

## Research article

# Immune Response in Women during Exercise in the Heat: A Spotlight on Oral Contraception

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## Abstract

This study compared the immune and stress response of oral contraceptive users (WomenOC;  $n = 9$ ) to normally-menstruating women (WomenNM;  $n = 9$ ) at rest and during exercise in temperate (TEMP; 22°C) and hot (HEAT; 35°C) conditions. Participants performed a 3-stage cycling trial in each condition at 90% (Stage 1; 30 min), 135% (Stage 2; 15 min), and 180% (Stage 3; 7.5 min) of lactate threshold 1. C-reactive protein (CRP) and immune cell counts were measured at rest, and serum cytokines (IL-1 $\beta$ , IL-1RA, IL-6, IL-8, IL-10, and TNF- $\alpha$ ) and salivary cortisol were evaluated before and after exercise in both the TEMP and HEAT conditions. There were no differences in resting immune cell counts between groups, nor any differences in cortisol or any of the pro- or anti-inflammatory cytokines measured at rest or after completion of the exercise trials ( $p > 0.05$ ). However, a trend for a higher resting CRP concentration was observed in WomenOC relative to WomenNM ( $1.102 \pm 1.182$  and  $0.326 \pm 0.228$ , respectively,  $p = 0.07$ ). The results obtained in the current study indicate similar immunoendocrine function in WomenOC and WomenNM both at rest and after exercise in temperate and hot environments.

**Key words:** Cytokines; cortisol; C-reactive protein; core temperature; ovarian hormones.

## Introduction

Regular physical activity enhances immune function (Gleeson, 2007; Petersen and Pedersen, 2005). However, acute bouts of intense endurance exercise or periods of intensified training can have an immunodepressive effect, subsequently resulting in an increased likelihood of illness and infection (Gleeson, 2007). This may be of particular consequence for athletes, as even minor infections may result in decrements to exercise performance and a reduced capacity for heavy training (Roberts, 1986). Heat exposure is another factor that can compromise immune function via an increase in pro-inflammatory cytokine concentrations (Bouchama and Knochel, 2002; Sawka et al., 2011), and there is some evidence to suggest that performing exercise in a hot environment can exacerbate the immune response (Peake et al., 2008). Exercising in the heat also stimulates the production of the stress hormone cortisol (Brenner et al., 1997; Niess et al., 2003), which has a known immunomodulatory effect (Walsh and Whitham, 2006). If prolonged or severe, these endocrine and immune disturbances can contribute to the pathology of heat stroke

(Lim and Mackinnon, 2006).

Sex hormones also modulate the immune system (Schuurs and Verheul, 1990), and it is well established that oral contraceptives (OC) change the hormonal milieu (Wiegratz et al., 2003). Given the relationship between sex hormones and immunity, it is perhaps surprising that relatively little research has investigated the role of exogenous female sex steroids on the modulation of immune function. Giraldo et al. (2008) previously demonstrated that OC use improves inflammatory status in untrained women (i.e., by lowering neutrophil activation and interleukin (IL)-8 concentrations and increasing the concentration of IL-13), and stated that this greater anti-inflammatory environment is likely related to the lower concentration of endogenous estrogen when compared to normally-menstruating women. Conversely, Timmons et al. (2005) observed increases in resting leukocyte counts in OC users compared to non-users, which could be indicative of inflammation. The same study also observed ~80% greater exercise-induced IL-6 concentrations in normally-menstruating women during the follicular phase when compared to women on OC. While IL-6 can have pro-inflammatory properties, increased IL-6 concentrations after exercise induce an anti-inflammatory environment by inhibiting pro-inflammatory (i.e., TNF- $\alpha$ ) cytokines and facilitating anti-inflammatory cytokine (i.e., IL-1ra, IL-10) production (Petersen and Pedersen, 2005). Thus, it is possible that the blunted IL-6 response among OC users in the Timmons et al. (2005) study reflects a disruption to the normal immune response to exercise. Oral contraceptive use is also associated with increased C-reactive protein (CRP; a biomarker of inflammation and tissue damage) concentrations in the general population (Cauci et al., 2008; Sørensen et al., 2014; van Rooijen et al., 2006) and in athletes (Cauci et al., 2017), which could predispose users to a higher inflammatory response to physical stress and elevate cardiovascular risk (Ridker et al., 2000). It is clear, then, that OC use has the potential to influence various aspects of the immune response, although further research is required to confirm the direction, magnitude, and implications of these changes. Importantly, no previous study has investigated the combined effect of heat and exercise on the immune response of women taking OC.

Previous studies investigating the stress response (i.e., cortisol) during exercise in the heat are limited by the exclusive use of male subjects (e.g., Brenner et al., 1997; Hoffman et al., 1997; Niess et al., 2003), or the use of

female subjects on and off OC but without the potentially additive effect of heat exposure (e.g., Boisseau et al., 2013; Bonen et al., 1991; Kirschbaum et al., 1996). Even in this literature the data are equivocal; one study observed increases in free cortisol at rest in OC users compared to non-users (Boisseau et al., 2013), while two reported no differences in pre-exercise cortisol concentrations (Bonen et al., 1991; Kirschbaum et al., 1996). Furthermore, while these three studies agree that the cortisol response to exercise is blunted in women on OC, Kirschbaum et al. (1996) nonetheless reported an increase in free cortisol in response to exhaustive cycling exercise in OC users (although to a lesser degree than non-users), whereas Boisseau et al. (2013) and Bonen et al. (1991) observed no exercise-induced changes in cortisol concentration in women taking OC. This may be important, as cortisol acts as a powerful natural immunosuppressant (Petrovsky, 2001). Thus, considerably more research is required before firm conclusions can be drawn in respect to OC and the stress response, and in particular, the cortisol response elicited during exercise in the heat.

This exploratory study investigated the immunoendocrine response to exercise in the heat in women taking combined monophasic OC when compared to normally-menstruating women. This an important consideration, as exercising in hot conditions is commonplace for both recreational and competitive athletes (e.g., the 2020 Olympic Games have been scheduled for the peak of the Tokyo summer). Specifically, CRP and immune cell counts were measured at rest, and pro- and anti-inflammatory cytokines (IL-1 $\beta$ , IL-1RA, IL-6, IL-8, IL-10, and TNF- $\alpha$ ) and cortisol were evaluated in OC users and non-users before and after exercise in both temperature and hot conditions.

## Methods

### Participants

Eighteen recreationally-active women (performing 300-500 min/wk of moderate-intensity exercise) voluntarily participated in this study. Nine of the women were normally-menstruating (i.e., every 25-32 days) for >12 months (WomenNM), while the remaining nine women were taking a low-dose combined monophasic OC for >12 months (WomenOC). WomenOC continued their OC use throughout the experimental period. None of the participants had ever knowingly been pregnant, and they had no documented history of pulmonary, cardiovascular, or metabolic disorders. Written informed consent was provided by participants prior to data collection, and ethical approval was granted by the Griffith University Human Ethics Committee.

### Experimental design

WomenNM were tested in the follicular phase of their cycle (day 2-6) when endogenous estrogen is low (Charkoudian and Johnson, 2000), whilst WomenOC were tested during the active phase of the OC pill (day 2-21; when exogenous estrogen is administered). As women on OC have suppressed endogenous hormone concentrations (Burrows and Peters, 2007), these phases were selected to

isolate the effects (if any) of the synthetic hormones provided by the OC pill (Cherney et al., 2007). The follicular phase was identified by self-report (the participants alerted researchers at the onset of menses) and was confirmed via blood concentrations of the female sex hormones (see Results).

Participants completed three exercise tests, the first of which was an incremental cycling test to exhaustion to determine the workloads for the subsequent two trials (based on lactate threshold 1 [LT1]). Two 3-stage cycling trials were then performed, one in a temperate environment (TEMP; 22°C) and the other under hot ambient conditions (HEAT; 35°C). Room temperature and relative humidity were measured continuously throughout all trials (Vantage Vue, Davis Instruments, Hayward, USA). The incremental cycling test was performed 4 wk before the 3-stage cycling trials, and the 3-stage cycling trials were then separated by 48 h to ensure adequate recovery time. The exercise tests were performed at the same time each morning (between 5:00-9:00 am), and trial order for the 3-stage cycling trials was randomised. Participants were instructed to refrain from alcohol, caffeine, and vigorous exercise in the 24 h prior to testing.

### Incremental cycling test to exhaustion

All cycling tests were performed on an electronically-braked cycle ergometer (Sport Excalibur, Lode B.V. Medical Technology, Groningen, The Netherlands). Heart rate was monitored continuously throughout the incremental test (RS300X, Polar, Finland), and blood was sampled from the earlobe for the determination of blood [La<sup>-</sup>] (Lactate Pro, ARKRAY Inc., Japan). Gas exchange variables were measured breath-by-breath using a calibrated metabolic measurement system (CosMed, Pulmonary Function Equipment, Quark CPET, Italy). Participants began the protocol by cycling at 15 W at a pedal cadence of 70-80 rpm. Power output was increased by 15 W every 4 min until blood [La<sup>-</sup>] reached a value of >4.0 mmol/L, after which the power output was increased by 15 W every 30 s until volitional exhaustion. When the required cadence could not be maintained despite strong verbal encouragement, the test was terminated. Peak HR and power output were recorded at this time. LT1 was calculated using the modified Dmax method (Cheng et al., 1992), and the final 30 s of oxygen consumption (VO<sub>2</sub>) data were averaged to determine peak VO<sub>2</sub>.

### 3-stage cycling trials

Participants ingested a core temperature (T<sub>c</sub>) sensor (CorTemp COR-100 Wireless Ingestible Temperature Sensor, HQ Inc., FL) ~8 h before each trial to allow adequate time for the pill to transit from the stomach to the small intestines (Lee et al., 2000). Participants arrived to the laboratory in a fasted state and emptied their bladder before the measurement of body mass. Baseline T<sub>c</sub> was recorded at this time. Participants then moved into the environmental chamber where they remained for 1 h to allow for passive acclimation. They were given a standardized breakfast in the chamber (1 piece of raisin toast per 50 kg of body mass and 0.3 piece for every 5 kg over 50 kg, and 5 mL water per kg of body mass), which they consumed within 30 min

to allow for particle digestion prior to the commencement of exercise. After this time, no fluid or food was consumed until after the trial. After 1 h in the environment, resting  $T_c$  was recorded. During the two 3-stage cycling trials, participants cycled at a cadence of 70–80 rpm at 90% (Stage 1), 135% (Stage 2), and 180% (Stage 3) of LT1, as determined during the incremental cycle ergometer test. The length of each stage shortened as the trial progressed (Stage 1 = 30.0 min, Stage 2 = 15.0 min, Stage 3 = 7.5 min; total = 52.5 min) in order to prevent premature fatigue.  $T_c$  data were recorded at 7.5-min intervals throughout the trials.

### Blood and saliva sampling

Blood samples (approximately 10 mL) were collected from the antecubital vein at baseline (room temperature), after 60 min in the environmental condition, and immediately after exercise. Using whole blood, total white cell count, lymphocyte and monocyte counts, as well as hemoglobin and hematocrit were determined from vacutainer tubes containing EDTA (Becton–Dickinson, BD, Juiz de Fora, MG, Brazil) in a hematology analyzer (Ac.T™ 5 diff analyzer, Beckman Coulter, NZ). Venous blood samples were also collected into serum-separator tubes (Becton–Dickinson, BD, Juiz de Fora, MG, Brazil), stored in the dark for 30 min and allowed to clot before being centrifuged for 10 min at 3000 rev/min. Serum was separated into 500  $\mu$ L aliquots and frozen at  $-80^\circ\text{C}$  for the subsequent analyses of pro- and anti-inflammatory cytokines (IL-1 $\beta$ , IL-1RA, IL-6, IL-8, IL-10 and TNF- $\alpha$ ), CRP, and ovarian hormones.

Cytokine analysis was completed using custom manufactured multiplex suspension array kits (Bio-Rad Laboratories Pty Ltd; Hercules, CA USA) according to the manufacturer's instructions, and a Bioplex 200 Suspension Array Reader (Bio-Rad Laboratories Pty Ltd). Standard curves of cytokine concentration versus fluorescence intensity were automatically generated by the Bioplex Manager Software (Bio-Rad Laboratories Pty Ltd) and sample concentrations for each cytokine extrapolated from respective standard curves. All samples were analysed in duplicate.

CRP concentrations were determined via an immunoturbidimetric assay using commercially available reagents and a COBAS Integra 400 system (Roche Diagnostics, Mannheim, Germany). Ovarian hormone analysis was performed by a commercial pathology laboratory (ANZAC Institute, Sydney, Australia) using a stable-isotope dilution methodology in combination with liquid chromatography–mass spectrometry.

Baseline, pre-, and immediately post-exercise saliva samples were also collected. Unstimulated, whole saliva was collected using an IPRO oral fluid collector (OFC, IPRO Interactive, Wallingford, UK). Participants placed a OFC on the top of their tongue and closed their mouth. When 0.5 mL of saliva had been absorbed by the OFC, an indicator line on the OFC stem turned bright blue at which point the OFC was removed and placed into a 3 mL buffer solution. Mixing of the sample was performed by inverting the bottle with the OFC and buffer for 2 min to facilitate extraction of the target analyte. Bottles were stored at room temperature until two drops (using the dropper cap on the

bottle) of the sample were used to determine salivary cortisol concentration using an IPRO Lateral Flow Device (LFD) and Lateral Flow Reader (IPRO Interactive, Wallingford, UK). The method and reliability of salivary cortisol analyses using the IPRO has been described in detail elsewhere (Fisher et al., 2016).

### Statistical analyses

All statistical analyses were carried out using the IBM Statistical Package for the Social Sciences (SPSS Inc., Version 24) software. Group characteristics were assessed using independent samples t-tests. As the data were normally distributed, fully factorial ANOVA with repeated measures (condition and time) were used to determine any main effects of, or interactions between, the three independent variables i. Group (WomenNM and WomenOC), ii. Condition (TEMP and HEAT), and iii. Time (Baseline, Pre, Post) for the core temperature, cytokine, and cortisol data. Where statistically significant differences were detected, post hoc tests with Bonferroni adjustments were performed to detect where the differences occurred. All data are reported as means  $\pm$  SD. p values are reported with significance set at  $< 0.05$ , and Cohen's d (d) and partial eta-squared ( $\eta_p^2$ ) effect sizes are presented to indicate the magnitude of observed effects for the outcome variables analysed using t-tests and ANOVA, respectively. When interpreting effect sizes, .2, .5, and .8 and .01, .06, and .14 are considered small, medium, and large effect sizes for d and  $\eta_p^2$ , respectively (Cohen, 1992; Richardson, 2011).

### Results

Subject characteristics and incremental cycling test data are presented in Table 1. There were no between-group differences in any of the subject characteristics or incremental cycling test values ( $p \geq 0.16$ ). However, 17 $\beta$ -estradiol ( $75.74 \pm 57.21$  pmol/L v  $18.13 \pm 20.18$  pmol/L,  $p < 0.01$ ,  $d = 1.34$ ) and progesterone ( $0.24 \pm 0.15$  pmol/L v  $0.12 \pm 0.06$  pmol/L,  $p < 0.01$ ,  $d = 1.05$ ) were significantly higher in WomenNM compared to WomenOC, respectively.

**Table 1.** Subject characteristics and peak values determined during incremental cycling to exhaustion in naturally-menstruating women (WomenNM) and women using oral contraceptives (WomenOC). Values are mean ( $\pm$ standard deviation).

|                                      | WomenNM<br>(n = 8) | WomenOC<br>(n = 8) |
|--------------------------------------|--------------------|--------------------|
| Age (yr)                             | 22 (3)             | 22 (4)             |
| Body mass (kg)                       | 63.1 (5.6)         | 59.5 (6.4)         |
| Height (m)                           | 1.68 (.07)         | 1.64 (.06)         |
| Body mass index (kg/m <sup>2</sup> ) | 22.3 (1.8)         | 22.2 (1.9)         |
| Oxygen uptake (L/min)                | 2.89 (.54)         | 2.66 (.45)         |
| Oxygen uptake (mL/kg/min)            | 44.8 (5.2)         | 44.5 (4.2)         |
| Power output (W)                     | 241 (54)           | 227 (37)           |
| Blood lactate (mmol/L)               | 11.2 (1.3)         | 10.5 (.9)          |
| Heart rate (b/min)                   | 193 (8)            | 195 (6)            |

There were no differences in resting blood and immune cell counts (Table 2) between WomenNM and WomenOC ( $p \geq 0.120$ ). A trend ( $p = 0.07$ ,  $d = 0.91$ ) for higher CRP concentrations was noted for WomenOC ( $1.102 \pm$

1.182 mg/L), with a value more than three-fold higher than that observed in WomenNM ( $0.326 \pm 0.228$  mg/L).

Table 3 presents the environmental data, as well as the exercise intensities utilised during the 3-stage cycling

trials. There were no differences between WomenNM and WomenOC for ambient temperature ( $p \geq 0.410$ ), humidity ( $p \geq 0.210$ ), or power output at Stages 1-3 ( $p \geq 0.420$ ).

**Table 2.** Resting blood and immune cell counts in naturally-menstruating women (WomenNM) and women using oral contraceptives (WomenOC). Values are mean ( $\pm$ standard deviation).

|   | WomenNM<br>(n = 8) | WomenOC<br>(n = 8) |
|---|--------------------|--------------------|
| White blood cells ( $5.34 \times 10^9/L$ )  | 5.47 (.85)         | 6.47 (1.60)        |
| Red blood cells ( $5.34 \times 10^{12}/L$ ) | 5.34 (1.07)        | 5.17 (.70)         |
| Hemoglobin (g/L)                            | 158.15 (29.42)     | 151.39 (26.34)     |
| Hematocrit (L/L)                            | .46 (.09)          | .45 (.08)          |
| Platelets ( $5.34 \times 10^9/L$ )          | 215.17 (71.42)     | 255.15 (62.51)     |
| Neutrophils ( $5.34 \times 10^9/L$ )        | 2.52 (.47)         | 3.17 (1.20)        |
| Lymphocytes ( $5.34 \times 10^9/L$ )        | 2.22 (.47)         | 2.56 (.72)         |
| Monocytes ( $5.34 \times 10^9/L$ )          | .43 (.12)          | .50 (.17)          |
| Eosinophils ( $5.34 \times 10^9/L$ )        | .22 (.14)          | .23 (.14)          |
| Basophils ( $5.34 \times 10^9/L$ )          | .11 (.10)          | .18 (.22)          |

**Table 3.** 3-stage cycling test conditions. Values are mean ( $\pm$ standard deviation).

|                                     |            | WomenNM<br>(n = 8) | WomenOC<br>(n = 8) |
|-------------------------------------|------------|--------------------|--------------------|
| Ambient temperature ( $^{\circ}C$ ) | TEMP trial | 22 (1)             | 22 (1)             |
|                                     | HEAT trial | 35 (1)             | 35 (1)             |
| Humidity (%)                        | TEMP trial | 65 (5)             | 60 (10)            |
|                                     | HEAT trial | 39 (4)             | 35 (10)            |
| Power output (W)                    | Stage 1    | 73 (24)            | 64 (16)            |
|                                     | Stage 2    | 109 (36)           | 96 (24)            |
|                                     | Stage 3    | 145 (48)           | 128 (31)           |

TEMP = 3-stage cycling test performed in temperate conditions; HEAT = 3-stage cycling test performed in hot conditions. There were no significant differences between WomenNM and WomenOC in any of the variables ( $p > 0.05$ ).

**Table 4.** Core temperature, cortisol, and cytokine concentrations before and after the 3-stage cycling tests in naturally-menstruating women (WomenNM) and women using oral contraception (WomenOC). Values are mean ( $\pm$ standard deviation).

|                       |          | WomenNM        |                 | WomenOC       |                 |
|-----------------------|----------|----------------|-----------------|---------------|-----------------|
|                       |          | TEMP           | HEAT            | TEMP          | HEAT            |
| $T_c$ ( $^{\circ}C$ ) | Baseline | 37.1 (.2)      | 37.0 (.4)       | 37.5 (.2) †   | 37.6 (.3) †     |
|                       | Pre      | 37.0 (.2)      | 37.0 (.3)       | 37.4 (.1) †   | 37.5 (.3) †     |
|                       | Post     | 38.1 (.3) #§   | 38.4 (.4) #§*   | 38.2 (.2) #§  | 38.5 (.3) #§*   |
| Cortisol (nmol/L)     | Baseline | 13.62 (7.82)   | 17.26 (9.08)    | 16.66 (5.85)  | 20.59 (8.71)    |
|                       | Pre      | 7.88 (4.19)    | 14.09 (11.35)   | 14.18 (3.47)  | 17.88 (3.66)    |
|                       | Post     | 6.57 (4.09) #§ | 12.76 (10.24) * | 11.89 (5.05)  | 15.11 (5.70)    |
| IL-1 $\beta$ (pg/mL)  | Pre      | .38 (.33)      | .43 (.36)       | .29 (.12)     | .36 (.15)       |
|                       | Post     | .42 (.33)      | .42 (.39)       | .32 (.12)     | .34 (.10)       |
|                       | Post     | .39 (.27)      | .37 (.38)       | .28 (.09)     | .31 (.13)       |
| IL-1ra (pg/mL)        | Baseline | 50.22 (38.81)  | 48.90 (38.43)   | 27.12 (13.79) | 35.05 (14.75)   |
|                       | Pre      | 45.22 (37.57)  | 49.36 (35.94)   | 32.28 (12.32) | 44.43 (28.34)   |
|                       | Post     | 50.46 (39.03)  | 54.86 (35.35)   | 32.11 (9.81)  | 41.90 (29.69)   |
| IL-6 (pg/mL)          | Baseline | 5.24 (9.71)    | 3.33 (7.41)     | .95 (.80)     | 1.44 (1.24)     |
|                       | Pre      | 4.92 (9.02)    | 3.07 (6.45)     | .77 (.87)     | 1.45 (1.18)     |
|                       | Post     | 5.82 (10.17)   | 3.88 (8.12)     | .84 (.83)     | 1.07 (.83)      |
| IL-8 (pg/mL)          | Baseline | 4.66 (.89)     | 9.56 (3.76) *   | 3.91 (1.40)   | 10.68 (10.33) * |
|                       | Pre      | 4.17 (.73)     | 8.78 (4.60) *   | 3.67 (1.41)   | 7.43 (4.10) *   |
|                       | Post     | 5.02 (1.02)    | 5.88 (1.94) #   | 4.28 (2.30)   | 5.69 (4.14) #   |
| IL-10 (pg/mL)         | Baseline | 4.58 (2.60)    | 4.20 (.62)      | 4.20 (1.78)   | 4.91 (2.33)     |
|                       | Pre      | 4.76 (2.47)    | 4.36 (1.38)     | 4.61 (2.26)   | 5.30 (2.40)     |
|                       | Post     | 4.98 (2.92)    | 3.99 (.55)      | 4.38 (1.96)   | 4.59 (2.01)     |
| TNF- $\alpha$ (pg/mL) | Baseline | 9.28 (11.48)   | 6.88 (10.28)    | 4.68 (41.79)  | 4.31 (1.49)     |
|                       | Pre      | 9.41 (11.92)   | 7.55 (8.21)     | 4.63 (3.03)   | 4.93 (1.42)     |
|                       | Post     | 9.42 (10.82)   | 7.02 (8.48)     | 3.25 (1.38)   | 4.12 (.65)      |

TEMP = 3-stage cycling test performed in temperate conditions; HEAT = 3-stage cycling test performed in hot conditions; Pre = after 1 hr resting in the testing conditions; Post = immediately after completion of the 3-stage cycling test;  $T_c$  = core temperature. † Significantly different from WomenNM at the same time point ( $p \leq 0.004$ ). \* Significantly different from the TEMP trial at the same time point ( $p \leq 0.004$ ). # Significantly different from baseline in the same trial ( $p \leq 0.024$ ). § Significantly different from pre-exercise values in the same trial ( $p < 0.001$ ).

All  $T_c$ , cortisol, and cytokine data are presented in Table 4. In both the TEMP and HEAT trials, respectively,  $T_c$  was higher in WomenOC than WomenNM at baseline ( $p \leq 0.002$ ,  $\eta^2_p = 0.530, 0.491$ ) and pre-exercise ( $p \leq 0.004$ ,  $\eta^2_p = 0.521, 0.452$ ) but not post-exercise ( $p \geq 0.259$ ,  $\eta^2_p = 0.090, 0.022$ ), and the  $T_c$  reached at the cessation of exercise was higher in the HEAT trial compared to the TEMP trial irrespective of group ( $p = 0.006$ ,  $\eta^2_p = 0.433$ ).

Main effects for condition ( $p = 0.019$ ,  $\eta^2_p = 0.335$ ) and time ( $p = 0.004$ ,  $\eta^2_p = 0.321$ ) were observed for salivary cortisol, in that cortisol was significantly higher in the HEAT trial, and decreased over time in both experimental conditions. No significant differences were observed between WomenNM and WomenOC ( $p = 0.097$ ), although WomenOC typically had higher cortisol concentrations as indicated by a large effect size ( $\eta^2_p = 0.184$ ).

There was no effect of group ( $p > 0.05$ ), condition ( $p > 0.05$ ), or time ( $p > 0.05$ ) for IL-1 $\beta$ , IL-1ra, IL-6, IL-10, and TNF- $\alpha$ . IL-8 was not different between WomenNM and WomenOC ( $p = 0.742$ ,  $\eta^2_p = 0.007$ ), however a condition  $\times$  time interaction was observed ( $p = 0.004$ ,  $\eta^2_p = 0.296$ ) whereby IL-8 was significantly elevated in both groups in the HOT trial at baseline ( $p = 0.004$ ,  $\eta^2_p = 0.415$ ) and after resting in the heat for 1 h ( $p = 0.001$ ,  $\eta^2_p = 0.512$ ), but not after completion of the 3-stage cycling test ( $p = 0.139$ ,  $\eta^2_p = 0.131$ ).

## Discussion

In the present study, no differences were found between long-term oral contraceptive users (during the active pill phase) and normally menstruating women (during the early follicular phase) in resting blood and immune cell counts, or the cortisol and cytokine response to performing exercise in temperate and hot ambient environments. There was a trend for elevated resting CRP in WomenOC ( $p = 0.07$ ), which could be indicative of inflammation. For the most part, however, the results obtained in the current study indicate similar immunoendocrine function in WomenOC and WomenNM at rest and after exercise in both the TEMP and HEAT conditions.

To the authors' knowledge, no previous research has examined the combined effect of heat and exercise on the immune and stress response of OC users when compared to normally-menstruating women. Previously, the select studies that have investigated immune function in OC users under temperate conditions have found varying results, with some indicating a protective anti-inflammatory environment (e.g., Giraldo et al., 2008) and others suggesting increased inflammation (e.g., Timmons et al., 2005). The present findings showed a comparable response between WomenNM and WomenOC for all of the pro- (IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$ ) and anti-inflammatory (IL-1ra, IL-6, IL-10) cytokines measured, both at rest and after exercise in the TEMP and HEAT environments. Surprisingly, there was no increase in cytokine concentrations in response to the exercise protocol (in either environmental condition), which contradicts a considerable body of research that reports increased cytokine concentrations post-exercise (see reviews: Pedersen, 2000; Pedersen et al.,

1998). It is unclear why the exercise protocol utilised in the present study did not induce immune changes, although previous research has shown considerable differences in the exercise-induced immune response depending on the exercise duration (Ostrowski et al., 1998), mode (Nieman et al., 1998), core temperature reached (Rhind et al., 2004), and the participants' training status (Evans et al., 1986). Indeed, a reduced IL-6 response has been observed when comparing cycling exercise (as was used in the present study) to running (Nieman et al., 1998). In support of this, Ullum et al. (1994) found no changes in plasma IL-1 $\alpha$ , IL-1 $\beta$ , and TNF- $\alpha$  in response to 1 h of bicycle exercise at 75%  $VO_{2max}$ . Starkie et al. (2005) also reported no change in plasma concentrations of IL-6 or TNF- $\alpha$  after cycling in cool conditions for 90 min at 70% peak  $VO_2$ , although cycling in a hot environment did elicit a cytokine response. The reason this was not replicated during the HEAT trial in the present study is not immediately clear, although it should be noted that Starkie and colleagues (Starkie et al., 2005) utilised a different protocol (i.e., fixed intensity), participant group (i.e., males), and participant training status (i.e., endurance-trained) to that employed in the current research, which may help to explain the inconsistent findings.

Rhind and colleagues (2004) highlighted the importance of elevated core temperature in instigating an immune response, reporting no increase in cytokine concentrations during or after cycling exercise when core body temperature was clamped at near-resting levels (via cold water immersion). Conversely, significant increases in IL-1ra, IL-6, IL-12, and TNF- $\alpha$  concentrations were observed in the same study when core temperature rose to  $39.1 \pm 0.20^\circ\text{C}$  during exercise in hot water ( $39^\circ\text{C}$ ). Interestingly, while significantly higher at the cessation of exercise, IL-1ra and IL-6 were not elevated after 20 min of hot-water exercise despite a core temperature increase from  $37.2 \pm 0.20^\circ\text{C}$  to  $38.2 \pm 0.36^\circ\text{C}$ . This suggests that a core temperature threshold has to be reached before certain cytokine concentrations will detectably increase. Thus, it is possible that the moderate hyperthermia achieved by participants in the current research ( $T_c \leq 38.5^\circ\text{C}$  in all trials), in concert with the low-impact cycling protocol, may not have been sufficient to induce a detectable cytokine response. It should also be noted that IL-8 was significantly elevated in both WomenNM and WomenOC in the HEAT trial at baseline and after resting in the heat, but not after completion of the 3-stage cycling test. It is unclear as to why this occurred, however this result is not likely to be practically meaningful as the concentrations reported upon completion of the cycling protocol were comparable to that observed in the TEMP trial.

There was no statistically significant difference in salivary cortisol between OC users and non-users ( $p = 0.97$ ), although a large effect size ( $\eta^2_p = .184$ ) indicated that cortisol concentrations were generally higher in WomenOC when compared to WomenNM. Previous studies have been highly variable when comparing resting cortisol values between eumenorrhic women and women on OC, with some showing an elevation (Boisseau et al., 2013; Timmons et al., 2005), others showing no difference

(Bonen et al., 1991; Kirschbaum et al., 1996), and one showing a decreased concentration (Reinberg et al., 1996) in OC users. However, of the three existing studies that have investigated the cortisol response of OC users to exercise (albeit in temperate conditions) (Boisseau et al., 2013; Bonen et al., 1991; Kirschbaum et al., 1996), all have observed a blunted cortisol response to exercise when compared to normally-menstruating women, which was not found in the present study. Rather, both groups reported similarly decreased salivary cortisol concentrations after the exercise protocol when compared to baseline and resting values. This is in opposition to most previous research (e.g., Niess et al., 2003; Rhind et al., 2004; Starkie et al., 2005), which reports exercise-induced increases in cortisol concentrations. While the current findings oppose the majority of previous literature, they are not entirely unprecedented; Hoffman et al. (1997) reported decreased cortisol concentrations immediately post a high-intensity cycling protocol in both temperate (22°C) and hot (35°C) conditions, and Francesconi et al. (1985) found that plasma cortisol decreased over the course of a light exercise protocol performed in a very hot environment (49°C). The authors of these studies suggest that the circadian variation in cortisol could have been at least partially responsible for the observed decrement, as there is evidence to suggest that it moderates the cortisol response to exercise (Thuma et al., 1995). Thus, it is possible that the morning testing sessions utilised in the present research coincided with a natural decline from participants' circadian peak. However, it should be noted that some earlier studies have reported increases in cortisol concentrations in response to morning exercise (Rhind et al., 2004; Starkie et al., 2005), and so the reasons behind this disparity remain unclear. Nevertheless, no differences in salivary cortisol concentrations were observed according to OC use, which was the focus of the present study. The paucity of research investigating the cortisol response to exercise in OC users, especially in hot ambient conditions, necessitates that considerably more research is conducted before the effects of OC use on the stress response can be fully understood.

Comparable immune cell counts were observed in OC users and non-users in the present study, which generally supports previous research that has found no apparent differences between OC users and non-users in resting neutrophil, monocyte, and lymphocyte counts (Timmons et al., 2005). However, this earlier study did observe higher leukocyte counts in OC users at rest (Timmons et al., 2005), whereas no differences in white blood cell counts were found between the current cohorts. The present study did observe a trend ( $p = 0.07$ ) for elevated resting CRP concentrations in WomenOC, which supports a number of previous studies that have reported elevated CRP in women on OC when compared to their normally menstruating counterparts (Cauci et al., 2008; Cauci et al., 2017; Sørensen et al., 2014; van Rooijen et al., 2006). Interestingly, a recent study found that CRP concentrations are significantly higher in the follicular phase of the menstrual cycle when compared to the luteal phase (Vashishta et al., 2017), and WomenNM in the present study were tested in the follicular phase of their cycle in order to isolate the effects of the exogenous hormones contained in the OC pill.

This cyclical variation in CRP likely reduced the magnitude of difference between WomenNM and WomenOC for this particular measure, and may help to explain why the CRP results fell short of reaching statistical significance. Importantly, however, a CRP value of  $> 1$  as observed in WomenOC indicates a moderate cardiovascular disease risk (whereas WomenNM fell into the low-risk category) (Ridker, 2003). Thus, the current results may be clinically relevant despite not being statistically different.

While the current study reflects a significant advancement in the scientific knowledge regarding OC use and immunoendocrine function, there are some limitations that should be addressed in future studies. Firstly, the timing of blood sampling (i.e., immediately post exercise) may not have captured the exercise- and heat-induced immune response in its entirety, with some cytokines (e.g., IL-1ra) showing peak concentrations  $> 1$  h after exercise (Ostrowski et al., 1998; Ostrowski et al., 1999). However, peak IL-6, IL-10, and TNF- $\alpha$  cytokine concentrations have been shown to occur immediately after exercise (Ostrowski et al., 1998; Ostrowski et al., 1999), and thus, increases in these cytokine concentrations should have been detected by the sampling protocol had they occurred. Future studies should also endeavour to use larger participant numbers to ensure all meaningful differences are captured during analysis. It is possible that the large difference in mean CRP observed in the present study was not statistically significant due to the relatively small number of subjects utilised ( $n = 9$  in each group). Lastly, given the cyclical variation that occurs across the menstrual cycle (i.e., in CRP) in eumenorrhic women, future research should include additional testing sessions at different points throughout the cycle, as well as during the inactive pill phase, in order to discern any changes that may occur.

## Conclusion

No previous studies have examined the combined effect of heat and exercise on the immunoendocrine response of OC users when compared to eumenorrhic women. WomenOC and WomenNM exhibited comparable immune (i.e., cytokines) and stress (i.e., cortisol) responses both at rest and during exercise in temperate and hot conditions. Moreover, there were no differences in resting blood and immune cell counts between WomenOC and WomenNM. However, resting CRP was more than three times higher in WomenOC compared to WomenNM, and their value of  $> 1$  indicates a moderate risk of developing cardiovascular disease. Future studies are required to provide a more comprehensive understanding of the effects of OC use (or lack thereof) on immunoendocrine function, particularly during exercise in the heat.

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### Key points

- No previous study has investigated the combined effect of heat and exercise on the immune response of women taking oral contraceptives
- Resting C-reactive protein was more than three times higher in the women taking the active oral contraceptive pills compared to naturally menstruating women during the early follicular phase
- There were no differences in resting immune cell counts between groups, nor any differences in cortisol or any of the pro- or anti-inflammatory cytokines measured at rest or after completion of the exercise trials
- With the exception of C-reactive protein, women using oral contraceptives exhibit similar immunoendocrine function to naturally menstruating women both at rest and after exercise in temperate and hot environments

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