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Full Length Article

# Polymorphonuclear leucocyte phagocytic function, γδ T-lymphocytes and testosterone as separate stress-responsive markers of prolonged, high-intensity training programs

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ABSTRACT

Excessive exercise with limited recovery may lead to detrimental states of overreaching or the overtraining syndrome. Chronic maladaptation in endocrine and immune mechanisms occur with the incidence of these states. Exercise-induced cortisol and testosterone responses have been proposed as biomarkers of overreaching, with blunted responses following intensified-training periods. Yet, limited information on the effects of overreaching in immunity is available. Healthy individuals completed a 30-min running protocol (the $RPE_{TP}$) before and after a 12-day intensified-training period. Blood and saliva were collected before, after and 30min after $RPE_{TP}$ at pre-training and post-training. Plasma and salivary cortisol and testosterone, leucocyte proliferation and polymorphonuclear leucocyte phagocytic activity were examined. Plasma and salivary cortisol were acutely unaffected pre-training (−14% and 0%, $p > 0.05$) and post-training (−14% and +46%, $p > 0.05$). Comparing pre-training with post-training, blunted responses were observed in plasma testosterone (43%–19%, $p < 0.05$) and salivary testosterone (55%–24%, $p > 0.05$). No acute or resting changes in total leucocyte counts or most leucocyte subsets occurred pre-training or post-training. Yet, a 194% acute elevation in γδ T-lymphocyte number occurred pre-training ($p < 0.05$), and average resting concentrations were 174% higher post-training. Baseline phagocytic activity was 47% lower post-training ($p < 0.05$). Intensified training was detrimental, significantly reducing phagocytic activity. Testosterone blunted post-training, indicating an excessive training-related hypothalamic-pituitary gonadal dysfunction. The γδ T-lymphocytes sensitivity to exercise was noted, rendering it as a potential stress-responsive cellular marker. The usefulness of the $RPE_{TP}$ to track the onset of overreaching is proposed.

## 1. Introduction

Excessive exercise training with limited recovery is normally associated with maladaptation, which has been reported to occur within a period as short as 7 days (Halson et al., 2002). Recovery from this may take days to weeks [referred to as functional overreaching (FOR)], weeks to months [non-functional overreaching (NFOR)], or even years [the overtraining syndrome (OTS)]. The incidence of these states has been reported to be between 60-64% in both males and females athletes (Birrer et al., 2013). Uncovering an early detection biomarker would help to reduce this prevalence. It has been suggested that intensified exercise training may lead to a dysfunction of the endocrine system, specifically with the hypothalamic-pituitary adrenal (HPA) and gonadal (HPG) axes. It has been proposed that blunted exercise-induced cortisol and testosterone responses would occur following periods of intensified training, potentially indicating a NFOR state, (Meeusen et al., 2004; Hough et al., 2013, 2015), with Hough et al. (2013) (Hough et al., 2013) observing a blunted exercise-induced response of salivary cortisol (~166%) and testosterone (~21%) to a 30-min cycle bout following an 11-day period of intensified training suspected to have induced overreaching. Despite the interest in the findings, these authors focused on cycling exercise only. To allow for a broader population to be tested, a self-paced, 30-min

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running bout (the $RPE_{TP}$) has been developed, which has been shown to induce acute reproducible physiological and endocrine responses with a significant elevation of plasma testosterone (Leal et al., 2019) and salivary testosterone (Leal et al., 2021) observed, suggesting that the $RPE_{TP}$ may also highlight blunted exercise-induced testosterone responses following an intensified-training period.

The immune system is sensitive to exercise, with the magnitude of response being dependent on the intensity and duration of the exercise imposed (Gleeson, 2007). Indeed, whilst daily moderate-to-vigorous intensity exercise has been proposed as a co-adjuvant in the enhancement of immune defence, excessive intensified-training periods have been associated with immune dysfunction and illness (Nieman and Wentz, 2019). There is considerable crosstalk between the HPA axis and immunity and therefore, a dysfunctional HPA may lead, in part, to an impaired immune response. The effect of cortisol in the regulation of immunity has long been reported (Jefferies, 1991). However, elevated cortisol levels exert catabolic mechanisms in the organism, such as immunosuppression (Tulzo et al., 2004; Van Voorhis et al., 1989). Elevated cortisol occurred alongside a reduction in T-lymphocyte subsets, and Natural Killer-(NK) cell numbers and activity following strenuous exercise of long-duration (Malm et al., 2004; Mackinnon et al., 1997; Pedersen et al., 1996), which has also been associated with an amplified risk of upper respiratory symptoms (URS) (Nieman, 2009). However, despite cortisol concentrations were proposed to be blunted in overreached athletes, intensified training leading to overreaching has been reported to cause a reduction in neutrophil phagocytic activity, independently of unchanged cortisol levels (Robson et al., 1999), and a 20-day pre-competition training period has induced an 80% increase in oxidative stress per cell associated with a consequent 20% decrease in phagocytic activity (PA) in resting samples in female judoists (Yaegaki et al., 2007), highlighting that prolonged intensified-training with limited recovery may reduce phagocyte (mainly neutrophils) function. Interestingly, to the author's knowledge only two studies have proposed the cytotoxic γδ T-lymphocytes as stress (either psychological or physical) responsive cells, one observing a significant 50% elevation in this cell population concentration in blood to an acute speech stress task and a ~200% increase to a 16-min cycling test at 85% maximal workload, in combination with increased numbers of NK ($CD56^{+}CD3^{-}$) and $CD8^{+}$ T-cells (Anane et al., 2009), and another study observing a noticeable rise in the circulating concentration immediately after completion of a 30-min cycling bout at a pre-determined resistance corresponding to 80–85% peak power output (Pistillo et al., 2013).

Therefore, the aim of this present study is to examine the influence of a 12-day intensified-training period on immunity, specifically neutrophil PA and leucocyte mobilisation, and on the acute plasma and salivary cortisol and testosterone responses to the $RPE_{TP}$. It was hypothesized that this intensified-training period would be stressful enough to induce a state of FOR, and that the $RPE_{TP}$ would be an adequate tool to highlight blunted acute hormonal (certainly testosterone) responses and an inconsistent leucocyte mobilisation and function following the 12 days, compared to before the intensified-training period.

## 2. Materials and methods

### 2.1. Participants and ethical approval

Fourteen recreationally-trained and trained active males (performance levels 2 and 3) (de Pauw et al., 2013) volunteered to participate. From these, 6 withdrew from the study due to excessive fatigue, and therefore, 8 participants [age 21 ± 5 years; height 178 ± 5 cm; body mass 72.8 ± 6.6 kg; maximal heart rate ($HR_{max}$) 187 ± 8 beats·$min^{-1}$; maximum oxygen uptake ($\dot{V}O_{2max}$) 59 ± 6 mL $kg^{-1}$·$min^{-1}$; velocity at $\dot{V}O_{2max}$ ($v\dot{V}O_{2max}$) 16.6 ± 2.2 km $h^{-1}$] completed the overall study protocol. This study was granted ethical approval by the University of Bedfordshire Research Ethics Committee (2016ISPAR007) and was done in accordance with the 2013 Declaration of Helsinki. After comprehensive verbal and written descriptions of the study, written informed consent was provided by participants.

### 2.2. Procedures

#### 2.2.1. Study design

All participants visited the laboratories on 17 separate occasions in a pre-post intervention, cross-over study design. A familiarization trial was completed at least three days prior to the start of the study. Then two main experimental trials were completed, one before and the other after the 12-day intensified training period. A maximal oxygen uptake ($\dot{V}O_{2max}$) test was completed the day after each main trial (see Fig. 1). The $\dot{V}O_{2max}$ test protocol used has been detailed elsewhere (Leal et al., 2019). Briefly, a 4-stage, 16-min submaximal test was used for determination of the exercise speed to be used on the subsequent slope-incremented $\dot{V}O_{2max}$ test. The oxygen consumption on the submaximal test was used to determine the $v\dot{V}O_{2max}$ values to be applied on some of the exercise days during the 12-day training period, and was determined by regressing oxygen consumption over exercise intensity as detailed in previous research (Leal et al., 2019; Jones et al., 2009).

During the 12 days of training, all participants were encouraged to continue normal training sessions. To compare the volume of training during a normal training period (before the training period) to the training volume during the 12-day intensified training period, training diaries were completed by the participants for a period of 12 days within 5 days of the intensified training period. Training load was measured via training impulse (TRIMP) scores using HR variability and a weighing factor using the formula TRIMP = t(min). ΔHR. y (Banister et al., 1991), where, $t$ is the duration of each training session, $y = 0.64e^{1.92x}$ (weighting factor), $e$ is the base of the Napierian logarithms, and x = ΔHR (Heart Rate Reserve). The ΔHR is the difference between maximal and resting heart rate (HR). TRIMP scores were 124% higher (713–1598), with the duration being 143% greater (7 h–17 h) in the 12 days of training, when compared to the normal training period. Participants' body mass, rating of sweat loss, heart rate (HR) and rating of perceived exertion (RPE) were monitored on every training day and water was provided *ad libitum*. Exercise, and alcohol and caffeine were not allowed, and a 24-hour food diary was also completed and replicated the day before each main trial.

#### 2.2.2. Main experimental trials

All participants arrived at the laboratories at approximately 11:30 a.m. A standard breakfast was consumed before 09:00 on the day of the trials and 500 mL of water were consumed during the morning to aid a euhydrated state. Upon arrival, a urine sample was collected, and the participant sat resting for 20 minutes before beginning the exercise bout. During this period, a 76-statement recovery-stress questionnaire (the RESTQ-76 Sport) (Kellmann and Kallus, 2001) and an upper respiratory symptoms (URS) log using the Jackson score (Hanstock et al., 2016) were completed. The RESTQ-76 is a self-report of stress and recovery events over the previous 3 days, with answers ranging from never (0) to always (6). The analysis has been completed according to its authors' guidelines (Kellmann and Kallus, 2001). Then, the $RPE_{TP}$ (a 30-min, self-paced running bout that has been detailed elsewhere (Leal et al., 2019; Leal et al., 2021) and in Fig. 1) was undertaken (~12:00 p.m.), and venous blood and saliva samples were collected pre-, post- and 30 min post-exercise. A 10-km time-trial test (10-km TT) was also completed 60 min post-$RPE_{TP}$, as a measure of physical performance.

#### 2.2.3. The 12-day intensified-training period

To decrease the risk of injury and to ensure the participants completed the intensified exercise training in full, all participants completed three different running sessions on four occasions each in an intercalated manner (Fig. 1). Training sessions were as follows: a) A 90-min, continuous treadmill-run subdivided in one block of 70 min at 55%

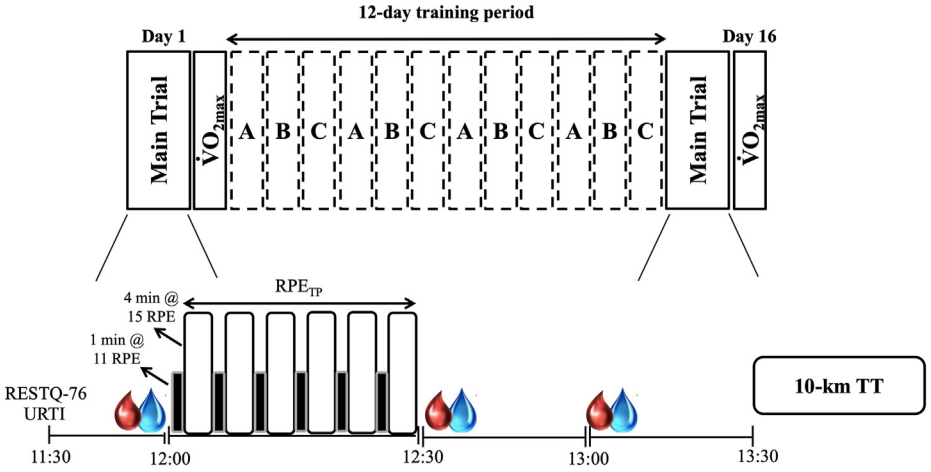


**Fig. 1.** Schematic presentation of the study design and $RPE_{TP}$ protocol. where A, B and C represent each training session type during the 12-day training period, specifically: A) a 90-min, intensity-specific, continuous treadmill-run; B) a 5-km time-trial run; and C) a 70-min self-paced, continuous treadmill-run.

of the individuals' $v\dot{V}O_{2max}$ and one 20-min block at 75% $v\dot{V}O_{2max}$; b) a 5-km time-trial; and c) a 70-min, self-paced, continuous treadmill-run at a speed corresponding to an RPE of 12 (light) on the 6–20 Borg scale for the first 30 min, at 13 (somewhat hard) during the following 30 min, and at 15 (hard) for the final 10 min.

### 2.3. Analytical procedures

#### 2.3.1. Blood and saliva collection, treatment and analysis

15 mL of whole blood were obtained at each time point. All samples were collected into 5 mL $K_3$EDTA-treated vacutainers (Vacuette, Greiner Bio-One, Stonehouse, UK), and plasma was obtained from one of these by centrifugation (1500 *g* for 10 min at 4 °C) (Leal et al., 2019) (Heraeus Multifuge X3R, Thermo Scientific, Loughborough, UK).

Unstimulated passive drool saliva collection was performed for 3 min into 7 mL polystyrene sterile aliquots (Sterilin, Thermo Scientific, Loughborough, UK) as detailed elsewhere (Leal et al., 2021). To avoid dilution of the sample water consumption was not allowed in the 10 min preceding this procedure. Briefly, samples were centrifuged at 14600 *g* for 10 min (Espresso Microcentrifuge, Thermo Scientific, Loughborough, UK) and the supernatant was transferred into 1.5 mL containers (Eppendorf, Hamburg, Germany) to be stored at −80 °C until further analysis.

The salivary (Salimetrics, PA 16803, USA) and plasma (IBL International, Hamburg, Germany) cortisol and testosterone concentrations were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits. The manufacturer reported sensitivity of the plasma cortisol and testosterone and salivary cortisol and testosterone kits as 6.3 $nmol.L^{-1}$ and 0.32 $nmol.L^{-1}$ and 6.1 $nmol.L^{-1}$ and 3.7 $pmol.L^{-1}$, respectively whilst the mean intra-assay CV were 3.4% and 3.2% (plasma and salivary cortisol, respectively) and 4.6% and 3.4% (plasma and salivary testosterone, respectively).

#### 2.3.2. Polymorphonuclear granulocytes (PMNs) isolation

Isolation of pure polymorphonuclear granulocytes (PMNs) from whole blood was accomplished using Polymorphoprep™ (PMP) (Axis-Shield, Oslo, Norway) containing sodium diatrizoate and polysaccharide. 5 mL of PMP was layered with 5 mL of whole blood avoiding mixing the contents. Centrifugation at 500 *g* for 30 min at 20 °C, allowing the rotor to decelerate without the brake. Two bands of cells were obtained. The obtained plasma and peripheral blood mononuclear cells (upper band) were discarded and the PMNs (lower band) harvested and transferred to a clean 15 mL tube.

The PMNs were subsequently washed in a 1:2 solution of Dulbecco's Modified Eagle's Medium containing glucose (4500 $mg{\cdot}L^{-1}$), sodium pyruvate (110 $mg{\cdot}L^{-1}$) and L-glutamine (Sigma-Aldrich, Gillingham, Dorset, UK) and sterile-filtered water in a separate 15 mL tube. Centrifugation at 400 *g* for 10 min with the acceleration and deceleration back to full speed was then performed. The supernatant was discharged and the pellets re-suspended in 1 mL of AIM-V® serum free medium (1X) containing L-Glutamine, streptomycin sulphate (50 μg $mL^{-1}$) and gentamicin sulphate (10 μg $mL^{-1}$), supplemented with bovine serum albumin (BSA, AlbuMAX®) (Gibco™, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The tubes were then set aside for further cell counts, phagocytic activity assay, and staining for flow cytometry.

#### 2.3.3. Total leucocytes from whole blood (WBL)

A 1X red blood cell lysis buffer containing ammonium chloride (eBioscience™, Thermo Fisher Scientific, Waltham, Massachusetts, USA) was mixed with whole blood in a 10:1 proportion by gentle inversion. A 10 min incubation period was completed, and the reaction was stopped by adding twice the volume of 10X, pH 7.4 Phosphate-Buffered Saline (PBS) (Gibco®, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The sample was then centrifuged at 400 *g* for 5 min at 4 °C. The supernatant was discharged, and the pelleted cells were re-suspended in 1 mL PBS.

#### 2.3.4. Cell counting

Cell counting of WBL and PMNs was performed using an automated handheld cell counter (Scepter™, Merck Millipore, Germany). Dilutions were prepared in 1.5 mL microcentrifuge aliquots in a ratio of 1:5 with 10X, pH 7.4 PBS. The minimal sample volume used and required for an accurate count was 100 μL. Disposable 60 μm (operating range between 10,000–500,000 $cells{\cdot}mL^{-1}$) microfluidic sensor tips (PHCC60050, Scepter, Merck Millipore, Billerica, Massachusetts, USA) were used. Total cell numbers were determined by multiplying the given cell number by the dilution factor.

#### 2.3.5. Polymorphonuclear granulocytes phagocytic activity assay

A 1.6 mM working stock dihydroethidium solution (wsDHE) was prepared by diluting dihydroethidium (DHE) in a 1:40 ratio in dimethyl sulfoxide (DMSO). Opsonized zymosan was obtained by suspending zymosan (~200 mg) in 1 mL of a 1:2 solution of PBS with sterile-filtered human male serum incubated for 1 h at 37 °C. The solution was then centrifuged at 7500 rpm for 5 min and the supernatant removed. The pellet was washed with 1 mL PBS and centrifuged again. The PBS wash was discarded and the opsonized zymosan was resuspended at 200 mg $mL^{-1}$ in fresh PBS. The isolated PMNs were resuspended to ~2 million/mL in AIM-V®. PMN sample was placed into 3 wells (3 x 100 μL) of a 96-well sterile, flat bottom plate, to serve as non-stain control, baseline and

treatment. 100 μL of AIM-V® were added to the non-stain well. 100 μL of a 2X DHE (16 μM) stain solution (5 μL of wsDHE with 250 μL of AIM-V®) were added to the other two wells. Finally, 2.5 μL of opsonized zymosan were added to the treatment well. The samples were incubated at 37 °C in a humid 5% $CO_2$ environment for 30 min to allow for sufficient time for the DHE to label the PMNs. Contents were then transferred into 1.5 mL microcentrifuge tubes and 1 mL PBS was added into each. All samples were centrifuged in a microcentrifuge at 5000 rpm for 5 minutes, supernatant removed with a vacuum pump and pellet re-suspended in 200 μL of a FACS buffer (1X PBS pH 7.2, containing calcium chloride, magnesium chloride, 2% foetal bovine serum and 0.01% azide), before acquisition via flow cytometry. Side scatter (SSC) *versus* forward scatter (FSC) plots of all acquired cells were used to gate on the isolated PMNs. Histograms for counts (cell number) plotted against FL2 were created with gates for examination of the unstimulated PMNs, and fluorescence of dihydroethidium (DHE)-stained PMNs, and DHE-stained and opsonized zymosan-stimulated PMNs (see Supplementary Fig. S1 online).

#### 2.3.6. Leucocyte subsets activation and acquisition

WBL and PMNs were stained with fluorochrome-conjugated anti-human monoclonal antibodies for flow cytometric analysis (BD Accuri™ C6 Plus, BD Biosciences, Oxford, UK), with further centrifugation at 500 *g* for 5 min. 30 μL of antibody cocktails (eBioscience™) (1:50 in FACS buffer) were added directly onto the cell pellets in each tube as follows: A) T-lymphocyte surface markers: FITC conjugated anti-CD3, PE conjugated γδ T-cell receptor, PerCP-Cyanine 5.5 conjugated anti-CD4, APC conjugated anti-CD8a; B) Monocyte/Macrophage, NK cells, B-cell and Granulocytes surface markers: FITC conjugated anti-CD11b, PE conjugated anti-CD56, PerCP-Cyanine 5.5 conjugated anti-CD19, APC conjugated anti-CD11c; C) Monocyte/Macrophage, Neutrophils, Eosinophils surface markers: FITC conjugated anti-CD16, PE conjugated anti-CD15, PerCP-Cyanine 5.5 conjugated anti-CD14, APC conjugated anti-CD23. Aliquots were incubated at 4 °C for 30 min and cells were subsequently washed in 300 μL of FACS buffer and re-suspended in 200 μL of the same buffer for immediate flow cytometer acquisition. All samples were gated by morphology using side scatter (SSC) *versus* forward scatter (FSC) plots and 50,000 events were acquired per sample. Data files were generated through the BD C6 Analysis Software for PC and were stored for later analysis. Side scatter (SSC) *versus* forward scatter (FSC) plots of all acquired cells were used to gate on the different populations. T-lymphocyte and granulocyte analysis is outlined in Supplementary Fig. S2 and Supplementary Fig. S3 online, respectively.

### 2.4. Statistical analysis

Data was checked for normality (Shapiro-Wilk test) and homogeneity of variance. If normality was not achieved, logarithmic transformations of base 10 were performed on the data prior to re-assessment of normality. When logarithmic data sets violated normality (WBL, CD19 B-cells, $CD11c^+$ dendritic cells and phagocytosis data), multiple Wilcoxon signed rank tests,. were completed on the original data. Logarithmic data sets were used for the examination of salivary cortisol, γδ T-lymphocytes, $CD4^+/CD8^+$ ratio, and NK cells. A one-way (trial) repeated measure ANOVA with Bonferroni adjustments were applied to urine osmolality, RESTQ-76 Sport, $\dot{V}O_{2max}$ and 10-km time-trial data. The URS questionnaires were analysed with Student's paired sample *t test* (pre-training vs. post-training for each scale). A two-way (trial x time) repeated measures ANOVA was used with Bonferroni corrections on all other variables. For all comparisons where the assumption of sphericity was violated, Greenhouse-Geisser corrections were used. When required, Student's paired sample *t tests* were used. The HR and speed data were examined over time at the end of each 1 min and 4 min stages, and average HR and speed for trial (pre- and post-Training) were determined taking into account the duration of each stage (i.e. ((average of data in the 1 min stages x 6) + (average of data in the 4 min stages x 24))/30). The presented effect sizes were calculated by hand, by dividing the difference between the means of the groups by the pooled SD (Nieman, 2009). Statistical significance was accepted at $p < 0.05$. All data were presented as mean ± SD unless stated otherwise.

## 3. Results

### 3.1. Recovery-stress questionnaire

There was a trial effect when comparing pre-training to post-training in the General Stress and in the Sport Stress scales, with an increase in these scales at post-training, compared to pre-training (Fig. 2). Further examination shows a 38% increase in injury perception (i.e. 'Fitness/Injury') subscales at post-training ($p < 0.05$).

### 3.2. Upper respiratory symptoms (URS)

There were no significant differences in most URS scores post-training, compared with pre-training ($p > 0.05$). However, *Headaches* scores increased post-training ($p < 0.01$). Additionally, five individuals reported overall increased symptoms of URS post-training, with three of them describing more severe URS symptoms compared to pre-training (Fig. 3).

### 3.3. Physiological responses to $RPE_{TP}$

There was no trial effect when comparing HR or speed responses to the $RPE_{TP}$ across time at pre-training and post-training. Average HR for trial was 154 ± 15 beats·min$^{-1}$ at pre-training and 150 ± 18 beats·min$^{-1}$ at post-training. Average speed for trial was 12.0 ± 2.9 km h$^{-1}$ at pre-training and 12.2 ± 2.5 km h$^{-1}$ at post-training. $\dot{V}O_{2max}$ was unaffected by the training period, being 59 ± 6 mL kg$^{-1}$·min$^{-1}$ (pre-training) and 59 ± 7 mL kg$^{-1}$·min$^{-1}$ (post-training).

### 3.4. 10-km time trial performance test

There was no trial effect when comparing the time to completion in 10-km TT at pre-training and post-training ($p = 0.577$). However, time to completion was 1:21 ± 00:39 (min:s) higher at post-training ($p > 0.05$), with ES = 0.3.

### 3.5. Total leucocytes and polymorphonuclear leucocytes

Total WBL were unaffected by exercise or the training period ($p > 0.05$ in all). However, an exercise-induced increased number of PMNs was seen at post-training ($p < 0.05$). Average cell numbers are presented in Table 1.

### 3.6. Leucocyte phenotyping and PMN phagocytic activity

The 12-day training period had no effect on any of the leucocyte populations at rest ($p > 0.05$). Yet, an exercise-induced effect was present pre-training in CD19 B-lymphocytes (6.4%–4.6%, which was a 28% decrease, $p < 0.05$) and γδ T-lymphocytes (3.1%–9.1%, which was a 194% elevation, $p < 0.05$). Elevated numbers of CD56 NK were observed at post-training ($p < 0.05$), and, though not significantly, average resting γδ T-lymphocytes were increased by ~174% post-training, compared with pre-training (Table 1).

An exercise-induced 20% reduction in PA was observed at pre-training ($p < 0.05$). However, despite no alteration in the acute PA at post-training, baseline PA was reduced by 47% post-training, when compared to pre-training ($p < 0.05$) (Fig. 4).

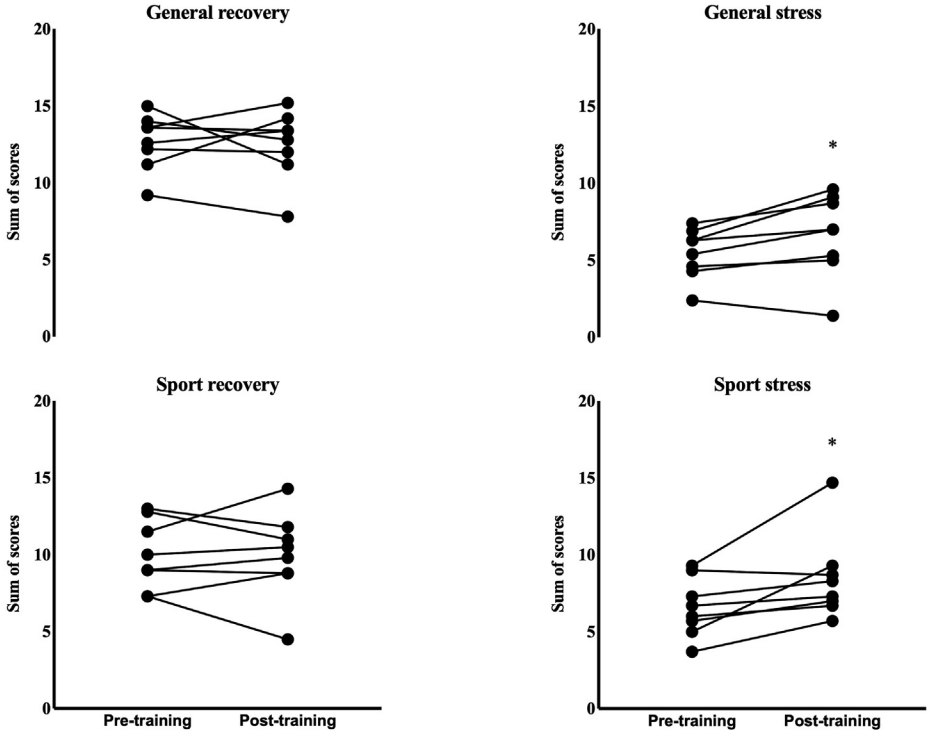


**Fig. 2.** RESTQ-76 Sport scores in all four main scales at pre-training and post-training. Values are means ± *SD*. *Different from pre-training ($p < 0.05$).

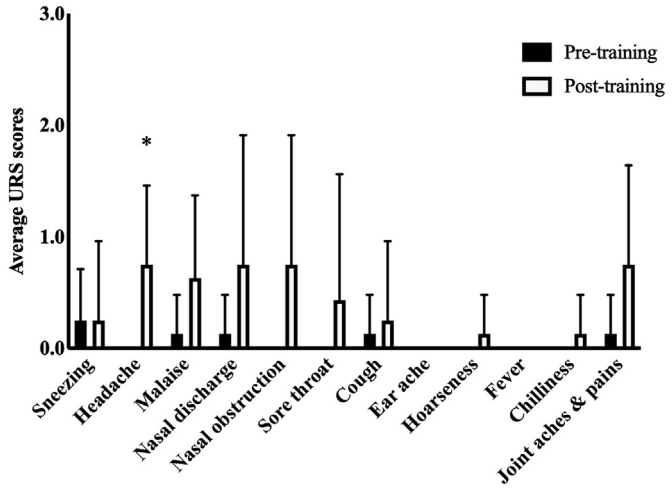


**Fig. 3.** Average upper respiratory symptoms (URS) and sum of individual scores at pre-training and post-training. Values are means ± *SD*. *Different from pre-training ($p < 0.01$).

### 3.7. Hormonal responses to exercise

#### 3.7.1. Plasma cortisol

No effects for trial ($p = 0.48$), or time ($p = 0.09$) were found. Absolute change from pre-to post-exercise was −41.7 nmol $L^{-1}$ (−14%) pre-training, and 31.7 nmol $L^{-1}$ (+15%) post-training. Individual responses varied, with absolute exercise-induced change ranging between −88.5 nmol $L^{-1}$ and +13.9 nmol $L^{-1}$ pre-training, and between −59.6 nmol $L^{-1}$ and +169.5 nmol $L^{-1}$ post-training (Fig. 5).

#### 3.7.2. Salivary cortisol

No trial effect was observed ($p = 0.84$), yet a time effect was present ($p < 0.05$). Salivary cortisol was lower at 30 min post-compared to post-exercise at pre-training (Fig. 5), with average absolute change from pre-to post-exercise being −0.5 nmol $L^{-1}$ pre-training (individual response ranging between −8.0 nmol $L^{-1}$ and +19.2 nmol $L^{-1}$, 0% average increase), and 2.3 nmol $L^{-1}$ post-training (individual response ranging between −2.4 nmol $L^{-1}$ and +19.2 nmol $L^{-1}$, +46% average increase) (Fig. 5).

#### 3.7.3. Plasma testosterone

No trial effect was found ($p = 0.24$). However, a time effect was observed ($p = 0.003$), with plasma testosterone acutely elevating from pre-to post-exercise at pre-training only (Fig. 6). Average absolute change from pre-to post-exercise significantly decreased ($p < 0.05$) from 4.6 nmol $L^{-1}$ (43%) at pre-training to 1.9 nmol $L^{-1}$ (19%) at post-training.

#### 3.7.4. Salivary testosterone

No trial effect was found ($p = 0.75$), yet a time effect was observed ($p < 0.05$), with salivary testosterone acutely elevating at pre-training only (Fig. 6). Despite not significantly ($p > 0.05$), absolute change in salivary testosterone decreased from 227.8 pmol $L^{-1}$ (55%) at pre-training to 105.3 pmol $L^{-1}$ (24%) at post-training.

## 4. Discussion

The main findings of this study were that plasma and salivary cortisol were acutely unaffected by the $RPE_{TP}$ both before and after a 12-day intensified training period. However, plasma and salivary testosterone acutely elevated pre-training, but this response was not observed post-training. Increased subjective injury perception (through RESTQ-76 scores) and URS were found (specifically for Headaches scores) post-training when compared with pre-training. $CD56^{+}$ NK-cells have acutely elevated by 50% in response to the $RPE_{TP}$ post-training, but no change was observed pre-training. An exercise-induced 194% elevation in γδ T-lymphocyte concentrations occurred pre-training, but no change was observed post-training. However, despite not significantly, a 174% higher resting concentration was observed post-training. Resting phagocytic function significantly decreased by 47% post-training, when compared to pre-training.

**Table 1**
Total and differential leucocyte counts, and leucocyte phenotyping at pre-training and post-training (50,000-cell population).

| | Pre-training | | | Post-training | | |
|---|---|---|---|---|---|---|
| | Pre-exercise | Post-exercise | 30 min post-exercise | Pre-exercise | Post-exercise | 30 min post-exercise |
| Leucocyte counts | | | | | | |
| Total (x $10^6 \cdot mL^{-1}$) | 3.6 ± 0.9 | 3.4 ± 0.7 | 3.4 ± 0.5 | 3.4 ± 0.2 | 3.4 ± 0.3 | 3.4 ± 0.3 |
| PMNs (x $10^6 \cdot mL^{-1}$) | 1.9 ± 0.5 | 2.2 ± 0.5 | 2.1 ± 0.6 | 1.9 ± 0.5 | 2.4 ± 0.5[b] | 2.0 ± 0.8 |
| **Leucocyte phenotyping (% in 50,000 events)** | | | | | | |
| γδ T-lymphocytes | 3.1 ± 3.8 | 9.2 ± 14.3[a] | N/A | 8.5 ± 15.1 | 8.8 ± 15.5 | N/A |
| $CD4^+$ | 28.9 ± 6.6 | 23.1 ± 10.9 | N/A | 27.9 ± 9.1 | 24.3 ± 8.0 | N/A |
| $CD8^+$ | 23.8 ± 8.6 | 21.9 ± 12.8 | N/A | 19.4 ± 7.1 | 21.5 ± 8.9 | N/A |
| $CD4^+/CD8^+$ | 1.3 ± 0.3 | 1.2 ± 0.3 | N/A | 1.6 ± 0.7 | 1.3 ± 0.8 | N/A |
| $CD19^+$ B-cells | 6.4 ± 3.1 | 4.6 ± 3.0[a] | N/A | 5.0 ±2.0 | 4.4 ± 2.2 | N/A |
| $CD56^+$ NK cells | 1.8 ± 2.3 | 1.9 ± 2.2 | N/A | 1.6 ± 1.2 | 2.4 ± 1.9[b] | N/A |
| $CD11c^+$ | 8.3 ± 4.5 | 9.3 ± 5.1 | N/A | 5.0 ± 3.5 | 9.4 ± 7.9 | N/A |
| $CD15^+$ | 55.7 ± 27.6 | 46.6 ± 32.6 | N/A | 55.8 ± 30.5 | 71.0 ± 22.6 | N/A |

PMN – Polymorphonuclear leucocytes; $CD4^+$ - Helper T-cells; $CD8^+$ - Cytotoxic T-cells; CD11c+ - Dendritic cells; $CD15^+$ - Neutrophils. Values are means ± SD.
[a] Different from pre-exercise values at pre-training ($p < 0.05$).
[b] Different from pre-exercise values at post-training ($p < 0.05$).

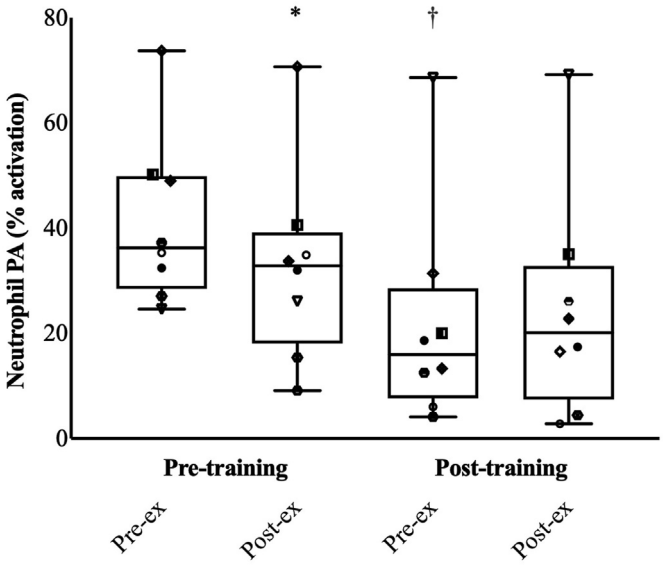


**Fig. 4.** Phagocytic activity of zymosan-stimulated PMNs at pre-training and post-training. Values are means ± *SD*, symbols represent individual responses. *Different from pre-exercise values at pre-training ($p < 0.05$). †Different from pre-training values ($p < 0.05$).

Increased scores of General-, and Sport-related stress were observed post-training when compared to pre-training, with further examination showing higher scores on the 'Fitness/Injury' subscale. In this present study, despite the greater scores in the stress-related scales and perceived susceptibility to injuries, the athletic performance levels examined through completion of a 10-km time-trial run were statistically unaffected, suggesting the increased psychological and social stress levels did not influence athletic performance. However, detailed examination of the data shows that time to completion was 1:21 ± 00:39 (min:s) higher at post-training compared to pre-training. Although the calculated ES = 0.3 represents a small effect, as proposed by Cohen (1988) (Cohen, 1988), it is recognized that a difference of this magnitude may be considered an impactful difference in athletic performance. To contextualise this, British Athletics data shows that a 1 min 21 sec difference separates a 1st from a 15th place in a 21-male run competition. Additionally, an overall increase in URS, specifically headaches in the participants at post-training compared with pre-training was observed. Individual examination of URS indicates that 5 participants have experienced increased URS, from which 3 have reported higher symptoms at post-training when compared to before the training period. These data suggest that the 12 days of training may have been intense enough to increase URS commonly observed in athletes suspected to be overreached (Meeusen et al. Prevention, 2013). However, no differences in any of the physiological characteristics examined have been observed, with HR and speed in the $RPE_{TP}$ being similar at pre-training and post-training. Participants' $\dot{V}O_{2max}$ was also unaffected, which suggests that any differences that have been observed in endocrine markers and immunity have not been influenced by physiological stress or fitness level. Furthermore, these findings also suggest that the $RPE_{TP}$ was of similar intensity before and after training, and consequently the imposed exercise-induced stress was also analogous. This is important as a change in intensity of exercise post-training would have resulted in a different exercise-induced response of the markers that were measured.

Plasma and salivary testosterone robustly and acutely elevated pre-training, as expected due to the testosterone responsiveness to short-duration, high-intensity exercise reported elsewhere (Hough et al., 2013; Leal et al., 2019). In addition, the absolute change in plasma and salivary testosterone presented in this study were blunted (significant for plasma responses only), with absolute changes of 4.6 $nmol \cdot L^{-1}$ (43%) and 227.8 $pmol \cdot L^{-1}$ (55%) at pre-training, and 1.9 $nmol \cdot L^{-1}$ (19%) and 105.3 $pmol \cdot L^{-1}$ (24%) at post-training, respectively. This is in line with the findings from Hough and colleagues (2013 & 2015) (Hough et al., 2013, 2015), who have reported a 21% and 44% blunted salivary testosterone response following an 11-day training period and a 10-day training camp in healthy male individuals and male elite triathletes, respectively. In this present study, the repeated exercises during the 12 days of intensified training may have continually induced a cortisol response, which is likely to have happened as supported by previous evidence reporting exercise above 60% $\dot{V}O_{2max}$ and 20 min in duration for cortisol to acutely elevate (Davies and Few, 1973). This continual elevation may lead to an alteration (reduction) in testosterone responsiveness. This may be hypothesized to have happened due to the dysfunctional gonad production of testosterone associated with high levels of circulating cortisol, possibly due to an inhibitory effect of cortisol upon the luteinizing hormone receptors present in the Leydig cells in the testes, as proposed elsewhere (Cumming et al., 1983).

However, cortisol did not acutely elevate in response to the $RPE_{TP}$ (observed elsewhere) (Leal et al., 2019) pre-training or post-training. For this reason, a blunted response of plasma or salivary cortisol to the $RPE_{TP}$ could not be expected, which is in contrast to what has been observed in similar cycling-based research design protocols (Hough et al., 2013, 2015). Specifically, an 11-day intensified-training period (Hough et al., 2013) reported a blunted (11.1–3.1 $nmol \cdot L^{-1}$, ~ −72%) exercise-induced salivary cortisol response to a 30-min high-intensity cycling bout in young males post-training, compared to pre-training. In this present study, the $RPE_{TP}$ was not stressful enough to elicit an elevated cortisol

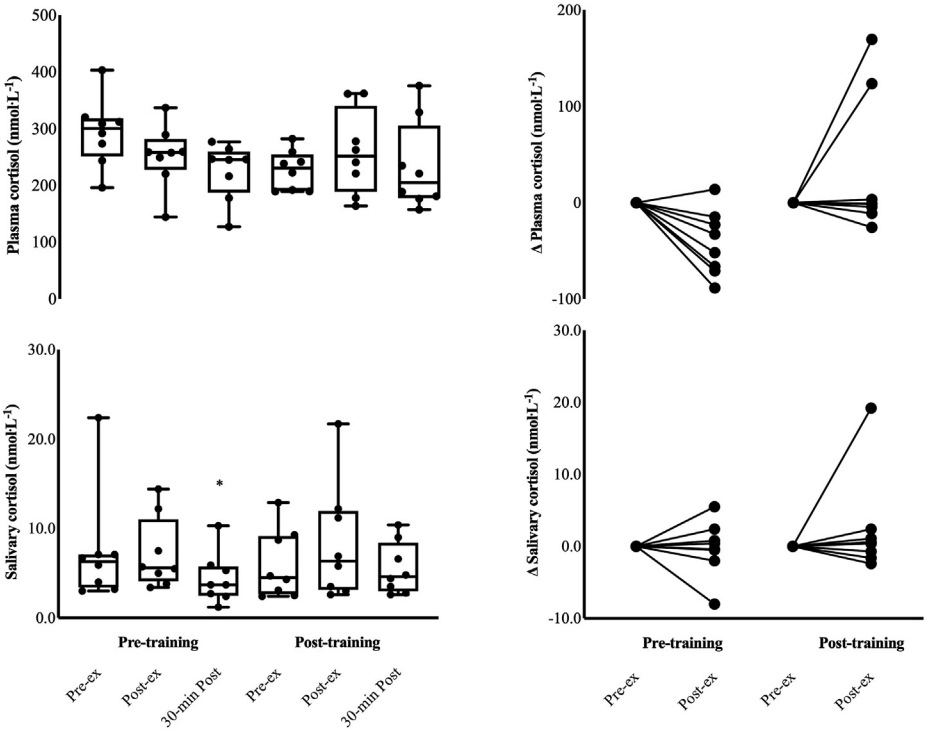


**Fig. 5.** Acute plasma and salivary cortisol responses and individual absolute change from pre-to post-exercise at pre-training and post-training. Values are means ± *SD*. *Different from pre-exercise values at pre-training ($p < 0.01$).

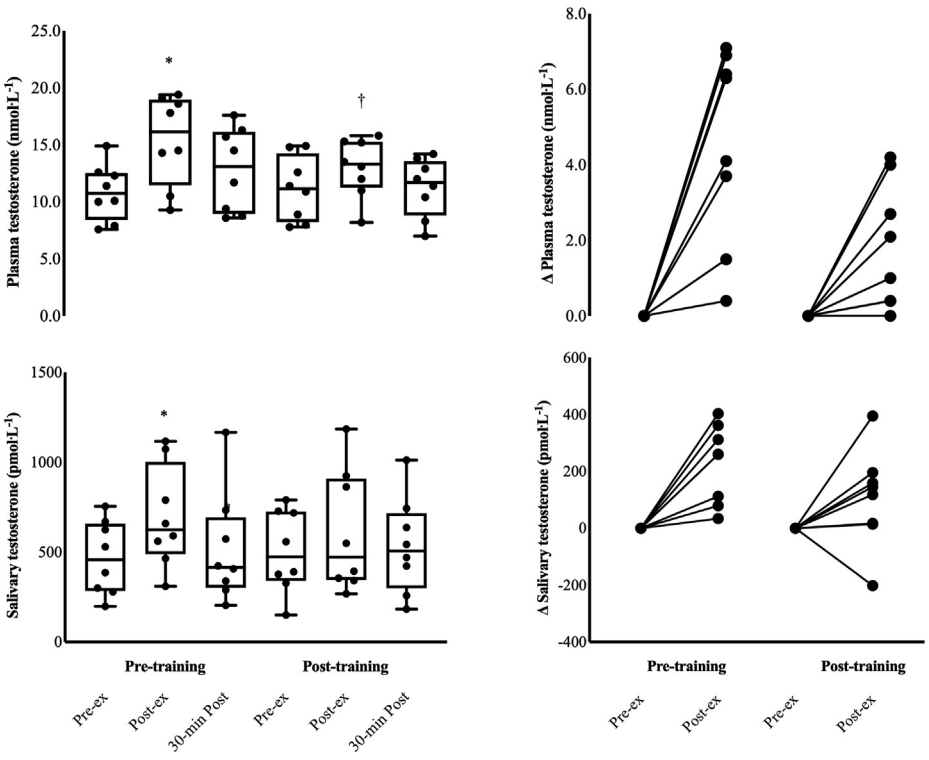


**Fig. 6.** Acute plasma and salivary testosterone responses and individual absolute change from pre-to post-exercise at pre-training and post-training. Values are means ± *SD*. *Different from pre-exercise values at pre-training ($p < 0.01$). †Different from pre-training values ($p < 0.05$).

response, as observed in previous research (Leal et al., 2019). However, the plasma and salivary cortisol showed a tendency to respond with higher sensitivity post-training, compared to pre-training. Despite not significantly, average absolute change of plasma and salivary cortisol to the $RPE_{TP}$ was −14% and 0% pre-training, and +15% and +46% post-training. Previous research has demonstrated the response of plasma cortisol to the $RPE_{TP}$ is reproducible (Leal et al., 2019), therefore these responses may not be due to individual variability but due to increased

stress levels associated with intensified exercise and limited recovery. It has been suggested that one of the HPA patterns during exercise-induced chronic stress may be a hyperresponsiveness of the pituitary gland inducing a higher secretion of ACTH levels with concomitant elevated levels of circulating glucocorticoids (Aguilera, 1994), which may be explanatory of the tendency to elevated cortisol sensitivity post-training.

Importantly, a robust increase in serum cortisol levels has been associated with immunosuppression through a marked decrease in T-lymphocyte subsets (specifically $CD3^+CD4^+CD8^+$ and $CD3^+CD4^-CD8^-$), and Natural Killer- (NK) cell numbers and activity following strenuous exercise of long-duration (Malm et al., 2004; Mackinnon et al., 1997; Pedersen et al., 1996), which has also been proposed to be associated with an amplified risk of upper respiratory symptoms (URS) (Nieman, 2009). It has been extensively reported that acute exercise-induced leucocytosis is expected to occur, depending on exercise intensity and duration (Nieman and Wentz, 2019; McCarthy and Dale, 1988). As an example, circulating leucocyte numbers have been reported to elevate by ~65% in healthy individuals in response to a self-paced running test to exhaustion (60–90 min), explained by a 97% significant elevation in PMNs, despite unaltered lymphocyte numbers (Risøy et al., 2003). Contrastingly to what would be expected, this leucocytosis was not observed in the present study, with undetected acute changes in total WBL counts pre-training and post-training. Indeed, cell numbers fluctuate on the balance of cell death/division/exit to tissues as a response of a dynamic homeostatic mechanism. Therefore, the observed WBL numbers may be considered as snapshots that may not directly reflect the influence of the exercise intervention. On the other hand, the unaltered numbers may also be interpreted as an effect of the apparently higher concentration levels of cortisol, reverting the expected rise in leucocyte mobilisation associated with exercise (Ronsen et al., 2001; Pedersen, 1991). To the authors' knowledge, this present study observes similar data to one study only, completed on 37 individuals who undertook a graded running test to exhaustion, in which it was speculated that the leucocyte irresponsiveness not seen in other studies may be due to the different exercise stimulus used (Wardyn et al., 2008).

When considering the differential blood counts, it was hypothesized that neutrophil numbers would significantly elevate in response to the $RPE_{TP}$ when in a healthy state, and that exercise-induced neutrophilia would be greater following a period of intensified training. Again, no significant changes were found in exercise-induced PMN relative numbers from whole blood at pre-training, but a modest 16% percentage increase was seen. However, a significant 26% acute elevation occurred post-training. No significant differences were found when examining the percentage of granulocytes expressing CD15, which are likely to be neutrophils. This increase in PMNs at post-training may be due to a more marked elevation in eosinophils and potentially even basophils numbers (despite the latter represent only approximately 1% of WBL) than neutrophils, as observed elsewhere (Schwindt et al., 2007), despite the recognized exercise-induced neutrophilia (Anane et al., 2009; Ronsen et al., 2001; Pedersen, 1991). In this present study, although not significantly, the percentage of $CD15^+$ cells examined in a population of 50, 000 cells increased by 27%. Therefore, the increase in PMNs reported after the 12-day period of intensified training may be due to an increase in the circulating numbers of neutrophils as well. Yet, an attenuation of exercise-induced leucocytosis may occur with regular exercise (Blannin et al., 1996), presumably by reducing the physical stress and fatigue levels provoked by the exercise bout itself (Vrabas et al., 1999). It has also been demonstrated that endurance trained individuals ($\dot{V}O_{2max}$ was 61 ± 9 mL $kg^{-1}{\cdot}min^{-1}$) had lower neutrophil counts when compared to sedentary individuals ($\dot{V}O_{2max}$ was 37 ± 7 mL $kg^{-1}{\cdot}min^{-1}$), with neutrophil numbers acutely increasing in both trained and sedentary, albeit a less pronouncedly in the trained population (Blannin et al., 1996). It may be reasonable to suggest that perhaps the modest increase in PMNs at pre-training in this present study may be due to the fitness level of the participants, and that a greater neutrophilia occurred as an effect of increased stress levels post-training. However, contrastingly to what was expected, we have not observed an acute effect of the $RPE_{TP}$ on NK-cell numbers at pre-training. A study completed on trained individuals who underwent an incremental cycling test to exhaustion has reported similar results, with no changes in the absolute number of NK-cells (Brahmi et al., 1985). However, an exercise-induced 50% increase in $CD56^+$ NK-cells was observed post-training, when compared to pre-training. This finding is in contrast with common findings from previous research, showing decreased total CD56 NK-cells in elite swimmers being observed over the course of a winter training season (Rama et al., 2013). Considering an elevation in overall stress in the participants occurring during the 12 days, the increased percentage of NK cells observed in the present study may be considered an adaptive effect caused by the training period.

Noticeably, this present study has observed a significant exercise-induced 194% elevation in cytotoxic γδ T-lymphocytes at pre-training only, highlighting the possible stress-responsiveness of this lymphocyte subset. These data are in line with what was firstly reported by Anane et al. (2009) (Anane et al., 2009) who have observed a significant elevation in this cell population to an acute speech stress task (~50% elevation) and to a 16-min cycling test at 85% maximal workload (~200% elevation), in combination with increased numbers of NK ($CD56^+CD3^-$) cells (~200% and ~900%, respectively) and $CD8^+$ T-cells (~100% change to exercise only), and later also observed by Pistillo et al. (2013) (Pistillo et al., 2013), who reported a markedly increase in γδ T-lymphocytes to a 30-min cycling at a pre-determined resistance corresponding to 80–85% peak power output. In our study, despite not significantly, this stress responsiveness of γδ T-lymphocytes was also observed post-training, with resting levels being approximately 174% higher than pre-training.

Interestingly, this present study observes a 20% decrease in PMNs phagocytic function in response to the $RPE_{TP}$ occurring at pre-training. Similar findings have been shown in 18 healthy male individuals who completed an exercise session at 55% $\dot{V}O_{2max}$ for up to 3 hours, with indices of decreased neutrophil function regardless of an elevation in circulating neutrophils (Robson et al., 1999). More importantly, the current study has shown that the 12-day intensified-training period has induced a significant 47% decrease in resting phagocytic function, which is corroborated with data from elsewhere (Robson et al., 1999; Yaegaki et al., 2007). Our data is supported by Robson et al. (1999) (Robson et al., 1999), who have observed that athletes who were overreached following prolonged periods of intensified training have shown substantial decrements in neutrophil function, independent of cortisol responses. Supporting these findings, Yaegaki et al. (2007) (Yaegaki et al., 2007) have also observed a resting 20% decrease in phagocytic activity associated with an 80% increase in oxidative stress per cell following a 20-day preparation for a competition. This is an impactful finding as it reinforces the negative effects that intensified periods of training with limited recovery may have in immunity.

However, the low sample size presented may be interpreted as a limitation of this study and must be addressed. A post-hoc computation of achieved power was completed on four of the main variables in this research study. These variables were the delta plasma testosterone pre-to post-training, the acute response of the γδ T-lymphocytes, the neutrophil phagocytic activity, and the total neutrophil counts. The analyses achieved a post-hoc power of 0.71 (plasma testosterone), 0.70 (γδ T-lymphocytes), 0.40 (neutrophil phagocytic activity), and 0.75 (neutrophil counts). These findings detail a 29%, 30%, 60%, and 25% risk of committing type II errors (i.e. missing an effect if it genuinely exists), respectively. It is commonly agreed that a power level of 80% is credible to determine actual effects. Therefore, the reader should be aware of this higher risk of missing an effect that exist in these main variables examined in this present study. However, the several control variables applied during data collection may strengthen a potential reduction of type II errors in the event of further examination of these variables following

periods of intensified training. Future studies may also consider the completion of a recovery period to help confirm overreaching (certainly FOR), and to understand the duration needed for full recovery and/or supercompensatory adaptation to occur. An examination of systemic inflammation, and monocyte- or neutrophil-derived inflammatory cytokines may also reinforce the impact intensified-training periods with limited recovery may have in immunity and inflammation.

## 5. Conclusion

The variable nature of cortisol may render it as an unreliable endocrine biomarker to be used to detect an exercise-induced endocrine dysregulation, certainly when using the $RPE_{TP}$ as a tool to highlight overreaching. In contrast, testosterone has shown to consistently acutely elevate to the $RPE_{TP}$, with blunted salivary (although not significant) and plasma values occurring at post-training. The responses observed in plasma testosterone and its more coherent character suggest that perhaps this may be a reliable indicator of an HPG dysfunction associated with an early-stage overreaching, and that the $RPE_{TP}$ may be a suitable running tool to be used to help detect the onset of overreaching, as a strategy to prevent the incidence of OTS.

Additionally, this present study has shown a significant 194% exercise-induced increase in γδ T-lymphocytes concentrations at pre-training, supporting previous research suggesting this leucocyte subset may be sensitive to physiological stress. The elevated resting concentrations in γδ T-lymphocytes observed post-training may render this leucocyte population as a novel possible cellular marker to detect dysregulated overall stress levels, which may be useful for the professionals from different areas associated with sports and health knowledge. This is also of interest to immunologists, for whom γδ T-lymphocytes have proved enigmatic for decades. It is only recently being realised that these cells act as stress sensors and that distinct sub populations of γδ T-lymphocytes patrol different tissues. This warrants further investigation in the context of intense exercise and possible associated tissue damage/repair.

Moreover, the observed decrease in the capability of PMNs (mainly neutrophils) to undergo phagocytosis suggests that the training period designed in this present study induced detrimental effects in immunity associated with overreaching, which may be extrapolated to what could happen following intensified exercise and stress events, with limited recovery.

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## Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2021.100234.

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