

Participants completed either a control (CON) or experimental (DEP; sleep deprivation) trial in a randomized, crossover design. Trials were separated by at least four weeks to allow for full recovery. An outline of the experimental protocol is presented in Figure 1. Forty-eight hours prior to each trial, participants were required to refrain from strenuous exercise, alcohol and caffeine. On the night of the trial (CON or DEP), standardized meals (pasta, tomato sauce) containing approximately 20% fat, 14% protein and 66% carbohydrate were provided to participants with water *ad libitum*.

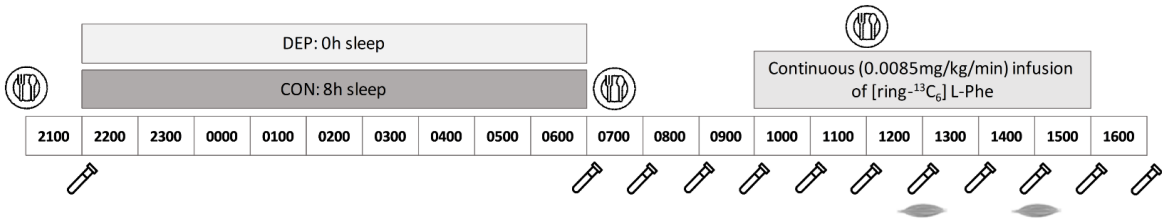
Procedure

At 2100 on the night of the sleep deprivation trial, the participants reported to the laboratory, received their standardized meal and were limited to sedentary activities (i.e., reading a book, watching a movie). Participants were constantly observed by research personnel and monitored by actigraphy (Actical MiniMitter/Respironics, Bend, OR) to ensure they did not fall asleep. They remained in a sound attenuated, light (>100 lux) and temperature (21±2°C)

controlled facility for the entire protocol. To comply with ethical requirements, participants were permitted to consume low-protein snacks (i.e., fruits and vegetable, rice crackers) and water *ad libitum* during the sleep deprivation period. Regardless of potential differences in insulinemia, adding a non-pharmacological dose of carbohydrates to a protein synthesis activating dose of proteins (15-30 g) has no additive effect on fractional synthesis rate (Glynn et al., 2013; Hamer et al., 2013), our primary outcome. The same protocol was repeated for the control trial. Participants consumed their standardized meal and were permitted to sleep from 2200 to 0700 at home. Spending the night of the control condition at home prevented the need to habituate participants to another sleeping environment.

At 0700 the following day, a venous blood sample was collected (at home or at the laboratory depending on the condition) before the participants consumed a standardized breakfast containing approximately 9% fat, 11% proteins and 80% carbohydrates, and a total of 20.3 ± 1.8 g of proteins. At 0800, an 18-gauge cannulae was inserted into the antecubital vein of each arm and blood was sampled every hour for the duration of the protocol. In the other arm, another cannulae was inserted for the continuous infusion of [ring-$^{13}C_6$]-L-phenylalanine (0.34 mg·kg^{-1} bolus followed by a constant infusion rate of 0.0085 mg·kg^{-1}·min^{-1}) (Cambridge Isotope Laboratories, Tewksbury, MA) from 1000 to the end of the protocol. At 1200, participants consumed a standardized lunch containing 12% fat, 21% protein and 67% carbohydrate, and a total of 20.6 ± 0.3 g protein. Skeletal muscle samples were obtained at 1300 and 1500 under local anaesthesia (1% Lidocaine) at separate locations from the belly of the *vastus lateralis* muscle using a percutaneous needle biopsy technique as previously described by our group (Lamon et al., 2016). Muscle samples were immediately frozen in liquid nitrogen and used for the measurement of isotopic enrichment and gene expression analysis.

The same protocol was repeated for the control trial, but participants were permitted to sleep from 2200 to 0700 at home, before reporting to the laboratory at 0800. Spending the night of the control condition at home prevented the need to habituate participants to another sleeping environment. An outline of the experimental protocol is presented in Figure 1.



Sleep measures

Participants sleep was recorded objectively using actigraphy (Actical MiniMitter/Respironics, Bend, OR). The Actical (28 × 27 × 10 mm, 17 g) device uses a piezoelectric omnidirectional accelerometer, which is sensitive to movements in all planes in the range of 0.5–3.0 Hz. Data output from activity monitors (actigraphy) provides an objective, non-invasive, indirect assessment of sleep and has been validated against polysomnography. Primary outcomes were total sleep time and sleep efficiency (total sleep time/time in bed).

Hormone measures

Venous blood samples were collected in EDTA-tubes, manually inverted and immediately centrifuged for 15 min at 13’000 rev·min^{-1} at 4°C. The supernatant (plasma) was then isolated and frozen at -80°C for further analysis. Plasma cortisol, testosterone and insulin growth factor-1 (IGF-1) concentrations were determined using a high-sensitivity enzyme immunoassay ELISA kit (IBL International, Hamburg, Germany) according to the manufacturer’s instructions. Insulin concentration was determined using the MILLIPLEX® MAP Human Metabolic Hormone Magnetic Bead Panel (Merck KGaA, Darmstadt, Germany) according to the manufacturer’s instructions.

Isotopic enrichment in plasma

After thawing, plasma was precipitated using an equal volume of 15% sulfosalicylic acid (SSA) solution and centrifuged for 20 min at 13,000 rev·min^{-1} at 4°C. Blood amino acids were extracted from 500 mL of supernatant by cation exchange chromatography (Dowex AG 50W-8X, 100–200 mesh H+ form; Bio-Rad Laboratories). Phenylalanine enrichments were determined by gas chromatography–mass spectrometry (GC-MS) using the tertbutyldimethylsilyl derivative with electron impact ionization as described previously (English et al., 2016). Ions 336 and 342 were monitored.

Isotopic enrichment in muscle proteins

A 30 mg piece of muscle was used for isolation of mixed muscle bound and intracellular protein fractions. Briefly, bound muscle proteins were extracted in perchloric acid and hydrolysed using 6N hydrochloric acid (110°C for 24 h). Isotopic enrichments of [ring-$_{13}C^6$]-L-phenylalanine in tissue fluid (intracellular fraction) were used as a precursor pool for the calculation of the fractional synthesis rate. Total muscle phenylalanine was isolated using cation exchange chromatography (50W-8X, 200–400 mesh H+ form; Bio-Rad Laboratories). Amino acids were eluted in 8 mL of 2N ammonium hydroxide and dried under vacuum. Muscle intracellular and bound protein [ring-$_{13}C^6$]-L-phenylalanine enrichments were determined by GC-MS with electron impact ionization using the tert-butyldimethylsilyl derivative. Ions 238 and 240 were monitored for bound protein enrichments; ions 336 and 342 were monitored for intracellular enrichments as described previously (English et al., 2016). Mixed muscle protein FSR (% / hour) was calculated by measuring the direct incorporation of [ring-$_{13}C^6$]-L-phenylalanine by using the precursor-product model (Paddon-Jones et al., 2006):

$$FSR = \frac{EP2 - EP1}{Em\ x\ t}\ x\ 60\ x\ 100$$

where EP1 and EP2 are the bound enrichments of [ring-$_{13}C^6$]-L-phenylalanine for the 2 muscle biopsies, Em is the mean enrichment of [ring-$_{13}C^6$]-L-phenylalanine in the muscle intracellular pool, and t is the time interval (min) between biopsies.

RNA extraction and gene expression analysis

Muscle biopsies collected at 1300 were used for gene expression analysis. RNA was extracted from ~15 mg of skeletal muscle samples using Tri-Reagent© Solution (Ambion Inc., Austin, TX, USA) according to the manufacturer's protocol. RNA was treated with DNase I Amplification Grade (Thermo Fisher Scientific, MA) and RNA concentration was assessed using the Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific). First-strand cDNA was generated from 1000 ng RNA using the High Capacity RT-kit (Applied Biosystems, Carlsbad, CA, USA). cDNA was then treated with RNase H (Thermo Fisher Scientific) according to the manufacturer protocol. Real-time PCR was carried out using an AriaMx real-time PCR system (Agilent Technologies, Santa Clara, CA) to measure mRNA levels. mRNA levels for *ARNTL (BMAL1), CRY1, PER1, IGF-1Ea, IGF-1Eb*, FBX032 (atrogin-1), TRIM63 (MuRF-1), FOXO1 and FOXO3 were measured using 1 × SYBR© Green PCR MasterMix (Applied Biosystems) and 5 ng of cDNA. All primers were used at a final concentration of 300 nM. Primer details are provided in Table 2. Single-strand DNA was quantified using the Quant it OliGreen ssDNA Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instruction and used for PCR normalization.

Table 2. Primer sequences

Gene	Accession number	Forward	Reverse

ARNTL (BMAL1)	NM_001030272.2	GGCAGCTCCACTGACTACCA	CCCGACGCCGCTTTTCAATC
CRY1	NM_004075.5	CCGTTCCCGGTCCTTTC C	CTAAAGACAAAACGGCCCGC
PER1	NM_002616.3	GAGGACACTCCTGCGACCAG	GCCATGGGGAGAACAGAACA
IGF-1Ea	XM_017019259.1	GACATGCCCAAGACCCAGAAGGA	CGGTGGCATGTCACTCTTCACTC
IGF-1Eb	XM_017019261.1	GCCCCCATCTACCAACAAGAACAC	CAGACTTGCTTCTGTCCCCTCCTTC
FBX032 (Atrogin-1)	NM_001242463.2	AGTTTCGTGAGCGACCTCAG	CTTTGAAGGCAGGCCGGA
TRIM63 (MuRF-1)	NM_032588.3	GGGAGGTGATGTCTTCTCTCTG	CTGACAATCGCAGGTCACCC
FOXO1	NM_002015.4	GCAGCCGCCACATTCAACAG	AGAACTTAACTTCGCGGGGC
FOXO3	NM_001455.4	CCGCACGTCTTCAGGTCCTC	CGACGAACATTTCCTCGGCT

Statistical analysis

Statistical analyses were conducted using SPSS 26.0 (IBM Corp, Armonk, NY). Diagnostic plots of residuals and fitted values were checked to ensure homogeneity of the variance. Hormonal levels were analysed using a two-way analysis of variance (ANOVA) with within-participant factors for time and condition (CON vs DEP) unless specified otherwise. The Sidak test was used to compare pairs of means when a main effect was identified. Paired t-tests were used to compare group means where there were single variables. Area under the curve (AUC) was computed using the trapezoidal method. The significance levels for the F-tests in the t-tests and ANOVA and the Sidak tests were set at $p<0.05$. All data are reported as mean ± SD.

Sample size

To the best of our knowledge, this is the first study to investigate the effect of sleep deprivation on muscle protein synthesis. Using data obtained for previous work conducted by

our group investigating the effects of a metabolic stimulus (immobilization or exercise) on changes in muscle protein synthesis rates in humans (Lamon et al., 2016; Paddon-Jones et al., 2006), power analyses conducted on the expected primary outcome (fractional synthesis rate) indicated that a sample size of 13 is sufficient to minimize the risk of type II error (β=0.2,α=0.05). Males and females were included as previous work demonstrated that muscle fractional synthesis rate, our primary outcome, is similar in both sexes (Knowles et al., 2019; West et al., 2012). When incidental findings prompted us to conduct exploratory analyses on the male and female cohort separately, individual effect sizes and β were computed and reported.

Results

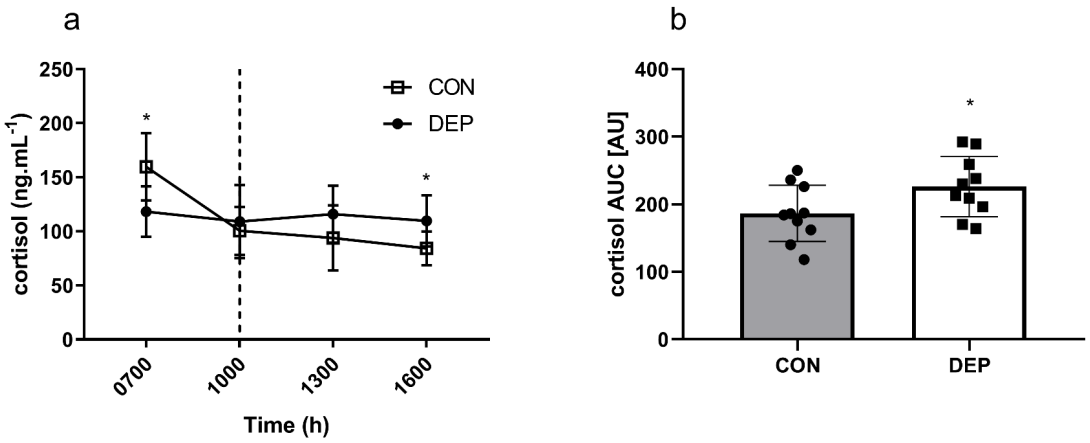
Sleep

In the week prior to the study, there was no difference in total sleep time (CON: 5.9 ± 0.5 h, DEP: 6.1 ± 1.4 h, $p = 0.718$) or sleep efficiency (CON: 78.5 ± 6.5 %, DEP: 79.4 ± 4.7 %, $p = 0.801$) between conditions. There was also no difference in total sleep time (CON: 6.8 ± 0.8 h, DEP: 7.4 ± 0.7 h, $p = 0.195$) and sleep efficiency (CON: 77.3 ± 6.3 %, DEP: 81.0 ± 8.6 %, $p = 0.424$) in the night directly preceding trial participation.

Plasma cortisol levels

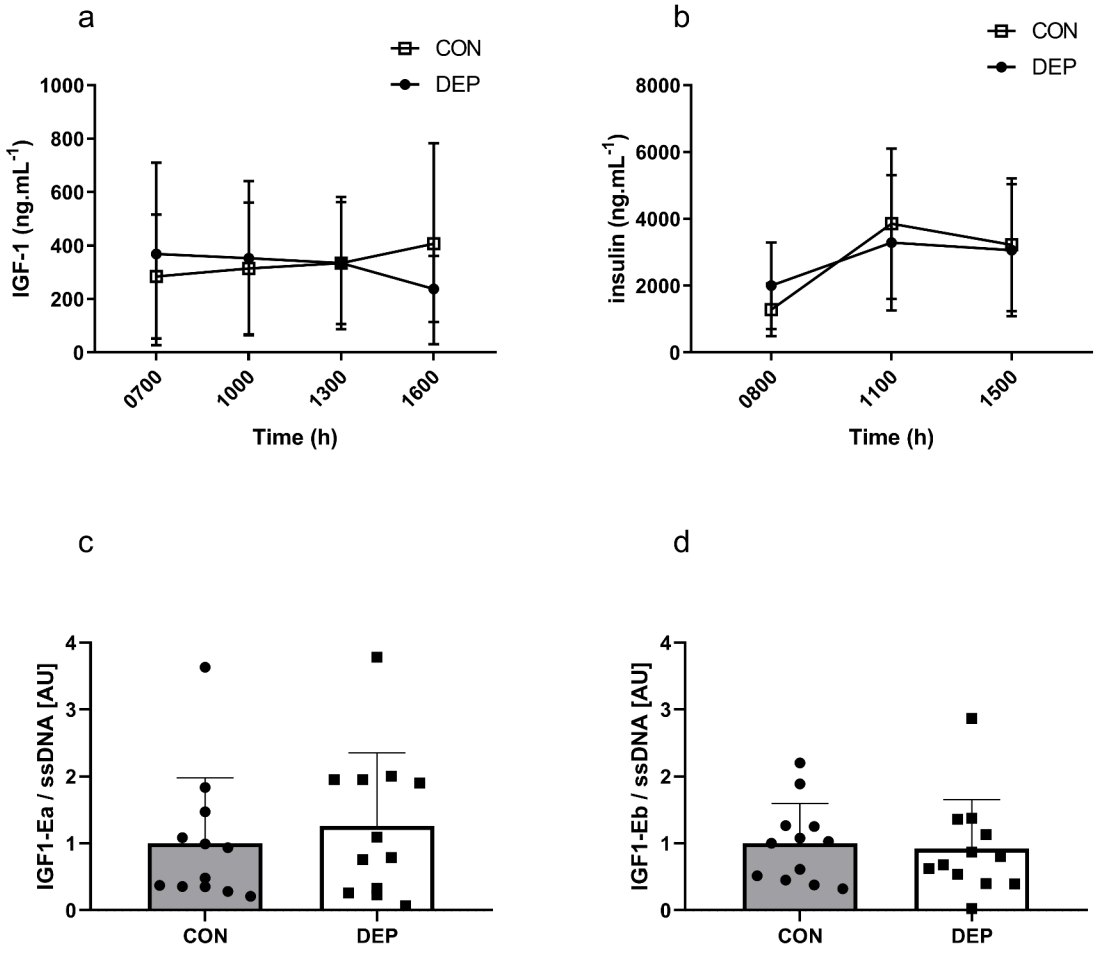
A significant interaction effect of sleep × time ($p<0.001$) was observed for plasma cortisol levels. Consistent with the typical increase in cortisol observed during the later stages of sleep (Vargas & Lopez-Duran, 2017), plasma cortisol levels were significantly higher ($p=0.014$) in the CON condition than in the DEP condition at 0700 (wake time for the control condition) (Figure 2a). At 1000, plasma cortisol was similar in both sleep conditions ($p=0.940$), but by 1600,

cortisol was significantly lower in the CON condition (*p=0.048)* (Figure 2a). The area under the curve was calculated without accounting for the cortisol awakening peak. Over the 6-h time period starting at 1000, cortisol levels were significantly higher during DEP than during the CON condition (*p=0.023)* (Figure 2b).



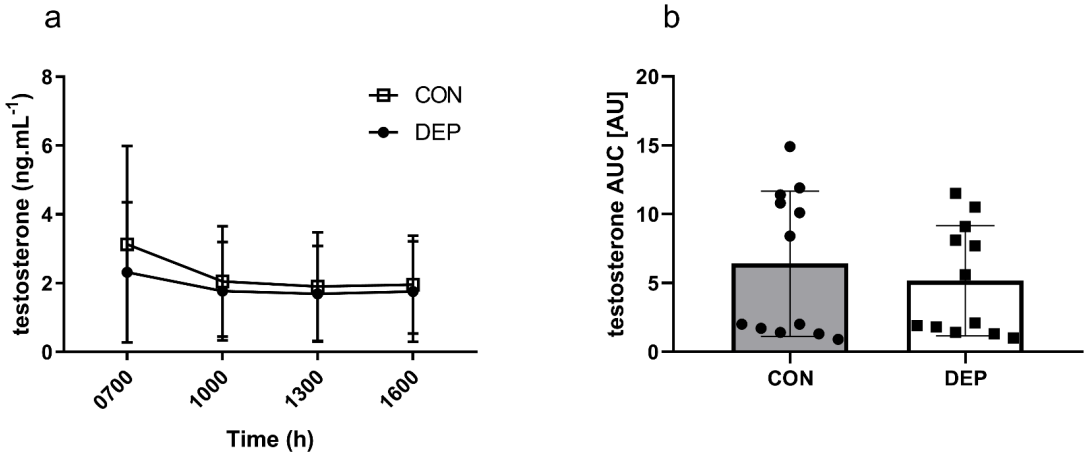
Insulin and IGF-1 levels

Plasma insulin concentrations varied across the day, but there was no effect of sleep or the combination of sleep and time (Supplementary figure 1a). Plasma IGF-1 concentrations did not vary with time, sleep or the combination of both factors (Supplementary figure 1b). In line with these results, one night of total sleep deprivation did not influence the muscle expression levels of IGF1 mRNA isoforms IGF1-Ea and IGF1-Eb when measured at 1300 (Supplementary figure 1c and 1d).

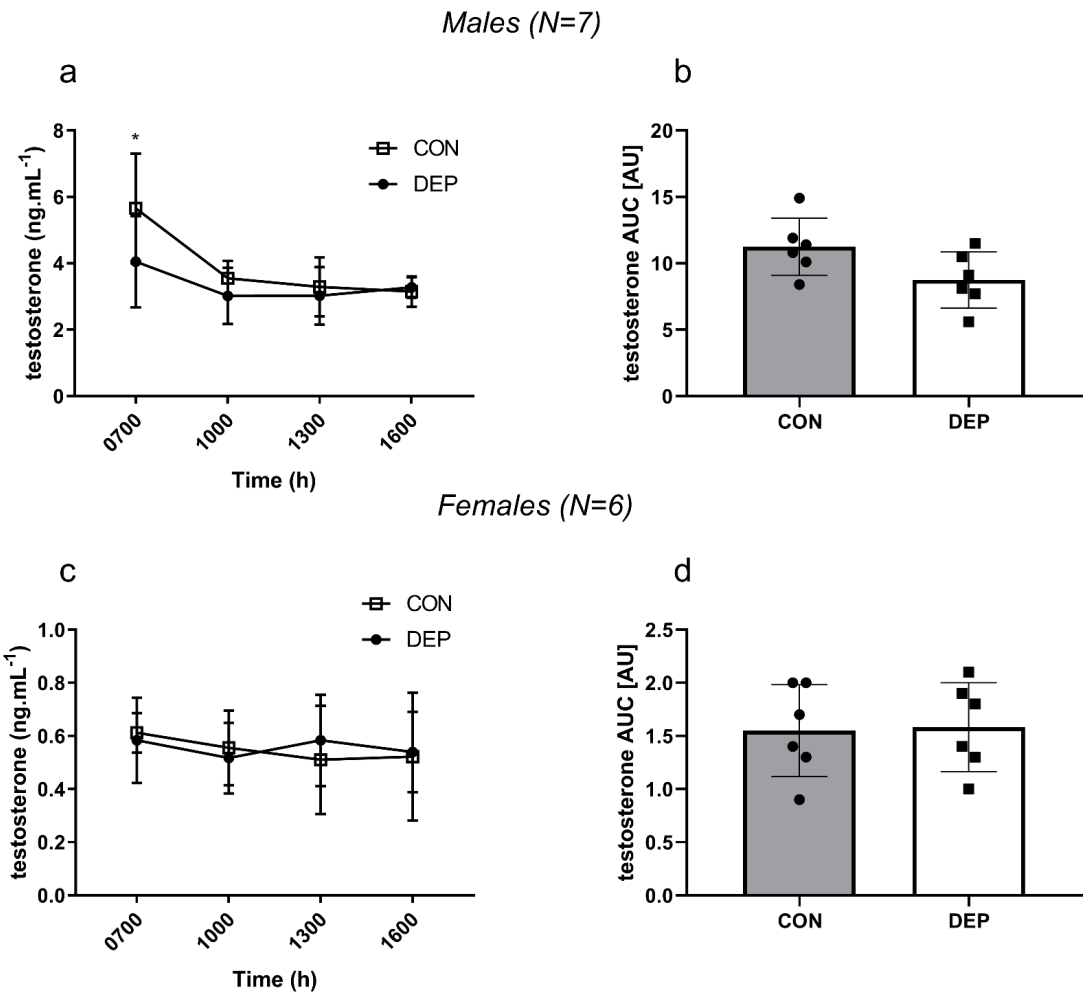


Plasma testosterone levels

There was a main effect of time (*p=0.002*) and a trend towards an effect of sleep × time (*p=0.063*) for plasma testosterone levels, where testosterone concentrations were lower in the DEP than in the CON condition (Figure 3a). The area under the curve displayed a trend for decreased testosterone levels in the DEP when compared to the CON condition (*p=0.090*) (Figure 3b).

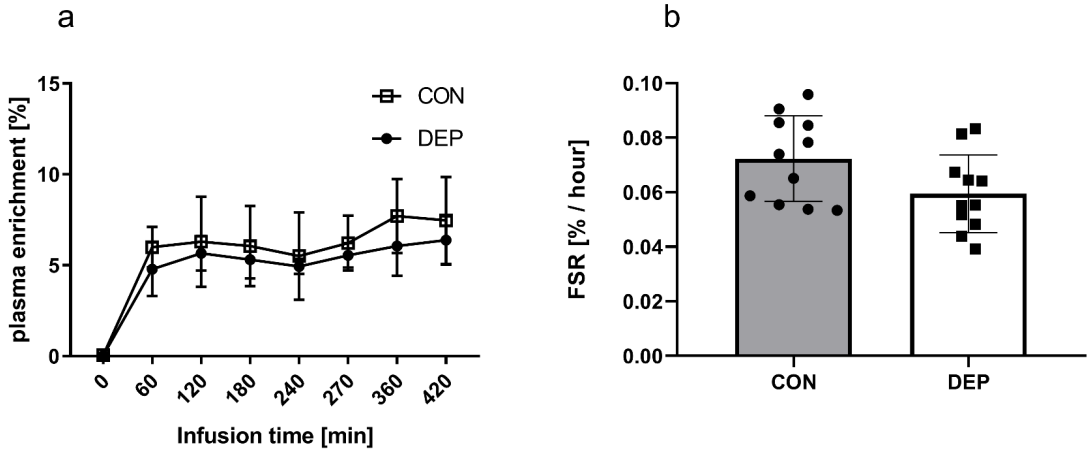


Since endogenous testosterone levels are 10-30-fold higher in males than in females (Vingren et al., 2010), we ran exploratory analyses (paired t-tests) for potential sex differences. We did not run ANOVAs due to the small size of each male and female cohort. However, in males, testosterone levels were lower in the DEP when compared to the CON condition at the 0700 time point (Figure 4a, *p=0.050*). No difference was observed in females at any time point (Figure 4c). These observations translated to the areas under the curve, where only the male population accounted for the trend (*p=0.080*) observed in the whole cohort (Figure 4b and 4d).

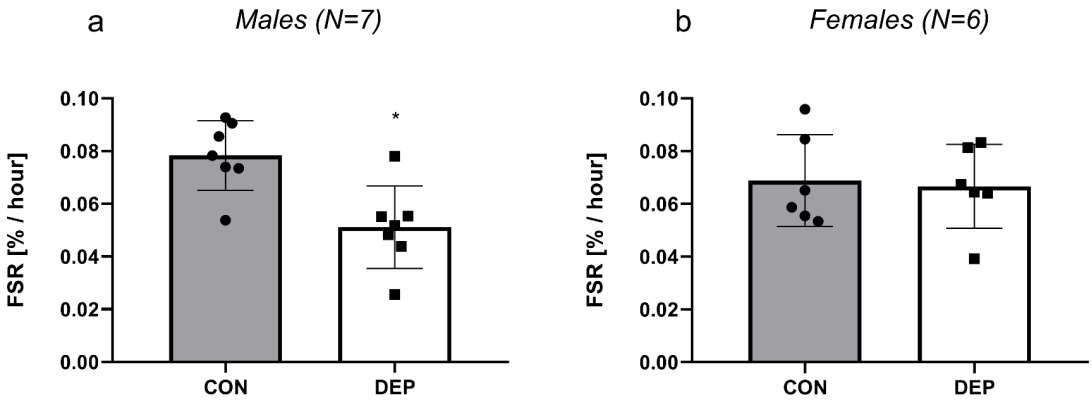


Muscle protein synthesis

Subjects remained in isotopic steady state during the duration of the isotope infusion, with no differences in plasma enrichment between the CON and DEP condition (*p=0.618*, Figure 5a). In CON, the fractional synthesis rate (FSR) was 0.075%·h^{-1}. There was a trend for FSR to be lower in the DEP than in the CON condition (*p=0.081*) (Figure 5b).

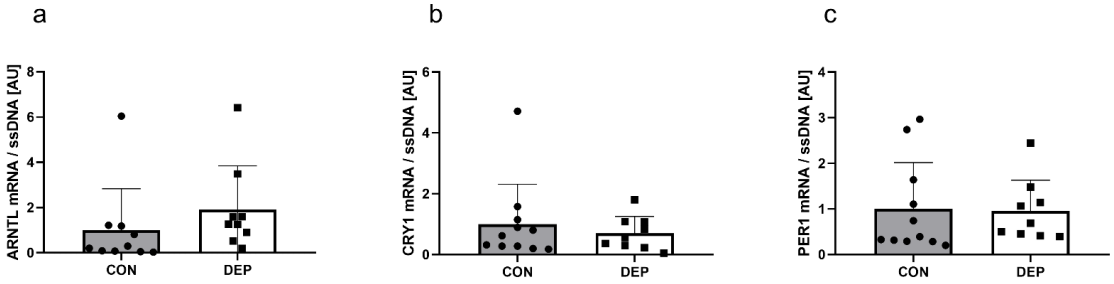


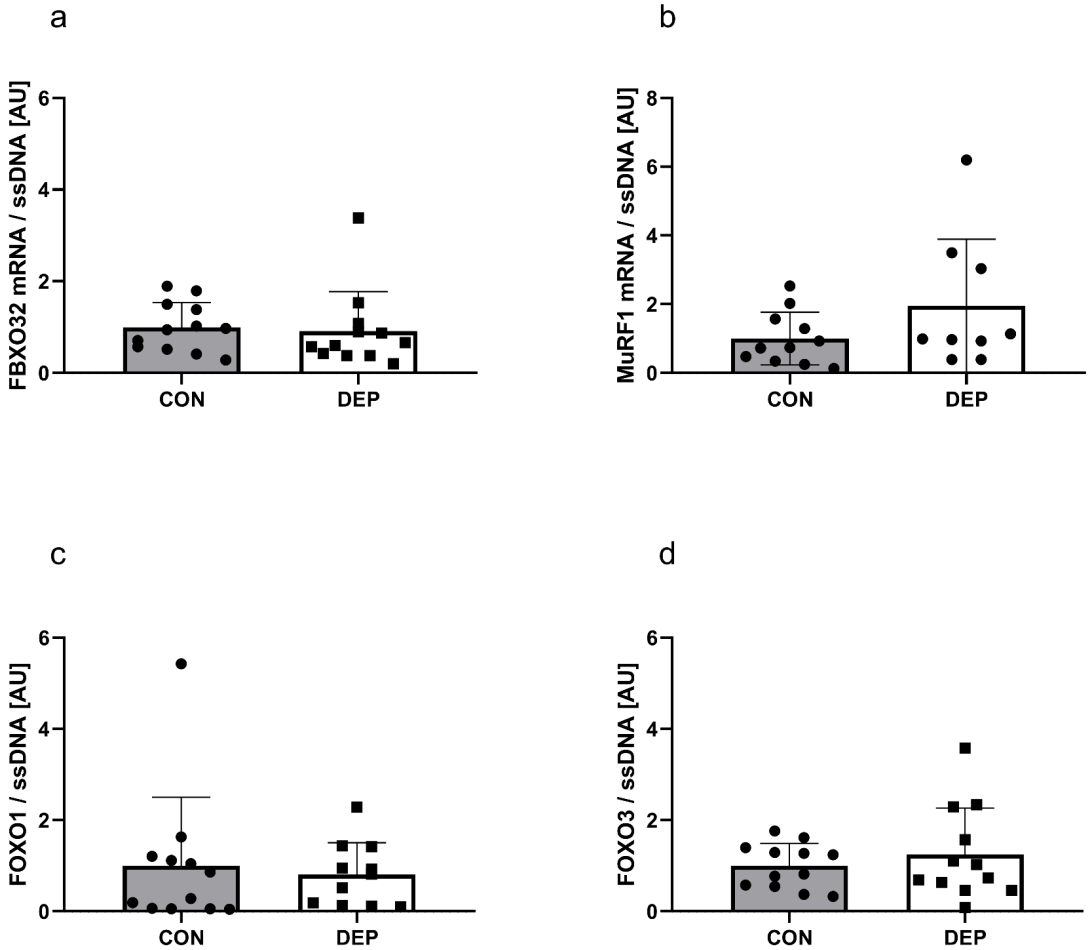
To determine whether sex-specific differences could contribute to variation in FSR, we ran further exploratory analyses (paired t-tests). To this end, the trend of a lower FSR during sleep deprivation in the whole cohort was exclusively driven by the male participants, who experienced a 33% decrease in FSR (*p=0.021*) (Figure 6a and 6b).



Gene expression

The muscle expression levels of the core clock genes ARNTL, CRY1 and PER1 did not change in response to one night of total sleep deprivation (Figure 7). The expression levels of FOXO1, FOXO3 and the muscle-specific atrogenes TRIM32 (MURF-1) and FBXO32 (Atrogin-1) did not display any difference between conditions (Supplementary figure 2).





Discussion

The aim of this study was to determine whether a 30-h sleep deprivation period would impair muscle protein synthesis in young, healthy males and females. Total sleep deprivation elicited a significantly higher cortisol AUC and a trend for a lower testosterone AUC when compared to the control condition. These results were paralleled by a trend for a lower muscle protein

fractional synthesis rate (FSR) in the sleep-deprived when compared to the control condition. Exploratory analyses of the separated male and female date indicated that the trend for sleep-deprivation-induced testosterone suppression became significant in the male cohort, and was driven by the male participants only. Further, males' FSR was significantly lower following sleep deprivation than following normal sleep. These exploratory sex-based differences should prompt a dedicated investigation to establish the causal links between inadequate sleep and impaired skeletal muscle health in male and female cohorts.

Consistent with previous studies (Vargas & Lopez-Duran, 2017), the cortisol response upon awakening was supressed following one night of acute sleep deprivation in both males and females. As shown previously (Cedernaes et al., 2018; Dattilo et al., 2020), this blunted cortisol response upon awakening was accompanied by a chronically higher cortisol release across the day in the sleep-deprived cohort. In rats, corticosterone reduced muscle protein synthesis while increasing myofibrillar protein breakdown (Kayali et al., 1987). In contrast, acute hypercortisolemia in humans did not affect muscle fractional synthesis rates but blunted the net muscle protein balance (Paddon-Jones et al., 2003). This suggests that cortisol makes a greater contribution to increased muscle protein breakdown than to decreased muscle protein synthesis. While others have reported a catabolic signature in skeletal muscle following a night of total sleep deprivation (Cedernaes et al., 2015), we did not observe any difference in the muscle expression levels of the proteolytic genes FOXO1 and FOXO3, or in the expression levels of the muscle specific atrogenes, Atrogin-1 and MURF1. Our results suggest that if sleep deprivation induces protein breakdown, it is not likely to be facilitated by activation of the muscle-specific E3 ubiquitin ligases, but may instead originate from other proteolytic pathways including lysosome dependant mechanisms.

Testosterone, the major androgenic hormone, activates muscle protein synthesis via the non-DNA binding-dependent activation of the Akt/mTOR pathway; the latter being the central regulator of protein synthesis in the muscle (Russell, 2010). Limited observational evidence describes how restricted sleep may alter testosterone daytime secretion patterns in males. Plasma testosterone levels display a circadian regulation, where concentrations rise during the night and gradually decrease during the day (Touitou et al., 1990). A minimum of three hours of normal sleep including paradoxical sleep opportunities (Luboshitzky, Zabari, Shen-Orr, Herer, & Lavie, 2001) is required to observe such increase. In healthy young men, one night of acute sleep deprivation did not decrease testosterone AUC when calculated over a 24-hour period; however, a pattern similar to ours could be observed across the day (Dattilo et al., 2020). Sustained sleep restriction (five hours of sleep per night during one week) lowered testosterone levels during waking hours (Leproult & Van Cauter, 2011). Five nights of sleep restriction (four hours of sleep per night) also led to a trend towards reduced total daytime testosterone in the same population (Reynolds et al., 2012). Our study supports these findings and suggests that, in males, one night of sleep deprivation elicits a reduction in daytime testosterone concentrations.

Testosterone is a potent regulator of muscle protein synthesis in males, both on the short (5 days) (Sheffield-Moore et al., 1999) and longer term (4 weeks) (Urban et al., 1995). However, acute exposure to testosterone is not sufficient to influence muscle protein synthesis or degradation rates over a 5-hour period (Church, Pasiakos, Wolfe, & Ferrando, 2019). Whether transiently low testosterone levels negatively impact muscle protein synthesis rates is unknown and constitutes a challenge to validate experimentally. However, our results suggest that low testosterone secretion during the sleep deprivation period (Luboshitzky et al., 2001) is followed by another low testosterone secretion period during the daytime. This

accumulation may therefore provide a mechanism to explain that, in males, muscle protein synthesis decreases following total sleep deprivation when compared to control sleep conditions.

Testosterone is also produced in females, who display endogenous testosterone levels that are 10-30-fold lower than in males. The role of testosterone on female muscle protein metabolism and muscle mass has however received limited attention. Sex-specific differences have primarily been investigated in animal models, where female mice lacking the androgen receptor, through which testosterone exerts its effects, do not display any difference in muscle mass when compared to wild type females (MacLean et al., 2008). In humans, a recent study corroborated these results by demonstrating that increasing testosterone concentrations around the upper limits of the female physiological range increased muscle lean mass but not strength or power (Hirschberg et al., 2019), suggesting that the effects of physiological testosterone concentrations on the female anabolic response are limited.

Taken together, our results suggest that acute sleep deprivation promotes a less anabolic and more catabolic environment the following day, where cortisol levels are elevated in both sexes, while testosterone levels are reduced in males. This hormonal dysregulation presents a potential mechanism for the observed decrease in muscle protein synthesis in males but not females. In line with this hypothesis, some authors recently suggested that poor sleep-induced hypercortisolemia might play a role in the development of sarcopenia (Piovezan et al., 2015), with potential sex-specific effects (Buchmann et al., 2016); however, further research is required to establish cause and effect relationships.

In contrast to some of the results reported by Cedernaes (Cedernaes et al., 2015), we did not observe a decrease in the muscle expression levels of the core clock genes following a night

of total sleep deprivation. Since the timing of muscle sample collection was different (0730 *versus* 1300 in our study), it can be hypothesised that the muscle circadian rhythm might have been able to realign over this time period. More likely, the subtle differences in core clock gene expression reported by the same group following qPCR (Cedernaes et al., 2015) and transcriptomic analysis (Cedernaes et al., 2018) might not be reflective of a physiologically significant change, and warrant the use of more functional measures.

Ethical limitations of this study included the decision not to fast participants during the night of total sleep deprivation. Instead, to improve compliance, comfort and retention, participants were allowed to consume low-protein snacks and water *ad libitum.* This strategy was effective in achieving similar insulin levels across the two conditions at all time points. Another limitation was the inability to obtain overnight blood samples due to the use of a home-based environment for the CON condition, which is an important strength of this study. Finally, in designing this clinical trial, we did not focus on sex-specific differences, nor did we power our study in order to detect such differences. Indeed, muscle fractional synthesis rates, our primary outcome, are similar in males and females at rest and in response to anabolic stimulation (West et al., 2012). Our incidental findings led us to perform further exploratory analyses on the male and female cohort separately. With these limitations in mind, any sex-specific effect should be interpreted with the appropriate caution.

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