Influence of biological sex on exercise-induced nuclear factor erythroid 2-related factor 2 and downstream targets

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Abstract

Objective: To evaluate whether exercise induces different nuclear factor erythroid 2-related factor 2 (NRF2) responses in males and females.

Methods: 22 males and females (n=11 per group; mean [SD]: age: 25 [6] years, height: 1.71 [0.10] m, weight: 69.6 [12.3] kg) performed 100 drop jumps and 50 squat jumps. NRF2/antioxidant response element (ARE) binding in peripheral blood mononuclear cells, glutathione peroxidase (GPX) activity, and immune markers influenced by NRF2 (interleukin-6 [IL-6], tumour necrosis factor-α [TNF-α], matrix metalloproteinase-9 [MMP-9], vascular cell adhesion molecule-1 [VCAM-1], total leukocytes, neutrophils, monocytes) were measured pre-, post-, and 1 h post-exercise.

Results: NRF2/ARE binding did not significantly alter following exercise (p=0.59) and no sex differences were evident (p=0.17). Similarly, GPX activity did not change post-exercise (p=0.74) and did not differ between sexes (p=0.61). IL-6 and TNF-α did not increase post-exercise (p>0.05 for both) but were lower in females (p<0.01 for both). MMP-9 increased post-exercise (p=0.02), but no group differences were found (p=0.27). VCAM-1 was unchanged after exercise (p=0.38) and did not differ between groups (p=0.11). Total leukocytes, neutrophil, and monocytes all increased post-exercise (p<0.01 for all); neutrophils were lower (p<0.01), and monocytes higher (p=0.03) in females vs. males.

Conclusion: There were no sex differences in NRF2 activity at rest and in response to an exercise bout, however, several immune markers displayed sex-specific differences, independent to NRF2.

Significance: Sex differences in NRF2 signalling were not evident, suggesting males and females may incur similar redox-specific adaptations post-exercise.

Exercise challenges whole-body homeostasis, stimulating a myriad of biological responses that, over time, confer adaptations that protect against future exercise stress (Hawley et al., 2014). Amongst these widespread responses is an increasingly pro-oxidant redox balance during exercise, consequent to increased reactive oxygen species (ROS) production in the contracting skeletal muscle through enzymes such as NADPH oxidases (NOX) (Henríquez-Olguin et al., 2019). Altered redox balance following exercise is increasingly recognized as a mediator of exercise adaptations, since small elevations in ROS can act as signalling molecules that augment various transcriptional pathways (He et al., 2016).

One transcriptional pathway that can be activated by exercise-induced ROS is the nuclear factor erythroid 2-related factor 2 (NRF2) pathway (Done & Traustadóttir, 2016). During unstressed conditions, NRF2 is sequestered within the cytosol by kelch-like ECH-associated protein 1 (KEAP1) through continual ubiquitination (M. Kobayashi & Yamamoto, 2006). Following a rise in ROS during exercise, the cysteine residues of KEAP1 are prone to oxidation, leading to a conformational change to its structure that frees NRF2 from ubiquitination (Dinkova-Kostova et al., 2017). NRF2 can then translocate to the nucleus where it binds with antioxidant response element (ARE) DNA sequences, subsequently promoting the transcription of cytoprotective genes involved in antioxidative, anti-inflammatory and detoxifying responses (Copple, 2012; Motohashi & Yamamoto, 2004).

Different exercise modalities, including moderate-intensity exercise (Ballmann et al., 2014; Done et al., 2016, 2017; Ostrom & Traustadóttir, 2020; Scott et al., 2015) and eccentric-bias exercise (Thorley, Thomas, Bailey, et al., 2023), have been shown to increase NRF2 protein content and NRF2/ARE binding activity in skeletal muscle and peripheral blood mononuclear cells (PBMCs).

NRF2 activation also competitively inhibits the transcription factor nuclear factor-κB (NF-κB) [12, 13] and subsequently NF-κB driven production of cytokines with pro-inflammatory properties, such as interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) (E. H. Kobayashi et al., 2016; Thimmulappa et al., 2006). Furthermore, NRF2 activation has been shown to attenuate vascular cell adhesion molecule-1 (VCAM-1), which increases leukocyte migration to sites of inflammation (Banning & Brigelius-Flohé, 2005; Kim et al., 2012), as well as matrix metalloproteinase-9 (MMP-9) which modulates leukocyte migration, chemotaxis, and inflammatory cytokine activation (Lee et al., 2007; Saw et al., 2014; H. Wang et al., 2010).
Thus, in addition to its antioxidant effects, NRF2 may help modulate immune responses to exercise.

There is some evidence in rodents to suggest that redox responses differ between male and female rats, with male rats exhibiting greater susceptibility to oxidative damage, likely explained by male rats possessing a greater number and diversity of ROS-producing NOX subunits than female rats (Borrás et al., 2003; Miller et al., 2007; Zhang et al., 2012). In humans, sex differences in redox responses remain equivocal. Indeed, while some studies suggest that biomarkers of oxidative damage are lower in pre-menopausal females than in males (Bloomer & Fisher-Wellman, 2008; Hermsdorff et al., 2014; Ide et al., 2002; Kerksick et al., 2008), other studies report no differences or lower levels of oxidative damage in males (Brunelli et al., 2014; Campesi et al., 2016). Nevertheless, there is rationale to suggest that several sex-specific dimorphisms, particularly dimorphisms to sex hormones, could lead to diverse redox responses in humans. Oestrogens, the primary class of sex hormone in females, are reported to possess antioxidative properties, notably through their ability to increase antioxidative proteins, such as glutathione peroxidases (GPX), reduced glutathione, and manganese-dependent superoxide dismutases (Borrás et al., 2010; Persky et al., 2000; Stepniak & Karbownik-Lewinska, 2016). This antioxidative effect is particularly noticeable during menopause in women, a phase marked by a decrease in oestrogen levels; indeed, postmenopausal women have been reported to exhibit higher levels of oxidative damage biomarkers compared to premenopausal women (Signorelli et al., 2006). Conversely, in males, androgens are the major sex hormones and are thought to exert pro-oxidative properties under certain conditions (Pinthus et al., 2007; Tenkorang et al., 2018).

These sex differences in hormones could consequently have downstream effects on the NRF2 axis, revealing differences in NRF2 activation between males and females at rest and after exercise. Indeed, oestrogen, specifically 17β-estradiol, is reported to activate NRF2 (Borrás et al., 2005; Chen et al., 2013; Wu et al., 2014), purportedly by regulating glycogen synthase kinase-3 beta activity (Wu et al., 2014), as well as upregulating NRF2 expression (Fão et al., 2019; Pomatto et al., 2018; Qu et al., 2020). Data on the effect of androgens on NRF2 remains disputed; work by Schultz et al. (2014) found that treating two types of cancer cell lines (LNCaP, C4-2B) with the androgen dihydrotestosterone led to increased NRF2 content in LNCaP cells, but decreased content in C4-2B cells. Other work supports the notion that androgens suppress NRF2 activity, as blunting androgen receptor binding via bicalutamide treatment led to increased NRF2 content in cancer cells (Tian et al., 2022).
Ultimately, the influence of biological sex on NRF2 activity in humans has been scarcely investigated; Galvan-Alvarez et al. (2023) recently reported no sex differences between basal levels of NRF2, NRF2/KEAP1 ratio, and NRF2 gene targets superoxide dismutase, catalase, and glutathione reductase in skeletal muscle. In rodents, however, there appears to be a clear distinction in sex-specific NRF2 responses, with female mice exhibiting lower basal NRF2 expression, augmented NRF2 responses to stress, and greater expression of downstream NRF2 gene targets (Rohrer et al., 2014; Rooney et al., 2018). Sex differences in exercise-induced NRF2 activation could impact the extent of pleiotropic adaptations to exercise and physical performance levels. Further research is, therefore, needed to elucidate potential sex differences in NRF2 signalling in humans.

The aim of this study was to examine whether biological sex influences NRF2 signalling, quantified by measuring NRF2/ARE binding in PBMCs, at rest and in response to strenuous exercise. As secondary outcomes, the circulating concentrations of IL-6, TNF-α, VCAM-1 and MMP-9, all of which may be modified by NRF2 [36], and GPX activity, were measured to discern whether possible sex differences in NRF2 signalling led to distinct changes in NRF2 regulated targets. It was hypothesised that exercise would increase NRF2 activity and some of its downstream targets to a greater extent in females than in males.

**Methods**

**Participants**

Twenty-two healthy male and female participants (n=11 per group; mean [SD]: age: 25 [6] years, height: 1.71 [0.10] cm, weight: 69.6 [12.3] kg) volunteered to participate in this study. Our sample size was based on a simulation-based power analysis for changes in NRF2/ARE DNA binding activity and calculated using the ANOVA_power app (Lakens & Caldwell, 2021). This calculation has been described previously (Thorley, Thomas, Thon, et al., 2023). Briefly, 11 participants per group, and a difference in means and SD of 0.01 and 0.0075 optical density units, gave us ≥80% power to detect a statistically significant time and interaction effect (effect size of ≥ 0.22 (partial eta squared).

Six females were eumenorrheic, and five females were monophasic oral contraceptive pill (mOCP) users. Females using mOCP reported taking this contraceptive for at least 2 years as prescribed (28 d cycle; 7 d break after every 21 d pill consumption period), whereas eumenorrheic women reported having regular cycles without using any form of hormonal contraceptives for >5 months. mOCP users reported using different brands of contraception;
these were Desogestiel®, Rigevidon® (x2), Dianette® and Cerelle®. All participants were
classified as Tier 1, recreationally active, according to a participant classification framework
(McKay et al., 2021). Prior to experimental testing, participants completed a health screening
survey to determine whether they were eligible to take part; those with a history of or current
cardiovascular or metabolic disease, musculoskeletal injury, food allergy, or were taking
medication, were excluded from the study. Throughout experimental procedures, participants
were instructed to refrain from using putative recovery aids or consuming any dietary
supplements. Exercise outside of experimental procedures was restricted in the 48 h prior to
the second visit until completion of the study.

**Experimental Design**

A between-subjects design was utilized for this study. Prior to data collection, this study was
pre-registered on the Open Science Framework (osf.io/5fpvn). Ethical approval was granted
by Loughborough University Research Committee, Human Participants Sub-Committee and
was in accordance with the Declaration of Helsinki. This study was a secondary aim of larger
investigation examining the influence of (poly)phenols on NRF2 activity (unpublished); some
participants in that project who were randomly allocated to the placebo control group also
took part in this study. To ensure participants in this present study underwent the same
experimental procedures, they all undertook a 4 d supplementation period with a placebo
supplement (inulin; 1000 mg/d for 4 d) and a low antioxidant breakfast, details of which are
provided in the Supplementary Material (Table S1). Due to the dietary interventions,
participants were deceived on the study aim by being told they were participating in a study
investigating the effects of (poly)phenols on exercise recovery.

Participants attended the laboratory twice. During the first visit, participants provided informed
consent following a thorough briefing of the study procedures, and then were familiarised with
all the procedures described below; anthropometric measurements were collected, and
participants were given 3 opportunities to achieve their maximal counter movement jump
(CMJ) and squat jump (SJ) height, which was used to monitor intensity during the exercise
intervention. Participants were then provided with their control supplements and meals, to
which they consumed for the next 4 d. During the second laboratory visit, participants arrived
at 09:00 am in a hydrated state following an overnight fast lasting 8-10 hrs, and a resting
blood sample was collected. They then consumed a final dose of their control supplement
and the final portion of their control meal. A 30 min rest period then commenced, with
participants remaining in a seated position. Participants then began the exercise intervention. Immediately post-, and 1 h post-exercise, further blood samples were collected. For the female eumenorrheic participants, the second visit was scheduled to take place during the early follicular phase of their menstrual cycle (first 10 d of bleed) to ensure oestrogen concentrations remained stable due to this hormone’s potential interference with oxidative damage markers (Davies et al., 2009). For the female mOCP participants, the second visit was scheduled to take place during an equivalent time of phase, which was within the first 10 d of pill withdrawal. A menstrual cycle questionnaire was completed by female participants during familiarisation to calculate cycle phase; this questionnaire has been previously used to calculate phase cycle in the absence of hormonal profiling (Brown et al., 2018).

**Exercise protocol**

The exercise protocol consisted of performing 100 drop jumps from a 0.6 m steel box, followed by 50 squat jumps. This protocol is a more metabolically challenging adaptation to the eccentric-heavy exercise previously shown to augment NRF2 activity in PBMCs (Thorley, Thomas, Bailey, et al., 2023). The exercise intensity was increased in the present study to impose greater metabolic and mechanical stress, with the expectation that this would trigger a more significant, measurable NRF2 response. Each of the 100 drop jumps was interspaced by a 5 s rest, and every 20 jumps with a 1 min rest. For drop jumps, participants were asked to drop from the 0.6 m box with no arm swing, then land and lower into a squat position (to ~90˚ knee angle) on a contact jump mat (JumpMat™, FSL Scoreboards, Cookstown, Northern Ireland), followed immediately by a vertical CMJ at maximal effort. After the drop jumps, a 1 min rest period commenced, followed by the performance of 50 consecutive SJ. For these, participants were told to stand on the jump mat, squat to a 90˚ knee angle, then immediately perform a maximal effort vertical jump. Each SJ was interspaced by a 5 s rest and were performed consecutively until completion. For both protocols, participants were instructed to reach a jump height that was within 20% of their previously recorded maximal effort CMJ and SJ recorded to standardize exercise intensity.

**Dietary restrictions**

In the 24 hrs prior to the second visit, participants recorded their dietary intake using a weighed food diary. Total energy, carbohydrate, fat, protein, Omega-3 fatty acids, vitamin C, D, and E intakes were assessed using online dietary analysis software (Nutritics Education...
Throughout the supplementation period, participants were instructed to maintain their usual diet but to refrain from increasing the consumption of foods rich in (poly)phenols. This precaution was taken because such foods could potentially activate NRF2 independently of exercise, as suggested previously (Clifford et al., 2021). A list of these foods was provided to participants before the supplementation period commenced.

### Blood sampling and processing

At pre-, post-, and 1 h post-exercise, venous blood was collected from the antecubital fossa using a 21-gauge butterfly needle by a trained phlebotomist and drawn into Vacuette containers treated with Ethylenediaminetetraacetic acid (EDTA) and lithium heparin (LH) (Vacuette, Greiner Bio-One, Austria). EDTA blood was first processed to measure the circulating concentration of immune cells (total leukocytes, neutrophils, lymphocytes, monocytes) using a Yumizen H500 cell counter (Horiba Medical, Montpellier, France) within 5 min of collection. The same sample was then centrifuged at 1500 x g for 10 min at 4°C, with the resulting plasma interphase pipetted into cryovials and stored at -80°C for later analysis. 10 mL of LH treated blood was used to isolate PBMCs as previously described (Thorley, Thomas, Bailey, et al., 2023; Thorley, Thomas, Thon, et al., 2023). Nuclear proteins were later fractionated from PBMCs using a commercial extraction kit (Nuclear extraction kit, Cat No. 40010, Active Motif, Waterloo, Belgium) according to manufacturer’s instructions. Protein content of nuclear fractions was measured using a commercial bovine serum albumin assay (Prostain™ Protein Quantification Kit, Cat No. 15001, Active Motif, Waterloo, Belgium).

### NRF2/ARE binding

NRF2/ARE binding was measured using a commercially available human NRF2 activity assay (Cat. No. TFEH-NRF2-1, RayBiotech, Georgia, United States) according to manufacturer’s instructions. To initiate binding, nuclear proteins were incubated overnight in wells containing immobilized oligonucleotides possessing the ARE consensus binding site (5′-GTCACAGTACTCAGCAGAATCTG-3′). Following wash procedures, anti-NRF2 antibodies were incubated in wells, followed by incubation with HRP conjugated secondary antibodies. Absorbance was read at 450 nm on a Varioskan™ LUX multimode microplate reader (ThermoFisher Scientific, Loughborough, UK). As detailed in our pre-registration, we
initially aimed to examine NF-κB DNA binding; however, this analysis was not completed due to technical faults with the assay and limited remaining sample for any further analysis.

**NRF2 target protein activity**

GPX activity was measured in plasma using a commercially available assay (Cat No. 703102, Cayman Chemical, Michigan, USA) according to manufacturer’s instructions. One unit of GPX activity is defined as the number of enzymes causing the formation of 1 nmol of NAPDH to NADP+ per min (nmol/min/ml). Concentrations of TNF-α, IL-6, MMP-9, and VCAM-1 were measured in plasma using SimplePlex™ Ella (ProteinSimple, Bio-Techne, Oxford, UK). GPX, TNF-α, and IL-6 values were read in duplicate, whilst MMP-9 and VCAM-1 were read in triplicate. Intra-assay CVs for these variables were 3.1%, 3.9%, 3.1%, 4.5% and 4.1% for GPX, TNF-α, IL-6, MMP-9 and VCAM-1, respectively.

**Statistical Analysis**

Data was analysed using jamovi v2.3.26. Normality was assessed by visually inspecting histograms and QQ plots of the residuals. Independent samples t-tests were used to examine group differences in physical characteristics, mean and maximal CMJ and SJ height, energy intake, and total area under the curve (tAUC) for blood variables. A linear mixed model (gamlj v.2.6.6) (2 [group: male vs. female] x 3 [time: pre-, post-, 1 h post-exercise]) was used to analyse differential leukocyte counts, antioxidant and cytokine activity, and NRF2/ARE binding). Linear mixed models were preferred to ANOVAs because some samples were missing at random due to missed blood samples or analytical issues. Where significant main effects were found, holm-Bonferroni post-hoc corrections were used to locate the differences. Partial eta squared ($\eta^2_p$) effect sizes were calculated for omnibus tests (small: 0.01, medium: 0.05, large 0.14 (Cohen, 1988). tAUC was used to examine for any sex differences across all time points; analysis was completed with an automated spreadsheet (Narang et al., 2020). If the first and/or last datapoint was missing, then these participants were excluded from tAUC analysis. Statistical significance was set at $p<0.05$ prior to analysis. GraphPad Prism (v9.4.1, Boston, USA) was used to create figures.

**Results**

The physical characteristics and total energy, macronutrient, and micronutrient intakes of the male and female participants are presented in Table 1. Food diaries showed that all
participants adhered to the imposed dietary restrictions. When monitoring exercise intensity, it was found that 7/11 males and 9/11 females achieved a mean CMJ height that was within 20% of their max recorded CMJ height. For SJs, 5/11 males and 6/11 females achieved a mean height that was within 20% of their recorded maximal SJ height.

Table 1. Group differences in physical characteristics and 24 h dietary intake.

NRF2 activity

More than 75% of time series data was missing for 2 participants (*n*=1 from each group), thus, they were excluded from analysis. Exercise did not increase NRF2/ARE binding (time *p*=0.59; η*²*=0.03). Furthermore, NRF2/ARE binding was not influenced by biological sex (group *p*=0.17; η*²*=0.05) and no time x group effect (*p*=0.56; η*²*=0.03) was found (Figure 1[a]). No group differences in NRF2/ARE binding tAUC responses were found (*p*=0.23) (Figure 1[b]). 9/66 data points were missing for this analysis.

Inflammatory markers regulated by NRF2

TNF-α (group *p*<0.01; η*²*=0.60) and IL-6 (group *p*<0.01; η*²*=0.32) concentrations were lower in females than males (Figure 2[a][c]). Exercise, however, did not alter TNF-α (time *p*=0.57; η*²*=0.03) or IL-6 (time *p*=0.47; η*²*=0.03) concentrations, and no time x group effect was observed for either cytokine ([TNF-α] *p*=0.72; η*²*=0.02 [IL-6] *p*=0.66; η*²*=0.02). Differences were found in TNF-α (*p*=0.02) and IL-6 (*p*=0.03) tAUC responses (Figure 2[b][d]) between males and females; 5/132 data points were missing for this analysis.

Figure 1. [a] Nuclear factor erythroid 2-related factor 2 (NRF2)/antioxidant response element (ARE) binding at pre-, post-, and 1 h post-exercise in males and females (males *n*=10; females *n*=10). Box and whisker plots are presented with symbols (triangles=males; circles=females) representing individual responses. Whiskers correspond to lowest and highest values. Horizontal line within boxes represents median values. [b] NRF2/ARE binding total area under the curve (tAUC) responses between sexes (males *n*=9; females *n*=8).

Figure 2. Tumour necrosis factor-alpha (TNF-α) [a] and interleukin-6 (IL-6) [b] concentrations at pre-, post-, and 1 h post-exercise in males and females (*n*=11 for both sexes). Box and whisker plots are presented with symbols (triangles=males; circles=females) representing individual responses. Whiskers correspond to lowest and highest values. Horizontal line within boxes represents median values. TNF-α [b] and IL-6 [d] total area under the curve (tAUC) responses between sexes (males *n*=10; females *n*=11).
There were no biological sex differences in MMP-9 (group $p=0.27$; $\eta^2_p=0.03$) and VCAM-1 (group $p=0.11$); however, MMP-9 did increase in response to exercise (time $p=0.02$; $\eta^2_p=0.16$), peaking immediately post-exercise ($p=0.02$) but VCAM-1 did not change (time $p=0.38$) (Figure 3[a][c]). No time x group effect was found for MMP-9 ($p=0.56$; $\eta^2_p=0.03$) or VCAM-1 ($p=0.75$). No differences for MMP-9 ($p=0.40$) or VCAM-1 ($p=0.25$) tAUC responses were observed (Figure 3[b][d]) between males and females. 4/132 data points were missing for this analysis. VCAM-1 $\eta^2_p$ could not be calculated due to denominator degrees of freedom <1.

Figure 3. Matrix metalloproteinase 9 (MMP-9) [a] and vascular cell adhesion protein 1 (VCAM-1) [b] concentrations at pre-, post-, and 1 h post-exercise in males and females (n=11 for both sexes). Box and whisker plots are presented with symbols (triangles=males; circles=females) representing individual responses. Whiskers correspond to lowest and highest values. Horizontal line within boxes represents median values *=different to pre-exercise ($p<0.05$). MMP-9 [b] and VCAM-1 [d] total area under the curve (tAUC) responses between sexes (males n=10; females n=11 for both).

GPX activity

GPX activity did not differ between biological sexes (group $p=0.74$; $\eta^2_p<0.01$) and did not change in response to exercise (time $p=0.61$; $\eta^2_p=0.03$) (Figure 4[a]). No time x group effect ($p=0.56$; $\eta^2_p=0.03$), or differences between biological sex in GPX tAUC ($p=0.89$) responses were found (Figure 4[b]). 2/66 data points were missing for this analysis.

Figure 4. Glutathione peroxidase (GPX) activity [a] at pre-, post-, and 1 h post-exercise in males and females (n=11 for both groups). Box and whisker plots are presented with symbols (triangles=males; circles=females) representing individual responses. Whiskers correspond to lowest and highest values. Horizontal line within boxes represents median values. [b] GPX total area under the curve (tAUC) responses between sexes (males n=9; females n=11).

Total and differential leukocyte counts

Total leukocytes (time $p<0.01$; $\eta^2_p=0.52$), neutrophils (time $p<0.01$; $\eta^2_p=0.45$), and monocytes (time $p<0.01$; $\eta^2_p=0.45$) all increased following exercise; specifically, total leukocytes and monocytes were elevated immediately post-exercise ($p<0.001$ for both) whilst neutrophils were elevated at all post-exercise timepoints ($p<0.001$ for all) (Figure 5[a][c][e]). No differences were observed in total leukocyte concentrations (group $p=0.09$; $\eta^2_p=0.06$) between biological sexes, however, neutrophils were lower in males (group $p<0.01$; $\eta^2_p=0.22$) and monocytes were lower in females (group $p=0.03$; $\eta^2_p=0.09$). No group x time effect was found for total leukocytes ($p=0.15$; $\eta^2_p=0.08$) or monocytes ($p=0.85$; $\eta^2_p<0.01$); a
A group x time effect was found for neutrophils ($p=0.01$; $\eta^2=0.19$), with concentrations lower in males 1 h post-exercise vs. females ($p=0.03$). There were no differences in total leukocyte ($p=0.82$), neutrophil ($p=0.31$), and monocyte ($p=0.25$) tAUC responses between males and females (Figure 5[b][d][f]). 3/198 data points were missing for this analysis.

**Figure 5.** Total leukocyte [a] neutrophil [c] and monocyte [e] concentrations at pre-, post-, and 1 h post-exercise in males and females ($n=11$ for both sexes). Box and whisker plots are presented with symbols (triangles=males; circles=females) representing individual responses. Whiskers correspond to lowest and highest values. Horizontal line within boxes represents median values *=different to pre-exercise ($p<0.05$). Total leukocyte [b] neutrophil [d] and monocyte [f] total area under the curve (tAUC) responses between sexes (males $n=10$; females $n=11$ for all).
Discussion

The primary aim of this study was to investigate whether there are differences in resting and exercise-induced NRF2 activity between males and females. The primary findings from this study were: (1) exercise did not increase NRF2/ARE binding in PBMCs, and there were no differences at rest or post-exercise between males and females; (2) IL-6 and TNF-α did not increase post-exercise but were lower in females than males; (3) VCAM-1 and MMP-9 levels did not differ between males and females; (4) neutrophil concentrations were lower and monocytes higher in females than males. These findings demonstrate that, contrary to our hypothesis, there are no sex differences in resting or exercise-induced NRF2 activity in young, healthy adults. However, likely independent of changes to NRF2 activity, this study reported sex-specific differences in several immune markers, regardless of exercise.

The exercise employed in this study did not increase NRF2/ARE binding in PBMCs. This was surprising considering that the exercise protocol was chosen to impose a greater physiological demand than the exercise employed in previous investigations, where NRF2 activity was greater post- and 1 h post-exercise vs. a non-exercise rest group (Thorley, Thomas, Bailey, et al., 2023). It was anticipated that the greater metabolic stress of this exercise protocol would have augmented the NRF2 response, since higher exercise intensities are thought to elevate ROS production (as commonly represented by transient elevations in oxidative damage markers) to a greater extent than lower-intensity exercise (Sureda et al., 2009; J. S. Wang et al., 2006). However, the lack of change in NRF2 compared to our previous investigation could be due to differences in study design. Firstly, the previous study compared the effects of exercise to a non-exercise control group, whereas no control group was employed in the present study. In that study, exercise did not increase NRF2 activity from pre-exercise, but rather there were significant differences compared to the resting non-exercise group. Because the changes in exercise-induced NRF2 activity were highly variable, not including a non-exercise group, where changes were more homogeneous (Thorley, Thomas, Bailey, et al., 2023), may have limited our ability to detect small differences. In addition, the sample size in this study was smaller, as due to technical issues, NRF2/ARE binding could not be analysed for all 22 participants. The variability in exercise-induced NRF2/ARE binding was also greater than that reported in the Ostrom et al. (2021) investigation, in which the sample size estimation for this study were based on. Further studies with higher samples sizes are therefore warranted.
No differences in NRF2/ARE binding were found between males and females in this study. This aligns with recent findings by Galvan-Alvarez et al. (2023) who showed that the muscle protein expression of NRF2, in addition to its negative regulator, KEAP1, does not differ between healthy males and females at rest. It was hypothesised that females would have lower basal, but greater exercise-induced NRF2 activity, since rodent studies have highlighted that female mice present with lower basal levels of oxidants (due to lower abundance of NOXs), but greater oxidant production in response to stressors (Rohrer et al., 2014; Rooney et al., 2018; Zhang et al., 2012). These diverging responses were hypothesised to be, in part, due to females having greater oestrogen concentrations than males. Indeed, oestrogen may have antioxidative and NRF2 stimulating effects via downstream interaction with the oestrogen receptor, Erα, which is abundantly expressed in PBMCs (Ansell et al., 2004; Brundin et al., 2021; Lo & Matthews, 2013; Persky et al., 2000; Pomatto et al., 2018; Yao et al., 2010), and by regulating glycogen synthase kinase-3 beta activity (Wu et al., 2014).

The lack of a hypothesised sex difference in NRF2 activity in this study could be related to the menstrual cycle. For consistency, this study measured eumenorrheic females during the early follicular phase of their menstrual cycle, when oestrogen levels are at their most stable, but also at their lowest, and mOCP females at an equivalent phase. As such, any potential stimulatory effect of oestrogen on NRF2 signalling could have been limited by the low oestrogen produced at this time. However, as oestrogen was not measured in this study, this remains speculative. While no research to date has investigated the possible effect of menstrual cycle phase on NRF2, the activity of the transcription factor NF-κB, which is known to competitively inhibit NRF2 (Saha et al., 2020), has been shown to fluctuate across the menstrual cycle in PBMCs isolated from eumenorrheic healthy females (Faustmann et al., 2016). Unfortunately, no similar data exists to understand the effects of mOCP on redox-sensitive signalling pathways. Future research is therefore needed to similarly investigate possible fluctuations in NRF2 activity at different phases across the menstrual cycle, in addition to understanding if any changes exist in mOCP users.

Another possibility for the lack of difference could be due to that fact that other dimorphisms specific to males, such as androgen concentration, could be equally as stimulating as oestrogen on NRF2 activity, thereby nullifying any differences. Moreover, this study recruited young adults, however the likelihood of sex-based differences in NRF2 activity might be more pronounced among older individuals. In older adults, the decline in endocrinal function,
notably the impact of menopause on oestrogen levels in women, coupled with prior findings showing diminished NRF2 activity after exercise in this cohort (Done et al., 2016), may accentuate any variations.

This absence of sex-based differences in NRF2 activity is supported by no change in the activity of the NRF2 gene target, GPX, between groups. This finding is in line with Galvan-Alvarez et al. (2023), who similarly found that the protein expression of NRF2-regulated antioxidants, CAT, SOD and GR, albeit in muscle, did not differ between males and females. Furthermore, in males and females undergoing orthopaedic surgery, no sex differences in SOD1/2, GPX, and CAT protein expression from 3 different muscles were found (Pansarasa et al., 2000). Collectively, these findings, and those of the present study, suggest there are no sex differences in NRF2 and antioxidant specific gene target activity at rest and in response to exercise in muscle or PBMCs. While more studies are needed to confirm these findings, there appears to be a disparity in sex-specific redox responses between rodent and human studies.

Interestingly, some inflammatory markers differed between males and females regardless of exercise. Since NRF2 activity did not differ between sexes, these immune-related differences are unlikely explained by the downstream effects of NRF2. Sex differences in immune function at rest and following exercise have been reported previously (Abbasi et al., 2016; Benini et al., 2015; Northoff et al., 2008; Timmons et al., 2006). However, these effects are not consistent with other studies citing no sex-related immune differences following exercise (Allgrove et al., 2009; Gleson et al., 2000; Stupka et al., 2001). In the present study, there were no sex differences in MMP-9 and VCAM-1, but the cytokines IL-6 and TNF-α were lower in females vs. males. IL-6 concentrations were previously lower in females 24 h after acute resistance exercise (Aragón-Vela et al., 2021) and 1 h after a similar whole-body resistance exercise session (Benini et al., 2015). However, other studies have reported no sex differences in IL-6 after marathon running (Nieman et al., 2001) or prolonged cycling (Timmons et al., 2005). Thus, it remains unclear whether exercise results in differential IL-6 responses between sexes. Regarding TNF-α, limited research has examined these responses between sexes after exercise; Benini et al. (2015) found no sex differences in TNF-α concentrations following resistance training. Conversely, one study found similar responses to the present study, reporting lower TNF-α levels in middle aged females vs. males after long-distance walking at moderate intensity on 4 consecutive days (Terink et al., 2018). The mechanisms to explain the lower cytokine response in females in this study are
unclear. One possible explanation is that the greater presence of oestrogen in females than
males blunted pro-inflammatory cytokine production (Lewis et al., 2008; Liu et al., 2005;
Smith et al., 2011) by interfering with the various oestrogen receptors in cytokine expressing
tissues. Alternatively, since contracting skeletal muscle is a pivotal cytokine-secreting tissue
(Pedersen et al., 2001), these differences could simply be due to the observed
anthropometrical differences as shown in Table 1; specifically, males likely had greater fat-
free mass, a phenotypical difference widely observed between sexes (Abe et al., 2003),
stimulating greater relative cytokine production. The modulating effects of oestrogen and lean
body mass on sex specific cytokine responses requires further research.

There were group differences in neutrophil and monocyte concentrations, which were higher
and lower, respectively, in females vs. males. The mechanisms to explain these differences,
and their clinical significance is unclear. Higher neutrophil concentrations in females post-
exercise have been reported in some (MacIntyre et al., 2000; Timmons et al., 2005) but not all studies (Ferrer et al., 2009; Stupka et al., 2001). There is also inconsistency in the effects of exercise on monocytes between the sexes; monocytes were higher in females vs. males after acute aerobic exercise (Lobo et al., 2022) and prolonged cycling (Timmons et al., 2005), but not different between sexes after a half-marathon race (Abbasi et al., 2016). However, this study only investigated the total population of circulating leukocytes, and not phenotypic changes in leukocyte subsets, which could have revealed whether changes in cell population was accompanied by distinct changes in cell function.

Neutrophils and monocytes play distinct roles in mediating the inflammatory response and
facilitating muscle repair following exercise, passively migrating from bone marrow to blood
and eventually to skeletal tissue to perform their pro-inflammatory roles (Malm, 2001). As
demonstrated in this study, neutrophils are abundantly mobilized from bone marrow and into
circulation where their concentration is sustained in the hours post-exercise (Suzuki et al.,
1996, 2003), due to the increased presence of soluble proteins, including cytokines,
glucocorticoids, and growth hormones. Monocytes, on the other hand, rise during and after exercise, but as they differentiate into tissue-resident macrophages, they soon leave the circulation and migrate towards damaged skeletal tissue in large numbers, resulting in reduced systemic concentrations post-exercise (Peake et al., 2005). The extent of muscle damage, the ensuing inflammatory response, and the subsequent mobilization of these immune cells after exercise is heavily dependent on exercise intensity (Gabriel et al., 1994; Gabriel & Kindermann, 1997). Thus, one potential reason why neutrophils were higher and
monocytes lower in females vs. males could be due to the fact females worked closer to the
desired intensity (within 20% of maximal CMJ and SJ) than males, as presented in Table 1. Thus, they may have incurred a greater acute inflammatory response than the males. While this is at odds with the lower inflammatory cytokine concentrations observed in females, the time course of leucocytosis and cytokine responses to exercise do not necessarily correlate (Paulsen et al., 2005). However, monocyte concentrations were predominantly lower in females vs. males at rest as opposed to post-exercise, inferring that the group difference may not have stemmed from post-exercise inflammatory responses, but instead from random differences at baseline. Measuring changes in leukocyte phenotypes may have revealed if these changes affected the magnitude of inflammation; however, that was beyond the scope of this study.

There are some key limitations to this research; firstly, our a priori sample size was not reached, and therefore we may have been underpowered to detect small changes. Secondly, we did not quantify oestrogen levels and can therefore can only speculate what effect this and other sex hormones may have had on the findings. Thirdly, we only quantified NRF2/ARE binding in PBMCs, and therefore cannot rule out any sex differences in other tissues. Lastly, differences in pro-inflammatory cytokine levels between the sexes may have been due to differences in skeletal muscle mass, since myocytes secrete a wide variety of cytokines in response to exercise; thus, instead of total body mass, fat free lean mass could have been measured to determine whether these differences were due to anthropometric variations.

In conclusion, NRF2/ARE binding did not differ between males and females at rest or in response to exercise. However, some sex-specific differences in inflammatory markers were observed, suggesting that males and females may possess differential immune responses.

**Statements and Declarations**

**Author Contribution**


**Funding**

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Disclosure Statement

The authors declare no conflict of interest.

Data Availability Statement

Data can be provided at reasonable request from the corresponding author.

References


suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription. *Nature Communications*, 7. https://doi.org/10.1038/ncomms11624


Rohrer, P. R., Rudraiah, S., Goedken, M. J., & Manautou, J. E. (2014). Is Nrf2 Responsible for Sex Differences in Susceptibility to Acetaminophen-Induced Hepatotoxicity? *Drug Metabolism and Disposition, 42*(10), 1663–1674. https://doi.org/10.1124/DMD.114.059006


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Table 1. Group differences in physical characteristics and 24 h dietary intake. Values are presented as mean ± SD.

<table>
<thead>
<tr>
<th>Physical characteristics</th>
<th>Males (n=11)</th>
<th>Females (n=11)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>25 ± 7</td>
<td>25 ± 5</td>
<td>0.92</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.78 ± 0.07</td>
<td>1.63 ± 0.06</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>79.0 ± 8.7</td>
<td>59.7 ± 4.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Maximal CMJ height (cm)</td>
<td>33.5 ± 5.5</td>
<td>22.9 ± 4.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Mean CMJ height across exercise (cm)</td>
<td>28.8 ± 4.2</td>
<td>20.2 ± 3.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Maximal SJ height (cm)</td>
<td>31.4 ± 3.7</td>
<td>25.1 ± 4.5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Mean SJ height across exercise (cm)</td>
<td>23.6 ± 6.0</td>
<td>20.2 ± 5.8</td>
<td>0.19</td>
</tr>
<tr>
<td>24 h energy intake</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy (kcal)</td>
<td>2268 ± 877</td>
<td>1743 ± 308</td>
<td>0.08</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>116.5 ± 57.8</td>
<td>84.1 ± 23.9</td>
<td>0.10</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>93.5 ± 45.6</td>
<td>67.0 ± 14.3</td>
<td>0.07</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>252.4 ± 131.3</td>
<td>197.6 ± 49.7</td>
<td>0.21</td>
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<tr>
<td>Omega-3 fatty acid (n-3) (g)</td>
<td>0.7 ± 1.2</td>
<td>1.14 ± 1.42</td>
<td>0.41</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>33.3 ± 31.6</td>
<td>50.9 ± 53.7</td>
<td>0.36</td>
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<tr>
<td>Vitamin D (µg)</td>
<td>3.1 ± 3.5</td>
<td>3.4 ± 3.2</td>
<td>0.86</td>
</tr>
<tr>
<td>Vitamin E (mg)</td>
<td>3.8 ± 3.0</td>
<td>4.0 ± 1.9</td>
<td>0.85</td>
</tr>
</tbody>
</table>

CMJ = counter movement jump. SJ = squat jump. Statistical significance set at \( p < 0.05 \).
Figure 1. [a] Nuclear factor erythroid 2-related factor 2 (NRF2)/antioxidant response element (ARE) binding at pre-, post-, and 1 h post-exercise in males and females (males n=10; females n=10). Box and whisker plots are presented with symbols (triangles=males; circles=females) representing individual responses. Whiskers correspond to lowest and highest values. Horizontal line within boxes represents median values. [b] NRF2/ARE binding total area under the curve (tAUC) responses between sexes (males n=9; females n=8).
Figure 2. Tumour necrosis factor-alpha (TNF-α) [a] and interleukin-6 (IL-6) [b] concentrations at pre-, post-, and 1 h post-exercise in males and females (n=11 for both sexes). Box and whisker plots are presented with symbols (triangles=males; circles=females) representing individual responses. Whiskers correspond to lowest and highest values. Horizontal line within boxes represents median values. TNF-α [b] and IL-6 [d] total area under the curve (tAUC) responses between sexes (males n=10; females n=11).
Figure 3. Matrix metallopeptidase 9 (MMP-9) [a] and vascular cell adhesion protein 1 (VCAM-1) [b] concentrations at pre-, post-, and 1 h post-exercise in males and females (n=11 for both sexes). Box and whisker plots are presented with symbols (triangles=males; circles=females) representing individual responses. Whiskers correspond to lowest and highest values. Horizontal line within boxes represents median values *=different to pre-exercise (p<0.05). MMP-9 [b] and VCAM-1 [d] total area under the curve (tAUC) responses between sexes (males n=10; females n=11 for both).
Figure 4. Glutathione peroxidase (GPX) activity [a] at pre-, post-, and 1 h post-exercise in males and females (n=11 for both groups). Box and whisker plots are presented with symbols (triangles=males; circles=females) representing individual responses. Whiskers correspond to lowest and highest values. Horizontal line within boxes represents median values. [b] GPX total area under the curve (tAUC) responses between sexes (males n=9; females n=11)
Figure 5. Total leukocyte [a] neutrophil [c] and monocyte [e] concentrations at pre-, post-, and 1 h post-exercise in males and females (n=11 for both sexes). Box and whisker plots are presented with symbols (triangles=males; circles=females) representing individual responses. Whiskers correspond to lowest and highest values. Horizontal line within boxes represents median values *=different to pre-exercise (p<0.05). Total leukocyte [b] neutrophil [d] and monocyte [f] total area under the curve (tAUC) responses between sexes (males n=10; females n=11 for all).

105x145mm (600 x 600 DPI)
Supplementary Table 1. Energy breakdown and antioxidant content of low (poly)phenol control breakfast.

<table>
<thead>
<tr>
<th>Serving, d⁻¹</th>
<th>Cadbury’s™ Milk Chocolate</th>
<th>Nature Valley™ Crunchy Granola Oats and Honey bar</th>
<th>Gatorade™ Sport Orange drink</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>53 g</td>
<td>30 g</td>
<td>400 mL</td>
</tr>
<tr>
<td>Energy per serving</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total energy (kcal)</td>
<td>283</td>
<td>140</td>
<td>96</td>
</tr>
<tr>
<td>Carbohydrates (g)</td>
<td>30</td>
<td>19.3</td>
<td>24</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>16</td>
<td>5.4</td>
<td>0</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>3.9</td>
<td>2.6</td>
<td>0</td>
</tr>
<tr>
<td>Antioxidant content (mmol/100g)</td>
<td>1.95</td>
<td>0.83</td>
<td>0</td>
</tr>
</tbody>
</table>