Title:

Concurrent adaptations in maximal aerobic capacity, heat tolerance, microvascular blood flow and oxygen extraction following heat acclimation and ischemic preconditioning

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List of abbreviations

Arterio-venous O_2 difference ($C_{a\text{-}v}O_2$)

Carbon dioxide (CO_2)

Cardiac output ($\dot{Q}$)

Control (CON)

Core body temperature (Tc)

Delta efficiency (DE)

Deoxyhaemoglobin (HHb)

Heat acclimation (HA)

Heart rate (HR)

Ischemic pre-conditioning (IPC)

Limb blood flow (LBF)

Maximal oxygen uptake ($\dot{V}O_{2max}$)

Muscle O_2 diffusional conductance (DmO_2)

Muscle oxygen consumption (m$\dot{V}O_2$)

Near-infrared spectroscopy (NIRS)

Oxygen (O_2)

Oxyhaemoglobin (O_2Hb)

Oxygen pulse (O_2 pulse)

Plasma volume (PV)

Relative humidity (RH)

Rating of perceived exertion (RPE)

Stroke volume (SV)

Thermal sensation (TS)

Total haemoglobin (tHb)

Ventilatory threshold (V_T)

Whole-body sweat rate (WBSR)

Abstract

We investigated the effects of: 1) Ischemic pre-conditioning (IPC) plus a concurrent five-day heat acclimation + IPC (IPC+HA), 2) five-day HA with sham IPC (HA), or 3) control (CON) on thermoneutral measurements of endurance performance, resting measures of skeletal muscle oxygenation and blood flow. Twenty-nine participants were randomly allocated to three groups, which included: 1) five-days of repeated leg occlusion (4 x 5-min) IPC at limb occlusive pressure, plus fixed-intensity (55% $\dot{V}O_{2max}$) cycling HA at ~36°C/40% humidity; 2) HA plus sham IPC (20 mmHg) or 3) or CON (thermoneutral 55% $\dot{V}O_{2max}$ plus sham IPC). In IPC+HA and HA, there were increases in maximal oxygen consumption ($\dot{V}O_{2max}$) (7.8% and 5.4%, respectively; $P < 0.05$), ventilatory threshold (V_T) (5.6% and 2.4%, respectively, $P < 0.05$), delta efficiency (DE) (2.0% and 1.4%, respectively; $P < 0.05$) and maximum oxygen pulse (O_2pulse-Max) (7.0% and 6.9%, respectively; $P < 0.05$) during an exhaustive incremental test. There were no changes for CON ($P > 0.05$). Changes ($P < 0.05$) in resting core temperature (T_C), muscle oxygen consumption ($m\dot{V}O_2$), and limb blood flow (LBF) were also found pre-to-post intervention among the HA and IPC+HA groups, but not in CON ($P > 0.05$). Five-days of either HA or IPC+HA can enhance markers of endurance performance in cooler environments, alongside improved muscle oxygen extraction, blood flow, exercising muscle efficiency and O_2 pulse at higher intensities, thus suggesting the occurrence of peripheral adaptation. Both HA and IPC+HA enhance the adaptation of endurance capacity, which might partly relate to peripheral changes.

Key words: hot training; maximal oxygen consumption; limb occlusion

1. Introduction

Repeated exposures to a hot environment, often in combination with exercise, results in a series of systemic physiological adaptations, collectively referred to as 'heat acclimation' (HA; Daanen et al., 2018). These adaptations can enhance one's capacity to thermoregulate in the heat, taking place rapidly within the first five-days and maximised within two-weeks (Daanen et al., 2018). This combination of physiological adaptations can enhance endurance performance in hot (Racinais et al., 2015) and thermoneutral environments (Lorenzo et al., 2010; Waldron et al., 2019).

A number of studies have demonstrated changes in sub-maximal markers of endurance performance (i.e. lactate threshold) and maximal oxygen consumption ($\dot{V}O_{2max}$) in a thermoneutral environment following HA (Nadel et al., 1974; Sawka et al., 1985; Pivarnik et al., 1987; Takeno et al., 2001; Lorenzo et al., 2010; Waldron et al., 2019), while others have not (Houmard et al., 1990; Gore et al. 1997; Karlsen et al., 2015; Keiser et al., 2015; Rendell et al., 2017; Sotiridis et al., 2019). It was reasoned in a recent study that insufficient post-acclimation adaptation periods could explain these discrepancies (Waldron et al., 2019), alongside other factors such as inter-individual differences in adaptation capacity (Corbett et al., 2018). Adaptation of $\dot{V}O_{2max}$ in response to training or HA is commonly ascribed to centrally-limiting factors, such as plasma volume (PV) expansion, increased cardiac output ($\dot{Q}$) and stroke volume (SV) (Bassett & Howley, 2000). However, these central cardiovascular changes often decay prior to $\dot{V}O_{2max}$ following HA. Indeed, peak $\dot{V}O_{2max}$ adaptations appear to occur between four (Waldron et al., 2019) and ~26-days after HA (Weller et al., 2007). During this period, almost complete decay of central cardiovascular

adaptation could occur (Daanen et al., 2018), which would indicate that adaptation in $\dot{V}O_{2max}$ (and possibly other determinants of endurance performance) could be attributed to factors distally located in the O_2 transport pathway. Preliminary findings indicate that adaptations in the peripheral microvasculature could contribute to the heat acclimated phenotype (Lorenzo & Minson, 2010). Furthermore, increases in mitochondrial protein content and function have also been demonstrated in animal models following heat therapy (Tamura et al., 2014) but are frequently overlooked in relation to endurance performance adaptations, such as exercise efficiency or aerobic capacity, perhaps owing to the contrary evidence in humans (Mang et al., 2020). On the understanding that muscle O_2 diffusion limitation has a significant effect on $\dot{V}O_{2max}$ (Wagner et al., 1996), it is possible that a HA-induced enhancement of skeletal muscle O_2 extraction or microvascular blood flow could lead to increases in endurance capacity or improved submaximal exercise efficiency but this has not been investigated.

Ischemic pre-conditioning (IPC) - typically applied via a series of intermittent bi-lateral occlusions to the lower-limbs - has been found to enhance endurance exercise performance (de Groot et al., 2010; Kido et al., 2015; Jeffries et al., 2019). Repeated IPC (between three and seven consecutive daily applications) appears to elicit the greatest effect on micro- and macro-vascular blood flow, muscle oxidative capacity and subsequent endurance performance in thermoneutral conditions (Jones et al., 2014; Jeffries et al., 2018; 2019). Repeated remote IPC of a single-limb has been shown to enhance nitric oxide-independent skin microvascular blood flow by between 20 and 50%, which was attributed to an early-phase structural (angiogenic) response (Lang et al., 2019). Collectively, each of these adaptations could be advantageous for

convective or evaporative heat dissipation among those exposed to hot environments and, in combination with HA, could ameliorate adaptations for endurance performance in thermoneutral environments.

Acute pre-exercise IPC interventions have been reported to enhance lower-limb oxygenation in hypoxia (Wiggins et al. 2019) and increase performance in hypoxic conditions (Paradis-Deschênes et al., 2018). Indeed, five-days of repeated IPC has been shown to enhance endurance performance by ~ 6.6% at high-altitude (Foster et al., 2014). However, to the best of the authors' knowledge, five-days repetition of IPC – a non-thermal intervention strategy - has not been investigated for the purpose of heat acclimation. This is a feasible strategy since systemic hypoxic exposure (which can also include local limb blood flow arrest via IPC) of sufficient magnitude has been theorised to induce protection from a secondary stressor (i.e. heat or *vice versa*), thus conferring 'cross-tolerance' (Ely et al., 2014). Indeed, stimulation of the Hypoxia Inducible Factor-1 pathway has been reported following both IPC (Albrecht et al., 2013) and heat exposure of varying duration (Assayag et al., 2012; Lee et al., 2016). This pathway initiates a cascade of cellular events that control oxygen-related gene expression (Ely et al., 2014), including vascular endothelial growth factors, which can respond in short time periods (seven single leg training sessions; Gustafsson et al., 2002) or alongside increased vessel density after three training sessions in rodent models (Amaral et al., 2001). The combination of daily local, passive ischemia, with endurance exercise in the heat, could therefore feasibly enhance the heat acclimated phenotype and provide more comprehensive preparation for subsequent hot or thermoneutral exercise capacity. Given the array of aforementioned physiological adaptations, it is therefore possible that IPC could be used as a remote, non-thermal

adjunct heat acclimation strategy to improve systemic tolerance of the heat and endurance performance, particularly for those preparing to perform in hot conditions or attempting to maximise endurance adaptations prior to athletic competition or military operation.

Based on the above reasoning, we investigated the effects of two experimental interventions: 1) IPC plus a concurrent five-day HA (IPC+HA) or 2) five-day HA with sham IPC (HA), on thermoneutral measurements of $\dot{V}O_{2max}$, ventilatory threshold (V_T), delta efficiency (DE) and measures of resting muscle microvascular blood flow and oxygenation *vs.* a sham IPC control (CON), among recreationally active males. It was hypothesised that both experimental groups (HA and IPC+HA) would improve measures of endurance performance compared to CON, and that the IPC+HA intervention would elicit the greatest physiological adaptations.

2. Methods

2.1. Participants

A total of 29 recreationally active male adults provided written informed consent to take part in this study. Ten of the participants were randomly allocated to a HA group (age 26 ± 3 years, stature 1.79 ± 0.81 m, body mass 77.9 ± 9.8 kg), 9 were allocated to a IPC+HA group (age 26 ± 4 years, stature 1.79 ± 0.80 m, body mass 83.0 ± 13.6 kg) and 10 were allocated to CON (age 25 ± 2 years, stature 1.81 ± 0.63 m, body mass 74.1 ± 6.0 kg). A-priori sample size was calculated using G*Power (Version 3.1.9.3), according to changes in $\dot{V}O_{2max}$ following repeated IPC intervention (*d* =

0.91; Lindsay et al., 2017). Ten participants per group was deemed a sufficient sample size, with power of 0.80 and α = 0.05. One participant withdrew from the control group during testing (*n* = 9). None of the participants had taken part in outdoor hot weather training in the previous 12-months, and were at least three months without exposure to environmental heat. Testing took place between November and May in the UK. Thus, all participants were deemed to be unacclimatised to the heat. All participants completed a food diary for two days prior to each test, which was replicated with similar content and volume for the remainder of the study. The participants were instructed not to use saunas or take hot baths during the study period. Participants were asked to refrain from alcohol and any supplementation during the study period and arrive at the laboratory having eaten a standardised meal and consumed 500 ml of fluid in the previous 2-h. Euhydration was verified via urine analysis using an osmometer (<600 mOsmol·kg^{-1} H_2O, Osmocheck, Vitech Scientific Ltd, UK). Ethical approval was provided by the institutional ethics committee (SMEC_2018-19_041), which was conducted in accordance with the 2013 Helsinki declaration, except for registration in a database.

2.2. Experimental design

This study followed a randomized, single-blind, independent groups design, comprising three study arms (HA, IPC+HA, CON). After baseline tests of thermoneutral (~ 17 °C) $\dot{V}O_{2max}$, ventilatory threshold (V_T) and measures of resting muscle blood flow and oxygenation, the participants were randomly allocated to their groups using a Microsoft Excel random number generator. Two-days after their baseline testing, the HA group visited the laboratory (an environmental chamber

[Sporting Edge UK, Basingstoke, UK]) to complete five consecutive days of 60-min fixed-intensity (55% $\dot{V}O_{2max}$) cycling at (36.0 ± 1.4°C, 40 ± 5% RH). This was preceded by a Sham IPC procedure. The IPC+HA group completed the same HA programme, except exercise was preceded by an IPC procedure. The CON group visited the laboratory on the same number of occasions but completed their cycling exercise at the same intensity in controlled conditions (17.0 ± 1.1 °C, 30 ± 10% RH), before which a sham IPC intervention was conducted. In accordance with a recent study (Waldron et al., 2019), precisely four-days after the final intervention session, all thermoneutral (~ 17 °C) re-testing took place. This also permitted sufficient time for a second adaptation window (+ 72-h) following IPC (Loukogeorgakis et al., 2005). The participants were instructed not to take part in any additional exercise outside of that prescribed during the study for the entire data collection period.

2.3. Incremental ramp tests for V_T and $\dot{V}O_{2max}$

Participants were familiarised with the cycle ergometer (Monark Exercise AB, Ergomedic 874E, Varberg, Sweden), and the saddle and handlebar position were recorded and repeated for all subsequent visits. Participants then completed a 5-min self-selected warm-up prior to completing an incremental ramp test. The test was conducted at 70-80 rev/min, starting at ~ 70-80 W and increasing by 24 W/min until volitional exhaustion. The same increments were used across all trials and for all participants. Pulmonary gas was measured continuously using a breath-by-breath gas analyser (Jaeger Vyntus CPX, Hoechberg, Germany). The gas analyser was calibrated before every trial with gases of known concentration (15.95% O_2, 4.97% CO_2, BAL. N_2) and the turbine volume transducer was calibrated automatically by the

system at flow values of 2 L/s and 0.2 L/s (Hans Rudolph, Kansas City, KS). Maximal oxygen consumption was determined as the mean value recorded over the final 30-s of the test. Criteria for achieving $\dot{V}O_{2max}$ was: [1] reaching volitional exhaustion, [2] respiratory exchange ratio > 1.15, [3] final HR within 10 beats/min of age-predicted maximum and [4] RPE > 19 (6-20 Borg scale). All criteria were met during the study. The same gas analyser was used throughout the study and calibrated identically. Heart rate was recorded throughout the tests (Polar FT1, Polar Electro Oy, Kempele, Finland). End-power output was measured as the highest external power output reached in the corresponding segment of the test. All tests were performed at the same time of day (± 2-h). In our laboratory, incremental tests of $\dot{V}O_{2max}$ have a coefficient of variation of 3.0%. Breath-by-breath $\dot{V}O_2$ and $\dot{V}CO_2$ data from the incremental cycling test was used to plot V_T using the simplified v-slope method (Schneider et al., 1993).

As a correlate of arterio-venous O_2 difference ($C_{a\text{-}v}O_2$) during latter stages of incremental exercise (Degani-Costa et al., 2019), the O_2 pulse (ml pulmonary O_2 / HR) was measured at 80% (O_2pulse-80%) and 100% of the incremental test (O_2pulse-Max). Delta efficiency (DE) was determined as the ratio of the change in work accomplished/min to the change in metabolic energy expended/min between the V_T and end of the incremental test (Perez et al., 2003). Delta efficiency was calculated from linear regression (y = ax + b) of energy expended/min (y, in kcal/min) and work accomplished/min (x, in kcal/min), where DE is equal to the reciprocal of the slope (1/a).

2.4. Resting m$\dot{V}O_2$, blood flow and reactive hyperaemia

Following 10-min of supine rest on a padded table, with both legs fully extended and raised onto a cushion, a near-infrared spectroscopy (NIRS) optode (Portamon, Artinis medical systems) was placed on the gastrocnemius medialis (2/3 distance from the calcaneus and anterior fossa), secured with an elastic bandage (Tiger Tear, Hampshire, UK) and covered with an optically dense black material. The system is a two-wavelength continuous wave system that simultaneously uses the modified Beer-Lambert law and spatially resolved spectroscopy methods. Changes in tissue oxyhaemoglobin (O_2Hb), deoxyhaemoglobin (HHb), and total haemoglobin (tHb) were measured using the differences in absorption characteristics of infrared light at 760 and 850 nm. Differential path factor of 4 was used throughout. NIRS data was connected to a computer by Bluetooth for acquisition at 10 Hz. The position of the probe was marked with indelible ink, which was reapplied at regular intervals during the intervention protocol, using the same anatomical location.

A rapidly inflating blood pressure cuff was placed around the mid-thigh (width: 15 cm; Hokanson SC12D, Bellevue, WA, United States), which was connected to a rapid cuff inflator (E20, Hokanson, Bellevue, WA, United States), supplied by an air compressor. Resting limb blood flow (LBF) was assessed by rapidly occluding the pressure cuff to 50 mm Hg for 10-s on two occasions, each separated by 2-min. The mean of the repeated LBF measures was reported. Blood flow was determined via the linear increase in tHb within the first two seconds of the venous occlusion (Van Beekelt et al., 2001). Concentration changes of tHb were expressed in μM/s and were converted to ml blood/100 mg tissue/min according to (Van Beekelt et al., 2001). Resting $m\dot{V}O_2$ was assessed by rapidly inflating the blood pressure cuff to 300 mmHg (arterial occlusion) for 30-s on two occasions, each separated by 5-min, with the

mean of the repeated measures reported. For $m\dot{V}O_2$ measures, a blood volume correction factor was applied (Ryan et al., 2012) and calculated by the slope of change in the corrected O_2Hb-HHb difference during the first 3-s of an arterial occlusion using linear regression. $m\dot{V}O_2$ data were expressed relative to a normalised range (see below) and reported as %/s.

Finally, a reactive hyperaemia test was conducted by inflating the cuff to 300 mmHg for 5-min. This represented maximal deoxygenation (0%) of the tissue under the optode. Following release of the cuff, a peak hyperaemic response (representing 100% oxygenation) was recorded. All $m\dot{V}O_2$ and hyperaemia data were normalized to this 'physiological' range to allow comparisons between individuals with differing tissue thicknesses (Ryan et al., 2012; Jeffries et al., 2018). Reactive hyperaemia was described as the normalised rate constant ($k; s^{-1}$) of the normalized O_2Hb signal from the point of cuff release until plateau, using a sigmoidal Gompertz model (Bopp et al., 2011). All raw data analysis and curve fitting procedures were performed on GraphPad Prism (GraphPad 5.0 Software, La Jolla, CA, United States).

2.5. Heat acclimation protocol

The power output corresponding to 55% of the participants' baseline $\dot{V}O_{2max}$ was set as the external work intensity for the intervention and was monitored using power output on the cycle ergometer. Cadence was self-selected and adjusted if necessary by the investigators using weights to maintain the target intensity. This intensity was maintained for all subsequent trials. Participants cycled for 60-min per session. This type of fixed-intensity HA protocol was selected based on previous studies but for a

shorter number of days (Houmard et al., 1990; Lorenzo et al., 2010; Waldron et al., 2019). The participants' nude body mass was recorded pre- and post-session on every day of the HA programme to estimate whole-body sweat rate (WBSR) by subtracting post-exercise body mass from pre-exercise values (MPMS-230, Marsden Weighing Group, Oxfordshire, UK). A rectal thermometer (Edale Instruments Ltd, Cambridge, UK) was self-inserted ~10 cm past the anal sphincter, as an indication of Tc, and recorded at rest (pre-exercise outside on the heat chamber) and then every 5-min once exercise commenced inside the heat-controlled chamber (Edale Instruments Ltd, Cambridge, UK). The resting Tc on day 1 and day 5, as well as the end Tc of each session was used for statistical analysis. The participants then entered the heat chamber wearing cycling shorts, socks and training shoes, where they sat upright on the same cycle ergometer used during the ramp test. HR was also recorded, alongside thermal sensation (TS) and RPE (6-20) at 5-min intervals throughout the exercising protocol. TS was recorded on an ASHRAE 9-point analogue scale, where -4 = "very cold", 0 = "neutral", and 4 = "very hot" (Zhang et al., 2004). In the control condition, the heat chamber was controlled at 19.0 ± 1.3°C, 30 ± 7% RH. No fans were used during the exercise trials and no fluid intake was permitted until after post-session measurements.

2.6. IPC protocol

Sixty-min prior to entering the environmental chamber, the participants lay in a supine position on a massage bed, with automatic inflation cuffs (14.5 cm width, Delfi Medical Innovations, Vancover, Canada) fitted to the proximal portion of both thighs. The inflatable cuffs were connected to a pressure gauge and were automatically inflated to

the participant's individual limb occlusive pressure, which ranged between 210 and 250 mmHg, thus ensuring full arterial occlusion. The HA and CON groups were given a lower pressure (20 mmHg) using the same automatic inflation cuffs. The protocol involved 5-min occlusion, followed by 5-min reperfusion, which was repeated four times (lasting 40-min). This procedure was repeated for five consecutive days (Foster et al., 2014).

2.7. Statistical analyses

Changes in all incremental test variables; $\dot{V}O_{2max}$, end-power output, O_2pulse-80%, O_2pulse-Max, DE, V_T (%), final HR, as well as the resting Tc and resting HR on day 1 and 5, were analysed using two-way (3 [group] X 2 [time]) within and between analyses of variance. End Tc, end HR, end RPE and end Ts recorded during the HA programme (3 [group] X 5[time]) were analysed using two-way within and between analyses of variance. Where relevant, assumptions of sphericity were assessed using Mauchly's test, with any violations adjusted using the Greenhouse-Geisser correction. When significant *F*-values were observed, Bonferroni *post-hoc* tests were used to determine differences. For pairwise effects, Cohen's *d* effect sizes were calculated, with thresholds set as: ≤ 0.2 small; ≤ 0.6 moderate; ≤ 1.2 large; ≥ 2.0 very large (Hopkins et al., 2009). Cohen's *d* was determined as pairwise mean differences / pooled standard deviation and eta squared (η_p^2) were calculated as the sum of squares of the effect / total sum of squares. Qualitative descriptors were not used for η_p^2 values. of Statistical significance was accepted at $P < 0.05$ for all tests and all analyses were performed on IBM SPSS Statistics (Version 21, IBM Corp., Armonk, NY, USA).

3. Results

3.1. Incremental exercise test measures

There were group x time interactions for $\dot{V}O_{2max}$ ($F_{(2,26)}$= 5.407, P = 0.011, η_p^2 = 0.57), with pre-to-post increases in the HA (37.3 ± 5.9 to 39.3 ± 5.3 ml/kg/min; P < 0.001; d = 0.37) and IPC+HA group (38.2 ± 8.1 to 41.4 ± 8.6 ml/kg/min; P < 0.001; d = 0.39). There were no changes in CON (41.7 ± 5.2 42.3 ± 5.4 ml/kg/min; P = 0.336) (Figure 1A). Similarly, there was a group x time interaction for mechanical power output ($F_{(2,26)}$= 23.098, P = 0.001, ηp^2 = 0.640), with pairwise increases between pre- and post-tests for HA (P < 0.001; d = 0.35) and the IPC+HA groups (P < 0.001; d = 0.75) (Figure 1B). No other pairwise differences within or between groups (P > 0.05) were found and there were no pre-to-post differences for CON.

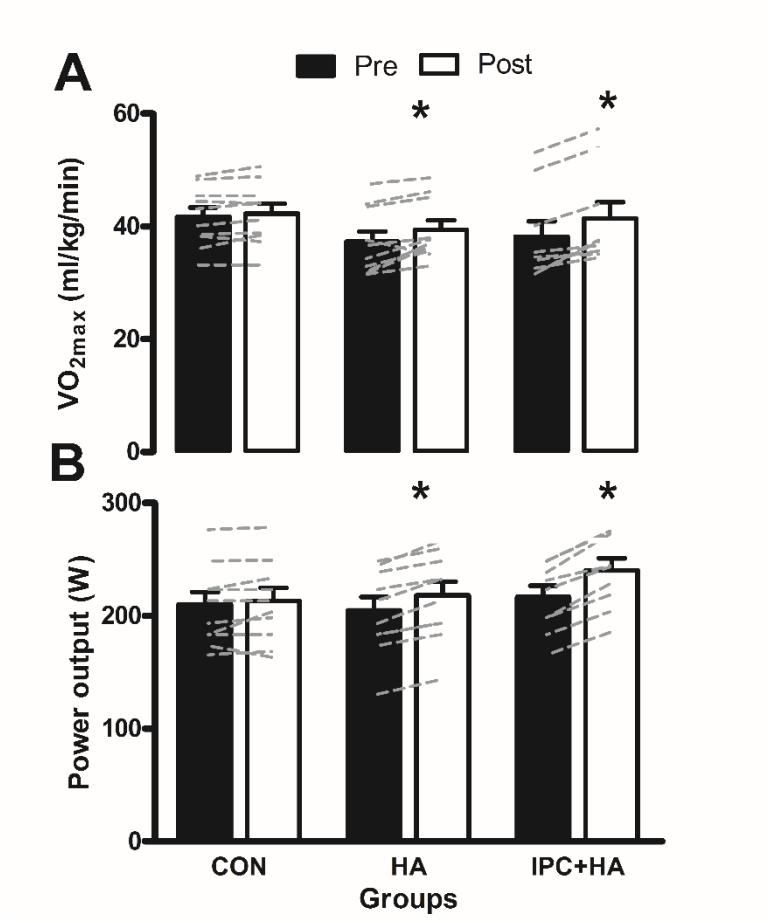

Figure 1. Pre and post values (mean ± SD) of (A) maximal oxygen consumption ($\dot{V}O_{2max}$) and (B) mechanical power output among the control (CON), heat acclimation (HA) and the ischemic pre-conditioning + heat acclimation (IPC+HA) groups. * = pre-to-post difference ($P < 0.05$) for that group.

As presented in Table 1, there were group x time interactions for V_T ($F_{(2,26)}$= 13.461, $P < 0.001$, $\eta p^2 = 0.51$) and DE ($F_{(2,26)}$= 18.890, $P < 0.001$, $\eta p^2 = 0.59$). Pre-to-post increases were identified in V_T and DE for the HA ($P = 0.004$ and $P < 0.001$, respectively) and IPC+HA group ($P < 0.001$ and $P < 0.001$, respectively). Both the O_2pulse-80% ($F_{(2,26)}$= 3.392, $P = 0.047$, $\eta p^2 = 0.21$) and the O_2pulse-Max ($F_{(2,26)}$= 3.297, $P = 0.050$, $\eta p^2 = 0.21$) demonstrated group x time interactions, which were explained by pre-to-post increases for the HA ($P = 0.010$ and $P = 0.011$, respectively) and the IPC+HA groups ($P = 0.004$ and $P = 0.006$, respectively) (Table 1). There were no group x time interactions ($F_{(2,26)}$= 0.914, $P = 0.414$, $\eta p^2 = 0.066$) or any main effects of time or group ($P > 0.05$) for final HR during the incremental test (Table 1). No other pairwise differences within or between groups ($P > 0.05$) were found, with no pre-to-post differences for CON.

Table 1. Pre and post values (mean ± SD) of resting and exercising physiological measures among the control (CON), heat acclimation (HA) and the ischemic pre-conditioning + heat acclimation (IPC+HA) groups.

	CON		HA		IPC+HA	
	Pre	***Post***	***Pre***	***Post***	***Pre***	***Post***
Resting						
Resting HR (beat/min)	63 ± 9	62 ± 8^{S}	65 ± 6	64 ± 5^{S}	63 ± 4	61 ± 5^{S}
Resting Tc (°C)	37.3 ± 0.2	37.3 ± 0.2$^{S\#}$	37.1 ± 0.3	36.9 ± 0.3^{M}*	37.1 ± 0.4	36.8 ± 0.4^{M}*
Incremental test						
V_T (%)	57.7 ± 3.8	57.8 ± 3.8^{S}	56.7 ± 3.1	58.0 ± 2.4^{S}*	56.6 ± 3.1	59.9 ± 2.1^{L}*
V_T (ml/kg/min)	24.1 ± 2.8	24.3 ± 2.9^{S}	21.2 ± 4.1	22.8 ± 3.7^{S}	21.5 ± 3.7	24.7 ± 4.5^{M}
Final HR (beats/min)	174 ± 10	176 ± 10^{S}	177 ± 11	176 ± 12^{S}	170 ± 8	172 ± 9^{S}
DE (%)	27.2 ± 1.7	27.2 ± 1.8^{S}	25.3 ± 2.1	26.7 ± 1.7^{M}*	26.0 ± 2.1	28.0 ± 2.3^{M}*
O_2pulse-80% (ml/beat)	17.8 ± 3.1	17.7 ± 2.6^{S}	16.2 ± 2.2	17.4 ± 3.0^{S}*	18.3 ± 3.2	19.7 ± 4.3^{S}*
O_2pulse-Max (ml/beat)	24.1 ± 3.9	24.1 ± 3.4^{S}	20.9 ± 2.6	22.4 ± 2.8^{S}*	22.4 ± 4.3	24.0 ± 4.9^{S}*

*Note: heart rate (HR), core temperature (Tc), ventilatory threshold (V_T), final exercising HR, delta efficiency (DE), and O_2 pulse at 80% and maximal O_2 consumption. * = pre-to-post difference (P < 0.05) for that group; # = different (P < 0.05) to post values for comparison groups. Cohens d: S = small, M = moderate, L = large.*

3.2. Resting measures

As presented in Table 1, there were group x time interactions for resting Tc ($F_{(2,26)}$= 11.322, P < 0.001, ηp^2 = 0.47). Pre-to-post decreases were identified in both the HA (P < 0.001) and IPC+HA groups (P < 0.001) but not CON (P = 1.000) (Table 1). There were lower values in resting Tc between CON and HA (P < 0.001) and CON and IPC+HA (P < 0.001) in the post-tests (Table 1). There were no group x time interactions ($F_{(2,26)}$= 0.713, P = 0.500, ηp^2 = 0.05) but there were main effects of time ($F_{(2,26)}$= 14.408, P < 0.001, ηp^2 = 0.36) for resting HR (Table 1). There were group x time interactions for m$\dot{V}O_2$ ($F_{(2,26)}$= 18.416, P < 0.001, ηp^2 = 0.58). Resting m$\dot{V}O_2$ decreased pre-to-post in both the HA (P < 0.001; d = 1.41) and IPC+HA groups (P < 0.001; d = 2.75) but not CON (P = 1.000; d = 0.08) (Figure 2C). There were further pairwise differences in m$\dot{V}O_2$ between CON and HA (P = 0.018; d = 0.65) and CON and IPC+HA (P < 0.001; d = 2.05) in the post-tests, with the lower values for the experimental groups. There were no interaction effects found for LBF ($F_{(2,26)}$= 2.064, P = 0.147, ηp^2 = 0.14) but there were main effects of group ($F_{(2,26)}$= 3.869, P = 0.034, ηp^2 = 0.23) and

time ($F_{(1,26)}$= 17.919, P < 0.001, ηp^2 = 0.41), with higher values for HA *vs.* CON (P = 0.039; d = 1.55) and for IPC+HA vs. CON (P = 0.038; d = 2.01) (Figure 2B). There were no main effects or interactions (P > 0.05) for rate constant during the reactive hyperaemia tests (Figure 2A).

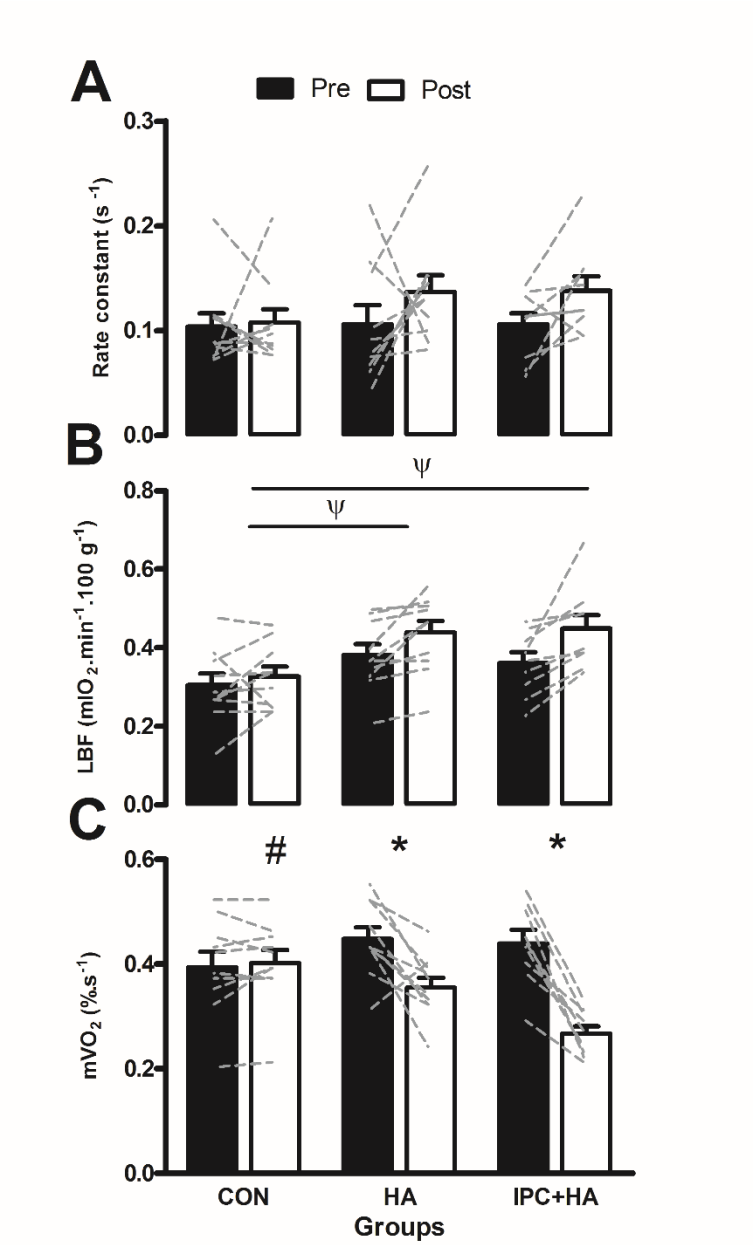

Figure 2. Pre and post values (mean ± SD) of (A) rate constant during reactive hyperaemia, (B) limb blood flow (LBF) and (C) muscle oxygen consumption ($m\dot{V}O_2$) among the control (CON), heat acclimation (HA) and the ischemic pre-

conditioning + heat acclimation (IPC+HA) groups. * = pre-to-post difference (P < 0.05) for that group; # = different (P < 0.05) to post values for comparison groups; φ = between-group effects (P < 0.05).

3.3. Heat acclimation measures

There were group x time interactions for end Tc during the acclimation sessions ($F_{(8,104)}$= 2.263, P = 0.028, ηp^2 = 0.15). End Tc was lower for CON *vs*. HA (P < 0.05) or IPC+HA groups (P < 0.05) at each day of acclimation but not between HA and IPC+HA (P > 0.05) (supplementary file, Table 1). There were pairwise differences in end Tc between day one of HA and day five of HA for the HA group (P = 0.017; d = 0.85) and the IPC+HA group (P = 0.010; d = 0.65), demonstrating a reduced Tc in response to the acclimation sessions across time, but this was not found for CON (P = 1.000; d = 0.15) (supplementary file, Table 1). There were also main effects of time ($F_{(4,104)}$= 10.302, P < 0.001, ηp^2 = 0.28) and group ($F_{(2,26)}$= 92.665, P < 0.001, ηp^2 = 0.87) for end Tc.

There were group x time interactions for end TS during the acclimation sessions ($F_{(8,104)}$= 3.031, P = 0.004, ηp^2 = 0.189). End TS was lower for CON *vs.* HA (P < 0.05) or IPC+HA groups (P < 0.05) at each day of acclimation but not between HA and IPC+HA (P > 0.05) (supplementary file, Table 1). There were pairwise differences in end TS between day one of HA and day 5 of HA for the HA group (P < 0.001; d = 2.19) IPC+HA group (P < 0.001; d = 2.18), indicating a lowered perception of the heat across days of acclimation; however, this did not occur in the CON group (P = 1.000; d = 0.16) (supplementary file, Table 1). There were also main effects of time ($F_{(4,104)}$= 14.919, P < 0.001, ηp^2 = 0.37) and group ($F_{(2,26)}$= 85.676, P < 0.001, ηp^2 = 0.868) for end TS.

There were group x time interactions for end RPE during the acclimation sessions ($F_{(8,104)}$= 2.950, P = 0.005, ηp^2 = 0.185). End RPE was lower for CON *vs.* HA (P < 0.05) or IPC+HA groups (P < 0.05) at each day of acclimation but not between HA and IPC+HA (P > 0.05) (supplementary file, Table 1). There were pairwise differences in end RPE between day one of HA and day 5 of HA for all groups, indicating a reduction in RPE across days of acclimation (P < 0.001) (supplementary file, Table 1). There were also main effects of time ($F_{(4,104)}$= 81.990, P < 0.001, ηp^2 = 0.76) and group ($F_{(2,26)}$= 58.870, P < 0.001, ηp^2 = 0.81) for end RPE.

There were group x time interactions for end HR during the acclimation sessions ($F_{(8,104)}$= 7.925, P < 0.001, ηp^2 = 0.38). End HR was lower for CON *vs.* HA (P < 0.05; d = 1.93 to 3.06) or IPC+HA groups (P < 0.05 d = 1.31 to 1.50) at day one and two of acclimation but not between HA and IPC+HA (P > 0.05; d = 0.30 to 0.50). On HA days three, four and five, end HR was lower for both the HA and CON *vs*. IPC+HA group (P < 0.05; d = 0.72 to 3.12) (supplementary file, Table 1). There were pairwise differences in end HR between day one of HA and day 5 of HA for all groups (P < 0.001), indicating a HR reduction across days of acclimation (supplementary file, Table 1). There were main effects of time ($F_{(4,104)}$= 40.249, P < 0.001, ηp^2 = 0.65) and group ($F_{(2,26)}$= 6.926, P = 0.004, ηp^2 = 0.35) for end HR.

There were group x time interactions for WBSR during the acclimation sessions ($F_{(8,104)}$= 12.125, P < 0.001, ηp^2 = 0.48). WBSR was higher by days four and five of HA compared to day one for both the HA (P < 0.05; d = 2.45 to 3.01) and IPC+HA groups

($P < 0.05$; $d = 1.89$ to 2.23) but not for CON ($P = 1.000$). On each day of HA, WBSR was higher in the HA and IPC+HA groups compared to CON ($P < 0.001$ for all comparisons), with no differences between HA and IPC+HA groups ($P > 0.001$) (supplementary file, Table 2).

4. Discussion

The primary findings of this study were that both HA and IPC+HA increased sub-maximal (V_T and DE) and maximal ($\dot{V}O_{2max}$) measures of endurance performance compared to CON. These improvements corresponded to enhanced local blood flow and reduced m$\dot{V}O_2$ at rest, as well as increased O_2 pulse at 80% and 100% of an incremental test. Despite some descriptively larger mean changes in the IPC+HA group compared to the HA group for $\dot{V}O_{2max}$ (3.2 ± 2.0 ml/kg/min [7.8%] and 2.6 ± 1.4 ml/kg/min [5.4%], respectively), DE (2.0 ± 0.8 % and 1.5 ± 0.6 %, respectively), V_T (3.3 ± 1.7 %$\dot{V}O_{2max}$ [5.6%] and 1.5 ± 1.0 %$\dot{V}O_{2max}$ [2.4%], respectively) and m$\dot{V}O_2$ (0.17 ± 0.07 %/s [40%] and 0.11 ± 0.05 %/s [21%], respectively), there were no statistical differences between the two experimental groups. Indeed, both HA and IPC+HA demonstrated comparable responses to the HA programme, with similar profiles of resting Tc, WBSR, end-exercise Tc, Ts and RPE. The time effects in both groups demonstrated the same classical adaptations to the hot environment during the five days of HA (see Periard et al., 2016; Dannen et al., 2018), with significantly lower HR on days four and five of the programme in the IPC+HA group compared to HA being the only indicator of a potential benefit of this strategy for acclimation purposes.

The magnitude of change in $\dot{V}O_{2max}$ reported herein is in accordance with a number of other studies, where HA-induced increases in thermoneutral $\dot{V}O_{2max}$ have ranged between 4% and 13% (Nadel et al., 1974; Shvartz et al., 1977; Sawka et al., 1985; Pivarnik et al., 1987; Takeno et al., 2001; Lorenzo et al., 2010; Waldron et al., 2019). However, we have shown here that these changes can be induced after a five-day HA dose, despite longer periods of HA likely to enhance the adaptation (Periard et al., 2016). Of note, the current sample was recreationally active, yet not engaged with any planned training or competition. Therefore, the efficacy of the shorter five-day intervention could be related to the current participants' training level. However, this is inconsistent with the notion that baseline $\dot{V}O_{2max}$ does not explain the variance in heat adaptation (Corbett et al., 2018) or explain its increase as a result of a HA programme (Benjamin et al., 2019). Therefore, the most probable reason could be a combination of characteristics inherent in the HA programme (i.e. the intensity) or the post-HA four-day adaptation window was observed (Waldron et al., 2019), thus permitting adequate time for a multi, 'in-series' system measurement such as $\dot{V}O_{2max}$ (Wagner et al., 2015) to super-compensate. Despite a 1.8% greater increase in $\dot{V}O_{2max}$ compared to HA, there was no statistical benefit of the IPC+HA; however, there have been inconsistent effects of repeated IPC on $\dot{V}O_{2max,}$ relating to a number of factors, including training level (Incognito et al., 2016). Given the descriptively greater adaption in IPC+HA group, our finding that IPC statistically provided no additive benefit requires further investigation, with longer training periods and those of different training backgrounds. The lower sample of the CON group (n = 9) could also explain the lack of difference in some variables.

Adaptations in endurance performance determinants, such as $\dot{V}O_{2max}$, are most commonly ascribed to centrally limiting factors (Bassett & Howley, 2000). Indeed, there is abundant evidence to demonstrate that increases in $\dot{V}O_{2max}$ can be explained by increased $\dot{Q}$ (Lundby et al., 2017), including after 10-days of HA (Nielsen et al., 1993; Lorenzo et al., 2010). However, there is also adequate evidence to support that microvascular O_2 extraction limitations equally explain the variability in $\dot{V}O_{2max}$ and, therefore, its adaptation capacity (Wagner et al., 1996). Careful consideration of both Fick's principle and Law of Diffusion indicate that $\dot{V}O_{2max}$ depends upon the combination of three factors: $\dot{Q}$, arterial O_2 content (CaO_2) and DmO_2 (muscle O_2 diffusional conductance) (Wagner et al., 2015). In regards to $\dot{Q}$, adaptation of $\dot{V}O_{2max}$ owing to endurance training is largely attributed to increased SV (Lundby et al., 2017). Although we did not measure it here, plasma volume expansion occurs rapidly during HA and is the most commonly reported reason for SV and $\dot{Q}$ increases, which can plausibly increase $\dot{V}O_{2max}$ (Périard et al., 2016). Indeed, while we did not measure SV directly, we report increases in O_2pulse at 80% and 100% of the incremental test. The O_2pulse can be used as a surrogate marker of SV and, therefore, could partly explain $\dot{V}O_{2max}$ increases (Bhambhani et al., 1994). However, it is well-known that SV plateaus near to 50% $\dot{V}O_{2max,}$ particularly in non-elite athletes (Stringer et al., 1997; Zhou et al., 2001) and that $Ca{-}vO_2$ linearly relates to pulmonary $\dot{V}O_2$ up to maximal exercise (Stringer et al., 2005). Thus, changes in O_2 pulse (mlO_2/beat) reported in the current study between 80% and 100% of the incremental test for both the HA and the IPC+HA groups are likely to be linked with peripheral muscle O_2 extraction and not solely central changes in SV. In support of this, concomitant increases in central ($\dot{Q}$), peripheral ($Ca{-}vO_2$) mechanisms and $\dot{V}O_{2max}$ in response to continuous endurance training (Daussin et al., 2007). Our results also provide further support to the recent evidence

that maximal rates of skeletal muscle respiration more completely explain maximal cycling endurance performance in comparison to traditional cardio-pulmonary determinants of central origin (Batterson et al., 2020). Further work is needed to determine the mechanistic basis of this adaptive response to HA but the current findings provide important direction for the potential sites of HA and/or IPC-induced adaptation.

In further support of the above changes in $\dot{V}O_{2max}$ and the theorised contributions from microvascular O_2 extraction, we report 40% (IPC+HA) and 21% (HA) reductions in resting m$\dot{V}O_2$. These changes are consistent with improved metabolic efficiency, as reported following repeated IPC (Jeffries et al., 2018), and are indicative of either a reduction in metabolic rate (reduced ATP requirement) or an increased mitochondrial efficiency (increased ATP per molecule O_2). However, we are the first to show this in response to HA protocol, thus indicating an effect on local muscle metabolism, which benefit could contribute to the acclimated phenotype. These reductions are larger than reported previously in healthy participants (Ryan et al., 2013; Jeffries et al., 2018); however, the baseline fitness of the current participants was low (supported by ~ 25% higher resting m$\dot{V}O_2$ at baseline compared to Jeffries et al. (2018)) and the aggressive nature of the interventions used. The reductions reported here in the resting skeletal muscle occurred alongside lower Tc at rest on day five compared to day one of acclimation in both the HA and IPC+HA groups. We also found increases in LBF following HA in both experimental groups, which can be caused by increased capillary growth (Hesketh et al., 2019) or nitric oxide availability (Brunt et al., 2019) following heat therapy. These changes are consistent with the suggestion that heat therapies elicit changes in skeletal muscle metabolism, the peripheral vasculature or a

combination of associated factors (Kim et al., 2020). However, given the shorter period of HA used here, only early-phase vascular adaptations can be expected, as demonstrated after seven single-leg training sessions (Gustafsson et al., 2002), following seven-days of remote single-arm IPC (Lang et al., 2019) or in response to brief three-day periods of exercise in animals (Amaral et al., 2001). Given the descriptively larger changes in microvascular flow and m$\dot{V}O_2$ (alongside exercising measures) in the IPC+HA group, it is possible that IPC augmented the effect of HA; however, no significant effects were found. This evidence partly questions the cross-adaptation hypothesis, assuming that the intermittent local hypoxic stimulus was sufficient to initiate the associated molecular pathways (Ely et al., 2014). Indeed, the only statistical inference that IPC assisted in the HA process above that of HA alone was the lower end-exercise HR on days four and five of the programme. We have no clear reason for this observation, besides the reported acute vagal stimulation elicited by IPC (Enko et al., 2011), which might have been carried over from the pre-exercise intervention. Based on the typical doses of HA (see Daanen et al., 2018) and IPC (see Incognito et al., 2016) interventions, it is possible that a longer intervention programme was required to elicit larger changes. Future research should investigate this possibility.

Both HA (Shvartz et al., 1977; Sawka et al., 1983; Young et al., 1985) and repeated IPC (Jeffries et al., 2019) can reduce the O_2 cost of exercise. Indeed, the 1.4% (HA) and 2.0% (IPC+HA) increases in DE found here are consistent with recent reports after seven-days of repeated IPC (3.1%; Jeffries et al. 2019) – although, it should be noted that the current calculations are based on linear slope during ramp exercise > V_T and could produce slightly different results. The current calculation of DE produces values

(~ 25-28%) that approximate muscle intracellular chemical-mechanical coupling efficiencies (Whipp & Wasserman, 1969), which can be enhanced by local changes in muscle mitochondrial content and function (Broskey et al., 2015). Therefore, the improvements in DE reported herein are consistent with our previous suggestions of distally located O_2 transport pathway mechanisms, such as enhanced muscle O_2 perfusion and/or metabolic efficiency. It should be noted that others have questioned the role of HA on thermoneutral DE, gross efficiency and $\dot{V}O_{2max}$ (Karlsen et al., 2015), despite reporting descriptive improvements across all of these variables.

In accordance with the magnitude of change reported previously in lactate threshold (Lorenzo et al., 2010; James et al., 2017), HA and IPC+HA improved V_T – a sub-maximal determinant of endurance performance - by 2.4% (HA) and 5.6% (IPC+HA). We partly attribute this finding to the lowered metabolic response at lower intensities (including resting muscle) and an increased O_2 pulse at higher intensities. The lower resting Tc found in the HA and IPC+HA groups following acclimation is also commonly overlooked as an important contributor to the increases in sub-maximal thresholds (i.e. V_T) and efficiency (i.e. DE), despite it dictating that thermoneutral incremental exercise will commence at a lower Tc. Assuming that Tc progresses in a similar manner to the pre-intervention incremental tests, the attainment of higher temperatures will occur at later stages. According to the Q_{10} effect, tissue $\dot{V}O_2$ should therefore proportionally decrease in the acclimated phenotype at baseline (Brooks et al., 1971) and could be reflected in pulmonary gas measures. Similarly, the ventilatory O_2 and CO_2 equivalents ($\dot{V}e/\dot{V}O_2$ and $\dot{V}e/\dot{V}CO_2$, respectively) are altered as a function of Tc (White & Cabanac, 1996), such that the determination of V_T will occur later in the cooler core of an acclimated participant. Therefore, along with increased efficiency/economy observed

during incremental cycling and possible changes in blood flow (inferred from resting LBF), the reduced baseline Tc provides some further explanation for the V_T improvement following the interventions.

4.1 Conclusion

We have demonstrated that five-days of either HA or IPC+HA can enhance markers of endurance performance in cooler environments. The changes observed in muscle O_2 extraction and blood flow at rest, coupled with preferable increases in exercising muscle efficiency and O_2 pulse at higher intensities, indicate that some of these adaptations occur peripherally. Despite similar responses to the HA programme, further research is required to understand the potential role of IPC as an adjunct, non-thermal intervention for heat adaptation and enhancement of endurance exercise as it is likely that longer intervention periods are needed to identify this.

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Conflicts of interest

There are no conflicts of interest or competing interests.

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