





## ORIGINAL ARTICLE

# Hepcidin response to interval running exercise is not affected by oral contraceptive phase in endurance-trained women

Víctor M. Alfaro-Magallanes<sup>1</sup>  | Laura Barba-Moreno<sup>1</sup> | Beatriz Rael<sup>1</sup>  |  
 Nuria Romero-Parra<sup>1</sup>  | Miguel A. Rojo-Tirado<sup>1</sup>  | Pedro J. Benito<sup>1</sup>  |  
 Dorine W. Swinkels<sup>2,3</sup> | Coby M. Laarakkers<sup>2,3</sup> | Ángel E. Díaz<sup>4</sup>  | Ana B. Peinado<sup>1</sup> 

<sup>1</sup>LFE Research Group, Department of Health and Human Performance, Faculty of Physical Activity and Sport Sciences, Universidad Politécnica de Madrid (UPM), Madrid, Spain

<sup>2</sup>Department of Laboratory Medicine, Translational Metabolic Laboratory (TML 830), Radboud University Medical Center, Nijmegen, The Netherlands

<sup>3</sup>Hepcidinanalysis.com, Radboud University Medical Center, Nijmegen, The Netherlands

<sup>4</sup>Clinical Laboratory, Department of National Center of Sport Medicine, Health and Sports, AEPSAD, Madrid, Spain

## Correspondence

Beatriz Rael, LFE Research Group, Department of Health and Human Performance, Faculty of Physical Activity and Sport Science (INEF), Universidad Politécnica de Madrid, Martín Fierro 7, 28040 Madrid, Spain.  
 Email: beanad16@gmail.com

## Funding information

Ministerio de Economía y Competitividad, Grant/Award Number: DEP2016-75387-P; Universidad Politécnica de Madrid

## Abstract

The use of oral contraceptives (OCs) by female athletes may lead to improved iron status, possibly through the regulation of hepcidin by sex hormones. The present work investigates the response of hepcidin and interleukin-6 (IL-6) to an interval exercise in both phases of the OC cycle. Sixteen endurance-trained OC users (age  $25.3 \pm 4.7$  years; height  $162.4 \pm 5.7$  cm; body mass  $56.0 \pm 5.7$  kg; body fat percentage  $24.8 \pm 6.0\%$ ; peak oxygen consumption [ $VO_{2peak}$ ]:  $47.4 \pm 5.5$  mL min<sup>-1</sup> kg<sup>-1</sup>) followed an identical interval running protocol during the withdrawal and active pill phases of the OC cycle. This protocol consisted of  $8 \times 3$  minutes bouts at 85%  $VO_{2peak}$  speed with 90 seconds recovery intervals. Blood samples were collected pre-exercise, and at 0 hour, 3 hours, and 24 hours post-exercise. Pre-exercise 17 $\beta$ -estradiol was lower ( $P = .001$ ) during the active pill than the withdrawal phase ( $7.91 \pm 1.81$  vs  $29.36 \pm 6.45$  pg/mL [mean  $\pm$  SEM]). No differences were seen between the OC phases with respect to hepcidin or IL-6 concentrations, whether taking all time points together or separately. However, within the withdrawal phase, hepcidin concentrations were higher at 3 hours post-exercise ( $3.33 \pm 0.95$  nmol/L) than at pre-exercise ( $1.04 \pm 0.20$  nmol/L;  $P = .005$ ) and 0 hour post-exercise ( $1.41 \pm 0.38$  nmol/L;  $P = .045$ ). Within both OC phases, IL-6 was higher at 0 hour post-exercise than at any other time point ( $P < .05$ ). Similar trends in hepcidin and IL-6 concentrations were seen at the different time points during both OC phases. OC use led to low 17 $\beta$ -estradiol concentrations during the active pill phase but did not affect hepcidin. This does not, however, rule out estradiol affecting hepcidin levels.

## KEYWORDS

estradiol, ferritin, inflammation, interleukin-6, iron, sex hormones

Alfaro-Magallanes and Barba-Moreno contributed equally to this study

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2020 The Authors. *Scandinavian Journal of Medicine & Science In Sports* published by John Wiley & Sons Ltd

## 1 | INTRODUCTION

Oral contraceptives (OCs) are used worldwide. Nearly 70% of female athletes use hormonal contraception during their reproductive life, with 68.1% of them using OCs.<sup>1</sup> Oral contraceptive formulations have changed over time, with later generations containing much lower doses of sex hormones. Monophasic OCs, which contain a set amount of estradiol and progesterin in each pill, are the most commonly used.<sup>2</sup> Certainly, they are frequently employed by female athletes to control their menstrual cycles, reduce menstrual symptoms, and shorten menstruation, and even to improve their iron status.<sup>3</sup> By reducing menstrual bleeding, ferritin—the primary form of stored iron in cells—and iron concentrations may be enhanced in OC users compared to women with eumenorrheic cycles.<sup>4</sup>

Iron homeostasis is regulated by hepcidin, an antimicrobial peptide synthesized in the liver. Hepcidin binds, internalizes, and degrades ferroportin, the cellular iron exporter, blocking the absorption of dietary iron into the circulation, and inhibiting iron recycling macrophages and the release of body iron stores.<sup>5</sup> Estradiol and progesterone may influence hepcidin regulation. Estradiol has been reported to inhibit hepcidin,<sup>6–8</sup> potentially via an estrogen-responsive element in the hepcidin promoter.<sup>6,7</sup> In contrast, Ikeda et al<sup>9</sup> reported hepcidin to be upregulated by estradiol through the induction of bone morphogenetic protein-6, one of the main activators of the major hepcidin production pathway (BMP-SMAD).<sup>5</sup> Progesterone has been proposed to induce hepcidin production via progesterone receptor membrane component-1.<sup>10</sup> However, the above studies were all performed in animals or in women under in vitro fertilization treatment and did not take into account eumenorrheic or OC-influenced cycles. Further study is therefore needed to clarify these hormones' roles in hepcidin regulation.

Regular exercise, especially endurance training, has been associated with iron deficiency and iron deficiency anemia.<sup>11</sup> Exercise is also known to increase inflammation-responsive cytokine levels, especially that of interleukin-6 (IL-6),<sup>12</sup> a major regulator of hepcidin expression.<sup>5</sup> Time-course hepcidin and IL-6 response studies report peak serum hepcidin concentrations around 3 hours after serum IL-6 reaches a peak (usually immediately after exercise).<sup>12,13</sup> One study has examined the response of hepcidin to running exercise during the active pill and withdrawal phases of monophasic OC-induced cycles,<sup>14</sup> with no changes in hepcidin or IL-6 reported between these phases. However, sex hormone concentrations were not measured in that study, precluding observations being made on the interaction between sex hormones and hepcidin. This would be of interest since OCs have been shown to reduce endogenous estradiol concentrations during the active pill phase.<sup>15</sup> With the hypothesis that the response of hepcidin to exercise may be stronger during this phase, the

present study investigates the hepcidin and IL-6 responses to interval running exercise in relation to the estradiol and progesterone profiles of women using monophasic OCs.

## 2 | MATERIALS AND METHODS

### 2.1 | Study subjects

The study subjects were 16 healthy endurance-trained female athletes taking monophasic OCs (age  $25.3 \pm 4.7$  years; height  $162.4 \pm 5.7$  cm; body mass  $56.0 \pm 5.7$  kg; percentage body fat  $24.8 \pm 6.0\%$ ; peak oxygen consumption [ $VO_{2peak}$ ]  $47.4 \pm 5.5$  mL·min<sup>-1</sup>·kg<sup>-1</sup>). All subjects took their active hormone pill at the same time each day for 21 days (active pill phase), followed by a 7-day withdrawal (non-active pill) phase. All subjects had used OCs for the last  $4.1 \pm 4.3$  years and had practiced endurance training for  $5.8 \pm 5.0$  years, which involved  $215 \pm 170$  min/wk over the six months prior to recruitment.

The inclusion criteria required all subjects to the following: (a) be female, healthy and between 18 and 40 years of age; (b) use monophasic OCs for a minimum of six months prior to the start of the study; (c) undertake endurance training between 3 and 12 hours per week; (d) be free of iron deficiency anemia (serum ferritin < 20 µg/L, hemoglobin < 115 g/L, and transferrin saturation < 16%); (e) have healthy thyroid function; (f) be a non-smoker; (g) be taking no medication other than the OC, nor any dietary supplement (including any iron supplementation); (h) not be pregnant; and (i) have not undergone ovariectomy. To verify these conditions were met, all potential subjects completed a questionnaire and underwent blood analyses prior to acceptance.

Twenty-nine subjects were initially enrolled, but an unexpected drop out rate (scheduling problems 6; afraid of needles 2; low performance in the maximal test [ $VO_{2peak}$  below the 50th percentile for the general population for age group<sup>16</sup>] 2; inability to obtain blood samples 1; menstrual bleeding during active pill phase 1; unable to complete the exercise protocol 1) reduced the final figure to 16.

This study was approved by the Institutional Review Board of the Research Ethics Committee of the Universidad Politécnica de Madrid. Subjects were informed of the risks and benefits of the study before participation, and each signed an institutionally approved document of informed consent. This trial was registered at clinicaltrials.gov (ID: NCT04458662).

### 2.2 | Oral contraceptives

The subjects used different monophasic OC preparations during the study, providing different doses of exogenous

hormones that ranged from 0.075 to 250 mg of progestogen and from 0.02 to 35 mg of ethinylestradiol (Table S1). All presentations included seven days worth of placebo pills with no hormonal load, corresponding to the withdrawal phase (ie, days 1-7 of the cycle), and 21 days worth of exogenous hormone pills corresponding to the active pill phase (ie, days 8-28 of the cycle).

### 2.3 | Study design

All subjects visited the laboratory on three occasions over two complete OC cycles (28 days per cycle). In the first session, they were subjected to a baseline analysis (see below) during the withdrawal phase (days 3-7 of the cycle). They then performed an interval running protocol under laboratory-controlled conditions, once during the withdrawal phase (day  $4.9 \pm 1.8$ , ie, “late withdrawal” to catch peak ovarian activity) and once during the active pill phase (day  $22.1 \pm 5.0$ , ie, “late pill consumption” to catch downregulating ovarian activity and increased circulation of exogenous hormone concentrations). Previous research has shown that in combined monophasic OCs, serum ethynyl estradiol concentrations triple with OC intake over day 1-21 of the active pill phase.<sup>17,18</sup> Similarly, serum progestin concentrations quadruple, reaching a steady concentration after 8-11 days of daily OC consumption.<sup>17,18</sup> The interval running tests were conducted at the same time of day to minimize any effect of diurnal rhythm. To reduce the influence of any learning effect among the subjects, half underwent examination of the withdrawal phase first, while the other half underwent examination of the active pill phase first.

### 2.4 | Baseline analyses

Resting blood samples were taken following an overnight fast and before any morning food intake. A complete blood count and biochemical and hormone analyses were performed to verify that none of the participants showed signs of inflammation, iron deficiency, or thyroid problems. A dual-energy x-ray absorptiometry (DXA) scan was then performed to assess body composition including body fat mass (%), total body fat mass (kg), and fat-free mass (kg); this was done using a GE Lunar Prodigy apparatus (GE Healthcare). Finally, after eating and resting for a minimum of 2 hours, all subjects performed a maximal incremental test to exhaustion on an H/P/COSMOS 3PW 4.0 computerized treadmill (H/P/Cosmos Sports & Medical GmbH, Nussdorf-Traunstein, Germany) to measure  $VO_{2peak}$ . They began with a warm-up of 3 minutes at 6 km/h, thereafter increased by 0.2 km/h every 12 seconds. A slope of 1% was maintained throughout the test. Expired gases were measured breath-by-breath

using a Jaeger Oxycon Pro gas analyzer (Erich Jaeger, Viasys Healthcare). Heart response was continuously monitored using a 12-lead ECG. The  $VO_{2peak}$  was determined as the mean of the three highest  $VO_2$  measurements recorded in the incremental test to exhaustion. The maximal aerobic speed ( $vVO_{2peak}$ ) was recorded as the minimum speed required to elicit the  $VO_{2peak}$ .

### 2.5 | Interval running protocol

Participants attended the laboratory between 08.00-10.00 hour on test days to avoid the diurnal variability of hepcidin.<sup>19</sup> Nutritional recommendations were provided to all subjects by a nutritionist in order to avoid the intake of pro-inflammatory foods; these recommendations were followed by all subjects from 48 hours before the exercise protocol to 24 hours after competing it. They ate breakfast 2 hours before the test and were instructed to abstain from alcohol, caffeine, and exercise for the prior 24 hours. All subjects ate the same breakfast before testing during both OC cycle phases. Weight and blood pressure were recorded, and blood samples were collected to analyze sex hormones and iron homeostasis variables. All subjects then performed the interval running exercise, which consisted of a 5 minutes warm-up at 60%  $vVO_{2peak}$  followed by 8 bouts of 3 minutes at 85%  $vVO_{2peak}$  with 90 seconds recovery at 30%  $vVO_{2peak}$  between bouts, followed by a final 5 minutes cooling down period at 30%  $vVO_{2peak}$ . This protocol was previously reported by Sim et al<sup>20</sup> to stimulate a hepcidin response at 3 hours post-exercise. Antecubital venous blood samples were collected at 0 hour, 3 hours, and 24 hours after finishing the exercise protocol.

### 2.6 | Blood collection

All venous blood samples were obtained using a 21-gauge (0.8 mm  $\times$  19 mm, Terumo®) needle. Blood samples for complete blood counts were collected in a 3 mL K3E EDTA K3 tubes (Vacuette®) and immediately sent to the clinical laboratory of the Spanish National Centre of Sport Medicine (Madrid, Spain) for analysis. Blood samples for serum variables were collected in a 9 mL Z serum separator clot activator tubes (Vacuette®) and allowed to clot at room temperature for 60 minutes. They were then centrifuged for 10 minutes at 1610 g to obtain the serum (supernatant), divided into 600  $\mu$ L aliquots, and stored at  $-80^\circ\text{C}$ . When data collection was complete, serum samples were sent to the Spanish National Centre of Sport Medicine (Madrid, Spain) for IL-6, tumor necrosis factor alpha (TNF- $\alpha$ ), iron, ferritin, transferrin, and C-reactive protein (CRP) analyses. Pre-exercise serum samples were also analyzed for  $17\beta$ -estradiol, progesterone,

	Withdrawal phase	Active pill phase	<i>z</i>	<i>P</i>	<i>r</i>
17 $\beta$ -Estradiol (pg/mL)	29.36 $\pm$ 6.45	7.91 $\pm$ 1.81*	-3.180	.001	-.56
Progesterone (ng/mL)	0.33 $\pm$ 0.05	0.32 $\pm$ 0.03	-0.465	.642	-.08
LH (mIU/mL)	4.17 $\pm$ 1.14	1.79 $\pm$ 0.70*	-2.981	.03	-.53
FSH (mIU/mL)	5.22 $\pm$ 1.05	1.51 $\pm$ 0.42*	-3.233	<.001	-.57
Prolactin (mIU/L)	418.68 $\pm$ 54.35	501.09 $\pm$ 94.57	-1.241	.215	-.22
TSH ( $\mu$ IU/mL)	2.63 $\pm$ 0.29	2.96 $\pm$ 0.35	-1.526	.127	-.27

Note: Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone; TSH, thyroid-stimulating hormone.

\*Significantly different from withdrawal phase.

luteinizing hormone (LH), and follicle-stimulating hormone (FSH). Duplicate serum samples were sent to the Department of Laboratory Medicine at Radboud University Medical Centre (Hepcidinanalysis.com, Nijmegen, The Netherlands) for hepcidin-25 analysis.

## 2.7 | Blood analysis

Samples were allowed to defrost at room temperature and homogenized in a vortex. Serum iron content was determined using colorimetry in the visible range, while ferritin, transferrin, and CRP were analyzed by turbidimetry. Both colorimetry and turbidimetry were conducted using an AU400 clinical analyzer (Beckman Coulter) with Beckman Coulter reagents. IL-6, 17 $\beta$ -estradiol, progesterone, FSH, and LH were determined by electrochemiluminescent immunoassay using Roche Diagnostics reagents in a Cobas E411 analyzer (Roche Diagnostics GmbH). TNF- $\alpha$  was determined using the IMMULITE<sup>®</sup> 1000 Immunoassay System (Siemens Healthineers, Malvern). Standard control samples were measured after calibration and after each analysis batch to ensure the continued accuracy of the results. Table S2 shows the inter- and intra-assay coefficients of variation (CV) for each variable.

Serum hepcidin was determined using a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (WCX-TOF MS) using a stable hepcidin-25<sup>+40</sup> isotope (a secondary reference material<sup>21</sup> as internal standard for quantification).<sup>22</sup> Peptide spectra were generated using a Microflex LT matrix-enhanced laser desorption/ionization TOF MS platform (Bruker Daltonics). Hepcidin-25 concentrations were expressed in nmol/L [nM]. The lower limit of quantification using this method was 0.5 nM. Inter-assay CVs for hepcidin were 4.6% at the 11.0 nM level and 8.3% at the 2.7 nM level. Reference values can be found at <http://www.hepcidinanalysis.com/provided-servi>

**TABLE 1** Resting serum concentrations of hormones related to the oral contraceptive cycle (mean  $\pm$  SEM)

ce/reference-values (accessed 22 April 2020). Reference levels for the WCX-TOF MS method are recalculated from those of our ELISA method,<sup>22</sup> based on the regression line: (ELISA-1.00)/1.52 = WCX-TOF MS that was derived from the results obtained by both methods for the same samples without hepcidin isoforms. All values were determined using secondary reference material for hepcidin assays, the value of which was assigned relative to the primary reference material, allowing traceability to the internationally recognized Système International.<sup>21</sup>

## 2.8 | Statistical analysis

Data are expressed as the mean  $\pm$  standard error of the mean ( $\pm$ SEM). The Shapiro-Wilk test was used to assess the normality of the variables. The non-parametric Friedman ANOVA test was used to analyze differences between time points (pre-exercise, 0 hour post-exercise, 3 hours post-exercise, and 24 hours post-exercise). The Wilcoxon signed-rank test was used to compare differences in hepcidin, IL-6, TNF- $\alpha$ , ferritin, iron, transferrin, and CRP concentrations between the withdrawal phase and the active pill phase. The same test was used to measure differences between OC cycle phases in terms of 17 $\beta$ -estradiol, progesterone, LH, and FSH concentrations. Effect sizes (*r*) were calculated and set to small ( $\geq 0.1$  and  $< 0.3$ ), moderate ( $\geq 0.3$  and  $< 0.5$ ), and large ( $\geq 0.5$ ).<sup>23</sup> Significance was set at *P* < .05. All calculations were made using SPSS software v.25 (IBM Corp.,).

## 3 | RESULTS

As expected, sex hormone concentrations (17 $\beta$ -estradiol, LH, and FSH) were significantly lower (Table 1 for *P* and *r* values) during the active pill phase of the OC cycle compared with the withdrawal phase.

No significant differences were seen in hepcidin, IL-6, CRP, or ferritin between the OC phases, although hepcidin and CRP showed a trend toward being higher in the withdrawal and active pill phase respectively. In contrast, significant differences were detected for TNF- $\alpha$ , iron, and transferrin, the former two being higher and the last being lower during the active phase (Table 2 for  $P$  and  $r$  values).

No significant differences were seen between the OC phases at each time point (baseline, 0 hour, 3 hours, and 24 hours post-exercise) for hepcidin (Figure 1A), IL-6 (Figure 1B), CRP (Figure 1D) or ferritin (Figure 2B). However, the hepcidin concentration showed a trend toward being higher in the baseline samples of the withdrawal phase ( $z = -1.863$ ;  $P = .063$ ,  $r = -.33$ ). Baseline TNF- $\alpha$  concentrations were significantly higher during the active phase than the withdrawal phase ( $z = -2.312$ ;  $P = .021$ ,  $r = -.41$ ) (Figure 1C), a difference that approached significance 3 hours after exercise as well ( $z = -1.915$ ;  $P = .056$ ,  $r = -.34$ ). Serum iron was higher at all time points during the active pill phase than during the withdrawal phase: pre-exercise ( $z = -2.223$ ;  $P = .026$ ,  $r = -.39$ ), 0 hour ( $z = -1.965$ ;  $P = .049$ ,  $r = -.35$ ), 3 hours ( $z = -1.862$ ;  $P = .063$ ,  $r = -.33$ ) and 24 hours post-exercise ( $z = -2.442$ ;  $P = .015$ ,  $r = -.43$ ) (Figure 2A). In contrast, transferrin concentrations were significantly lower during the active pill phase than the withdrawal phase at pre-exercise ( $z = -2.172$ ;  $P = .03$ ,  $r = -.38$ ), 0 hour ( $z = -2.690$ ;  $P = .007$ ,  $r = -.48$ ), and at 3 hours ( $z = -2.121$ ;  $P = .034$ ,  $r = -.38$ ) and 24 hours post-exercise ( $z = -2.386$ ;  $P = .017$ ,  $r = -.42$ ) (Figure 2C).

Taking the results for the two phases together, hepcidin ( $\chi^2 = 35.500$ ;  $P < .001$ ), interleukin-6 ( $\chi^2 = 66.675$ ;  $P < .001$ ), TNF- $\alpha$  ( $\chi^2 = 13.641$ ;  $P = .003$ ), CRP ( $\chi^2 = 9.391$ ;  $P = .025$ ), iron ( $\chi^2 = 10.837$ ,  $P = .013$ ), transferrin ( $\chi^2 = 23.530$ ,  $P < .001$ ), and ferritin ( $\chi^2 = 21.059$ ,  $P < .001$ ) showed significant increases at different times over the study period, revealing an effect of time of measurement. Table S3 shows the results of pairwise comparisons.

The time of measurement also influenced the hepcidin result (Figure 1A) during both the withdrawal ( $\chi^2 = 20.333$ ,

$P < .001$ ) and active pill phases ( $\chi^2 = 15.370$ ,  $P = .02$ ). However, post-hoc pairwise comparisons showed the hepcidin concentration to be significantly higher at only 3 hours post-exercise compared with pre-exercise ( $P = .005$ ,  $r = -.59$ ) and 0 hour post-exercise ( $P = .045$ ,  $r = -.47$ ) during the withdrawal phase. In the active pill phase, a trend toward a higher hepcidin concentration was seen at 3 hours post-exercise compared with pre-exercise ( $P = .068$ ). IL-6 showed the same pattern in both phases (withdrawal:  $\chi^2 = 31.879$ ,  $P < .001$ ; active pill:  $\chi^2 = 34.971$ ,  $P < .001$ , Figure 1B), with higher concentrations at 0 hour post-exercise than at pre-exercise (withdrawal:  $P = .001$ ,  $r = -.65$ ; active pill:  $P = .002$ ,  $r = -.64$ ), 3 hours (withdrawal:  $P = .003$ ,  $r = .62$ ; active pill:  $P = .001$ ,  $r = 0.67$ ), and 24 hours post-exercise (withdrawal:  $P < .001$ ,  $r = .71$ ; active pill:  $P < .001$ ,  $r = -.73$ ), as did ferritin (withdrawal:  $\chi^2 = 9.444$ ,  $P = .024$ ; active pill:  $\chi^2 = 12.333$ ,  $P = .006$ , Figure 2B), with higher concentrations at 0 hour post-exercise than pre-exercise (withdrawal:  $P = .022$ ,  $r = -.51$ ; active pill:  $P = .010$ ,  $r = -.56$ ). Only during the withdrawal phase did TNF- $\alpha$  concentrations show significant differences over the time-course ( $\chi^2 = 12.402$ ,  $P = .006$ ; Figure 1C), being higher at 0 hour post-exercise compared with pre-exercise ( $P = .016$ ,  $r = -.53$ ). In contrast, transferrin concentrations were significantly different only in the active pill phase ( $\chi^2 = 23.058$ ,  $P < .001$ ; Figure 2C), being higher at 0 hour post-exercise than at pre-exercise ( $P = .002$ ,  $r = -.63$ ) and 24 hour post-exercise ( $P < .001$ ,  $r = .76$ ). No differences were seen in CRP or iron concentrations between any time points (Figures 1D and 2A, respectively) in either the withdrawal or active pill phase.

## 4 | DISCUSSION

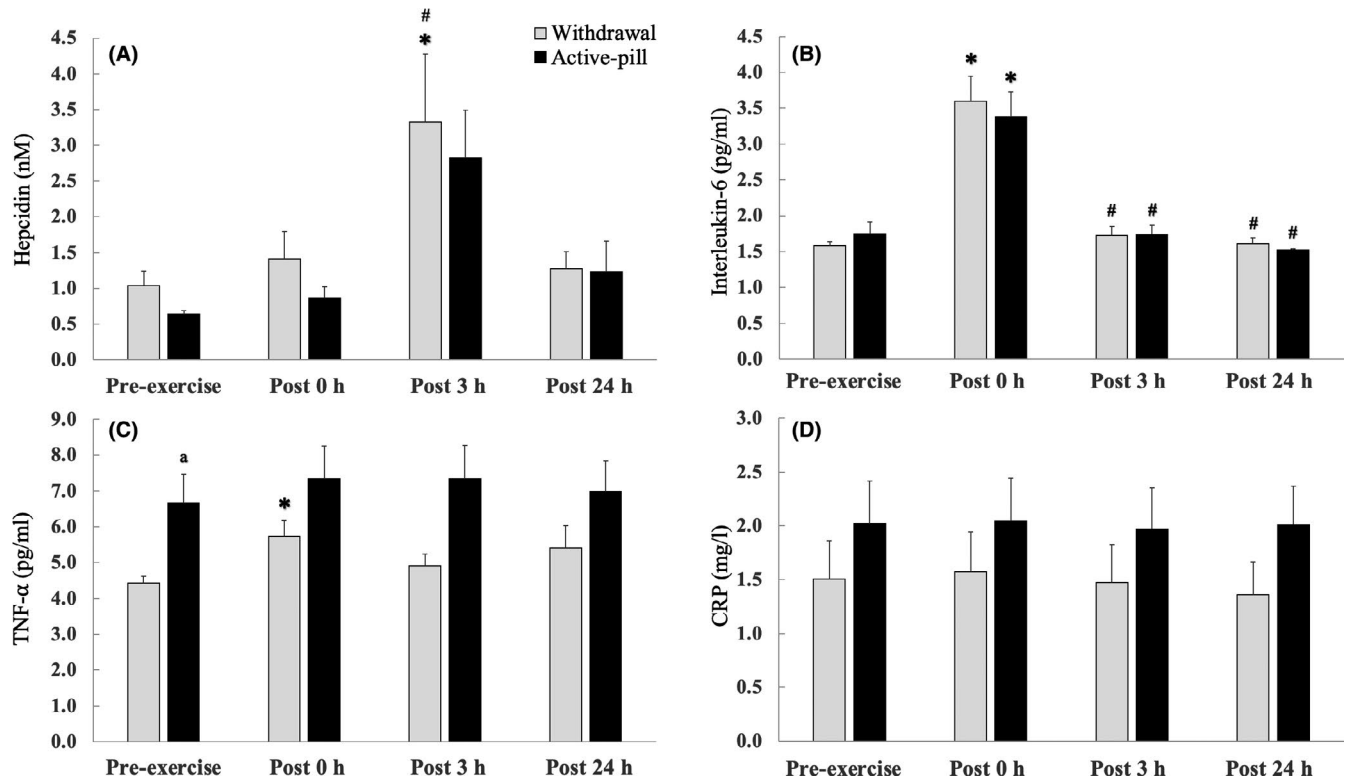
The present results show that low 17 $\beta$ -estradiol concentrations induced by OCs likely explain the previously reported<sup>14</sup> lack of difference between the phases of the OC cycle in terms of the response of hepcidin to running exercise—an

**TABLE 2** Iron physiology and inflammation variables in each oral contraceptive cycle phase (mean  $\pm$  SEM)

	Withdrawal phase	Active pill phase	$z$	$P$	$r$
Hepcidin (nM)	1.76 $\pm$ 0.56	1.39 $\pm$ 0.44	-1.886	.059	-.17
Interleukin-6 (pg/mL)	2.13 $\pm$ 0.26	2.10 $\pm$ 0.26	-0.551	.581	-.05
TNF- $\alpha$ (pg/mL)	5.12 $\pm$ 0.42	7.08 $\pm$ 0.86*	-3.339	.001	-.3
CRP (mg/L)	1.48 $\pm$ 0.34	2.01 $\pm$ 0.37	-1.770	.077	-.16
Iron ( $\mu$ g/dL)	105.68 $\pm$ 11.09	131.54 $\pm$ 10.29*	-4.094	<.001	-.36
Ferritin (ng/mL)	28.15 $\pm$ 3.33	28.13 $\pm$ 3.39	-0.212	.832	-.02
Transferrin (mg/mL)	346.33 $\pm$ 13.20	328.17 $\pm$ 11.94*	-4.516	<.001	-.4

Note: Abbreviations: CRP, C-reactive protein; TNF- $\alpha$ , tumor necrosis factor alpha.

\*Significantly different from withdrawal phase.



**FIGURE 1** A, Serum hepcidin, B, serum IL-6, C, serum TNF- $\alpha$ , and D, serum CRP concentrations (mean  $\pm$  SEM) in the withdrawal and active pill phases at specific times before and after exercise. \*Significantly different from pre-exercise. #Significantly different from 0 h post-exercise. <sup>a</sup>Significantly different from withdrawal phase. Abbreviations: Post 0 h, 0 h post-exercise; Post 3 h, 3 h post-exercise; Post 24 h, 24 h post-exercise; CRP, C-reactive protein; TNF- $\alpha$ , tumor necrosis factor alpha

observation previously proposed due to estradiol having no effect on hepcidin concentrations in humans.

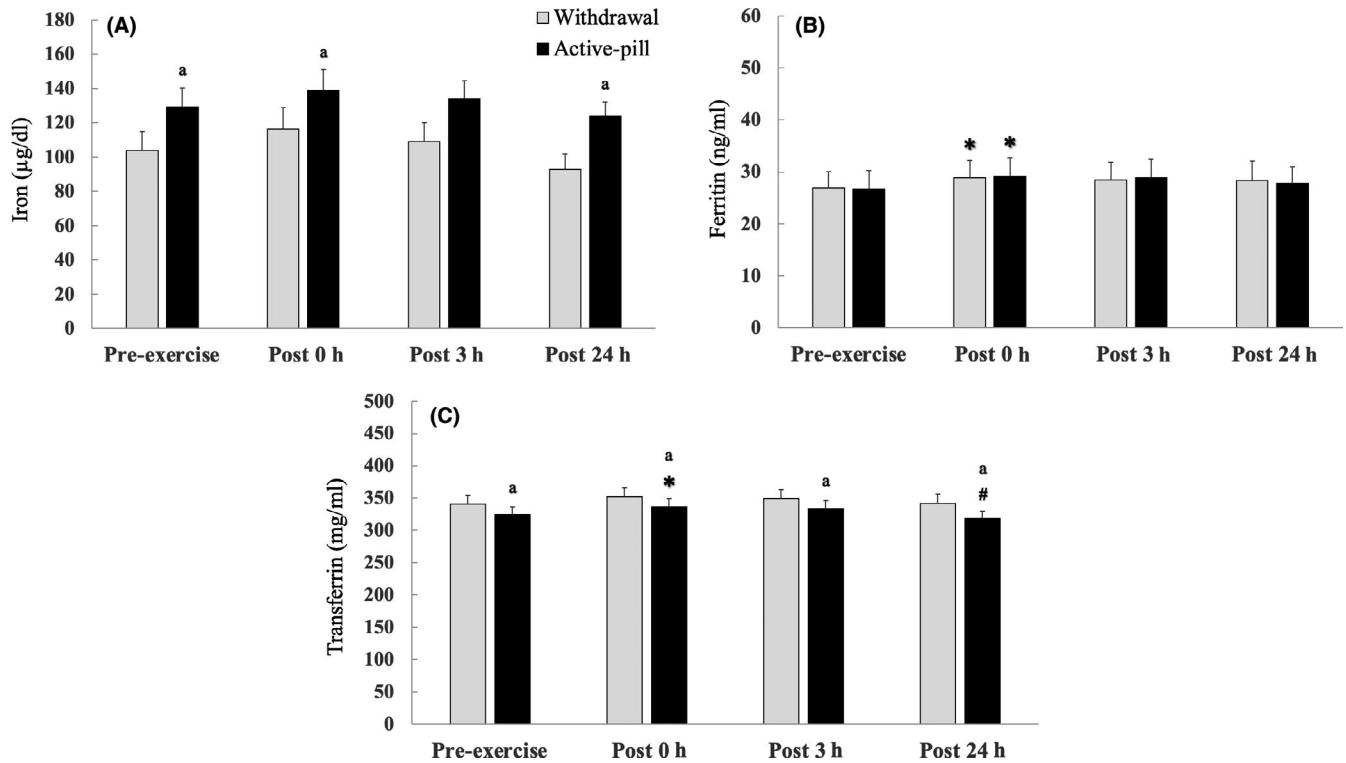
The present hormone analysis results confirm that the subjects' OC consumption was adequate; endogenous estradiol and progesterone synthesis were therefore suppressed. The hepcidin concentrations showed a trend toward lower concentrations during the active pill phase. Higher hepcidin concentrations were observed during the withdrawal phase when estradiol was higher than during the active pill phase. In support of this, Ikeda et al<sup>9</sup> reported ovariectomized mice to show lower hepcidin concentrations than sham-operated littermates. Interestingly, hepcidin concentrations in the ovariectomized mice were restored after estradiol supplementation. However, results from in vivo studies in mice<sup>6,7</sup> and on human liver cells<sup>6</sup> indicate that 17 $\beta$ -estradiol treatment inhibits hepcidin mRNA expression. Lehtihet et al<sup>8</sup> found that gonadotropin-stimulated endogenous estradiol noticeably reduced circulating hepcidin in women receiving treatment for in vitro fertilization. However, the estradiol concentrations in the women of the latter study were approximately 130 times the highest concentrations found in the present subjects, and 20 times those commonly seen in a natural menstrual cycle.

Earlier studies on human OC users reported no differences between OC phases for either hepcidin or IL-6, either when at rest<sup>14,24</sup> or after performing a running exercise.<sup>14</sup> While the

present results are in agreement with this, the latter authors' arguments indicate a supposed increase in estradiol concentration during the active pill phase had no effect on the above variables. However, in the present work, the estradiol concentration during the active pill phase was low. Clearly, this cannot preclude that higher estradiol concentrations would have no effect. Indeed, the present and other authors' results<sup>15</sup> show that 17  $\beta$ -estradiol concentrations are lower during the active pill phase than during the late withdrawal phase, and similar during the early withdrawal phase. In fact, serum hormone concentrations were not measured in the above earlier work,<sup>14,24</sup> yet the authors concluded that any downregulation of hepcidin via estradiol in animal models might not apply in the typical post-exercise response in humans.

IL-6 production has also been reported inhibited by 17 $\beta$ -estradiol in vitro<sup>25</sup> and in vivo in animals.<sup>26</sup> However, IL-6 was also previously reported to be unaffected by low 17  $\beta$ -estradiol concentrations, in agreement with studies showing neither resting IL-6<sup>24</sup> nor post-exercise IL-6<sup>14</sup> to be affected.

The present time-course results for both hepcidin and IL-6 after exercise are consistent with those of many other studies performed in men and women.<sup>13</sup> In the present work, a peak in IL-6 concentrations was observed at 0 hour post-exercise, while 3 hours later a significant increase in hepcidin was



**FIGURE 2** A, Serum iron, B, serum ferritin, and C, serum transferrin concentrations (mean  $\pm$  SEM) in the withdrawal and active pill phases at specific times before and after exercise. <sup>#</sup>Significantly different from 0 h post-exercise. <sup>a</sup>Significantly different from withdrawal phase. Abbreviations: Post 0 h, 0 h post-exercise; Post 3 h, 3 h post-exercise; Post 24 h, 24 h post-exercise; CRP, C-reactive protein; TNF- $\alpha$ , tumor necrosis factor alpha

seen—a response similar in both the withdrawal and active pill phases. Despite the hormonal differences between these phases, IL-6 increased after exercise, which may explain the lack of difference seen for hepcidin between the two phases.

Significantly higher concentrations of TNF- $\alpha$  were observed during the active pill phase, accompanied with a trend toward greater CRP concentrations. This may indicate that, during the active pill phase, an inflammatory state is present. Several studies have shown that the administration of a physiological dose of estrogen to immature or ovariectomized rodents induces an acute inflammatory-like response.<sup>27–29</sup> CRP is a well-known general marker of pathological processes including infection, tissue damage, cancer, and chronic inflammatory disease.<sup>30</sup> Further, TNF- $\alpha$  is a proinflammatory cytokine involved in the regulation of tissue homeostasis affecting cell proliferation, differentiation, and death.<sup>31</sup> It has also been reported that serum CRP increases during treatment with combined OCs.<sup>32</sup> This may be the result of increased CRP synthesis by hepatocytes rather than an effect of IL-6- or TNF- $\alpha$ -mediated inflammation. In another study,<sup>33</sup> a high prevalence of low-grade inflammation was detected among premenopausal women who used combined OCs, and it was therefore suggested that such usage might lead to an elevated inflammatory profile. However, none of the aforementioned studies compared proinflammatory variables in women with different hormonal profiles, or during the different phases

of a combined OC cycle. Therefore, even though proinflammatory markers were higher (or showed a trend toward this) in the active pill phase compared to the withdrawal phase, it cannot be definitely concluded that the exogenous hormones present during the active pill phase induce a stronger inflammatory state.

In the present work, TNF- $\alpha$  was elevated at 0 hour post-exercise in the withdrawal phase. This agrees with the results of Peake et al<sup>34</sup> who reported a slight increase after similar exercise. However, this finding does not necessarily imply increased inflammation; the TNF- $\alpha$  concentrations detected in the current study may be compatible with a non-inflammatory state since the CRP and IL-6 patterns over time do not correspond to inflammation produced by exercise as reported in the literature.<sup>12</sup>

Ferritin also influences plasma hepcidin. Interestingly, plasma ferritin before exercise influences the hepcidin response 3 hours post-exercise, which becomes stronger the higher the pre-exercise ferritin concentration.<sup>35</sup> However, in the present study, ferritin concentrations were similar in both OC phases, as previously reported by Sim et al<sup>14</sup> and could not, therefore, have unduly influenced the post-exercise hepcidin response observed in the present subjects. However, the present subjects show lower concentrations of iron during the withdrawal phase compared to the active pill phase, and the opposite with respect to transferrin.

Transferrin is reduced under inflammation and iron overload conditions and increases in iron-deficient situations.<sup>36</sup> Therefore, during the withdrawal phase, the liver may increase circulating transferrin in an attempt to bind more iron and thus promote its transport to iron-demanding cells. This may be related to the occurrence of menstrual bleeding during this phase. Despite menstrual losses being smaller in OC cycles than eumenorrheic cycles,<sup>37</sup> lower plasma iron concentrations are still observed during menses. In addition, the present results showed a trend for hepcidin concentrations to be higher at baseline than during the withdrawal phase, which along with menstrual bleeding may reduce the availability of iron in plasma during that phase.

The present work takes into consideration the main hormonal fluctuations during an OC cycle via biomarker verification, as suggested in the literature.<sup>38,39</sup> The exercise protocol chosen may be similar to a daily training session, making results more applicable to the athletes' day to day. Further, the authors excluded women with iron deficiency anemia rather than women with iron-depleted stores in order to more closely resemble the population of physically active women.<sup>40</sup> However, the fact that some of the present subjects showed iron depletion in terms of their ferritin status (<35 µg/L)<sup>40</sup> might have affected the hepcidin response at 3 hours post-exercise, and have masked the potential effects of OC on hepcidin. This potential problem is a concern since many active women have low iron stores,<sup>40</sup> which could limit the response of hepcidin to exercise. Future research should examine other variables related to erythropoiesis and hypoxia, such as circulating erythropoietin, erythroferrone, and hemoglobin levels, among others. Additional measurement time points close to and after the hepcidin peak would also more accurately record the effects of the hepcidin response to exercise on plasma iron. Finally, despite the severity of menstrual bleeding being reduced in OC users, the amount of blood lost should be examined for a clearer picture of the regulation of iron homeostasis in this population. Understanding these variables would add valuable information on hepcidin behavior at rest and in response to exercise in the different phases of the OC. Finally, future studies should measure further inflammation markers in the different phases of the OC cycle.

## 5 | CONCLUSION

An interval running exercise performed in the withdrawal and active pill phases of a monophasic OC cycle was associated with similar increases in IL-6 at 0 hour post-exercise, and in the size of the consequent peak of hepcidin recorded at 3 hours post-exercise. Therefore, a similar iron absorption is expectable during the two OC phases after exercise. Low

endogenous sex hormone concentrations induced by use of monophasic OCs do not appear to affect hepcidin.

## 6 | PERSPECTIVE

The role of estradiol as a modulator of hepcidin<sup>6-8</sup> may offer therapeutic advantages in improving iron status. Taking advantage of estradiol supplementation may help improve iron status in female athletes, who commonly have depleted iron stores. Earlier research investigating the effect of OC use on hepcidin<sup>14,24</sup> suggests that any downregulation of the latter via estradiol in animal models may not be mirrored in the typical post-exercise response in humans. The present study shows that OCs do not influence the response of hepcidin to exercise—but this is due to the low estradiol concentrations seen during OCs consumption, and not to a lack of effect of estradiol on hepcidin regulation. Further work should take into account hormonal environments with higher estradiol concentrations, such as in the eumenorrheic cycle or those induced by doses of exogenous hormone supplementation higher than those provided by OCs. The latter would help elucidate the potential effects of estradiol on hepcidin and its response to exercise.

## ACKNOWLEDGEMENTS

The authors thank the subjects who volunteered for this study, as well the students who helped in data collection. Special thanks are owed to Marcos Bodoque-Barbero for his commitment during data collection. Finally, we would like to thank the rest of the members of the IronFEMME Study group for their input: Javier Butragueño-Revenga, Jorge Coucerio-Canalejo, Rocío Cupeiro-Coto, Eliane Aparecida-De Castro, Alberto García-Bataller, Antonio García de Alcaraz-Serrano, Cristina Maestre-Cascales, Carmen Patricia Ortega-Santos, Jesús Javier Rojo-González, Francisco Javier Calderón Montero, and Elena Santiago-Romero. VMAM and NRP were supported grants provided by the Universidad Politécnica de Madrid. The IronFEMME Study is funded by the Spanish Ministerio de Economía y Competitividad, Convocatoria de ayudas I + D 2016, Plan Estatal de Investigación Científica y Técnica y de Innovación 2013-2016 (Contract DEP2016-75387-P).

## CONFLICT OF INTEREST

None to declare.

## AUTHOR CONTRIBUTIONS

VMAM, LBM, and ABP made substantial contributions to the conception and design of the study. VMAM, NRP, BRD, and MRT collected the data. VMAM, NRP, AED, DS, and CML performed the laboratory analyses. VMAM, LBM, ABP, and PJB interpreted the data. VMAM and LBM wrote




the initial draft. All authors critically reviewed the content and approved the final version.


## ORCID

Victor M. Alfaro-Magallanes  <https://orcid.org/0000-0002-0456-1222>

Beatriz Rael  <https://orcid.org/0000-0002-9331-4564>

Nuria Romero-Parra  <https://orcid.org/0000-0001-9754-5565>

Miguel A. Rojo-Tirado  <https://orcid.org/0000-0003-3217-7786>

Pedro J. Benito  <https://orcid.org/0000-0002-1631-8182>

Ángel E. Díaz  <https://orcid.org/0000-0002-6215-0093>

Ana B. Peinado  <https://orcid.org/0000-0002-4871-8682>

## REFERENCES

- Martin D, Sale C, Cooper SB, Elliott-Sale KJ. Period prevalence and perceived side effects of hormonal contraceptive use and the menstrual cycle in elite athletes. *Int J Sports Physiol Perform.* 2018;13:926-932.
- Rechichi C, Dawson B, Goodman C. Athletic performance and the oral contraceptive. *Int J Sports Physiol Perform.* 2009;4:151-162.
- Frassinelli-Gunderson EP, Margen S, Brown JR. Iron stores in users of oral contraceptive agents. *Am J Clin Nutr.* 1985;41:703-712.
- Milman N, Kirchhoff M, Jørgensen T. Iron status markers, serum ferritin and hemoglobin in 1359 Danish women in relation to menstruation, hormonal contraception, parity, and postmenopausal hormone treatment. *Ann Hematol.* 1992;65:96-102.
- Xiao X, Alfaro-Magallanes VM, Babbitt JL. Bone morphogenic proteins in iron homeostasis. *Bone.* 2020;138:115495.
- Yang Q, Jian J, Katz S, Abramson SB, Huang X. 17 $\beta$ -Estradiol inhibits iron hormone hepcidin through an estrogen responsive element half-site. *Endocrinology.* 2012;153:3170-3178.
- Hou Y, Zhang S, Wang L, et al. Estrogen regulates iron homeostasis through governing hepatic hepcidin expression via an estrogen response element. *Gene.* 2012;511:398-403.
- Lehtihet M, Bonde Y, Beckman L, et al. Circulating Hepcidin-25 is reduced by endogenous estrogen in humans. *PLoS One.* 2016;11:e0148802.
- Ikeda Y, Tajima S, Izawa-Ishizawa Y, et al. Estrogen regulates hepcidin expression via gpr30-bmp6-dependent signaling in hepatocytes (C Hermenegildo, Ed.). *PLoS One.* 2012;7:e40465.
- Li X, Rhee DK, Malhotra R, et al. Progesterone receptor membrane component-1 regulates hepcidin biosynthesis. *J Clin Invest.* 2015;126:389-401.
- Peeling P, Dawson B, Goodman C, Landers G, Trinder D. Athletic induced iron deficiency: new insights into the role of inflammation, cytokines and hormones. *Eur J Appl Physiol.* 2008;103:381-391.
- Pedersen BK, Febbraio MA. Muscle as an endocrine organ: focus on muscle-derived interleukin-6. *Physiol Rev.* 2008;88:1379-1406.
- Domínguez R, Sánchez-Oliver AJ, Mata-Ordoñez F, et al. Effects of an acute exercise bout on serum hepcidin levels. *Nutrients.* 2018;10:1-22.
- Sim M, Dawson B, Landers G, et al. Oral contraception does not alter typical post-exercise interleukin-6 and hepcidin levels in females. *J Sci Med Sport.* 2015;18:8-12.
- Rechichi C, Dawson B. Oral contraceptive cycle phase does not affect 200-m swim time trial performance. *J Strength Cond Res.* 2012;26:961-967.
- Kaminsky LA, Arena R, Myers J. Reference standards for cardiorespiratory fitness measured with cardiopulmonary exercise testing: data from the fitness registry and the importance of exercise national database. *Mayo Clin Proc.* 2015;90:1515-1523.
- Blode H, Kowal K, Roth K, Reif S. Pharmacokinetics of drospirenone and ethinylestradiol in Caucasian and Japanese women. *Eur J Contracept Reprod Heal Care.* 2012;17:284-297.
- Endrikat J, Blode H, Gerlinger C, Rosenbaum P, Kuhn W. A pharmacokinetic study with a low-dose oral contraceptive containing 20 microg ethinylestradiol plus 100 microg levonorgestrel. *Eur J Contracept Reprod Heal Care.* 2002;7:79-90.
- Troutt JS, Rudling M, Persson L, et al. Circulating Human hepcidin-25 concentrations display a diurnal rhythm, increase with prolonged fasting, and are reduced by growth hormone administration. *Clin Chem.* 2012;58:1225-1232.
- Sim M, Dawson B, Landers G, et al. Effect of exercise modality and intensity on postexercise interleukin-6 and hepcidin levels. *Int J Sport Nutr Exerc Metab.* 2013;23:178-186.
- Diepeveen LE, Laarakkers CMM, Martos G, et al. Provisional standardization of hepcidin assays: creating a traceability chain with a primary reference material, candidate reference method and a commutable secondary reference material. *Clin Chem Lab Med.* 2019;57:864-872.
- Laarakkers CMM, Wiegerinck ET, Klaver S, et al. Improved mass spectrometry assay for plasma hepcidin: detection and characterization of a novel hepcidin isoform (HS Randeva, Ed.). *PLoS One.* 2013;8:e75518.
- Cohen J. *Statistical power analysis for the behavioral sciences.* Erlbaum Associates: Hillsdale, NJ; 1988:40.
- Sim M, Dawson B, Landers G, et al. Interleukin-6 and hepcidin levels during hormone-deplete and hormone-replete phases of an oral contraceptive cycle: a pilot study. *Ann Nutr Metab.* 2017;70:100-105.
- Pottratz ST, Bellido T, Mocharla H, Crabb D, Manolagas SC. 17 beta-Estradiol inhibits expression of human interleukin-6 promoter-reporter constructs by a receptor-dependent mechanism. *J Clin Invest.* 1994;93:944-950.
- Jilka R, Hangoc G, Girasole G, et al. Increased osteoclast development after estrogen loss: mediation by interleukin-6. *Science (80-).* 1992;257:88-91.
- Hunt JS. Immunologically relevant cells in the uterus1. *Biol Reprod.* 1994;50:461-466.
- Kaushic C, Frauendorf E, Rossoll RM, Richardson JM, Wira CR. Influence of the estrous cycle on the presence and distribution of immune cells in the rat reproductive tract. *Am J Reprod Immunol.* 1998;39:209-216.
- Russo LA, Peano BJ, Trivedi SP, et al. Regulated expression of matrix metalloproteinases, inflammatory mediators, and endometrial matrix remodeling by 17beta-estradiol in the immature rat uterus. *Reprod Biol Endocrinol.* 2009;7:124.
- Pepys MB, Hirschfield GM. C-reactive protein: a critical update. *J Clin Invest.* 2003;111:1805-1812.
- Zaldivar V, Magri ML, Zárate S, et al. Estradiol increases the expression of TNF- $\alpha$  and TNF receptor 1 in lactotropes. *Neuroendocrinology.* 2011;93:106-113.
- Van Rooijen M, Hansson LO, Frostegård J, Silveira A, Hamsten A, Bremme K. Treatment with combined oral contraceptives induces

- a rise in serum C-reactive protein in the absence of a general inflammatory response. *J Thromb Haemost.* 2006;4:77-82.
33. Sørensen CJ, Pedersen OB, Petersen MS, et al. Combined oral contraception and obesity are strong predictors of low-grade inflammation in healthy individuals: results from the Danish Blood Donor Study (DBDS). *PLoS One.* 2014;9:e88196.
  34. Peake JM, Della Gatta P, Suzuki K, Nieman DC. Cytokine expression and secretion by skeletal muscle cells: regulatory mechanisms and exercise effects. *Exerc Immunol Rev.* 2015;21:8-25.
  35. Peeling P, Sim M, Badenhorst CE, et al. Iron status and the acute post-exercise hepcidin response in athletes. *PLoS One.* 2014;9:e93002.
  36. Morton AG, Tavill AS. The role of iron in the regulation of hepatic transferrin synthesis. *Br J Haematol.* 1977;36:383-394.
  37. Weisberg E, McGeehan K, Hangan J, Fraser IS. Potentially effective therapy of heavy menstrual bleeding with an oestradiol-nomegestrol acetate oral contraceptive: a pilot study. *Pilot Feasibility Stud.* 2017;3:1-7.
  38. Janse de Jonge XAK, Thompson B, Han A. Methodological recommendations for menstrual cycle research in sports and exercise. *Med Sci Sport Exerc.* 2019;51:2610-2617.
  39. Schaumberg MA, Jenkins DG, Janse de Jonge XAK, Emmerton LM, Skinner TL. Three-step method for menstrual and oral contraceptive cycle verification. *J Sci Med Sport.* 2017;20:965-969.
  40. Sim M, Garvican-Lewis LA, Cox GR, et al. Iron considerations for the athlete: a narrative review. *Eur J Appl Physiol.* 2019;119:1463-1478.

## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

**How to cite this article:** Alfaro-Magallanes VM, Barba-Moreno L, Rael B, et al. Hepcidin response to interval running exercise is not affected by oral contraceptive phase in endurance-trained women. *Scand J Med Sci Sport.* 2021;31:643–652. <https://doi.org/10.1111/sms.13894>