CROSS ADAPTATION AND CROSS TOLERANCE IN HUMAN HEALTH AND DISEASE

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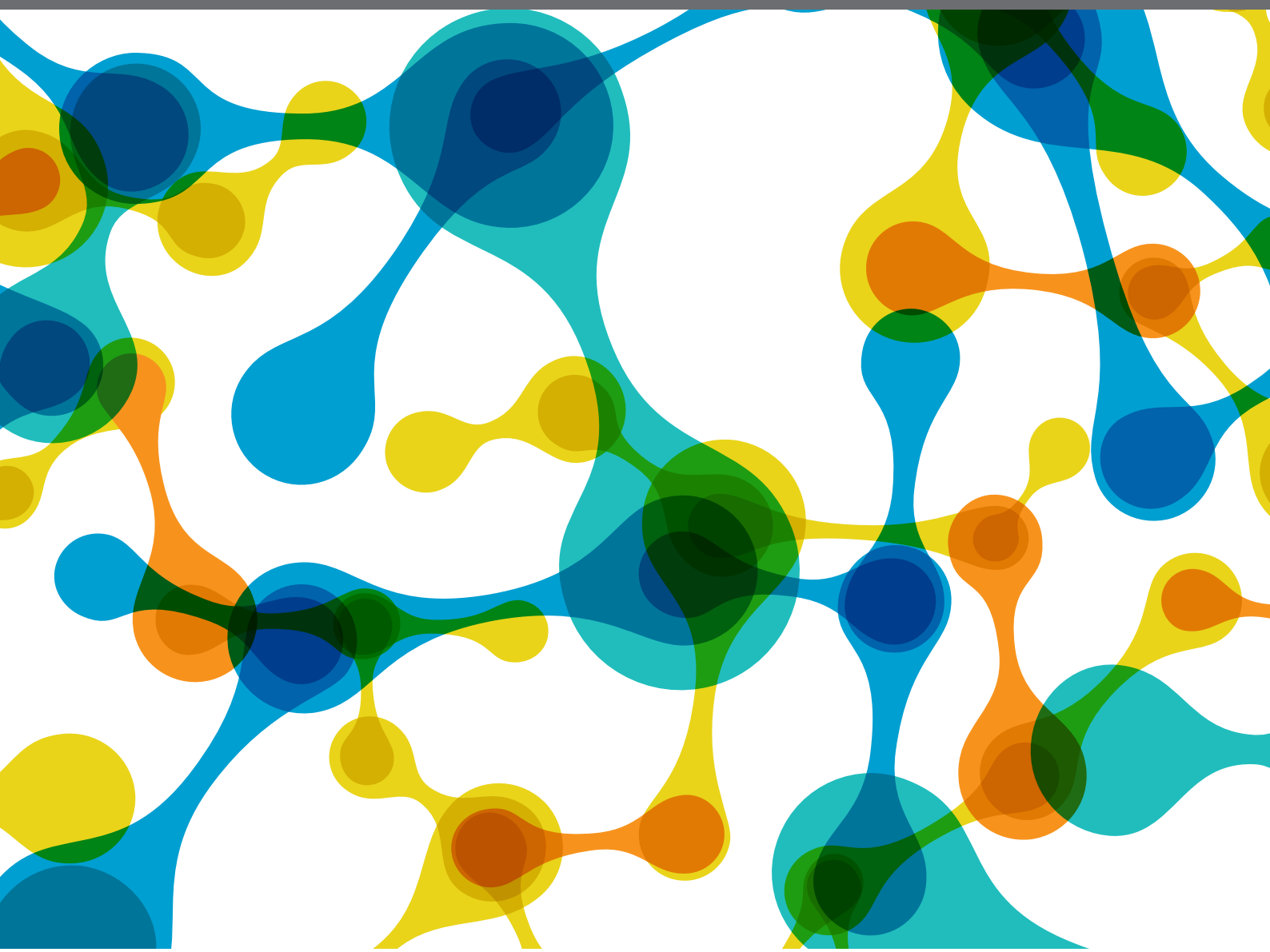

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CROSS ADAPTATION AND CROSS TOLERANCE IN HUMAN HEALTH AND DISEASE

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EDITORIAL
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Editorial: Cross Adaptation and Cross Tolerance in Human Health and Disease

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Editorial on the Research Topic

Cross Adaptation and Cross Tolerance in Human Health and Disease

Human physiological responses to heat, cold, hypoxia, microgravity, hyperbaria, hypobaria, and fasting are well-studied in isolation. However, in the natural world these stressors are often combined or experienced sequentially (Tipton, 2012). Studies examining human responses to these more realistic, yet relatively complex, circumstances remain sparse, but could provide important insights into an emerging area within human physiology: cross-adaptation **(Figure 1)** (Lunt et al., 2010; Gibson et al., 2017). Much of the current state of knowledge involves data demonstrating benefits of exercising in hot conditions, prior to performance in hypoxia (Heled et al., 2012; Lee et al., 2014a,b, 2016; Gibson et al., 2015; White et al., 2016; Salgado et al., 2017), with cold to hypoxia (Lunt et al., 2010), hypoxia to heat (Sotiridis et al., 2018), combined stressors (Takeno et al., 2001; Neal et al., 2017), and more mechanistic (signaling) data from animal models exposed to substantive volumes of stress (Maloyan and Horowitz, 2002, 2005). The role of nutrient availability and the nutrient-exercise interactions which drive phenotypic adaptations to skeletal muscle exposed to a multitude of stressors is also a growing field of interest (Hawley et al., 2018). This research topic includes publications which address both clinical and exercise-centric aspects allied to Cross-adaptation and Cross-tolerance in Human Health and Disease.

An excellent primer covering aspects of preconditioning, short and long term heat acclimation cross-tolerance, and heat acclimation memory in both animal and human experimental models has been provided by Professor Horowitz of the Hebrew University. This extensive review of the cellular and molecular responses underpinning cross-adaptation concludes that the dynamic epigenetic phenomenon not only induces long-lasting cross-tolerance, but enables preservation of its physiological beneficial features in a dormant manner. This conclusion is based on an increasing understanding of epigenetic mechanisms of cross-adaptation and the notion of Heat Acclimation-Mediated Cross-Tolerance vs. Preconditioning-Induced Cross-Tolerance, with a specific focus on the extensive publications from the Horowitz laboratory providing evidence for the role of hypoxia inducible 1-alpha (HIF-1α) and heat shock proteins (HSPs) in cytoprotection in the ischemic heart. In short, a rapid, short acclimation stimulus re-establishes the physiological, protected heat acclimation phenotype. The transcriptional machinery, however, is a continuum and as such our knowledge of the epigenetic within-life dynamic mechanisms involved, and their rates of both development and decay, is still in its infancy.

The review by Horowitz sets up three original investigations with a clinical focus. Pollak et al. provide original experimental data that evidences the protective effect of heat acclimatization

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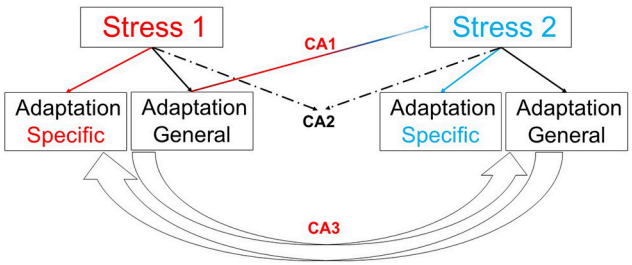

FIGURE 1 | Overview of cross-adaptation. Adaptation to one stimulus provides cross tolerance to another (CA1). For example, heat acclimation enhances tolerance to hypoxia, and may enhance exercise performance under temperate conditions. CA2 highlights the combined adaptive effects of two stimuli in providing beneficial responses to a third variable, e.g., heat acclimation alongside exercise training may enhance left ventricular function. The potential for adaptation to one stressor enhancing adaptation to other stressors (CA3), such as the manipulation of nutrient availability to enhance or amplify skeletal muscle responses to training, remains an interesting area for future work. Finally, the use of one stimulus to model the responses to the same or similar stimulus in another circumstance (e.g., high altitude hypoxia as model to examine physiological responses in an intensive care unit) follows the same conceptual premise as cross adaptation.

combined with modest exercise training, performed in "free living" conditions, against diastolic dysfunction imposed by ischemic/reperfusion insults in humans. The surgical theme is continued by Barrington et al., who demonstrate the efficacy of using orthopedic perioperative hypoxic air inhalation to prime the local HSP system and attenuate reperfusion stress in a manner similar to that of the current ischemic preconditioning intervention used prior to knee replacement surgery. The benefit of hypoxic air inhalation being that it comes without negative post-surgical side-effects, e.g., delayed wound healing, vascular injury, muscular damage, and greater post-operative pain that can arise as a consequence of ischemia. Hypoxic air inhalation was also the subject of investigation for Morrison et al.; they identify that during 21 days of bed rest, sleep macrostructure is negatively affected, the apnoea-hypopnea index increases, and breathing stability worsens. Each of these metrics become independently exacerbated by continuous exposure to hypoxia. Given the importance of sleep for many clinical and exercise applications where cross-adaptation/tolerance is investigated, interventions using chronic stress such as prolonged hypoxia should be mindful of the potential confounding/maladaptive issues arising from its use.

Exercise related cross-adaptation is studied most extensively within this Research Topic. Leckey et al. present interesting experimental work describing the effects of a 1,3-butanediol acetoacetate (ketone) diester on performance in professional cyclists. In comparison to a placebo, pre-exercise ingestion of the diester results in an impairment in time trial performance that is associated with gut discomfort and higher perception of effort concurrent with elevated serum β-hydroxybutyrate, serum acetoacetate and urine ketone concentrations. Continuing the exercise and nutrition cross-adaptive theme, papers by Akerman et al. and Neal et al., investigate the role of fluid balance, and more specifically dehydration as an additional stressor during exercise-heat stress. To understand mechanisms by which manipulating fluid balance may augment adaptation to exercise-heat stress, Akerman et al. prescribed calisthenics in temperate conditions, in hot conditions whilst euhydrated, and in hot conditions with dehydration. Following the three conditions the authors conclude that transient dehydration with heat potentiates short-term (24-h) hematological (hypervolemic) and cardiovascular (hypotensive) outcomes. Neal et al.'s work extends understanding of the relevance of fluid intake during heat acclimation protocols (repeated exercise heat stress) by identifying that when thermal-strain is matched, permissive dehydration which induces a mild, transient, hypohydration does not reliably affect the acquisition or decay of heat acclimation, or endurance performance parameters. Consequently, the current guidance relating to inducing heat adaptation using acclimation remains salient i.e., irrespective of hydration status, trained individuals require >5 days to optimize heat acclimation.

Since the emergence of seminal work in the field, heat has been the most widely considered stressor for cross-adaptation. Lee and Thake follow a previous publication in the area (Lee et al., 2016) with important insights into the responses of circulating inflammatory markers during acclimation to control, hypoxic or hot conditions, and subsequent hypoxic stress tests. Whilst heat acclimation induces more immediate and greater changes in monocyte HSP72 in comparison to equivalent training in hypoxia, neither regime attenuated the systemic cytokine response or intestinal damage following acute exercise in hypoxia. This experiment identified that 10 days of fixed-work acclimation does not induce full cytoprotective adaptation, and cellular acclimation homeostasis had yet to be achieved. Further studies are therefore warranted to determine the optimal heat "dose" to maximize the constitutive HSP72 reserves and achieve the potent cross-tolerance effects observed in rodent models (Maloyan and Horowitz, 2002, 2005). Once the optimal dose and associated rates of development and decay are better understood in humans, cross-adaptive interventions aimed at enhancing hypoxic tolerance and operational effectiveness in human occupational, military, and sporting scenarios can be explored. These applied fields can draw upon the data of Tuttle et al. who demonstrate that an acute bout of hot downhill running is an effective preconditioning strategy that ameliorates physiological strain, soreness, and Hsp72 and Hsp90α mRNA responses of muscle and leukocytes, to a subsequent bout of hot downhill running — these responses being indicative of cross-adaptation. The ability to elicit physiological and cellular protection to exercise heat stress following just a single bout of exercise is appealing for those individuals where extensive periods of time prior to exercise in extreme environments is not possible. For example, cross-adaptation and preconditioning principles could be considered when examining interventions aimed at preparing vulnerable populations, such as the elderly and those with chronic non-communicable diseases, before the onset of debilitating heatwave events that increase morbidity and mortality in these populations (Kenny et al., 2010). For the researcher, these data also valuably demonstrate

the efficacy of utilizing circulating tissue (leukocytes) in place of muscle samples to infer changes in the heat shock response.

The final inclusion in this research topic brings the collection full circle by considering exercise and clinical cross overs. Sahl et al. investigate how excessive repeated prolonged exercise influence low-grade inflammation and adipose tissue anti-inflammatory macrophage content in six older male recreationally-trained cyclists. The data support the conclusion that regular prolonged exercise does not influence abdominal adipose tissue inflammation, but a higher plasma IL-6 concentration concurrent with a trend toward higher insulin resistance and decreased VO_{2peak}, implies that excessive exercise probably attenuates its potential anti-inflammatory effects. The balance between successfully inducing an adaptation and providing too great a stimulus and thereby mitigating the desired response is a delicate balance, and all research into cross-adaptation should be mindful of the challenges of prescribing appropriate exercise intensities, particularly when dealing with individuals of widely varying levels of fitness and when seeking to complement exercise with environmental stress such as heat and hypoxia, or nutritional and dehydration manipulations.

These articles add to our understanding of the whole body, systemic and molecular responses to a multitude of stressors that interact with one another under the cross-adaptation paradigm. The sporting, occupational, and clinical relevance of these areas is only recently being discovered and we look forward to new applied and mechanistic data in this growing area.

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BL and OG: design and scripture. CT, MT, JH and JC: completion and correction.

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Heat Acclimatization Protects the Left Ventricle from Increased Diastolic Chamber Stiffness Immediately after Coronary Artery Bypass Surgery: A Lesson from 30 Years of Studies on Heat Acclimation Mediated Cross Tolerance

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During the period of 1986–1997 the first 4 publications on the mechanical and metabolic properties of heat acclimated rat's heart were published. The outcome of these studies implied that heat acclimation, sedentary as well as combined with exercise training, confers long lasting protection against ischemic/reperfusion insult. These results promoted a clinical study on patients with coronary artery disease scheduled for elective coronary artery bypass operations aiming to elucidate whether exploitation of environmental stress can be translated into human benefits by improving physiological recovery. During the 1998 study, immediate-post operative chamber stiffness was assessed in patients acclimatized to heat and low intensity training in the desert (spring in the Dead Sea, 17–33°C) vs. patients in colder weather (spring in non-desert areas, 6–19°C) via echocardiogram acquisition simultaneous with left atrial pressure measurement during fast intravascular fluid bolus administration. We showed that patients undergoing "heat acclimatization combined with exercise training" were less susceptible to ischemic injury, therefore expressing less diastolic dysfunction after cardiopulmonary bypass compared to non-acclimatized patients. This was the first clinical translational study on cardiac patients, while exploiting environmental harsh conditions for human benefits. The original experimental data are described and discussed in view of the past as well as the present knowledge of the protective mechanisms induced by Heat Acclimation Mediated Cross-tolerance.

Keywords: heat acclimation, heat acclimatization, coronary bypass, diastolic stiffness, cross-tolerance

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INTRODUCTION

A variety of environmental factors are known to influence cardiovascular morbidity and mortality among which climate and ambient temperature appear to have a considerable effect. Multiple reports from throughout the world suggest a seasonal pattern in the occurrence of acute ischemic syndromes and their complications. Higher environmental temperatures appear to reduce the risk of the occurrence and severity of cardiovascular events (Cech et al., 1977; Marchant et al., 1993; Xu et al., 2013). In contrast, however, in most studies, an increase in the prevalence of acute myocardial infarction and a higher infarction-related mortality rate were observed in winter time (Cech et al., 1977; Ornato et al., 1996; Spencer et al., 1998; Chen et al., 2016). Thus, cold weather, by influencing multiple hemodynamic, rheologic and hematologic variables (e.g., Gordon et al., 1987; Kawahara et al., 1989; Stout and Crawford, 1991; Woodhouse et al., 1993), has been suggested as trigger to acute cardiovascular morbid events.

Animal data have shown that prolonged exposure to moderate ambient heat, heat acclimation (33–34°C, upper limit of the thermoneutral zone-TNZ) induces structural and biochemical changes in the rat heart. These changes are related to the improved systolic and diastolic function and the increased metabolic efficiency observed in heat-acclimated hearts during normoperfusion (Horowitz et al., 1986, 1993; Levi et al., 1993; Levy et al., 1997). Furthermore, heat-acclimated hearts, when compared to non-acclimated hearts, preserve their mechanical and metabolic performance better during hypoperfusion and demonstrate a markedly improved recovery of contractile function and energy stores on reperfusion (Levi et al., 1993; Levy et al., 1997). These beneficial effects of heat acclimation on the ischemic heart are markedly enhanced when rats undergo combined heat acclimation and exercise training (Levy et al., 1997).

The advantages of the trained heat acclimated groups are qualitatively similar to those observed subsequent to sedentary acclimation, but greater in magnitude, on diastolic function in particular (Levy et al., 1997). Thus, in experimental animals, heat acclimation combined with exercise appears to induce efficient protective mechanisms against both ischemic and reperfusion insults. The potential benefits of these protective features, which are currently attributed to "heat acclimated cross-tolerance mechanisms" (Horowitz, 2016) was never evaluated in humans in real clinical settings except for our 1998 pioneering study (Pollak et al., 1998). Here we describe our 1998 collaborative study, attempted to translate the beneficial effects conferred by heat acclimation into a real clinical setting. Notably, the term acclimation describes adaptive changes occurring within an organism in response to experimentally induced changes. When adaptive responses are caused by stressful changes in the natural climate, (e.g., seasonal or geographical) the term acclimatization is used (IUPS Thermal Commission, 2001). The latter terminology was used here for the studied patient.

Abbreviations: CPB, cardiopulmonary bypass; LV, left ventricular; LAP, left atrial pressure; LVA, left ventricular end-diastolic area.

Cardiopulmonary bypass (CPB) during coronary artery bypass surgery imposes severe myocardial ischemia followed by reperfusion. Both ischemia and reperfusion are known causes of diastolic dysfunction. For example, an intraoperative study using transesophageal echocardiography has shown an increase in diastolic chamber stiffness immediately after coronary artery bypass surgery reflecting worsening diastolic dysfunction (McKenney et al., 1994).

In this investigation we aimed to prospectively study the influence of heat acclimatization with modest exercise training on diastolic dysfunction imposed by CPB in patients with coronary artery disease undergoing coronary artery bypass surgery. We utilized transesophageal echocardiography in conjunction with simultaneous hemodynamic monitoring during fluid challenge to evaluate diastolic function before and immediately after CPB.

METHODS

The study was approved by the Institutional Review Board for human's experimentation of Hadassah University Hospital, Jerusalem, Israel.

Male patients with coronary artery disease scheduled for elective coronary artery bypass operations were enrolled to participate in this study. All patients resided in between zero (sea level) to 700 m elevation. To focus on the effects of CPB and coronary revascularization on diastolic function in a clinically homogenous group we excluded patients for the presence of more than mild (grade 1) mitral or aortic regurgitation, any valvular disease requiring valve replacement, echocardiographic evidence of left-ventricular (LV) hypertrophy, and a significantly reduced LV ejection fraction (less than 45%). Eligible patients were divided into two groups: heat acclimatized (study) group and non-acclimatized (control) group.

Heat Acclimatization Combined with Exercise Training

Eight patients were prospectively and consecutively included in the acclimatized study group as they agreed to leave their home and spend 2 to 3 weeks on the Dead-Sea seashore (lowest point on earth, 410 m below sea level) immediately prior to operation. On location they were accommodated in caravans, devoid of air-conditioning, at an ambient temperature (day-night range) of 17–33°C. These patients exercised twice a day as a group under the supervision of a certified physiotherapist and a physician. An individual training program was developed for each patient. To assess training levels, maximal workload capacity of each patient was determined by a modified "sprint" test (Jennings et al., 1986; Meredith et al., 1990) on a bicycle ergometer, beginning with zero load. The workload was increased every minute by 15 W until further increase was prevented by fatigue. In every case, training exercise load was not increased beyond 65% of maximal heart rate determined by the test. Exercise was terminated earlier if symptoms of chest pain, dyspnea, fatigue and dizziness developed. Each exercise morning session consisted of 5 min. warm-up (stretching exercises of upper and lower extremities), and three stations of aerobic training on a treadmill,

stationary ergometer and APT (Active Passive Trainer). The patients exercised between 1 and 5 min at each station. The time for each exercise session was dependent on the time required for each patient to achieve elevated heart rate. After completing each station the patients cooled down until heart rate returned to baseline. Rotation among the 3 aerobic stations was repeated twice. The last 10 min was an extended cool-down period. Afternoon 45 min. session included general upper and lower extremity strengthening exercises, breathing exercise and relaxation. Other than the structured exercise sessions, study patients were free to do as they wished. Patients were operated upon within 3 days after completion of the 14 to 21 day conditioning period.

Non-acclimatized Control Group

Eight patients were prospectively matched to those included in the acclimatized group by age, sex, severity of coronary artery disease, diastolic LV dimensions, LV mass and LV systolic function. They were scheduled for elective coronary artery bypass grafting during the same time period as their matched study patients. However, they were not asked to leave their home, thus being exposed to an ambient temperature (day-night range) of 6–19°C. These patients' daily routine was not changed by any means prior to operation.

Intraoperative Protocol

Preparation of patients for open-heart surgery, including endotracheal intubation and general anesthesia was carried out using standard methods. The transesophageal probe was placed after the patient was fully anesthetized. At the time of aortic and venous cannulations, a left atrial line was also placed. While the patient was in steady state, a baseline echocardiogram (M-mode and 2-D, transgastric short-axis view of the left ventricle at mid-ventricular level) was recorded simultaneously with mean left atrial pressure (LAP) measurement. Subsequently, 500 to 1,000 cc of fluid was administered as a fast intravascular bolus over 5 to 10 min; this consisted of crystalloid solution (Standard bypass solution: PlasmaLite® with 2% mannitol). The infusion was continued until the LAP was increased by, at least, 5 mmHg over baseline. Echocardiogram acquisition simultaneous with LAP measurement was continuously recorded until fluid administration ceased. Thereafter, surgical routines were continued uninterrupted throughout the bypass period.

After discontinuation of CPB but before decannulation, while the patient has been fully warmed-up and has re-achieved steady state (i.e., systolic blood pressure of, at least, 100 mmHg and regular sinus or atrially-paced rhythm of less than 100 bpm), a new baseline echocardiogram was recorded simultaneously with mean LAP measurement. Subsequently, 500 to 1,000 cc of fluid was administered as a fast intravascular bolus over 5 to 10 min; this was likely to consist of whole blood and/or plasma but may have been supplemented by crystalloid solution (Standard bypass solution: PlasmaLite® with 2% mannitol and/or Hemacel). This infusion was continued until the LAP was increased by, at least, 5 mmHg over the new baseline. Echocardiogram acquisition simultaneous with LAP measurement was continuously recorded during volume load. Thereafter, surgical routines were continued as usual for decannulation and closure of chest wall. It is important to note that extreme care was taken to assure that the transesophageal echocardiography probe was kept at precisely the same place between the two echograms.

Data Analysis

Off-line echocardiographic analysis was performed with the interpreter being blinded to the group a particular patient belonged to. End-diastolic frames of the LV short axis view were identified. The LV area was traced at end-diastole and related to the corresponding LAP. Traced LV areas of 4 to 7 consecutive cardiac cycles were averaged for each increment of LAP to account for respiratory variations in LV size. LAP measurements were plotted against corresponding mean LV area to generate diastolic pressure-area curves from data obtained before and after CPB, one pair of curves for each patient. To assess changes in LV diastolic properties, LV area was compared at similar left atrial pressures (within 1 mmHg) for the range of LAP changes with volume loading. Thus, for a given LAP, a smaller LV area implies a leftward shift of the LV pressure-volume relationship while a larger LV area suggests a rightward shift of the pressure-volume curve.

Statistical Analysis

In view of the relatively small sample size of both the intervention and control groups of patients, data was expressed as median values and their ranges and non-parametric tests were used for statistical calculations. The Mann-Whitney rank-sum test was used to compare variables between the groups. Proportions were compared by Fisher exact test. The Wilcoxon signed-rank test was utilized in comparing pre- and post-bypass data, with each patient serving as his own control. A P-value of less than 0.05 was considered statistically significant.

RESULTS

Patient characteristics are presented in **Table 1**. Acclimatized and control groups were well matched for baseline variables (i.e., age, risk factors, LV size and mass) as well as intraoperative parameters (i.e., number of grafts, bypass time, intraoperative body cooling). Acclimatized patients were subject to an average daily temperature of 12°C higher than non-acclimatized patients during the immediate preoperative period (**Table 1**).

There was no significant difference in baseline LV end-diastolic area (LVA) between the study and the control groups, both before and after volume loading. The ratio of the difference in LAP to the difference in LVA before and after fluid loading is strongly related to the "slope" of the LV pressure-volume curve. This ratio was virtually identical in both the study and control groups, suggesting no difference in baseline (pre-CPB) diastolic function of acclimatized vs. non-acclimatized patients (**Table 1**, **Figure 2**).

Diastolic pressure-area curves before and after CBP in a representative patient from each group is depicted in **Figure 1** (individual diastolic pressure-area curves of all patient appear as in the Appendix, **Figure A1**).

Left ventricular area decreased after CPB in all control patients at comparable preloads. Before fluid loading, at mean left atrial pressure 6.5 mmHg, there was a 1.8 ± 0.3 cm^2 decrease in LVA

TABLE 1 | Baseline and intraoperative variables in acclimatized and non-acclimatized patients.

	Acclimatized group ($n = 8$)	Control group ($n = 8$)	*P*-value
Age (years)	65 (44–85)	67 (49–73)	0.88
Hypertension	5/8	4/8	1.00
Diabetes mellitus	4/8	2/8	0.61
LV end-diastolic diameter (mm)	47 (40–63)	48 (42–59)	0.96
LV wall thickness (mm)	10.4 (7.7–11.3)	10.5 (8.4–11.8)	0.87
LV mass (gr.)	199 (136–312)	208 (145–310)	0.87
Number of vessels grafted	3 (2–4)	3 (2–5)	0.88
Cardiopulmonary bypass time (min)	87 (54–121)	96 (57–203)	0.55
Aortic cross-clamp time (min)	57 (31–86)	66 (34–93)	0.23
Systemic cooling temperature (C°)	30 (27–34)	31 (28–34)	0.67
LVEDA before bypass and before loading (cm^2)	17.02 (10.6–26.1)	19.71 (13.9–28.6)	0.11
LVEDA before bypass and after loading (cm^2)	20.53 (15.5–29.7)	23.31 (18.5–30.44)	0.23
Ratio of ΔP/ΔA before CPB	1.48	1.49	
Range of day-night temperatures (C°)	17–33	6–19	0.0006

CPB, cardiopulmonary bypass; LV, left ventricular; LAP, left atrial pressure; LVA, left ventricular end-diastolic area; LVEDA, left ventricular end-diastolic area; ΔP, difference in left-atrial pressure before and after fluid loading; ΔA, difference in left-ventricular area before and after fluid loading Data presented as median (range) or proportion, as needed.

($p = 0.008$). After fluid loading, at mean left atrial pressure 11.9 mmHg, there was a 1.7 ± 0.6 cm^2 decrease in LVA ($p = 0.02$). Thus, a distinct leftward shift of the diastolic pressure-area curve was noted in non-acclimatized patients (**Figure 2**).

However, in patients who underwent heat acclimatization, no change in left ventricular area measured at comparable preloads before and after CPB was noted. Both before and after fluid loading, at mean left atrial pressures of 5 and 10.3 mmHg, respectively, mean left ventricular area pre-bypass was similar to left ventricular area post-bypass. Thus, a virtual overlap of the pre- and post-CPB diastolic pressure-area curves was noted (**Figure 2**).

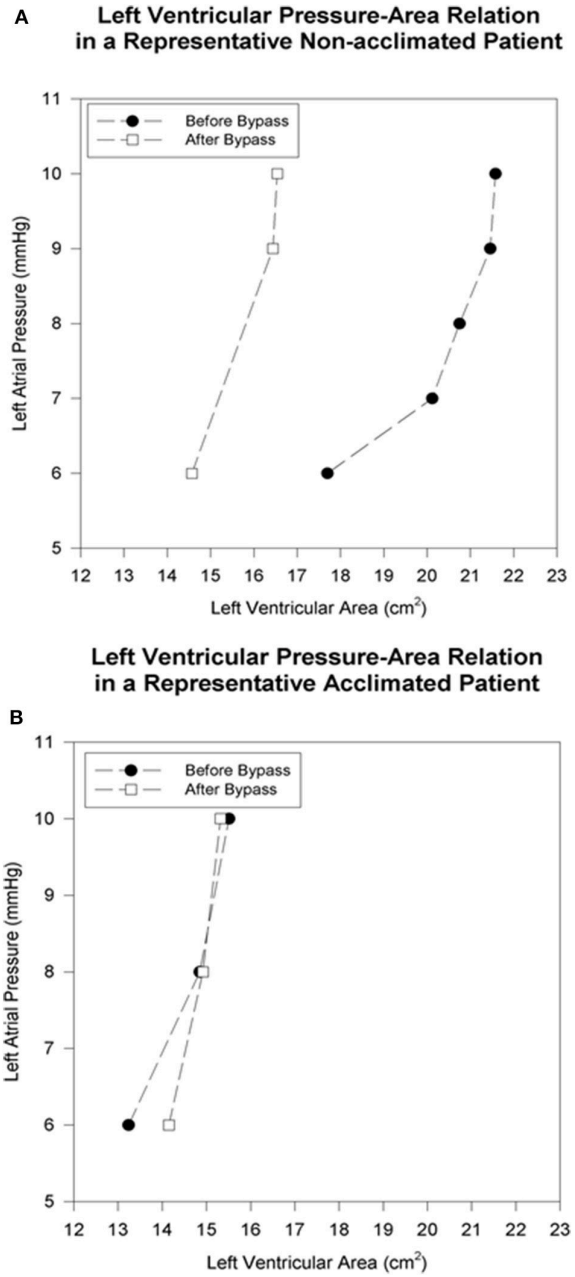

FIGURE 1 | Left ventricular pressure-area relation in representative patients before and immediately after cardiopulmonary bypass. The baseline left atrial pressure of 6 mmHg is progressively increased by volume loading. **(A)** In the non-acclimatized patient, the left-ventricular end-diastolic area is smaller after bypass at each pressure level, as reflected by a leftward shift of the pressure-area relation. **(B)** In the acclimatized patient, the pressure-area relation maintains its position after bypass, without significant change in left-ventricular end-diastolic area at comparable pre- and post-bypass pressures.

DISCUSSION

This study was the first clinical research, which exploited environmental stressor to improve human function/health by establishing controlled clinical setting, using a model of ischemia and reperfusion during coronary artery bypass surgery. We showed that patients undergoing "heat acclimatization combined with exercise training" were less susceptible to ischemic injury, therefore expressing less diastolic dysfunction after CPB compared to non-acclimatized patients. The relative contributions of heat vs. exercise were not studied. However, based on contemporary knowledge, this issue will be discussed. CPB imposes a multi-factorial burden on the myocardium resulting, among other things, in diastolic dysfunction. McKenney et al. (1994) have shown that a leftward shift in the LV diastolic pressure-area curve occurs immediately after CPB in patients undergoing coronary artery bypass surgery. This increase in LV diastolic chamber stiffness may be related to several mechanisms including ischemia, cooling and interstitial edema. Subsequent work done by the same group suggests

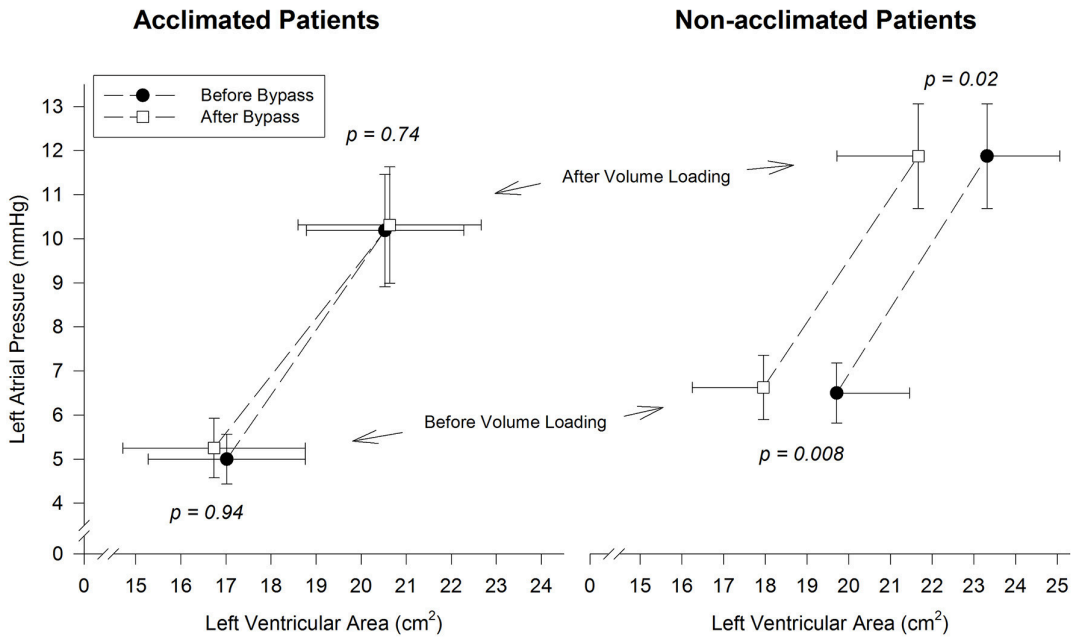

FIGURE 2 | Left ventricular end-diastolic pressure-area relation before and immediately after cardiopulmonary bypass. Pre- and post-bypass left-ventricular area is compared at similar left-atrial pressure throughout the range of volume manipulation. A parallel leftward shift of the pressure-area curve is evident in non-acclimatized patients, consistent with reduced left-ventricular diastolic distensibility after bypass. In contrast, the slope and position of the pressure-area curve is maintained after bypass in acclimatized patients, indicating no significant change in left-ventricular diastolic properties.

that the deterioration in diastolic properties persists into the post-operative period for at least 3 h (Ekery et al., 2003) and may indeed have clinical relevance in the post-operative hemodynamic management of these patients.

Without exception, there was a decrease in LV distensibility in each of our control patients immediately after CPB. This is in complete accordance with the previous work cited (McKenney et al., 1994; Ekery et al., 2003). It supports the universal applicability of our group of patients (i.e., this is not an unusual or improperly selected patient population). In contrast, pre-bypass diastolic LV distensibility did not changed in the acclimatized patient group. Both end-diastolic area and the slope of the pressure-area curve were practically identical before and after bypass, suggesting preservation of the pre-bypass diastolic properties throughout all loading conditions. Thus, our results indicate that 2 to 3 weeks of exposure to heat in combination with modest exercise training abolished the worsening in diastolic chamber distensibility, which is commonly related to bypass operative techniques.

Environmental factors can influence the structure and physiological characteristics of the myocardium as well as its ability to withstand ischemia and reperfusion. In animal experiments, heat acclimation with/without exercise proved to have a significant cardioprotection and to enhance recovery from ischemia/reperfusion insults. The beneficial effects were manifested by an improved chamber compliance (Horowitz et al., 1986) under normoxic conditions as well as greater systolic pressure generation with lower O_2 consumption (Horowitz et al., 1993; Levi et al., 1993; Levy et al., 1997; Eynan et al., 2002; Horowitz, 2003). Upon global ischemia and reperfusion insults, there was a delayed appearance of ischemic contracture, and better recovery of diastolic and systolic functions (Levi et al., 1993; Levy et al., 1997). This was concomitant with ATP preservation during ischemia in the heat acclimated hearts with and without exercise. This preservation of ATP, in both sedentary and trained hearts, implied the predominant contribution of the prolonged heat exposure *per se* on the recovery of diastolic function (Levi et al., 1993). To-date, our knowledge of the mechanism of heat acclimation mediated cross tolerance and cardioprotection has been largely advanced. There is heat acclimation induced decrease in Ca^{2+} sensitivity, thus the heart is less susceptible to Ca^{2+} overload (Kodesh et al., 2011). Concomitantly, gene chip analysis demonstrated profound upregulation of Nebulin, a giant, actin-binding protein that stabilizes the thin filaments for length maintenance and is also associated with Ca^{2+} sensitivity, increasing contractile strength and efficiency (Tetievsky et al., 2014). Collectively, these changes most likely underlie the qualitative alterations in the electrical component of the excitation-contraction coupling (Kodesh et al., 2011) and the structural modifications that enhance cardiac compliance and improve the ability to cope with the peripheral volume overload seen after heat acclimation or heat stress (Horowitz, 2003). Both HSP72 and the hypoxia transcriptional factor HIF-1α reserves are constitutively elevated and play an important role in cytoprotection under ischemic conditions (Maloyan et al., 1999, 2005; Horowitz, 2016).

Recently, heat acclimation induced cross tolerance was "navigated" to thermotherapy of vascular diseases. Brunt et al. (2016a,b) showed that, relative to a sham group which participated in thermoneutral water immersion, passive heat therapy for 8 weeks increased flow-mediated dilatation, reduced arterial stiffness, reduced mean arterial and diastolic blood pressure and that the vasodilation was NO dependent. Although these data are linked to major blood vessels rather than to the heart, the outcome fits with our postulate, based on animal studies, that heat *per se* enhances cardiovascular compliance, leading to improved exercise capacity and diastolic function in the ischemic heart.

Our recent studies shows that heat acclimation involves epigenetic control of gene expression (Tetievsky and Horowitz, 2010) and includes metabolic switch leading to mitochondrial upregulation of PDK1 and a shift to enhanced glycolytic ATP utilization (Alexander-Shani et al., 2017).

The cross-tolerance between heat acclimation and ischemic injury appears to be a long-lasting effect, for at least 2 to 3 weeks (Cohen O, PhD Thesis, The HU; Eynan M, PhD Thesis, The HU; Horowitz M, personal communication and Arieli et al., 2003).

Increasing number of studies emphasize the role of exercise training in cardioprotection. However, the knowledge of the impact of exercise training on ventricular diastolic function/dysfunction is rather limited and controversial. Bhella et al. (2014) demonstrated that lifelong committed exercise (5–6 sessions/week) prevented decreased compliance in aged healthy subject while Fujimoto et al demonstrated no training effects on compliance unless Alagebrium, which reverses stiffness of blood vessels wall was taken as well. Ades et al. (2013) in their meta-analysis on exercise and cardiac rehabilitation report inconsistencies and reciprocal effects regarding exercise impact on chamber and aortic compliance. Collectively, however, exercise intensity above 65% for at least 2 mo, was needed to impact diastolic function and aortic remodeling. Diastolic function in elite endurance athletes, however, correlated enhanced distensibility with exercise (Levine et al., 1991). In cardiac rehabilitation, exercise intensity above 65% of maximal heart rate determined by sprint test was needed to impact diastolic function or aortic remodeling.

We thus postulated that in our patients undergoing "heat acclimatization combined with exercise training" the heat exposure *per se* mitigates diastolic dysfunction following ischemia-reperfusion. This group would be less susceptible to ischemic injury not only because of changes in elasticity, which likely impact on diastolic dysfunction after CPB compared to non-acclimatized patients, but also because of better energy state and large reserves of cytoprotective proteins.

Study Limitations

A major limitation of this study is that the control group was assigned to "non-committed" to exercise group, which does not allow unequivocal conclusion regarding the relative contributions of heat and endurance exercise to protect post operation diastolic function. However, the low level of exercise in the acclimatized group, together with recent findings on the impact of sedentary thermotherapy support our conclusion that heat exposure *per se* is a major contributor to the results obtained.

A point of note is the question of whether the slight hyperbaric pressure in the Dead Sea has an impact on cardiac stiffness. Abinader et al. (1999), in a pilot study, measuring wall motion in patients with coronary artery disease prior to and 5 days after descending to the dead sea. These subjects demonstrated ∼8% decrease WMSI (wall motion score index) vs. their WMSI under normobaric pressure. Matched healthy controls did not show changes in WMSI under similar conditions. The Dead Sea area is known for its high ambient temperatures, suggesting perhaps that high ambient temperature was one beneficial parameters in the improvement observed in these patients. No conclusion statements by the authors was provided.

This work is too small to assess the importance of heat acclimatization on morbidity/mortality after coronary artery bypass surgery. The extension of the deleterious effect of bypass into the post-operative period supports the assumption that enhancement of diastolic function immediately after bypass may eventually prove to be beneficial to improve operative results.

CONCLUSION

This investigation is the first clinical study supporting the experimental evidence for a protective effect of heat acclimatization combined with modest exercise training against diastolic dysfunction imposed by ischemic/reperfusion insults. Although this study could not distinguish the relative contribution of each factor on the gained benefit, we believe that this effect is real and chronic heat exposure plays a major role in it. Further clinical studies are justified to expand on this preliminary data and evaluate the longevity of this effect as well as its relevance to the clinical outcome of patients undergoing coronary artery bypass surgery.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Hadassah committee for Human's experimentation with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The study was approved by the Institutional Review Board for human's experimentation of Hadassah University Hospital, Jerusalem, Israel.

AUTHOR CONTRIBUTIONS

AP: Ecocardiology, interpretation of the results, drafter the paper and the Figures; GM: coronary by-pass surgery; MH: conception of the study, Interpretation of the results in light of the current knowledge of the topic, drafted the paper; MS: Organization of exercise and physiotherapy; DG: Ecocardiology; YH: Conception of the study, Interpretation of the results.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

APPENDIX

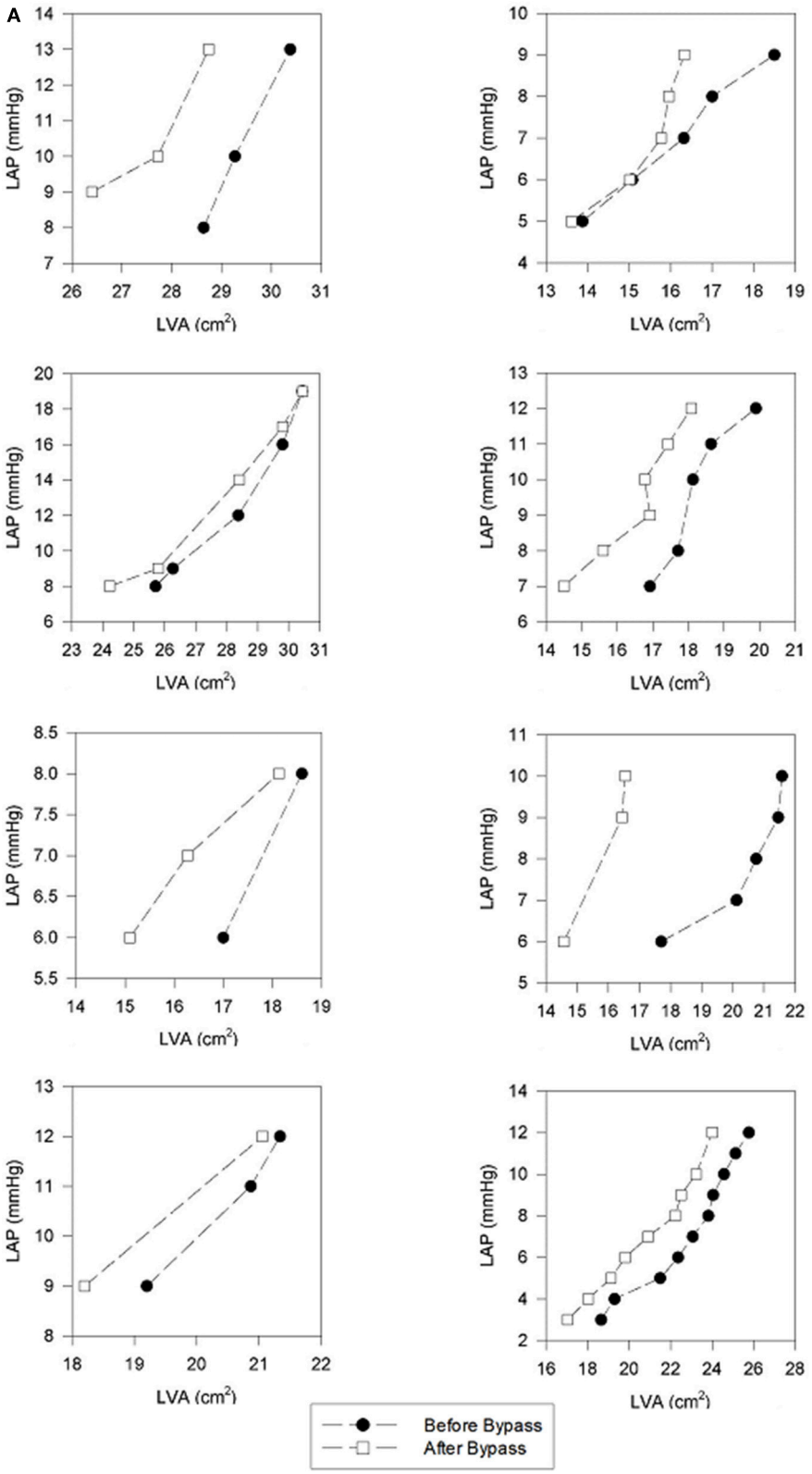

FIGURE A1 | Continued

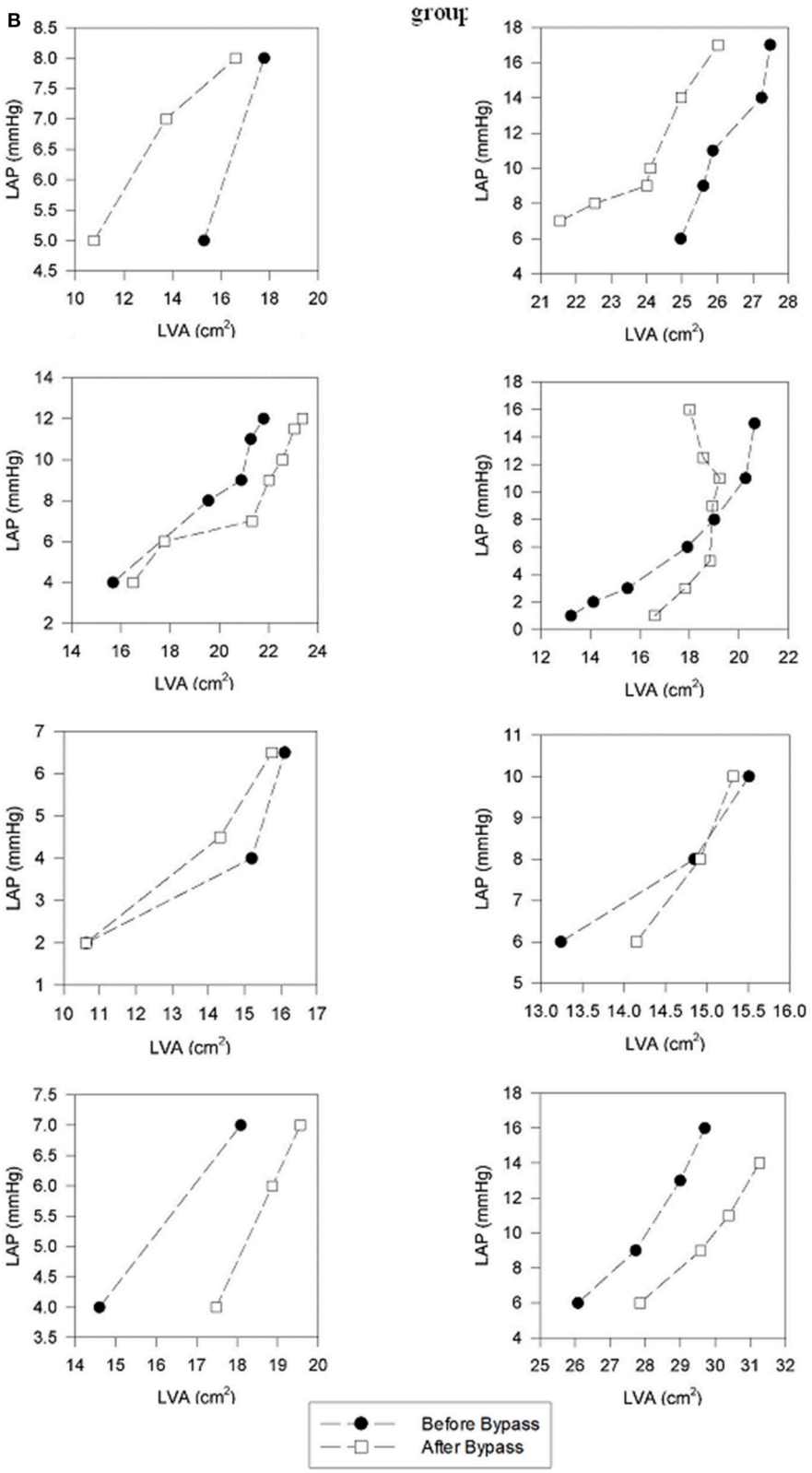

FIGURE A1 | Individual LV Left ventricular pressure-area relations pre- and post-bypass surgery. **(A)** Individual LV pressure-area relation pre- and post-bypass in the control patient; **(B)** Individual LV pressure-area relation pre- and post-bypass in acclimatized patients.

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Ketone Diester Ingestion Impairs Time-Trial Performance in Professional Cyclists

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We investigated the effect of pre- "race" ingestion of a 1,3-butanediol acetoacetate diester on blood ketone concentration, substrate metabolism and performance of a cycling time trial (TT) in professional cyclists. In a randomized cross-over design, 10 elite male cyclists completed a ∼31 km laboratory-based TT on a cycling ergometer programmed to simulate the 2017 World Road Cycling Championships course. Cyclists consumed a standardized meal [2 g/kg body mass (BM) carbohydrate (CHO)] the evening prior to a trial day and a CHO breakfast (2 g/kg BM CHO) with 200 mg caffeine on the morning of a trial day. Cyclists were randomized to consume either the ketone diester (2 × 250 mg/kg) or a placebo drink, followed immediately by 200 mL diet cola, given ∼ 30 min before and immediately prior to commencing a 20 min incremental warm-up. Blood samples were collected prior to and during the warm-up, pre- and post- TT and at regular intervals after the TT. Urine samples were collected pre- and post- warm-up, immediately post TT and 60 min post TT. Pre-exercise ingestion of the diester resulted in a 2 ± 1% impairment in TT performance that was associated with gut discomfort and higher perception of effort. Serum β-hydroxybutyrate, serum acetoacetate, and urine ketone concentrations increased from rest following ketone ingestion and were higher than placebo throughout the trial. Ketone ingestion induces hyperketonemia in elite professional cyclists when in a carbohydrate fed state, and impairs performance of a cycling TT lasting ∼50 min.

Keywords: ketone ester, cycling, time-trial, performance, ketones

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INTRODUCTION

Substrate utilization during exercise is influenced by several factors including the relative intensity and duration of exercise, an individual's training status and the effect of the preceding diet on both the substrate pool and the prevailing hormonal milieu (Hawley et al., 2015). As exercise intensity increases, there is a greater reliance on carbohydrate (CHO) based fuels (i.e., muscle and liver glycogen, blood glucose, lactate) and a reduction in the utilization of fat substrates (Brooks and Mercier, 1994). Ketone bodies provide another potential source of readily oxidized fuel for skeletal muscle, but are predominately associated with conditions of metabolic stress such as starvation, where they are needed to preserve essential function of peripheral tissues including the brain and heart (Robinson and Williamson, 1980; Veech, 2004). However, there has been recent interest

in the notion that increasing blood ketone concentrations could contribute to an enhancement of exercise performance by providing a readily available, alternative oxidative substrate for working muscle, sparing the limited stores of muscle glycogen (Pinckaers et al., 2017). As such, models of hyperketonemia from endogenous origin such as chronic exposure to a ketogenic diet (Burke et al., 2017) as well as introduction of exogenous sources of ketone bodies from ketone ester supplements (Cox et al., 2016) have been investigated. Nevertheless, there is some concern that the presence of high circulating concentrations of ketone bodies could inhibit the flux of other muscle substrates, either by impairing (rather than "sparing") muscle CHO oxidation and/or inhibiting adipose tissue lipolysis (Evans et al., 2017). As such, the situations in which an available ketone supply may benefit exercise capacity or performance may be determined by the duration and/or intensity of exercise and the need for combinations of muscle substrate to meet the metabolic demands.

Ketone bodies, namely D-β-hydroxybutyrate (βHB), acetone and acetoacetate (AcAc), are produced in the liver mitochondria from acetyl-CoA in response to an increased mobilization of free fatty acids (FFA) from adipose tissue in situations of reduced CHO availability (Robinson and Williamson, 1980). As summarized in recent reviews (Egan and D'Agostino, 2016; Pinckaers et al., 2017), under conditions of high CHO availability, circulating concentrations of ketone bodies are low, but are slightly elevated (0.1–0.5 mmol/L) by an overnight fast and further raised by exercising in a fasted state (0.5–1.0 mmol/L). Prolonged fasting/starvation (5 days) causes a maximal increase in rates of ketone body production (1–2 mmol/min or 140–280 g/day), leading to increased plasma concentrations that plateau under normal physiological conditions at ∼7–10 mmol/L. Meanwhile, chronic exposure to a ketogenic diet [low CHO (<50 g/d), low-moderate protein (∼15% of energy), high fat (75–80% of energy)] raises plasma ketone bodies to 1–2 mmol/L after several days, with concentrations reaching the apparent plateau achieved by prolonged fasting, according to the level of CHO restriction and duration of "keto-adaptation" (Pinckaers et al., 2017). Exogenous forms of ketone bodies include ketone salts, and more recently, ketone esters. Ingestion of the former appears to be less effective in increasing circulating ketone body concentrations and carries a significant salt load (Balasse and Ooms, 1968). Recently, a newly produced ketone monoester, R-3-hydroxybutyl R-3-hydroxybutyrate (Clarke et al., 2012), increases in plasma ketone concentrations (3–6 mmol/L) within the hours following its ingestion (400–600 mg/kg BM), although this may be altered by concomitant intake of food (Evans et al., 2017; Pinckaers et al., 2017).

To investigate the potential benefits to metabolism and sports performance, Cox et al. (2016) studied the effects of ingesting either CHO or CHO plus ketone ester (573 mg·kg^{-1} BM) on performance in trained cyclists. Their ingestion protocol induced a higher blood D-βHB concentration during submaximal cycling (ranging between ∼1.5 and 3 mmol/L) and lead to a subsequent improvement in time-trial (TT) performance by ∼2% following ketone ester and CHO ingestion compared to the ingestion of only CHO. However, aspects of the study design are inconsistent with conditions of "real world" cycling competition. Accordingly, we examined the effect of pre-"race" ingestion of a ketone diester on blood ketone body concentrations, substrate metabolism and performance under conditions of elite professional cycling; ingestion of a pre-race CHO-rich meal, inclusion of a warm-up, involvement of world-class cyclists and simulation of a real-life course. We hypothesized that this protocol would result in acute nutritional ketosis but that no performance improvement would be observed due to the high intensity nature of a real-life TT event which is dependent on the high rates of energy production from the oxidation of CHO-based fuels.

MATERIALS AND METHODS

Ethical Approval

This study conformed to the standards set by the *Declaration of Helsinki* and was approved (#20161005) by the Ethics Committee of the Australian Institute of Sport (AIS). After comprehensive details of the study protocol were explained to the participants verbally and in writing, all participants provided written informed consent.

Overview of Study Design

The study was a randomized crossover, double-blind, placebo-controlled design using elite (professional) cyclists attending a pre-season camp at the AIS, Canberra. On two separate occasions, three days apart, participants completed a 20-min standardized warm-up and rested for 5 min prior to completing a 31 km TT performed on a cycling ergometer (**Figure 1**). Participants were randomized to consume a 1,3-butanediol acetoacetate diester (described subsequently; KET; two doses of 250 mg/kg BM) or a viscosity and color-matched (PLAC) drink, given ∼30 min before and immediately prior to commencing the warm up. It was not possible to completely replicate the taste of the KET drink, but a comparably novel and bitter-tasting PLAC was prepared from a mixture of flavor essences (rum, almond, and bitters Angostura). Pilot testing revealed that the intake of a small volume of diet cola immediately after the KET and PLAC was able to quickly mask the taste and texture of the previous drink. In any case, none of the participants had previously ingested a ketone ester supplement and were therefore unable to recognize its characteristics. In recognition of the World Anti-Doping Code under which these cyclists compete, it was ascertained that ketone supplements are not considered a prohibited substance by the World Anti-Doping Agency.

Participants

Eleven internationally competitive male cyclists [age, 25 ± 7 (*SD*) y; body mass (BM), 73.7 ± 7.6 kg; $\dot{V}O_2$peak, 71.4 ± 5.6 mL/kg/min, 5.3 ± 0.3 L/min; Maximal Aerobic Power (MAP), 494 ± 20 W] from the ORICA-BikeExchange UCI World Tour (Road Cycling) team participated in this study. Participants included world class elite (n = 8; e.g., 2016 Paris-Roubaix winner, stage medalists from Tour de France, Tour Down Under, Giro d'Italia, Vuelta a España and Australian National championship Time Trial medalists) and highly trained under 23 riders contracted to the team (n = 3).

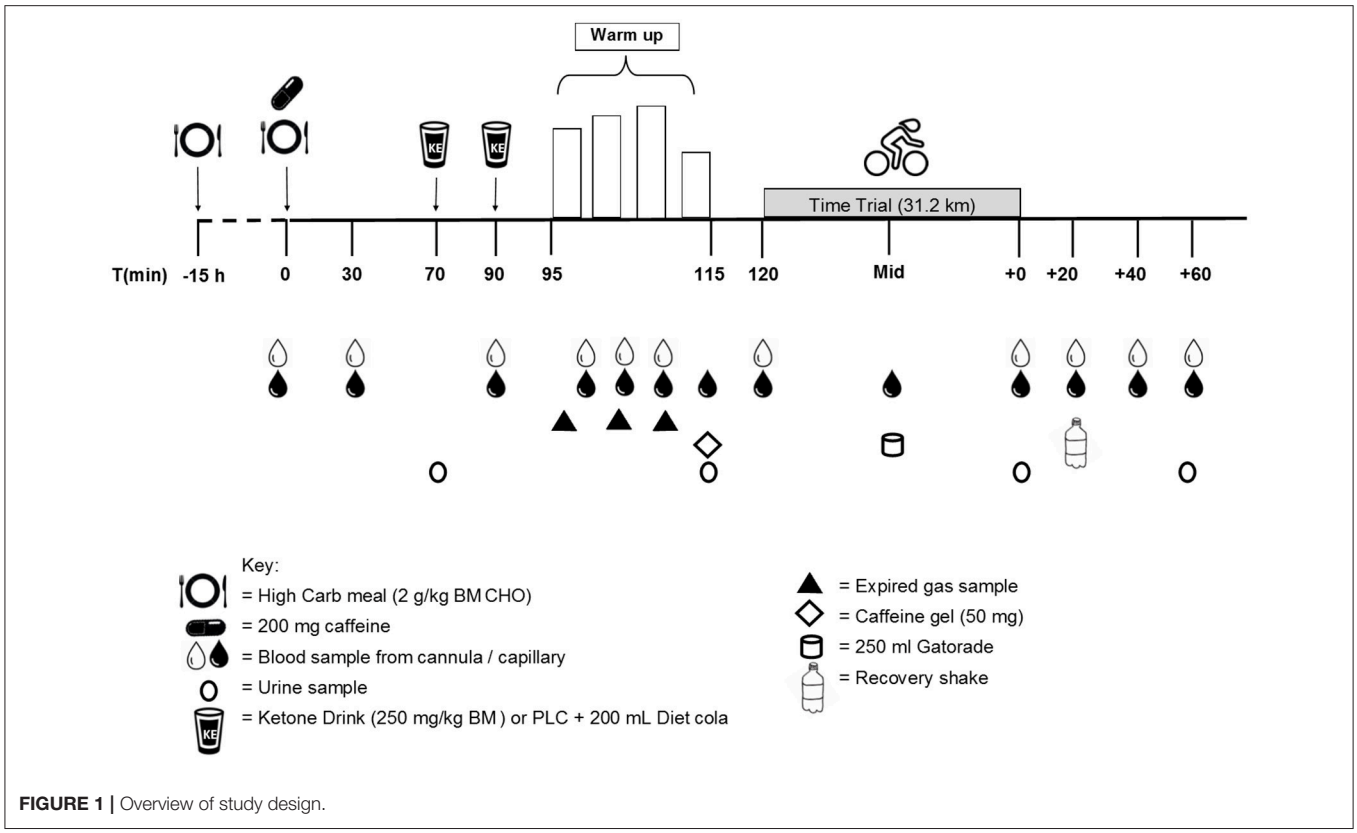

FIGURE 1 | Overview of study design.

Preliminary Testing and Familiarization

Before commencing the experimental phase, participants visited the laboratory to complete an incremental exercise test and a familiarization with the cycling ergometer (Velotron, Racermate Inc., Seattle, WA, USA) and the experimental exercise protocol (simulated 2017 World Championships time trial course, Bergen Norway).

Incremental Cycling Test

Participants completed a 5-min warm-up at 150 W on the cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands), thereafter the test protocol started at 180 W and increased 30 W every 60 s until volitional exhaustion. MAP was determined as the power output (PO) of the highest stage completed. If the participant finished partway through a 60 s stage, MAP was calculated in a pro-rata manner. During the maximal test, expired gases were collected into a calibrated and customized Douglas bag gas analysis system, as previously described (Russell et al., 2002). Peak aerobic capacity ($\dot{V}O_2$peak) was calculated as the highest oxygen consumption recorded over a 30-s period. $\dot{V}O_2$peak and MAP were used to calculate the PO for the individualized warm up on subsequent trial days, described below.

Dietary Control

CHO and caffeine intake were standardized the evening before and morning of a trial day and participants were also instructed to abstain from alcohol during the 24 h period prior to a trial day. Participants consumed an evening meal, snack and breakfast prepared by the team chef, providing a CHO content of 2, 1, and 2 g/kg BM, respectively. Participants were also provided with a post-exercise recovery drink, 20 min after the completion of the TT (1 g/kg BM CHO and 25 g protein). The composition and timing of all meals was repeated prior to trial two.

Synthesis of Ketone Ester

The ketone ester synthesized, 1,3-butanediol acetoacetate diester, is a non-ionized sodium-free and pH-neutral precursor of AcAc (D'Agostino et al., 2012). The ketone ester was synthesized by transesterification of *t*-butylacetoacetate with *R,S*-1,3-butanediol (Savind, Seymour, IL). The resultant product consisted of a mixture of monoesters and diester, the ratio of which could be adjusted by varying the stoichiometry of reactants. After synthesis the crude product was distilled under reduced pressure to remove all solvents and starting materials and the resultant ketone ester was obtained and assessed for purity using gas chromatography-mass spectrometry (GC-MS).

Trial Day Procedure

Participant Preparation

On each of the trial days, participants reported to the laboratory in a rested and overnight (10 h) fasted state, with the timetable creating a ∼30 min time between individuals that was repeated on the subsequent trial day. The trial day protocol commenced with the placement of an indwelling cannula (22G; Terumo, Tokyo, Japan) into a cephalic vein while lying in a supine position to allow for repeat blood sampling. A fingertip sample

of capillary blood was collected concomitantly with each cannula sample throughout each trial for immediate measurement of blood ketones (ß-hydroxybutyrate; FreeStyleOptium Neo, Abbott Diabetes Care, Doncaster, Australia). Following a resting blood sample ($t = 0$ min), participants were provided their standardized CHO breakfast including 200 mg caffeine (NO-DOZ Awakeners, Key Pharmaceuticals Pty Ltd, Macquarie Park, Australia), to mimic typical race preparations. Participants were instructed to consume the breakfast within 10 min, with a second blood sample being collected at $t = 30$ min. At $t = 70$ min, participants provided a urine sample, were weighed and fitted with a heart rate (HR) monitor. At this time, they ingested the first dose (250 mg/kg BM) of KET or PLAC, followed immediately by 200 mL diet cola. At $t = 90$ min participants were seated on the Velotron ergometer, blood samples (4 mL) were collected and participants consumed their second dose in the same manner.

Warm Up Protocol

Following the second KET or PLAC drink, participants completed a standardized 20-min warm up on the cycle ergometer. The set-up of the bike was performed by team mechanics to replicate each rider's unique bicycle position and was fitted with a calibrated (Gardner et al., 2004) SRM cycling power meter (scientific version, 8 strain gauge, Schoberer Rad Meβtechnik; Jülich, Germany), set to sample at 1-s intervals. The warm up consisted of 3 × 5 min at 50% ventilatory threshold (VT), VT1, and VT1 plus 50% of the difference between VT1 and VT2 (156 ± 14, 312 ± 28, 355 ± 29 W, respectively), followed by 5 min self-paced cycling. Venous and capillary blood samples were collected every 5 min and expired gas was collected continuously during the first 15 min of the warm up. Immediately following the warm up participants provided a urine sample and ingested an energy gel containing 50 mg caffeine (27 g CHO, PowerBarPowerGel). During this time (5 min), participants were free to complete their own preparations during which pre-TT blood samples were collected, participants were provided with standard pre-race instructions and the zero offset of the SRM crank was set according to manufacturer's instructions.

Cycling Time-Trial (World Championship Road Cycling Time Trial Simulation)

The TT consisted of a simulation of the 2017 Bergen World Championship TT course, based on global positioning system (GPS) mapping data (road altitude and distance) collected by the Orica cycling team staff (M. Quod, unpublished observations). Cyclists completed the 31.17 km TT as fast as possible and during the TT the only feedback provided to the participant was the distance covered (km), cycling gear-ratio (12–27/48–54) and road gradient (%). Participants were only informed of their TT results following the completion of both trials. HR was collected every 5 km and ratings of perceived exertion (RPE) using the Borg 6–20 scale and capillary blood samples were collected at 15.74 km and immediately post TT. Participants ingested 250 mL of commercially available 6% CHO drink (Gatorade) at 15.74 km, as this distance corresponded to the point identified by the cyclists as the most appropriate opportunity to drink on the actual course. Samples of venous and capillary blood, and urine, were collected immediately following the TT and participants were weighed. At $t = \text{TT} + 20$ min, blood samples were collected and participants consumed a recovery drink (1 g/kg BM CHO) and continued to rest quietly for a further 40 min. Blood samples were collected at $t = \text{TT} + 40$ min and $t = \text{TT} + 60$ min, with a final urine sample being collected at $t = \text{TT} + 60$ min. Following the removal of the cannula, participants participated in a semi-structured interview with a single researcher using a series of standard questions to probe perceived effort, motivation and comfort rating during the TT. When symptoms (e.g., gut discomfort and problems) were identified, a standardized Likert scale was used to quantify them into mild, moderate, or severe levels. On completion of the second trial, participants were asked whether they could identify the trial in which they received the ketone ester, and the trial in which they had performed best. The interview technique was used to probe levels of interest in using a ketone ester supplement in real competition.

Analytical Procedures

Capillary blood samples were analyzed for concentration of ketones and lactate (Lactate Pro 2, Akray, Japan). Venous blood samples were collected into 4 mL SST vacutainers with immediate analysis of a small aliquot for blood glucose concentrations (Cobas Integra 400 plus, Roche Diagnostics, Switzerland). This venous sample was then centrifuged at 1,500 g for 10 min at 4°C, and aliquots of serum were stored at −80°C for later analysis. Samples were analyzed for FFA concentrations using a non-esterified-fatty acid (NEFA) assay kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan), βeta-hydroxybutyrate concentrations using a β-hydroxybutyrate assay kit (Sigma-Aldrich, Ltd, Australia) and acetoacetate (AcAc) concentrations using an acetoacetate assay kit (Abcam, Cambridge, UK), as per the manufacturer's instructions. Urine samples were analyzed for urine ketones (namely AcAc) using ketone reagent strips (Keto-Diastix, Bayer).

Data Analysis

Statistical analysis was completed using SPSS (version 20 for Windows; SPSS, Chicago, IL). Paired t-tests were used to analyze average PO, cadence, HR and change in BM in the TT. Blood, urine, PO, HR, cadence, RPE, and respiratory data from the two trials were analyzed using a linear mixed model (treatment × time; $n = 10$) with the exception of respiratory data which includes ($n = 9$). When analyzing respiratory gases, an RER > 1.0 was not included in analysis as participants were not in steady state ($n = 1$, stage 3 for KET and PLAC). Statistical significance was set at $P < 0.05$ and data is presented as mean ± standard deviation (*SD*). TT performance was also analyzed for magnitude-based effect sizes between conditions using a custom spreadsheet (Hopkins, 2006). Data were log-transformed to account for non-uniformity and effect sizes with 90% confidence intervals [effect size (ES) ± 90% CI] were calculated and classified as either trivial (−0.2 to 0.2 ES) small (0.2–0.6, ES), moderate (0.6–1.2 ES), or large (1.2–2.0 ES). Where the 90% CI overlapped small positive (0.2) and negative (0.2) values, the effect was considered to be unclear.

RESULTS

Participant Experiences

Eleven cyclists commenced this study, but one participant experienced severe side effects from KET ingestion during and after the warm-up, including prolonged vomiting and dizziness and was unable to complete the TT. This participant withdrew from further participation in the study. However, data for this participant have been provided in the following analysis in comparison to those of the other riders to investigate a possible explanation for the occurrence of these side effects. All participants reported gastrointestinal discomfort associated with the intake of the ketone diester. Symptoms ranged from major (dry retching and nausea; $n = 2$), to moderate nausea ($n = 5$) or moderate reflux ($n = 1$), and minor discomfort (mild nausea; $n = 2$). No similar symptoms were reported with the PLAC trial. All participants correctly nominated the trial in which they received KET, identifying it via the gastrointestinal side-effects. However, only four of the cyclists correctly identified the trial in which they completed the TT in the fastest time, with one cyclist equivocal. Although each of the riders nominated their gut symptoms as a distraction or interference to performance, six participants identified an "unusual" centrally-derived feeling during the TT in the KET trial that they thought might be associated with better performance. When asked if they would use the current KET supplement in actual competition, prior to the unmasking of performance results, only one participant (who reported the least degree of discomfort during his KET trial) nominated being "possibly" interested. The remaining participants identified the need to remove the potential for illness and gut upset as well as to be sure of a robust performance effect before KET would be of value; "racing is hard enough without adding this complication."

Performance

All cyclists completed the TT in a faster time in PLAC compared with KET, with the crossover allocation of treatments meaning that there was no order effect on performance. **Figure 2** displays the results of the cycling TT including group mean and individual performances. There was an impairment to overall performance time with KET (2 ± 1%, 58.2 s; small ES –0.42 ± 0.1, $P < 0.001$). There was an impairment in cycling performance time in the first segment of the course (0–18 km; $P < 0.001$) and second segment with the climb included (18–32 km; $P = 0.004$) with KET ingestion compared to PLAC. Overall the KET condition was associated with a 3.7% reduction in average PO (KET 339 ± 37 W vs. PLAC 352 ± 35 W, $P < 0.001$, **Figure 3B**) and a lower cadence (KET 93 ± 6 rpm, PLAC 95 ± 6 rpm, $P = 0.06$, **Figure 3C**) compared to PLAC. There was an effect of time ($P < 0.001$) for power output and cadence during the TT, as displayed in **Figure 3**. A time × treatment interaction was reported for HR ($P = 0.001$) and average HR was significantly lower in the KET compared to PLAC condition (163 ± 7 vs. 167 ± 9 bpm, respectively, $P < 0.01$; **Figure 3D**). RPE increased in both the KET (16 ± 2 to 19 ± 2) and the PLAC (15 ± 2 to 19 ± 1) trials from mid- to post-TT ($P < 0.001$). There was no difference in RPE between trials despite the lower HR and PO in the KET condition.

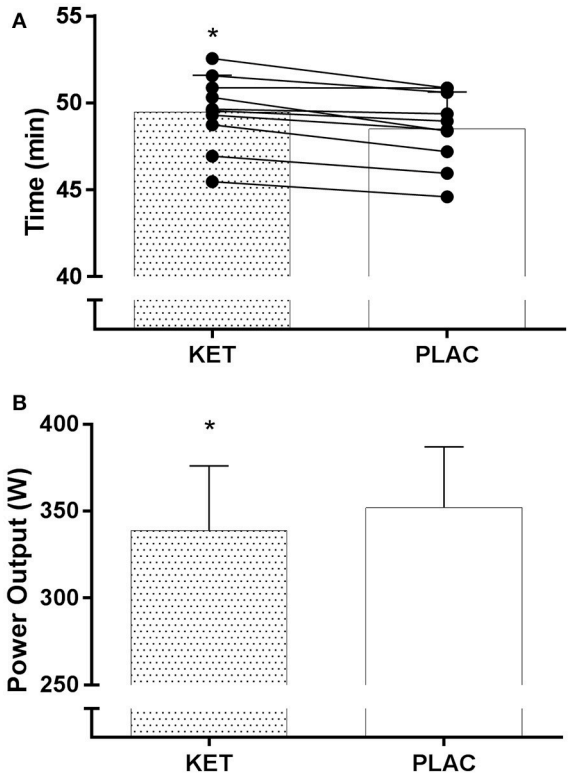

FIGURE 2 | Mean and individual TT performance time **(A)** and mean power output **(B)** following exogenous KET or PLAC ingestion. Values are mean ± *SD*. *KET different to PLAC.

Serum Metabolites

There was an effect of time ($P < 0.001$) and treatment ($P = 0.021$) for serum FFA concentrations (**Figure 4A**). FFA concentrations reduced from $t = 0$ during the 90 min following the CHO breakfast in both KET and PLAC trials (0.37 ± 0.10 mmol/L to 0.27 ± 0.04 mmol/L, $P < 0.02$). FFA concentrations were higher in PLAC vs. KET from pre- to post-TT ($P < 0.05$). A condition × time interaction was reported for serum βHB concentrations ($P < 0.001$; **Figure 4B**). There was an increase in βHB concentrations in the KET trial following dose one of KET ingestion ($t = 90$ min) and βHB remained significantly higher than PLAC trial until $t = \text{TT} + 60$ min. An increase in βHB concentrations from $t = 0$ was measured in the PLAC trial at the onset of the warm up ($t = 100$ min; **Figure 4B**) however βHB remained lower than in the KET trial. Serum AcAc concentration significantly increased from $t = 0$ min following dose one of KET ingestion ($P = 0.001$) and remained higher until $t = \text{TT} + 60$ min (**Figure 4C**).

Capillary Blood and Urine Metabolites

There was a condition × time interaction for both urine ketone and capillary blood βHB concentrations ($P < 0.001$; **Figures 5A,B**). No differences were observed at $t = 0$ between KET and PLAC for urine ketone concentration, but following KET ingestion, urine ketones were higher at pre-TT, post-TT, and at $t = \text{TT} + 60$ min for KET. Blood βHB concentrations increased

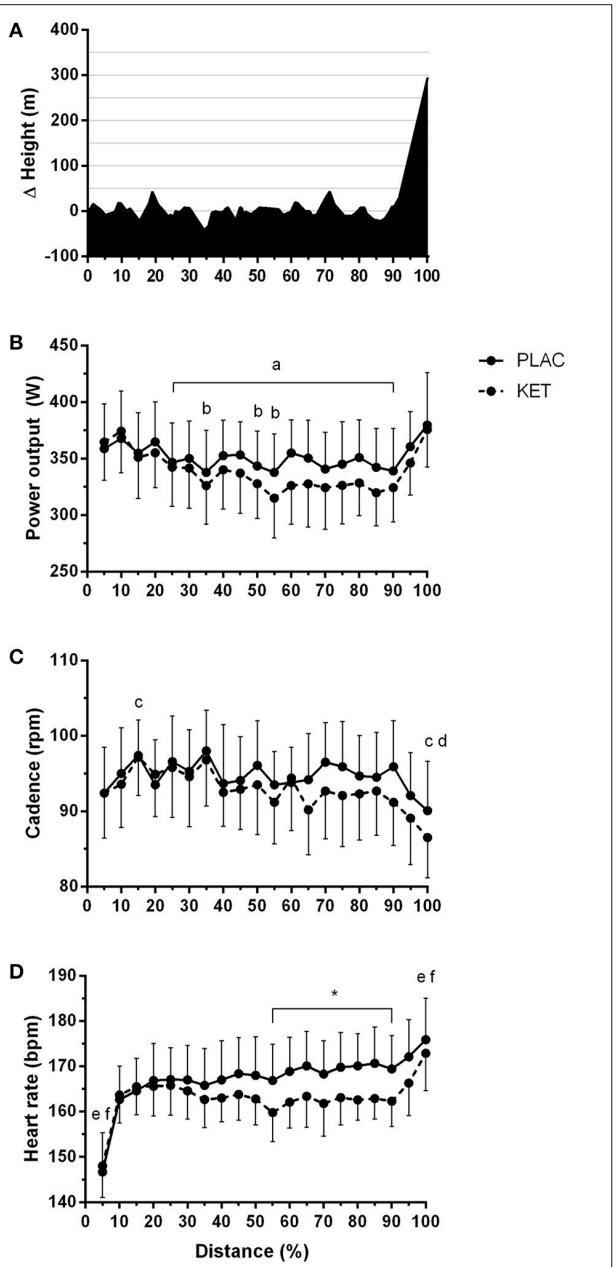

FIGURE 3 | Course profile of World Championships TT course (Bergen, Norway) **(A)**, average power output **(B)**, cadence **(C)**, and heart rate **(D)** during TT as a percentage of total distance, following exogenous KET or PLAC ingestion. Values are mean ± *SD*. a different to 5% in KET; b different to 5% in PLAC; c different to 10% in KET; d different to 10% in KET; *KET different to PLAC, e different to all other time points in KET, 50% different to f.

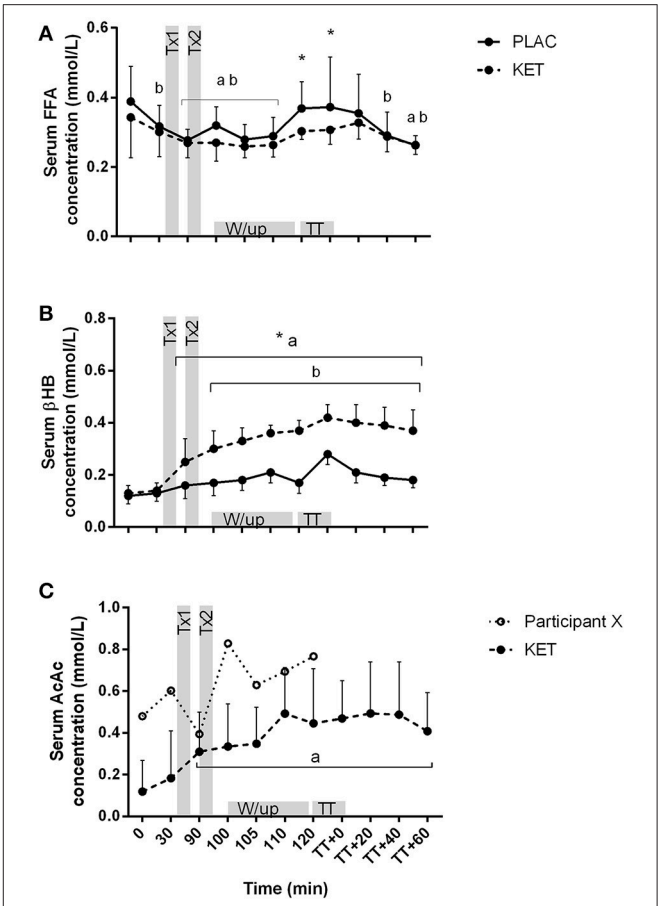

FIGURE 4 | Serum FFA **(A)**, βHB **(B)**, and AcAc **(C)** concentrations following exogenous KET or PLAC ingestion whilst completing a cycling TT. Values are mean ± *SD*. Tx1 and Tx2 refer to dose one and two of KET or PLAC drink. *KET different to PLAC at time point; a different to $t = 0$ min within KET; b different to $t = 0$ min within PLAC.

following the first dose of KET, compared with PLAC ingestion (0.32 ± 0.16 mmol/L, $P = 0.001$). Blood βHB concentrations increased from pre-TT to post-TT in the KET trial ($P < 0.001$) and this increase was maintained until t = TT + 60 min ($P = 0.03$; **Figure 5B**).

There was a significant condition × time interaction for blood glucose ($P = 0.036$) and lactate concentrations ($P < 0.001$; **Figures 6A,B**). Blood glucose concentrations were lower in KET following the first dose of KET ingestion, pre-TT and t = TT + 40, compared to PLAC. Blood lactate concentrations increased from pre-warm up at the end of stage 3 ($t = 110$ min) in both the KET and PLAC trials but had returned to resting values pre-TT for both trials. Post-TT, blood lactate concentrations were significantly lower in the KET trial compared to the PLAC trial (8.6 ± 3.2 vs. 13.1 ± 4.3 mmol/L, $P < 0.001$, respectively).

Respiratory Parameters and BM

There was a main effect of time in the KET and PLAC trials for VO_2, VCO_2, and RER ($P < 0.001$), where an increase was observed throughout the incremental warm up from stage 1 to stage 3 (**Table 1**). There was a main effect of time for BM ($P < 0.001$) where a similar loss was measured in the KET (1.6 ± 0.7 kg) and PLAC (1.4 ± 0.4 kg) trials.

DISCUSSION

This is the first study to report the effect of pre-exercise supplementation with a ketone diester on the performance of a cycling TT under conditions simulating real-life competition:

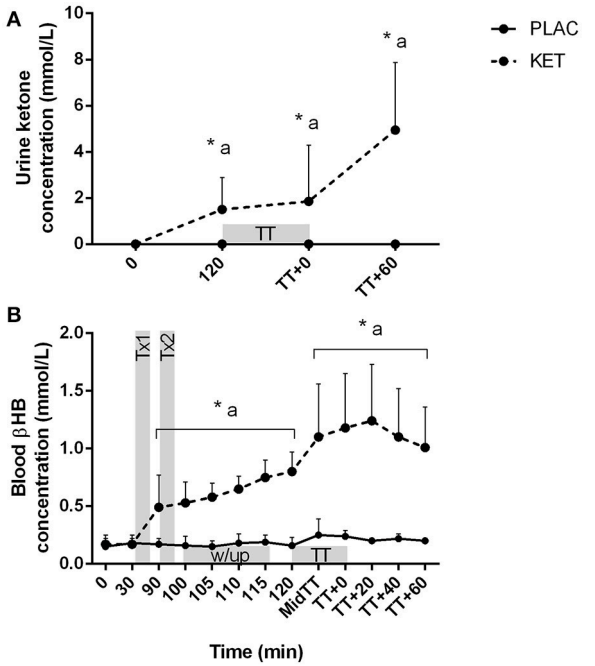

FIGURE 5 | Urine ketone **(A)** and blood βHB **(B)** concentrations following exogenous KET or PLAC ingestion whilst completing a cycling TT. Values are mean ± *SD*. Tx1 and Tx2 refer to dose one and two of KET or PLAC drink, w/up refers to warm-up. *KET different to PLC at time point; a different to $t = 0$ min within KET.

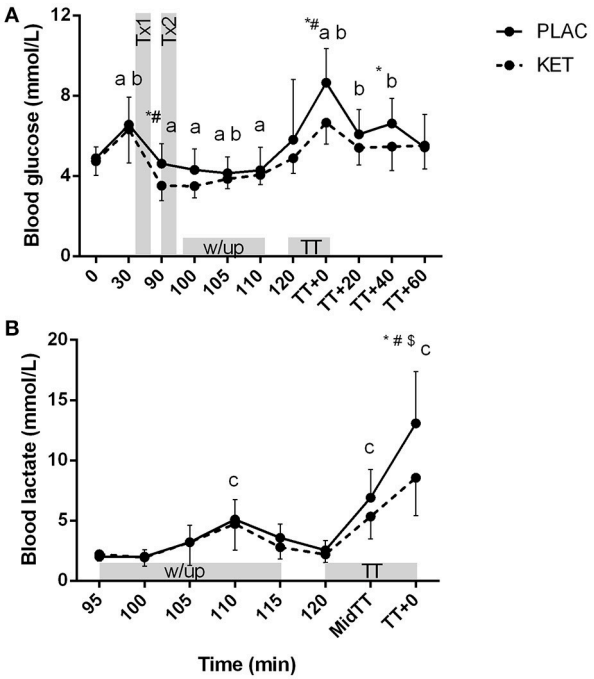

FIGURE 6 | Blood glucose **(A)** and lactate **(B)** concentrations following exogenous KET or PLAC ingestion whilst completing a cycling TT. Values are mean ± *SD*. Tx1 and Tx2 refer to dose one and two of KET or PLAC drink. *KET different to PLAC at time point; a different to $t = 120$ min within KET; b different to $t = 120$ min within PLAC; c different to 95 min within and PLAC; # different to all other time points within PLAC; $ different to all other time points within KET.

laboratory simulation of a World Championship TT course in world-class male road cyclists who undertook nutritional strategies mimicking competition practices with respect to CHO and caffeine supplementation. Although, our protocol achieved hyperketonemia, as evidenced by increases in serum βHB and AcAc concentrations, there was an impairment of TT cycling performance in these elite cyclists. This outcome appears to be linked to the general observation of gut discomfort and intolerance among the study participants, with symptoms ranging from mild to severe. Our investigation adds important information to sports nutrition, by adding a real-world element.

The primary aim of our study was to address recent reports of enhanced sports performance associated with an acute increase in blood ketone concentrations following the intake of a ketone ester drink (Cox et al., 2016), by re-examining this concept in a more ecologically valid protocol. The investigation was undertaken as a collaborative project with a World Tour professional team of the International Cycling Union (UCI), offering an opportunity for them to make an evidence-based decision regarding the potential use of a highly discussed performance aid (Abraham, 2015). A number of features were included in the study design to optimize the reliability and validity of data, including opportunities to mimic the conditions under which the performance aid (ketone ester supplement) would be used. Team sports scientists were able to provide a simulation of the profile of the 2017 World Championships TT course (Bergen, Norway) on a cycle ergometer. Furthermore, the world-class cyclists from the team who participated in the study were personally motivated to receive individual and group results, and were highly experienced in TT cycling as well as familiarized to the specific laboratory-based course simulation. Race nutrition strategies (24 h prior, pre-race and during race) were standardized and made realistic by involving meal preparation by the team chef to suit both sports nutrition guidelines (Thomas et al., 2016) and the cultural practices of the riders. This included attention to achieve adequate CHO availability in pre-race meals (as opposed to the overnight fasted state involved in other studies such as Balasse, 1979; Fery and Balasse, 1986; Cox et al., 2016), as well as the real-life intake of caffeine and CHO supplements during the pre-race and within-race practices.

Our primary finding of a 2.0 ± 1% (58 s) longer time to completion in the TT following ketone ingestion supports our initial hypothesis that ketone ingestion would not enhance TT performance (**Figure 2**). Although, the gut disturbances were the likely cause of the performance impairment, we note that none of the cyclists achieved a faster time in the ketone trial, even when they reported very minor symptoms. Furthermore, the RPE in the ketone trial were similar to those on the placebo trial despite a lower power output and heart rate. This suggests that the gastrointestinal discomfort and/or some direct effect of ketones on the brain increased the perception of effort, and in accordance

TABLE 1 | Metabolic measures collected during 3 stage incremental warm up following exogenous KET or PLAC ingestion.

Variable	Treatment	Stage 1	Stage 2	Stage 3
VO_2 (L/min)	KET	2.27 ± 0.27	3.95 ± 0.37*	4.42 ± 0.37
	PLAC	2.33 ± 0.26	4.01 ± 0.38*	4.45 ± 0.42
VCO_2 (L/min)	KET	1.94 ± 0.21	3.66 ± 0.37*	4.28 ± 0.38
	PLAC	2.02 ± 0.24	3.70 ± 0.28*	4.31 ± 0.43
RER	KET	0.86 ± 0.04	0.93 ± 0.03*	0.96 ± 0.02
	PLAC	0.87 ± 0.03	0.93 ± 0.04*	0.96 ± 0.03

Different to stage 1 and stage 3; Values are mean ± SD.

with the psychobiological model of pacing (Pageaux et al., 2014), our highly experienced cyclists reduced their work output to enable the TT to be completed without premature exhaustion. This finding warrants further investigation to confirm and explore the mechanisms. In the meantime, we note that the outcome of impaired performance with ketone ingestion is in contrast to previous studies (Clarke and Cox, 2015; Cox et al., 2016). Indeed, Clarke and Cox (2015) and Cox et al. (2016) reported 1–2% improvements in 30 min rowing performance and 30 min TT performance, respectively, following ingestion of a similar ketone dose to the current study (573 vs. 500 mg/kg BM, respectively) in combination with CHO, compared to CHO alone.

Due to the lack of a commercial supply, we were unable to obtain the ketone monoester supplement used in the study of Cox et al. (2016). However, we were able to source a diester that is currently being investigated as a potential treatment for seizures resulting from central nervous system oxygen toxicity (D'Agostino et al., 2013) and used in a similar dose to Cox et al. (2016). This diester contains a racemic mixture of βHB (i.e., contains both D- and L- enantiomers of the βHB) and has the ability to elevate both βHB and AcAc in a 1:1 ratio. The use of enzymatic analysis in the current study measures only the D-enantiomer which is the main circulating form of βHB and the most likely to have a direct effect on substrate metabolism and skeletal muscle responses (Yamada et al., 2010).

We provided the ketone drink in two doses, with the first bolus ingested 70 min prior to the TT. Based on previous research, our aim was to reach peak βHB concentrations at ∼1 h following ingestion (i.e., prior to the TT; Pinckaers et al., 2017). We measured a modest increase in serum βHB concentrations in the ketone trial, reaching >0.3 mmol/L following the warm-up, but to our surprise serum βHB concentrations peaked immediately following the TT (>0.4 mmol/L; **Figure 4B**). The capillary whole blood samples analyzed for βHB concentrations measured values 2- to 3-fold greater than the serum samples (**Figure 5B**). This variation in D-βHB concentrations via enzymatic analysis (serum) and whole blood is consistent with previous literature reporting a –0.5 to 0.6 mmol/L higher concentration with handheld monitors (Pineda and Cardoso, 2015). This variation in measuring blood ketones in a controlled laboratory setting highlights the challenges athletes face in the field when aiming to reach and stay within the "optimal" range for a performance benefit (Egan and D'Agostino, 2016). We also measured a peak in serum AcAc concentrations following the warm-up, reaching ∼0.5 mmol/L (**Figure 4C**). Therefore, when we consider total circulating ketones measured (i.e., βHB and AcAc) and the L- enantiomer that has not been measured, it is likely that athletes would be in the "optimal" range of 1–3 mmol/L for a proposed performance benefit (Egan and D'Agostino, 2016). Although "nutritional ketosis" was achieved, βHB concentrations reported in the current study are much lower than those reported previously (Cox et al., 2016). Cox et al. (2016) reported an increased in βHB concentrations to ∼2 mmol/L within 20 min of ketone ester ingestion when co-ingested with CHO or ∼4 mmol/L when ingested alone. This variation in serum βHB is likely explained by a range of factors including the different ketone esters used, the elite training status of cyclists in the current study and the different pre-ingestion nutritional strategies were the current study focused on appropriate race preparation practices.

Although we have not measured the same increase in circulating ketone concentrations as (Cox et al., 2016), ketones increased appropriately to alter metabolic responses compared to when a placebo was ingested. Blood glucose concentration was lowered in the ketone trial by ∼1 mmol/L within 30 min following ingestion of the first ketone dose, and following the TT blood glucose was ∼2 mmol/L lower than in the placebo trial. Additionally, we reported a 4.5 mmol/L (35%) reduction in blood lactate concentration following the TT in the ketone trial compared to the placebo trial. These findings of reduced blood glucose and blood lactate concentrations are consistent with the data of Cox et al. (2016) during 60 min of exercise at 75% Wmax and following a 30 min TT, respectively. We also measured lower circulating FFA during the TT following ketone ingestion compared to a placebo. Participants were cycling at 340–350 W during the TT and thus estimated contribution of FFA oxidation to total energy expenditure would likely be low as at this intensity, the muscle relies predominately on CHO-based fuels (Hawley and Leckey, 2015). However, the difference in FFA concentration between the ketone and placebo trials could be related to circulating ketone bodies having the ability to suppress lipolysis via inhibition of catecholamine's (Bjorntorp and Schersten, 1967).

As ketone bodies can be readily oxidized by skeletal muscle, expired gas was collected during the incremental warm-up. No differences in RER were measured between the ketone and placebo trials, although this could be related to the absolute exercise intensities attained by our elite subjects (155, 310, 355 W). Alternatively the high ketone concentrations in the urine suggest that the ketones are not being oxidized at the skeletal muscle. We have not estimated rates of substrate oxidation rates due to βHB and AcAc yielding respiratory exchange quotient values of 0.89 and 1.00, respectively and thus without appropriate correction factors for CO_2 displacement and urine volume this would lead to an inaccurate representation of substrate utilization (Frayn, 1983; Pinckaers et al., 2017). We also reported a modest reduction in heart rate during the TT in the ketone trial compared to the placebo trial (5 bpm) which may be associated with a slightly reduced average power output and in the ketone trial.

Of the 10 participants who completed the trials, all reported gastrointestinal discomfort associated with the intake of the ketone ester including dry retching, mild to moderate nausea, reflux and minor discomfort. Furthermore, one participant was unable to start the TT due to prolonged vomiting and dizziness. This participant also experienced the highest concentrations of serum AcAc concentration when compared to the other 10 participants (**Figure 4C**, participant X), suggesting bioavailability of the ketone diester may impact individual responses following ketone ingestion. Although the side effects of ketone esters are not frequently discussed in the literature, Clarke et al. (2012) has provided evidence that participants have experienced a range of adverse effects including vomiting, nausea, diarrhea, and abdominal pain. These side effects have been associated with high dose of ketone ester and the consumption of the ester with a milk-based drink (Clarke and Cox, 2015). While it is possible that different dosing strategies, or the use of a different ketone ester product might eliminate or greatly reduce the gut problems seen in the current study, it is unclear whether a performance enhancement could be expected with exogenous ketone use in sporting events undertaken under the conditions employed in our study.

In conclusion, the results of the current study show that ingestion of a 1,3-butanediol acetoacetate diester under conditions of optimal race nutrition (i.e., CHO fed) results in increases in βHB and AcAc concentrations. The diester was associated with gut discomfort and intolerance among the cyclists with symptoms ranging from mild to severe. Despite optimal nutritional support (i.e., CHO breakfast, feeding during the TT and caffeine ingestion) for performance, ketone ingestion was associated with an increase in perception of effort, leading to an impairment of TT performance in elite professional cyclists.

AUTHOR CONTRIBUTIONS

JL, MR, MQ, and JH: Conception and design, Collection and assembly of data, Data analysis and interpretation, Manuscript writing, Final approval of manuscript (required). LB: Conception and design, Financial support, Collection and assembly of data, Data analysis and interpretation, Manuscript writing, Final approval of manuscript (required).

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Heat and Hypoxic Acclimation Increase Monocyte Heat Shock Protein 72 but Do Not Attenuate Inflammation following Hypoxic Exercise

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Acclimation to heat or hypoxic stress activates the heat shock response and accumulation of cytoprotective heat shock proteins (HSPs). By inhibiting the NF-κB pathway HSP72 can preserve epithelial function and reduce systemic inflammation. The aim of this study was to determine the time course of mHSP72 accumulation during acclimation, and to assess intestinal barrier damage and systemic inflammation following hypoxic exercise. Three groups completed 10 × 60-min acclimation sessions (50% normoxic VO_2peak) in control ($n = 7$; 18°C, 35% RH), hypoxic ($n = 7$; $F_iO_2 = 0.14$, 18°C, 35% RH), or hot ($n = 7$; 40°C, 25% RH) conditions. Tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), interleukin 10 (IL-10), and intestinal fatty acid binding protein (I-FABP) were determined at rest and following a cycling normoxic stress test (NST; ∼2 weeks before acclimation), pre-acclimation hypoxic stress test (HST1; $F_iO_2 = 0.14$, both at 50% normoxic VO_2peak; ∼1 week before acclimation) and post-acclimation HST (48 h; HST2). Monocyte HSP72 (mHSP72) was determined before and after exercise on day 1, 3, 5, 6, and 10 of acclimation. Accumulation of basal mHSP72 was evident from day 5 ($p < 0.05$) of heat acclimation and increased further on day 6 ($p < 0.01$), and day 10 ($p < 0.01$). In contrast, basal mHSP72 was elevated on the final day of hypoxic acclimation ($p < 0.05$). Following the NST, plasma TNF-α (–0.11 ± 0.27 ng·mL^{-1}), IL-6 (+0.62 ± 0.67 ng·mL^{-1}) IL-10 (+1.09 ± 9.06 ng·mL^{-1}) and I-FABP (+37.6 ± 112.8 pg·mL^{-1}) exhibited minimal change. After HST1, IL-6 (+3.87 ± 2.56 ng·mL^{-1}), IL-10 (+26.15 ± 26.06 ng·mL^{-1}) and I-FABP (+183.7 ± 182.1 pg·mL^{-1}) were elevated ($p < 0.01$), whereas TNF-α was unaltered (+0.08 ± 1.27; $p > 0.05$). A similar trend was observed after HST2, with IL-6 (+3.09 ± 1.30 ng·mL^{-1}), IL-10 (+23.22 ± 21.67 ng·mL^{-1}) and I-FABP (+145.9 ±123.2 pg·mL^{-1}) increased from rest. Heat acclimation induces mHSP72 accumulation earlier and at a greater magnitude compared to matched work hypoxic acclimation, however neither acclimation regime attenuated the systemic cytokine response or intestinal damage following acute exercise in hypoxia.

Keywords: HSP72, I-FABP, Plasma cytokines, cross-tolerance, acclimation

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INTRODUCTION

Repeated exercise-heat exposures, leading to heat acclimation, can induce within lifetime phenotypic adjustments which enhance heat loss mechanisms and improve physiological responses to heat stress (Sawka et al., 2011; Horowitz, 2014). Heat acclimation improves tolerance to a diverse array of non-thermal stressors without a prior exposure to the new stressor-termed "cross tolerance" (Horowitz, 2016). Heat-acclimation mediated cross tolerance (HACT) to ischemia-reperfusion in the heart, brain hyperoxia, traumatic brain injury, and hypoxia have all been documented in animal models (Arieli et al., 2003; Horowitz, 2007; Shein et al., 2007) with increasing evidence for HACT in human models (Lee et al., 2014, 2015a, 2016; Gibson et al., 2015a,b; White et al., 2016).

Cross-tolerance effects are mediated by shared cellular adaptation, with the actions of the heat shock response (HSR) and upregulation of key heat shock protein family members being the most widely studied component of cross-tolerance in humans (Horowitz, 2016; Gibson et al., 2017). Heat acclimation is characterized as either having short term (STHA) and long term (LTHA) induction periods. STHA has been characterized as <5 daily heat exposures (Pandolf, 1979), and LTHA > 10 daily heat exposures (Garrett et al., 2011). Experimental evidence supports increases in basal heat shock protein 72 (HSP72) in response to exercise models of STHA; (Lee et al., 2015a), LTHA, (McClung et al., 2008; de Castro Magalhães et al., 2010; Gibson et al., 2015a,b; Lee et al., 2016) and both resting and exercising hypoxic exposures (Taylor et al., 2010, 2011, 2012; Lee et al., 2014). The enhancement of cytoprotective networks via progressive transcriptional activation and a buildup of HSP72 reserves occurs progressively throughout the acclimation period (Horowitz, 2016). Increased basal HSP72 is an integral adaptive mechanism that reduces the need for *de-novo* protein synthesis in response to later cellular stressors (Horowitz and Assadi, 2010; Horowitz, 2014, 2016). Heat acclimation has been shown to induce a greater daily physiological stress and increase in post-exercise and post-acclimation monocyte HSP72 compared to a matched absolute-intensity period of hypoxic acclimation (Lee et al., 2015a, 2016). This data suggests that heat *per se* is a more potent stimulator of the HSR and may be a more accessible method for enhancing both cellular and physiological tolerance. However, to date no study has examined the kinetics of the HSP72 response throughout the adaptive period of both heat and hypoxia.

Intracellular HSP72 (iHSP72) can induce anti-inflammatory effects by blocking nuclear factor-κB (NF-κB) activation, and the induction of iHSP72 via heat stimulation reduces the expression of inflammatory genes TNF-a, IL-1, IL-10, IL-12, and IL-18 (Ghosh et al., 1998). The HSR and blocking of NF-κB has been shown to enhance epithelial barrier resistance and reduces cytokine production through inhibition of NF-κB (Zuhl et al., 2014, 2015). Cellular injury to the intestinal tract has been observed followed a multitude of physiological stressors (heat stress, hypoperfusion/ischemia, oxidative stress, mechanical damage), which contribute to intestinal barrier disturbances (Pires et al., 2016; March et al., 2017). During exercise-heat stress a loss of epithelial integrity, increased GI permeability, and bacterial translocation induce a strong inflammatory response, which is characterized by activation, production, and release of cytokines, which are linked to exertional heat stroke (Bouchama and Knochel, 2002; Leon and Helwig, 2010). Acute hypoxia (< 2 h at 4,800 m) has been shown to reduce splanchnic perfusion via a reduction in blood flow to the superior mesenteric artery (Loshbaugh et al., 2006), however this finding is not consistent across studies (Mekjavic et al., 2016), with perfusion increasing during prolonged (3 day) exposure (Kalson et al., 2010). However, an acute reduction in oxygen availability may lead to intestinal cell damage and a loss of epithelial integrity (Ohri et al., 1994; Derikx et al., 2008). A hypoxic stimulus combined with intestinal ischemia activates NF-κB in intestinal epithelial cells, increasing the production of the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) (Eltzschig and Carmeliet, 2011). It is therefore plausible that heat or hypoxic-acclimation mediated activation of the HSR and inhibition of the NF-κB pathway may attenuate intestinal damage and reduce the inflammatory response following a later bout of exercise in acute hypoxia.

The aim of this study was to examine the rate of basal HSP72 accumulation throughout a 10-day period of exercising heat or hypoxic acclimation. We hypothesized that both modes of exercise-acclimation would elevate resting monocyte HSP72, and that this would be reflected in a shift toward an anti-inflammatory status as determined via eHSP72–mHSP72 ratio (*R;* Krause et al., 2015), and change in systemic cytokine balance (TNF-α, IL-6, IL-10). A secondary aim of the present study was to determine whether a period of heat or hypoxic acclimation would attenuate intestinal damage and reduce systemic inflammation following acute hypoxic exercise.

METHODS

This study is part of a recently published larger study of heat acclimation and hypoxic performance (Lee et al., 2016). However, this study focuses on the inflammatory response and intestinal cellular damage following a hypoxic exercise bout completed after acclimation to either heat or hypoxia.

Participants

Participants (n = 21 males; age 22 ± 5 years; stature 1.76 ± 0.07 m; mass 71.8 ± 7.9 kg; VO_2 peak 51 ± 7 $mL{\cdot}kg^{-1}{\cdot}min^{-1}$) provided written informed consent to take part in the study, which was approved by the Coventry University Ethics review panel. Participants were asked not to undertake any other exercise training in the 72 h leading up to a testing bout and throughout the intervention period and were blinded to the hypotheses of the study. All data collection was conducted in accordance with the standards set out in the Declaration of Helsinki of 1996.

Study Design

As depicted in **Figure 1**, participants completed two preliminary assessments of normoxic and hypoxic $\dot{V}O_2$peak, followed by a familiarization visit, a normoxic stress test (NST) and a hypoxic

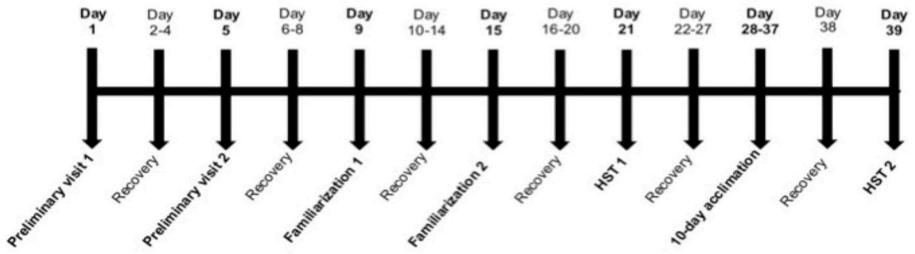

FIGURE 1 | Schematic of the experimental design. Participants attended the laboratory a total of 16 times over ∼39 days. Preliminary assessments of normoxic and hypoxic $\dot{V}O_2$peak were performed in a counterbalanced order separated by at least 3 days. After at least 3 days' recovery (range 3–7 days) participants completed a full familiarization visit under normoxic conditions, followed 6 days (range 6–11) later by a second familiarization, from which the normoxic stress test (NST) data was obtained. At least 6 days after the NST (range 6–9 days), participants completed the pre-acclimation hypoxic stress test (HST1). Following a "wash out" period of 7 days (range 7–11 days), participants commenced 10 consecutive days of exercise acclimation. A second, post-acclimation HST (HST2) was performed 48 h after the final acclimation session in all participants.

stress test (HST1). At least 7 days after HST1, participants completed a 10-day heat ($n = 7$), hypoxic ($n = 7$), or control ($n = 7$) acclimation period. A final HST (HST2) was conducted 48 h after the final acclimation session. On each familiarization, NST and HST session, as well as throughout the acclimation period, participants reported to the laboratory after an overnight fast to consume a standardized breakfast 2 h prior to the exercise bout. The energy content of the breakfast was 386kcal, made up of 15.6 g protein, 44.4 g carbohydrate and 16.4 g fat. Participants drank 400 ml of water with the breakfast. Each familiarization session, NST, HST, and acclimation session was preceded by 15 min of seated normoxic rest (after instrumentation) to collect baseline data and an additional 15 min of seated rest within the defined environment.

The familiarization, NST and HST sessions each consisted of 40 min of cycle exercise at 50% normoxic $\dot{V}O_2$peak (CON = 137 ± 28 Watts; HYP = 138 ± 18 Watts; HOT = 136 ± 16 Watts), and a 5 min recovery period during which a venous blood sample was obtained for the assessment of mHSP72, TNF-α, IL-6, IL-10, and I-FABP. The 10-day acclimation protocol consisted of once daily exposures of cycle ergometer exercise (60 min) within the defined environment, either CON (18°C, 35% RH), HOT (40°C, 25% RH) or HYP (18°C, 35% RH, $F_IO_2 = 0.14\%$) and performed at the same absolute power outputs described above (Castle et al., 2011). The control and hypoxic groups were blinded to their treatment groups by each breathing via a "hypoxic" reservoir filled with either hypoxic gas, or room air. Blinding to condition was not achievable for the heat group.

Preliminary Visits

Height was measured in the Frankfurt plane using a Harpenden stadiometer (Harpenden Instruments, Burgess Hill, UK), nude body mass determined on an electronic scale (Seca Body, Cranlea and Company, Birmingham, UK) and sum of skinfolds determined from 4 sites using a skinfold caliper (Harpenden Instruments, Burgess Hill, UK) as described by Durnin and Womersley (1974).

Peak $\dot{V}O_2$ was determined in both normoxic and hypoxic conditions on separate days (preliminary visits 1 and 2) using an incremental exercise test to volitional exhaustion on a calibrated SRM cycle ergometer (Schoberer Rad Meßtechnik, Welldorf, Germany). Hypoxia was generated via a Hypoxicator unit (Hypoxico HYP123 Hypoxicator, New York, USA), that was used to fill a reservoir of three 1,000 L Douglas bags in series. Participants inspired via a mouthpiece attached to a two-way non-rebreathable valve (Harvard Ltd, Eldenbridge, UK) connected to the gas reservoir with clear ethylene vinyl tubing. Inspired oxygen content was monitored throughout each trial via a Servomex 1,400 gas analyzer (Servomex, Crowborough, UK) and sample line introduced through the Douglas bag sample port.

Resting blood lactate (BLa; Biosen C-Line analyser, EKF Diagnostics, Sailauf, Germany) was determined from a finger capillary whole blood sample following a 10-min seated rest period. The test began at a workload of 70 W for 4 min and was then increased by 35 W every 4 min until a blood lactate value of >4 mmol·L^{-1} was reached. Thereafter, workload increased 35 W every 2 min until volitional exhaustion. A cadence of 70 rev·min^{-1} was maintained throughout. Expired gases were collected using 200 L Douglas bags (Cranlea & Co, Birmingham, UK) during the final minute of each stage. Heart rate (Polar FT1, Polar Electro OY, Kempele, Finland) and perceived exertion (Borg, 1982) were recorded at the end of each gas collection. Respiratory gas analysis was completed as previously described (Lee et al., 2014, 2015a).

Measurements

Prior to each testing session participants provided a urine sample for the assessment of urine specific gravity (USG; Atago Refractomer, Jencons Pls, Leighton Buzzard, UK) and urine osmolality (U_{OSMO}; Advanced 3,300 Micro-Osmometer, Advanced Inc, Massachusetts, USA), determined their nude body mass (Seca, Bodycare, UK) and inserted a rectal thermistor (Grant Instruments, UK) to a depth of 10 cm. Heart rate (HR) was monitored throughout each trial via telemetry (Suunto, T6c, Finland). Blood lactate (Biosen C-Line analyser, EKF Diagnostics, Sailauf, Germany) was determined from a finger capillary whole blood sample at the end of the resting period and at the end of exercise for both HST and acclimation sessions.

During all hypoxic sessions, arterial oxygen hemoglobin saturation (S_PO_2) was measured throughout via a pulse oximeter (WristOx, Nonin Medical Inc, Minnesota, USA). Ratings of perceived exertion (RPE; Borg, 1982) and thermal sensation (TS; Young et al., 1987) were collected at 10 min intervals during the 40 min exercise tolerance phase of the test session with the mean exercise value reported.

Blood Sampling and Analysis

Venous blood samples (∼7 mL) were collected from an antecubital vein into an EDTA treated vacutainer (Vacuette, Greiner Bio-One, Stonehouse, UK) following the 15 min seated stabilization period before the familiarization session and each HST. Post-exercise samples were collected immediately after the exercise phase of the 60 min HST exposure was completed. Hemoglobin and haematocrit were determined in triplicate via a B-Hemoglobin Photometer (Hemocue Ltd, Angleholm, Sweden) and centrifuged capillary tubes (Hawksley Micro Haematocrit Centrifuge, Hawksley and Son, Lancing, UK), measured using a haematocrit reader. Plasma volume changes were then calculated according to the equations of Dill and Costill (1974). All later analysis for TNF-α, IL-6, IL-10, and I-FABP were corrected for any changes in plasma volume from pre to post-exercise. The remaining blood sample was centrifuged for 10 min at 3,000 RPM and plasma aliquoted for storage at −80°C prior to analysis.

Circulating TNF-a, IL-6, and IL-10 were determined in duplicate using commercially available high sensitivity sandwich ELISA kits (R&D systems, Minneapolis, USA) which were sensitive to 0.19 $pg{\cdot}mL^{-1}$, 0.18 $pg{\cdot}mL^{-1}$ 0.11 $pg{\cdot}mL^{-1}$ and respectively. The inter-assay variability for TNF-a, IL-6 and IL-10 was 3.1, 1.5, and 1.8%, respectively. I-FABP concentration was determined in duplicate via an ELISA kit (Hycult, Biotechnology, Uden, The Netherlands). Circulating eHSP70 was assessed using the commercially available Amp'd® HSP70 high sensitivity ELISA kit (ENZ-KIT-101-001; Lee et al., 2015b) according to the manufacturer's instructions (Enzo Lifesciences, Lausen, Switzerland). The ENZ-KIT is sensitive to 0.007 $ng{\cdot}mL^{-1}$ with a working range of 0.039–5.00 $ng{\cdot}mL^{-1}$.

mHSP72 Determination

An IgG1 isotype and concentration-matched FITC-conjugated negative control were used in order to assess non-specific binding. Briefly, cells obtained after red cell lysis were fixed and permeabilised (AbD Serotec, Kidlington, UK) and a negative control (FITC, AbD Serotec, Kidlington, UK) or anti-HSP72 antibody (SPA-810, Enzo lifesciences, Exeter, UK) was added to a final concentration of 100 $\mu g{\cdot}ml^{-1}$, this was used to label 1×10^6 cells according to the manufacturer's instructions and then incubated for 30 min in the dark. Samples were then analyzed on a BD FACSCalibur (BD Biosciences, Oxford, UK) by flow cytometry with monocytes gated for forward/side scatter properties and further discriminated by CD14 expression (Selkirk et al., 2009). Mean florescence intensity (MFI) was then calculated using CellQuest Pro software (BD Biosciences, Oxford, UK) with a total of 15,000 cells counted.

eHSP72 to iHSP72 Ratio

The ratio of eHSP72 to iHSP72 was determined by dividing eHSP72 by iHSP72 (Krause et al., 2015). An increase in the ratio score was classed as a transition to a pro-inflammatory state, and a decrease in ratio score classed as a transition to an anti-inflammatory state.

Statistical Analysis

The primary outcome measure of this study were an assessment of the monocyte HSP72 response throughout the 10-day acclimation period. Secondary outcomes were the eHSP72, IL-6, IL-10, TNF-α, I-FABP responses before and after exercise on day 1 and day 10 of the acclimation period. Three families of hypotheses were tested according to the method of Benjamini and Hochberg (1995); (1) the absolute and percent changes in resting and post-exercise mHSP72 responses throughout acclimation; (2) the absolute and percent changes in eHSP72, TNF-α, IL-6, IL-10, and I-FABP before and after exercise; and (3) eHSP72/mHSP72 ratio before and after the acclimation period. The resting, mean exercise, and peak exercise physiological data have been reported in detail elsewhere (Lee et al., 2016) and are briefly presented over the course of acclimation for descriptive purposes. Mixed linear modeling with fixed effect for group (3), day (5) and time (2) was used to analyze mHSP72 data throughout the acclimation period. The same approach was used to analyze eHSP72, IL-6 and IL-10 before and after exercise on days 1 and day 10, with fixed effects for group (3), time (4) and day (2). In each instance main effects were explored using Tukey's HSD test and *post-hoc* results shown as $p < 0.05$ or $p < 0.01$. Precise *p*-values for main effects are reported alongside Cohen's D effect sizes (95% confidence intervals) to indicate the magnitude of any observed effects (Colquhoun, 2014). Effect sizes of 0.2, 0.5, and 0.8 are considered small, medium and large, respectively.

RESULTS

For complete performance results of the experimental and control groups, see our previously published study (Lee et al., 2016). All participants completed the full 60-min exercise bout during the acclimation period. **Table 1** illustrates mean-exercise and peak-exercise data for heart rate, rectal temperature, physiological strain index, SpO_2, change in body mass and change in plasma volume for days 1, 3, 5, 6, and 10 of the control, heat, and hypoxic acclimation period.

Intracellular and Extracellular Responses to Acclimation

No changes in mHSP72 or eHSP72 were observed in the control group at any phase of the acclimation period. Both hypoxic and heat acclimation increased resting concentrations of mHSP72 (time × trial interaction, $f = 2.55$, $p = 0.003$; **Figure 2A**). Specifically, basal mHSP72 MFI was increased in relation to day 1 on days 5 ($p < 0.05$; $d = 2.1$, 95% $CI = 0.7$–3.2), 6 ($p < 0.05$; $d = 2.2$, 95% $CI = 0.8$–3.4) and 10 ($p < 0.01$, $d = 3.1$, 95% $CI = 1.4$–4.4) in the heat acclimation group, and on day 10 in hypoxic group ($p < 0.05$; $d = 1.3$, 95% $CI = 0.04$–2.3). A post-exercise increase in mHSP72 was observed on day

TABLE 1 | Mean and peak exercise physiological responses throughout the 10-day acclimation period in the CON ($n = 7$), HYP ($n = 7$), and HOT ($n = 7$) experimental groups.

	Mean exercise SpO_2 (%)	Mean HR (bts·min^{-1})	Peak HR (bts·min^{-1})	Mean exercise T_{rectal} (°C)	Peak T_{rectal} (°C)	Mean PSI (AU)	Peak PSI (AU)	Change in Body mass (kg)	Change in PV (%)
CON									
Day 1	98 ± 1	133 ± 21	137 ± 10	37.73 ± 0.27	37.86 ± 0.24	3.5 ± 1.3	4.0 ± 1.0	0.6 ± 0.2	–
Day 3	98 ± 1	133 ± 11	139 ± 14	37.74 ± 0.28	37.99 ± 0.24	3.8 ± 0.9	4.7 ± 0.6	0.6 ± 0.2	0.2 ± 4.7
Day 5	97 ± 1	129 ± 12	131 ± 17	37.61 ± 0.22	37.85 ± 0.09	4.3 ± 1.2	3.9 ± 1.1	0.9 ± 0.3	−1.0 ± 4.4
Day 6	98 ± 1	130 ± 10	136 ± 11	37.65 ± 0.22	37.89 ± 0.22	3.6 ± 0.9	4.5 ± 1.0	0.8 ± 0.3	3.3 ± 4.6
Day 10	98 ± 1	134 ± 12	137 ± 17	37.70 ± 0.28	37.86 ± 0.21	3.7 ± 0.9	4.2 ± 1.3	0.7 ± 0.3	2.4 ± 4.6
HYP									
Day 1	81 ± 2^{b,f}	149 ± 16^{a}	159 ± 15^{b}	37.82 ± 0.51	38.16 ± 0.46	4.7 ± 1.5	5.9 ± 1.5^{b}	0.8 ± 0.3	–
Day 3	81 ± 2^{b,f}	151 ± 11^{a}	157 ± 10^{b}	37.89 ± 0.37	38.14 ± 0.29	5.2 ± 1.2^{a}	6.0 ± 1.0^{a}	0.7 ± 0.4	−2.9 ± 3.8
Day 5	81 ± 2^{b,f}	150 ± 13^{a}	157 ± 12^{b}	37.84 ± 0.40	38.16 ± 0.38	5.2 ± 1.2^{a}	6.2 ± 1.0^{b}	0.7 ± 0.2	−3.1 ± 4.6^{a}
Day 6	82 ± 2^{b,f}	148 ± 11^{a}	154 ± 8^{b}	37.90 ± 0.39	38.20 ± 0.25	4.4 ± 1.3	5.3 ± 1.1	0.7 ± 0.4	−3.6 ± 3.6^{a}
Day 10	82 ± 3^{b,f}	136 ± 11*	142 ± 7*	37.66 ± 0.33	37.92 ± 0.22	4.1 ± 0.9	4.9 ± 0.5*	0.9 ± 0.3	−5.6 ± 3.7*
HOT									
Day 1	97 ± 1	151 ± 16^{a}	164 ± 11^{b}	37.91 ± 0.44	38.68 ± 0.26^{c,e}	5.0 ± 1.7^{a}	7.3 ± 1.2^{c,e}	1.0 ± 0.6	–
Day 3	98 ± 2	145 ± 16^{a}	157 ± 16^{b}	37.81 ± 0.49	38.22 ± 0.38$^{\#}$	4.7 ± 1.4	6.2 ± 1.1$^{\#,b}$	1.2 ± 0.5	2.9 ± 1.7
Day 5	97 ± 1	147 ± 12^{a}	156 ± 12^{b}	37.86 ± 0.44	38.26 ± 0.37$^{\#,b}$	4.9 ± 1.3^{a}	6.2 ± 1.1^{*,b}	1.1 ± 0.3	4.3 ± 2.3
Day 6	98 ± 2	143 ± 13^{*a}	150 ± 8*	37.77 ± 0.41	38.18 ± 0.29$^{\#}$	4.9 ± 1.3	6.2 ± 1.1$^{\#}$	1.5 ± 0.3^{*b,d}	7.5 ± 3.6*
Day 10	98 ± 2	137 ± 14*	144 ± 9$^{\#}$	37.71 ± 0.33	38.06 ± 0.22$^{¥}$	4.2 ± 1.0*	5.4 ± 0.5$^{¥}$	1.9 ± 0.3^{*,b,d}	8.3 ± 3.5*

*Data are mean ± SD. Within group differences to day 1 of acclimation are denoted by *$p < 0.05$, #$p < 0.01$, ¥$p < 0.0001$. Within day differences to the control condition are denoted by $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.0001$. Within day differences to hypoxia are denoted by $^{d}p < 0.05$, $^{e}p < 0.001$. Within day differences to hot are denoted by $^{f}p < 0.0001$.*

1 of heat acclimation ($p < 0.01$), and on days 1 ($p < 0.05$), day 3 ($p = 0.01$), and day 6 ($p < 0.01$) of hypoxic acclimation (**Figure 2B**). A negative correlation between the recorded resting mHSP72 and fold change following exercise in both the heat ($r = -0.87$, $p = 0.009$) and hypoxic ($r = -0.52$, $p = 0.22$) groups was observed. A similar relationship was observed post-exercise on day of the hypoxic acclimation period ($r = -0.55$, $p = 0.20$), whereas a much weaker relationship between resting mHSP72 and the fold change post-exercise was observed after the heat acclimation period ($r = -0.05$, $p = 0.92$).

No main effects were observed for absolute eHSP72 concentrations (group $f = 1.52$, $p = 0.23$; time $f = 0.14$, $p = 0.71$; acclimation day $f = 0.38$, $p = 0.54$; **Figure 3A**). When reported as post-exercise percent change (**Figure 3B**), heat induced a 62 ± 50% (range 18–160%; $p < 0.01$, $d = 0.7$, 95% $CI = -0.4$–1.8) increase in eHSP72 following exercise on day 1 of acclimation, whereas hypoxia (31 ± 52%, range −43–106%, $d = 0.1$, 95% $CI = -0.9$–1.2) and control exercise (–2 ± 10.4%, range −18–14%, $d = -0.1$, 95% $CI = -1.2$–1.0) had little effect on eHSP72 concentrations. Following heat-acclimation the post-exercise fold change in eHSP72 was attenuated (−3.6 ± 50%, range −80–82%). The ratio of eHSP72 to mHSP72 was no different on day 10 ($R = 0.47$) of acclimation compared to day 1 ($R = 0.54$) in the control group and hypoxic group (day 1, $R = 0.93$; day 10, $R = 0.48$), but was reduced in the heat group (day 1, $R = 0.28$; day 10, $R = 0.07$; $p = 0.007$).

Cytokine and I-FABP Responses to Acclimation–Day 1

Cytokine data for before and after exercise on day 1 and day 10 of acclimation are shown in **Table 2**. Plasma IL-6 concentration increased with exercise on day 1 of acclimation in the HOT (+ 5.01 [2.78–7.24]′ ng·mL^{-1}; $p = 0.015$) and HYP (+ 3.44 [2.43–4.45] ng·mL^{-1}, $p = 0.003$) groups, with minimal changes observed after CON (+ 0.84 [0.09–1.60] ng·mL^{-1}, $p = 0.216$). Plasma IL-10 also increased with exercise in the HOT (+ 67.14 [35.46–98.81] ng·mL^{-1}; $p < 0.001$) and HYP (+ 24.17 [9.63–38.71] ng·mL^{-1}; $p < 0.001$) groups, and was minimally affected after exercise in CON (+ 3.21 [−0.54–6.95] ng·mL^{-1}, $p = 0.145$). TNF-α was undetectable at rest in 4/21 participants (1 CON, 1 HYP and 2 HOT) and unaffected by exercise in all groups at all phases of the study (**Table 2**). Plasma I-FABP concentrations were unaffected following exercise in CON (+ 28 [–61–118] pg·mL^{-1}, $p = 0.56$), and was increased from resting values after HYP (+201 [128–273] pg·mL^{-1}, $p = 0.0013$) and HOT (+282 [157–406], $p = 0.004$).

Cytokine and I-FABP Responses to Acclimation–Day 10

The plasma IL-6 response to exercise remained consistent with that observed on day 1 of acclimation in the CON and HYP groups, with post-exercise IL-6 concentrations increasing by 0.95 [−0.29–2.18] and 3.52 [2.49–4.55] ng·mL^{-1} respectively. Moderate evidence for a decrease in post-exercise IL-6 concentration was observed for the HOT group (+ 2.74 [1.03–4.44] ng·mL^{-1}, $p = 0.036$, vs. day 1). A similar pre to post-exercise change for IL-10 was observed in the CON (+ 3.56 [0.75–6.39] ng·mL^{-1}) and HYP groups (+28.94 [6.75–51.14] ng·mL^{-1}). In contrast to day 1, weak evidence for a reduction in post-exercise IL-10 concentrations were observed in the HOT group (+32.28 [7.61–56.95] $p = 0.058$, vs. day 1). Post-exercise I-FABP concentrations were unaffected by 10 days of CON (+73 [−54–201] pg·mL^{-1}), HYP (+211 [155–267 pg·mL^{-1}) or HOT (+209 [51–368], $p = 0.16$) exercise acclimation.

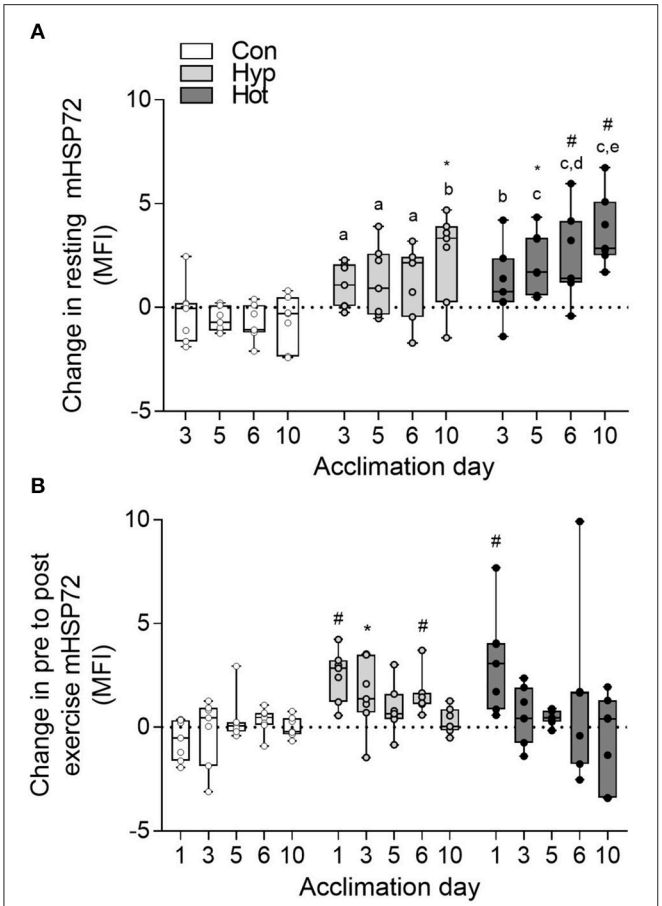

FIGURE 2 | Delta change in resting monocyte HSP72 MFI relative to the resting value obtained on day 1 of acclimation **(A)**; and delta change in pre to post-exercise mHSP72 MFI relative to daily resting mHSP72 MFI **(B)**. Box plots show all individual data points (dots), the 25 and 75th interquartile ranges (boxes), and the median (mid-line). Whiskers illustrate the highest and lowest value. Letters illustrate changes from the resting mHSP72 value obtained on day 1 of acclimation between experimental groups. a, b, c illustrates $p < 0.01$, < 0.001, and < 0.0001 compared to control respectively; d and e illustrate $p < 0.01$ and < 0.001 compared to hypoxia respectively. Within group changes in mHSP72 relative to the resting mHSP72 value obtained on day one of acclimation **(A)** or pre to post-exercise **(B)** are illustrated by $*p < 0.05$ and $\#p < 0.01$).

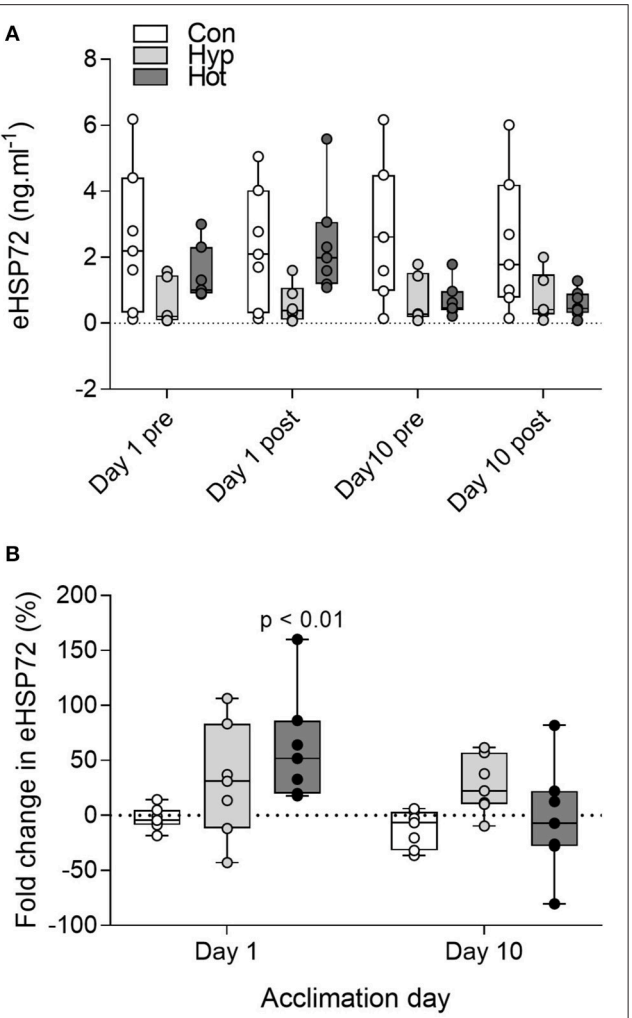

FIGURE 3 | Extracellular HSP72 before and after exercise on day 1 and day 10 of the acclimation intervention for all individual participants **(A)**; and percent change in eHSP72 from pre to post-exercise on day 1 and day 10 of acclimation **(B)**. Box plots show all individual data points (dots), the 25 and 75th interquartile ranges (boxes), and the median (mid-line). Whiskers illustrate the highest and lowest value. For display purposes one participant with high eHSP72 values is not included in panel A (day 1 pre, 17.8 ng·mL^{-1}, day 1 post, 24.4 ng·mL^{-1}; Day 10 pre, 15.6 ng·mL^{-1}, Day 10 post, 19.1 ng·mL^{-1}). *P*-values indicate differences relative to pre-trial resting values within each experimental group **(B)**.

Cytokine and I-FABP Responses to Hypoxic Exercise

Time × trial interaction effects were observed for IL-6 ($f = 18.513$, $p < 0.001$; **Figure 4A**) and IL-10 ($f = 15.037$, $p < 0.001$; **Figure 4B**). Data for TNF-α are shown in **Figure 4C**. Following the NST plasma IL-6 (pooled data for $n = 21$; +0.62 [0.34–0.91] ng·mL^{-1}) and IL-10 (+1.1 [−2.78–4.97] ng·mL^{-1}) exhibited minimal change ($p > 0.05$). After HST1, IL-6 (pooled data for $n = 21$; +3.87 [2.77–4.96] ng·mL^{-1}) and IL-10 (+26.15 [15.01–37.30] ng·mL^{-1}) were elevated from rest ($p < 0.01$), with no main effect for acclimation group ($p > 0.05$). A similar trend was observed after acclimation (HST2), with IL-6 (+3.09 [2.53–3.65] ng·mL^{-1}) and IL-10 (+23.22 [13.95–32.48] ng·mL^{-1}) increased following exercise ($p < 0.01$), and no main effect for trial or acclimation group observed (all $p > 0.05$).

Figure 5A shows the fold change from rest for I-FABP after the normoxic trial, pre-acclimation HST, and post-acclimation HST for all 21 participants (A). **Figures 5B–D** show the fold change from rest for I-FABP for the control (B), hypoxic (C) and heat (D) groups. For absolute I-FABP concentration there was no main effect for trial ($f = 0.495$, $p = 0.61$). There was a main effect for time ($f = 18.245$, $p < 0.001$) and a trial × time interaction ($f = 10.389$, $p < 0.001$). *Post-hoc* analysis showed that pre-exercise I-FABP concentrations were similar at the onset of all trials ($p = 0.248$). No change in post-exercise I-FABP

TABLE 2 | Mean ± SD differences before and after exercise for systemic cytokines and intestinal fatty acid binding protein on day 1 and day 10 of the acclimation period.

	Day 1		Day 10	
Variable	**Before exercise**	**After exercise**	**Before exercise**	**After exercise**
IL-6 (ng·mL^{-1})				
CON	1.18 ± 0.65	2.02 ± 1.40	1.27 ± 0.60	2.21 ± 1.67
HYP	1.06 ± 0.84	4.49 ± 1.54$^{\#}$	1.10 ± 0.73	4.62 ± 1.74*
HOT	1.20 ± 0.81	6.22 ± 3.23*	1.26 ± 1.35	4.00 ± 2.72^{*,a}
IL-10 (ng·mL^{-1})				
CON	3.61 ± 2.32	6.82 ± 5.39	3.21 ± 2.44	6.77 ± 4.53
HYP	12.39 ± 19.52	36.87 ± 26.35*	11.03 ± 14.60	39.97 ± 37.30$^{\#}$
HOT	18.56 ± 28.51	85.70 ± 59.35$^{\#}$	21.43 ± 36.66	53.71 ± 40.84$^{\#}$
TNF- α (ng·mL^{-1})				
CON	0.79 ± 0.62	1.26 ± 0.61	0.75 ± 0.39	0.40 ± 0.18
HYP	3.73 ± 7.30	4.32 ± 7.04	1.32 ± 1.02	0.97 ± 0.65
HOT	5.33 ± 6.16	4.88 ± 6.17	4.14 ± 7.71	4.16 ± 3.35
I-FABP (pg·mL^{-1})				
CON	352.3 ± 249.0	380.5 ± 240.0	331.6 ± 242.8	405.3 ± 202.1
HYP	449.6 ± 234.2	643.9 ± 232.3$^{\#}$	440.3 ± 179.5	651.7 ± 178.9$^{\#}$
HOT	370.1 ± 392.4	652.7 ± 100.4$^{\#}$	345.2 ± 350.8	554.7 ± 143.9$^{\#}$

*Within group differences from pre to post-exercise are denoted by *p < 0.05, #< 0.01. Within group differences between trials are denoted by [a]p < 0.05. IL-6; Interleukin-6, IL-10; Interleukin-10, TNF-; Tumor necrosis factor-alpha, I-FABP; Intestinal fatty acid binding protein.*

was observed after normoxic exercise (NST pre-exercise; 427 [247–606] pg·mL^{-1}, post-exercise; 464 [258–670] pg·mL^{-1}, $p = 0.153$). In contrast, strong evidence for a post-exercise increase in I-FABP was observed following hypoxic exercise (HST1 pre-exercise; 381 [209–553]. post-exercise; 565 [329–800] pg·mL^{-1}, $p < 0.001$). We found no evidence of blunted I-FABP following either the heat ($p = 0.44$) or hypoxic ($p = 0.16$) acclimation intervention.

DISCUSSION

The primary aim of the present study was to determine the mHSP72 response throughout both heat and hypoxic acclimation. We hypothesized that both modes of exercise acclimation would induce increases in resting mHSP72. In accordance with this, we show that heat acclimation produces a gradual and progressive increase in resting mHSP72 throughout the 10-day period, whereas the accumulation of resting mHSP72 was only evident on the final day of hypoxic acclimation. These data suggest that the greater physiological strain experienced throughout heat acclimation compared to both control and hypoxic acclimation, is a sufficient stimulus for rapidly enhancing the cytoprotective intracellular HSP72. Hypoxic acclimation at the same absolute work rate (50% normoxic $\dot{V}O_2$peak), but higher relative work rate (∼64 % hypoxic $\dot{V}O_2$peak) also enhanced resting mHSP72, however this appears to require a longer duration of acclimation. The greater length of time required to increase mHSP72 in the hypoxic group may be due to reduced level of total physiological strain imparted during each session as a result of a smaller thermal stimulus compared to that achieved in the heat group. A secondary aim was to determine whether a period of heat or hypoxic acclimation would reduce intestinal injury and the systemic cytokine response following hypoxic exercise. Moderate evidence for a reduction in post-exercise IL-6 and IL-10 were observed on day 10 of heat acclimation, with an adjustment to a more anti-inflammatory state further indicated by the reduced eHSP72/iHSP72 ratio, though no alterations in intestinal damage markers were observed after the acclimation period. We show that intestinal fatty acid binding protein is increased following moderate intensity hypoxic exercise, and that period of heat or hypoxic acclimation does not attenuate either the post-exercise I-FABP response, or the post-exercise increase in IL-6 and IL-10 after moderate intensity hypoxic exercise.

Accumulation of Monocyte HSP72 during Acclimation

The increase in basal intracellular HSP72 reserves is an established outcome of human-heat acclimation (McClung et al., 2008; de Castro Magalhães et al., 2010; Gibson et al., 2015a,b; Lee et al., 2015a, 2016). In the present study we show that monocyte HSP72 begins to increase from the third day of acclimation, and progressively increases over the 10-day acclimation period. These results are similar to those observed in our laboratory, with increased basal mHSP72 found after 3 days of STHA (Lee et al., 2015a). We did not observe a plateau in basal mHSP72 at the end of the 10-day acclimation period, which may suggest that complete cellular adaptation to heat stress, and therefore acclamatory homeostasis was not achieved. During the transition from STHA to LTHA a progressive transcriptional activation and consequent stockpiling of cytoprotective reserves occurs until cellular homeostasis is acquired (Horowitz, 2016). Interestingly, our data may suggest that from a cellular/cross-tolerance perspective, our participants were still in the STHA phase of adaptation. STHA animals have been shown to have a lower tolerance to novel stressors (Assayag et al., 2010, 2012; Yacobi et al., 2014) than their more efficient LTHA counterparts. Therefore, when assessing HACT effects, confirming participants

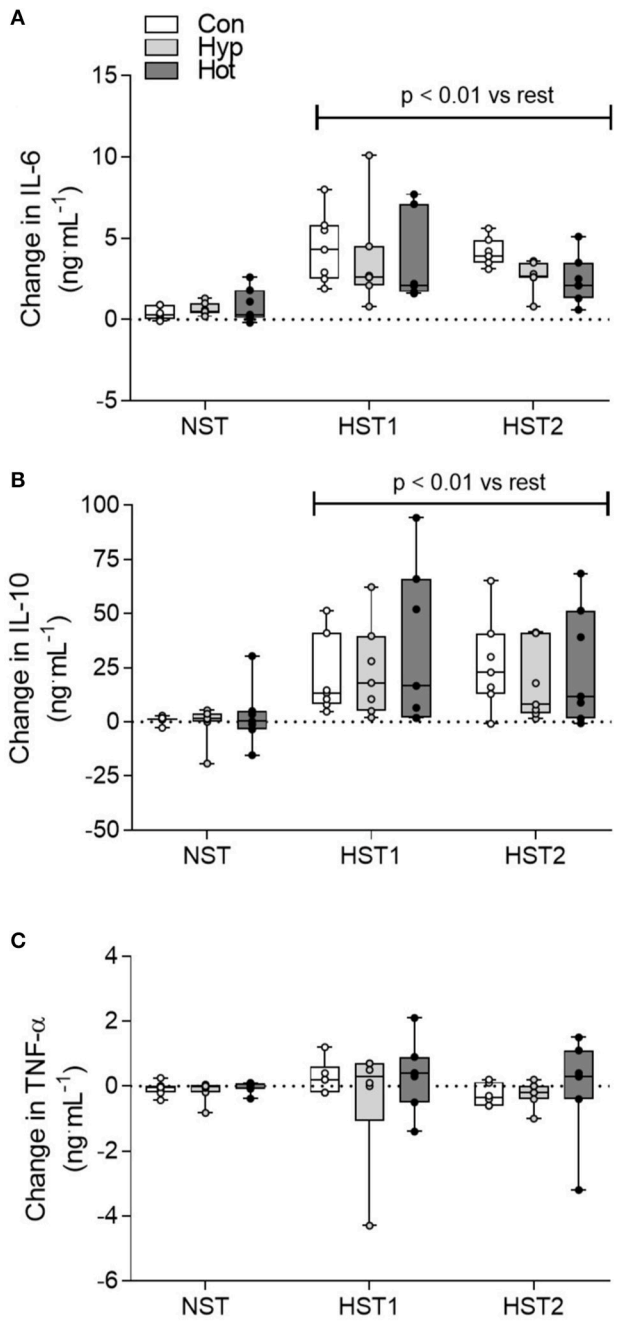

FIGURE 4 | Delta change (ng.mL^{-1}) in IL-6 **(A)**, IL-10 **(B)** and TNF-α **(C)** from pre to post-exercise for the NST, HST1, and HST2 trials. Box plots show all individual data points (dots), the 25 and 75th interquartile ranges (boxes), and the median (mid-line). Whiskers illustrate the highest and lowest value. *P*-values indicate differences relative to the pre-trial resting value within each experimental group and between normoxic and hypoxic conditions.

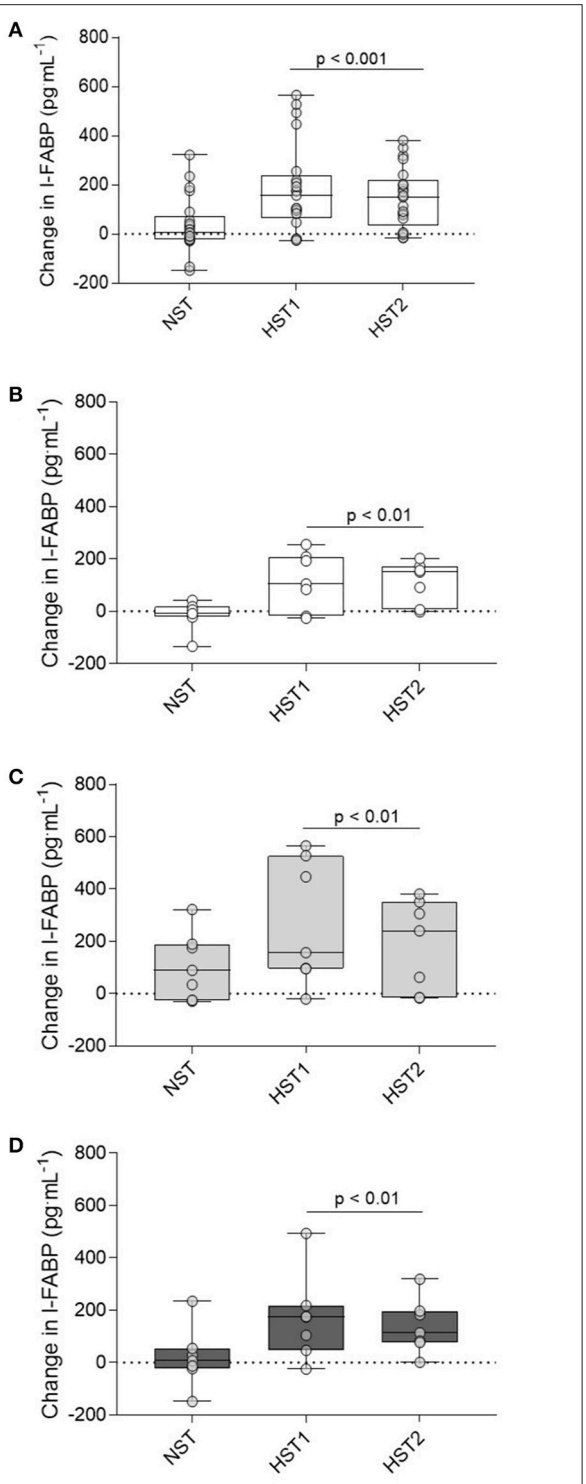

FIGURE 5 | Delta change in I-FABP from pre to post-exercise measurements for the NST, HST1, and HST2 trials for the total participant cohort ($n = 21$; **A**), and the delta change from pre to post-exercise for the CON **(B)**, HYP **(C),** and HOT **(D)** groups**.** Box plots show all individual data points (dots), the 25 and 75th interquartile ranges (boxes), and the median (mid-line). Whiskers illustrate the highest and lowest value. *P*-values indicate differences relative to the pre-trial resting values within each experimental group and between normoxic and hypoxic conditions.

are in the LTHA phase is experimentally important, yet no method exists to confirm this status in humans. Gibson et al. compared the efficacy of both fixed workload and isothermic heat acclimation regimes on HSP72 and HSP90 gene expression, with no differences in gene expression or physiological responses

found across the different heat acclimation regimes (Gibson et al., 2015a). However, the maintenance of daily physiological strain afforded by isothermic protocols make this an attractive methodology for attempting to determine whether a plateau in mHSP72 accumulation occurs. Future work is required in order to determine whether a final "plateau" in mHSP72 accumulation represents the complete transition to LTHA, and is therefore the optimal time period to assess cross-tolerance effects.

In contrast, the hypoxic acclimation period induced a slower accumulation in mHSP72, with increased reserves only observed by the final day of acclimation. The slower accumulation throughout acclimation could be due to the lower total physiological strain experienced by participants and reduced thermal component to the exercise stressor. Greater increases in HSP72 are observed when an increase in deep body temperature >38.5°C is induced (Gibson et al., 2015b), therefore, it is likely a combination of the numerous cellular and molecular stressors elicited by exercise were the inductive stimulus for HSP72 in the hypoxic group (Gibson et al., 2017). A hypoxia-mediated induction of monocyte-expressed HSP72 has been suggested to provide protection to the disturbances to redox balance associated with human sub-maximal aerobic exercise (Taylor et al., 2012). Mechanistically, evidence exists that elevations in oxidative stress are a trigger for increases in HSP72 concentration (Kukreja et al., 1994; Ahn and Thiele, 2003) with similar findings shown after both acute (Taylor et al., 2010) and repeated daily resting hypoxic exposures (Taylor et al., 2011, 2012). These authors suggest the repeated disturbance to redox balance from daily hypoxic exposures may act as a stimulus for elevated HSP72 expression (Taylor et al., 2012).

Exercise intensity is an important factor impacting on redox state in monocytes (Wang et al., 2006). Heavy exercise (∼80% $\dot{V}O_2$max) has been shown to increase the production of reactive oxygen species (ROS), and reduced mitochondrial superoxide dismutase and reduced γ-glutamylcysteinyl (GSH) in monocytes. The increases in monocyte ROS production could theoretically act as a cell-type specific stimulus for monocyte HSP72 induction. However, lower workloads (40 and 60% $\dot{V}O_2$max), which are similar to the relative intensity experienced by participants in the present study (∼64% hypoxic $\dot{V}O_2$peak), were shown not to increase monocyte ROS production (Wang et al., 2006). More recently, daily 2 h exercise bouts at a moderate exercise intensity (50% $\dot{V}O_2$peak) over a 10 day period enhanced antioxidant capacity, thereby blunting any hypoxia related oxidative stress (Pialoux et al., 2009), a finding that has been corroborated by others using low-to-moderate intensity exercise protocols (Debevec et al., 2016, 2017). In the present investigation relative exercise intensity was not matched between the control group and the hypoxic group, therefore the contribution of a higher relative workload cannot be separated from the hypoxic stimulus. Two experimental approaches could be employed to mitigate this: The incorporation of a matched relative workload control condition would better delineate the contribution of increased relative exercise intensities verses hypoxia *per se*. Alternatively, a passive heating and passive hypoxic acclimation protocol would remove any potential exercise intensity confounds by removing the multifaceted stimuli caused by exercise, thereby allowing the separate effects of each environment to be studied. It should also be acknowledged that an elevated rectal temperature was observed in the hypoxic acclimation group on days 1–6 of acclimation (peak T_{rectal} = 38.1–38.2°C). Therefore, the increase in mHSP72 observed in this group may simply reflect an elevated temperature, albeit to a lesser degree to that observed in the heat group. In addition, the present investigation does not allow for the separate effects of relative workload and thermal stimulus to be compared.

Heat or Hypoxic Acclimation Does Not Reduce Intestinal Damage following Hypoxic Exercise

In the present study we were unable to demonstrate an acclimation mediated reduction in intestinal barrier damage, as assessed by I-FABP. I-FABP is a robust biomarker of enterocyte damage and intestinal injury, and correlates well with the lactulose/rhamanose sugars gut permeability test (March et al., 2017; Pugh et al., 2017). No change in post-exercise I-FABP was observed on the final day of acclimation relative to day 1, and no change was observed following hypoxic exercise after the acclimation intervention. As such, the primary finding from this aspect of the study is that moderate exercise in hypoxic conditions (reducing arterial oxygen saturation to <85%) is sufficient to induce enterocyte damage and increase circulating I-FABP. However, this data needs confirming with the inclusion of a matched relative exercise intensity control group, as the effects of increasing exercise intensity are known to induce greater intestinal damage (Pires et al., 2016).

A HSP72 mediated inhibition of NF-κB translocation into the nucleus of intestinal epithelial cells has been demonstrated to reduce the synthesis of pro-inflammatory cytokines (Dokladny et al., 2010). This action limits stress-mediated inflammation by preventing endotoxin translocation into the portal circulation, attenuating systemic inflammatory responses, and is another mechanism by which HSP72 accumulation may protect the intestinal barrier (Kuennen et al., 2011; Zuhl et al., 2014). The lack of any observed positive effect on enterocyte damage following the acclimation mediated increase in mHSP72 may be explained by the comparatively short hypoxic exposure period employed (60 min) not being sufficiently stressful to induce severe disturbances to gastrointestinal permeability and pro-inflammatory responses. By employing a more intense hypoxic exercise bout (e.g., >80% $\dot{V}O_2$max), which is known to severely compromise the epithelial wall and increase gut permeability (Sessions et al., 2016; March et al., 2017), it is possible that any beneficial effects of acclimation induced mHSP72 may be more readily observed. Alternatively, extending the exercise duration to be in line with those observed in occupational or military settings may also warrant additional investigation when determining whether heat/hypoxic acclimation can maintain intestinal permeability. It should however be noted that prolonged resting hypoxic exposures of 3 days (3,300 to 4,392 m; Kalson et al., 2010), and 3 weeks (2,800 to 3,400 m; Mekjavic et al., 2016) have not demonstrated a hypoxia-mediated effect on gastrointestinal permeability or blood flow. Therefore, the

relative contribution of both hypoxia and exercise intensity on gut permeability require further investigation.

In the present study, IL-6 and IL-10 were elevated to a similar extent following day 1 of both heat and hypoxic acclimation, with the increase in each being greater than observed in the control condition. The results for IL-6 and IL-10 in the heat group are similar to those observed after a session of isothermic heat acclimation in which T_{core} was clamped at 39.0°C for the final 50 min of a 100-min protocol (Kuennen et al., 2011). The increase in post-exercise IL-6 and IL-10 in the hypoxic group is in accordance with Caris et al. (2016), and could reflect the greater exercise intensity and increased reliance on glucose as a substrate, with IL-6 released from the muscle in order to maintain glucose metabolism (Carey et al., 2006). On the final day of acclimation, there was no alteration in the post-exercise IL-6 or IL-10 in either the control or hypoxic group. However, we provide weak-to-moderate evidence for reductions in both IL-6 and IL-10 following heat acclimation, and a concomitant shift in the ratio of eHSP72 and iHSP72 which further indicates a shift toward an anti-inflammatory status (Krause et al., 2015). However, despite this shift toward an anti-inflammatory state in the heat-acclimation group, no differences in the systemic cytokine response were observed following the post-acclimation hypoxic exercise bout. We acknowledge that the relatively small sample size within each group may have limited the statistical power, and therefore ability to detect small changes in cytokine balance after acclimation, is a limitation of the present study. Further experiments are therefore warranted in order to confirm our observations.

The functional relevance of HSP72 during human heat acclimation was first indicated by using quercetin, a standard laboratory blocker of heat shock transcription factor-1 (HSF-1; Kuennen et al., 2011). By supplementing with quercetin throughout the acclimation phase, Kuennen and colleagues were able to disrupt the normal cellular accumulation of HSP72 in PBMCs. The blunted HSP72 response was associated with impairments to gastrointestinal barrier function, as determined by the lactulose/rhamanose sugars test. The inhibition of HSP72 accumulation and impaired post-acclimation GI function and endotoxin translocation into the systemic circulation disturbed the pro-inflammatory and anti-inflammatory cytokine profiles, which may be indicative of an overactive NF-κB system (Kuennen et al., 2011). In contrast to heat acclimation, the functional relevance of HSP72 in the context of hypoxic acclimation has yet to be fully elucidated. HSP72 has been shown to increase the stability of hypoxia-inducible factor-1a (HIF1a), and may improve both erythropoietic (Taylor et al., 2011) and angiogenic responses (Shiota et al., 2010; Bruns et al., 2012) which are key adaptive responses to hypoxia (Levine and Stray-Gundersen, 1997; Vogt et al., 2001). HSP72 has recently been identified as a specific regulator of both angiogenesis (Bruns et al., 2012; Kim et al., 2016) and erythropoiesis (Ribeil et al., 2004, 2007). However, at this time these proposed mechanisms lack experimental support in human studies, and therefore functional role of increased HSP72 during repeated hypoxic exposures cannot be causally determined. In order to clearly determine role played by increasing HSP72 in the development of heat/hypoxic acclimation and the conferring of cross-tolerance effects, methods that manipulate intracellular HSP72 content are required. Pharmacological interventions that augment HSP72 (e.g., quercetin; glutamine; O-(3-piperidin 2-hydroxy-1-propyl) nicotinic amidoxime-BGP-15) would allow for independent effects of the physiological and cellular adaptations in cross tolerance, and the functional significance of HSP72 in the context of hypoxic acclimation, to be investigated (Gibson et al., 2017).

CONCLUSIONS

This study presents data which show that heat acclimation per-se is a more time efficient means of increasing constitutive stores of monocyte HSP72 when compared to the same period of hypoxic acclimation. In contrast hypoxic acclimation produced a slower accumulation of mHSP72. Although we provide further evidence for heat-hypoxic cross-tolerance, our data show that the benefits of HACT do not affect pro/anti-inflammatory balance and nor do they protect against enterocyte damage. Alternative acclimation modalities, in which the daily stress stimulus is maintained via core temperature clamping may prove to be more effective in preserving intestinal integrity. An intriguing consequence of this experiment is the suggestion that 10 days of fixed work acclimation did not provide full cytoprotective adaptation and that, at least from a cellular perspective, acclimation homeostasis had yet to be achieved. Further studies are therefore warranted to determine the optimal heat "dose" in order to maximize the constitutive HSP72 reserves and potentiate the potential for cross-tolerance effects. From a practical perspective, heat acclimation is a more time-efficient and accessible method for enhancing cytoprotection than hypoxic acclimation. The application of HACT as a means to enhance hypoxic tolerance and operational effectiveness in human occupational, military, and sporting scenarios requires further study, but remains an interesting prospect.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Coventry University local Ethics Committee, with written informed consent obtained from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Coventry University local ethics Committee.

AUTHOR CONTRIBUTIONS

BL contributed to the conception, data collection sample analysis, manuscript preparation CT contributed to the conception, data collection, manuscript preparation.

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Heat and Dehydration Additively Enhance Cardiovascular Outcomes following Orthostatically-Stressful Calisthenics Exercise

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Exercise and exogenous heat each stimulate multiple adaptations, but their roles are not well delineated, and that of the related stressor, dehydration, is largely unknown. While severe and prolonged hypohydration potentially "silences" the long-term heat acclimated phenotype, mild and transient dehydration may enhance cardiovascular and fluid-regulatory adaptations. We tested the hypothesis that exogenous heat stress and dehydration additively potentiate acute (24 h) cardiovascular and hematological outcomes following exercise. In a randomized crossover study, 10 physically-active volunteers (mean ± SD: 173 ± 11 cm; 72.1 ± 11.5 kg; 24 ± 3 year; 6 females) completed three trials of 90-min orthostatically-stressful calisthenics, in: (i) temperate conditions (22°C, 50% rh, no airflow; CON); (ii) heat (40°C, 60% rh) whilst euhydrated (HEAT), and (iii) heat with dehydration (no fluid ~16 h before and during exercise; HEAT+DEHY). Using linear mixed effects model analyses, core temperature (T_{CORE}) rose 0.7°C more in HEAT than CON (95% CL: [0.5, 0.9]; $p < 0.001$), and another 0.4°C in HEAT+DEHY ([0.2, 0.5]; $p < 0.001$, vs. HEAT). Skin temperature also rose 1.2°C more in HEAT than CON ([0.6, 1.8]; $p < 0.001$), and similarly to HEAT+DEHY ($p = 0.922$ vs. HEAT). Peak heart rate was 40 b·min^{-1} higher in HEAT than in CON ([28, 51]; $p < 0.001$), and another 15 b·min^{-1} higher in HEAT+DEHY ([3, 27]; $p = 0.011$, vs. HEAT). Mean arterial pressure at 24-h recovery was not consistently below baseline after CON or HEAT ($p \geq 0.452$), but was reduced 4 ± 1 mm Hg after HEAT+DEHY ([0, 8]; $p = 0.020$ vs. baseline). Plasma volume at 24 h after exercise increased in all trials; the 7% increase in HEAT was not reliably more than in CON (5%; $p = 0.335$), but was an additional 4% larger after HEAT+DEHY ([1, 8]; $p = 0.005$ vs. HEAT). Pooled-trial correlational analysis showed the rise in T_{CORE} predicted the hypotension ($r = -0.4$) and plasma volume expansion ($r = 0.6$) at 24 h, with more hypotension reflecting more plasma expansion ($r = -0.5$). In conclusion, transient dehydration with heat potentiates short-term (24-h) hematological (hypervolemic) and cardiovascular (hypotensive) outcomes following calisthenics.

Keywords: hypotension, hypervolemia, aldosterone, erythropoietin, calisthenics, adaptation, orthostasis, hypohydration

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INTRODUCTION

Exercise is a multi-factorial, pluripotent stress that produces strain in several physiological systems. The nature of the stress dictates the profile of strain incurred, and thus the adaptive stimulus. The adaptations that occur as a result of this strain profile accrue as fitness, which improves an individual's physiological and functional capacities, and quality of life. The necessary stressors within exercise that are required to elicit a given response, or adaptation, are only partially resolved. Often the complex nature of exercise makes the delineation of these stressors difficult, and thus their individual and combined roles in adaptation also remain unclear.

Changes in blood pressure (BP) and blood volume (including plasma volume; PV) are principal components of the cardiovascular benefit of regular physical activity. However, the optimal means of facilitating these adaptations is elusive. The anti-hypertensive benefits associated with exercise (Pescatello et al., 2004, 2015) might be partly facilitated by repeated bouts of acute post-exercise hypotension (PEH; Thompson et al., 2001), and contribute to subsequent PV expansion (i.e., PVE/hypervolemia; Nagashima et al., 1999, 2000; Hayes et al., 2000). Assessing the acute cardiovascular outcomes to a conditioning stimulus, which are also important in their own right, may therefore also provide an indication of long-term cardiovascular adaptations (Liu et al., 2012). Exercise intensity moderates both PEH (Eicher et al., 2010; Halliwill et al., 2013; Graham et al., 2016) and PVE (Nadel et al., 1979; Convertino et al., 1981), such that a higher intensity elicits larger PEH and PVE, however finding an appropriate and effective exercise intensity to afford these benefits to all individuals is convoluted. Indeed, low-intensity exercise may be insufficient to confer these benefits (Wallace et al., 1999; Forjaz et al., 2004; Farinatti et al., 2011) despite its accessibility to the population. Thus, complementing low-intensity exercise with additional independent stressors may exacerbate the physiological strain, and provide an avenue for acute cardiovascular outcomes.

Heat stress and dehydration are by-products of exercise and stressors in their own right. The imposition of heat (Francesconi et al., 1983a; Ray and Gracey, 1997; Wright et al., 2010) and dehydration (Finberg et al., 1974; Nadel et al., 1980; Francesconi et al., 1983b; Brandenberger et al., 1986) increases strain in multiple systems at rest and in exercise, particularly when there is concurrent orthostatic stress (Beetham and Buskirk, 1958; Saltin and Stenberg, 1964; Ray et al., 1993; Gonzalez-Alonso et al., 1999). An increase in strain, and potential augmented adaptive stimulus, occurs despite low exercise intensity in dynamic aerobic (Finberg et al., 1974; Francesconi et al., 1983b), and isometric exercise (Ray and Gracey, 1997; Binder et al., 2012). Orthostasis and heat appear to be important stressors in stimulating PVE (Convertino et al., 1981; Nagashima et al., 1999, 2000) and potentially PEH (Halliwill et al., 2013), however the role of dehydration is equivocal and largely unexplored (Akerman et al., 2016). Considering that dehydration exacerbates the cardiovascular and hormonal (especially sympathetic and fluid regulatory) pathways associated with both PVE and PEH (Melin et al., 1997, 2001; Gonzalez-Alonso et al., 1999), it may also moderate these outcomes somewhat. Thus, identifying the separate and combined roles of these exercise- and environment-related stressors in driving or impairing adaptive processes is important for basic understanding and may aid in exercise prescription for a range of populations; especially those unable (or unwilling) to undertake moderate- and higher-intensity exercise.

The aim of the current study was therefore to determine the cumulative effects of orthostatically stressful, low-intensity exercise (dynamic body weight exercises, i.e., calisthenics), exogenous heat stress, and dehydration, on acute (24-h) cardiovascular and fluid regulatory outcomes. The separate effects of these stressors were not delineated *per se*, but rather their cumulative effects were examined in forming a composite stimulus (i.e., low-intensity exercise + heat stress + dehydration). This exercise type was chosen as an accessible mode and intensity for a majority of the population, whilst providing substantial cardiovascular strain, and force through all major muscle groups. We hypothesized that the addition of each stressor would provide a further stimulus for acute cardiovascular outcomes. Namely, plasma volume expansion at 24 h after exercise would be larger with the addition of each stressor, by virtue of inducing additional thermal and cardiovascular strain.

METHODS

Participants

Ten habitually-active, young healthy individuals participated; six females and four males (mean ± SD, aged 24 ± 3 year, mass 72.1 ± 11.5 kg, and height 173 ± 11 cm). All were non-smokers, recreationally physically active on 3 to 5 days per week, and unacclimatised to heat. They were required to refrain from alcohol, caffeine and physical activity for 24 h before each experimental trial. All female participants had been taking the oral contraceptive pill for at least the previous 2 years, and undertook experimental trials in their self-reported early follicular phase. Written informed consent was provided by all individuals prior to participation, and all procedures were approved by the Institutional Human Ethics Committee of the University of Otago (Project No. 12/125), and conformed to the *Declaration of Helsinki.*

Experimental Protocol

Design

The study was a randomized, counter-balanced crossover design. Following familiarization to the exercise protocol (one occasion in CON conditions, approximately 1 week prior to first exercise condition) and all experimental procedures and instrumentations, participants reported to the laboratory for three experimental trials, or "exercise conditions." The three exercise conditions were: calisthenics in a temperate environment [22.2 ± 0.3°C, 51.2 ± 1.5% relative humidity (RH)] in a euhydrated state (i.e., CON); calisthenics in a hot, humid environment (40.4 ± 0.4°C, 60.5 ± 1.9% RH) in a euhydrated state (HEAT); and calisthenics in a hot, humid environment in a mildly-hypohydrated state, with further dehydration during the session (HEAT+DEHY). No exogenous airflow was provided.

Each condition was at the same time of day (08:00 h) and included baseline measurements, a 90-min exercise protocol, and four follow-up measurement periods over 24 h of recovery (2, 3, 5, and 24 h after exercise; **Figure 1**). Exercise conditions were separated by 2–7 days, with a minimum of 2 days separating a temperate to hot condition, and 4 days between hot conditions. Participants recorded physical activity, food and fluid intake, and menstrual cycle logs for the 24 h prior to, and throughout the first exercise condition, and then replicated this for the remaining two conditions.

The three exercise conditions were completed on days that required similar 24-h physical activity requirements and often similar duration in the same posture (i.e., walking to and from appointments, and sitting in meetings or lectures). Participants were encouraged to consume 500 mL of water with dinner the night before, and another 500 mL with breakfast on the morning of the euhydrated conditions (CON and HEAT). Further to *ad libitum* fluid intake (water) during the euhydrated trials, body mass (BM) was measured at 15-min intervals during exercise, and sufficient water was provided to replace any BM deficit. The dehydration protocol in HEAT+DEHY consisted of ∼16-h fluid restriction (from 17:00 h the night before, to 09:00 h on day of testing; i.e., hypohydration), followed by no fluid replacement during the exercise.

Procedure

Participants arrived at the laboratory following a (self-reported) standardized fluid and meal intake. After voiding their bladder (second void of the morning) and inserting a rectal thermistor, participants were weighed semi-nude (underwear only) then rested supine before blood pressure (BP) was recorded and venous blood was drawn. Participants were then seated for 10 min to stabilize fluid compartment volumes (Ahlgrim et al., 2010) before capillary blood samples were obtained for tracking changes in plasma volume (PV). During this rest period participants were also instrumented with skin thermistors and a heart rate (HR) monitor.

Participants then entered an environmental chamber and sat for 5 min before recording of their resting HR, core (T_{CORE}) and skin temperatures (T_{SKIN}), and psychophysical perceptions (detailed below). This 5-min period was replicated at the end of the protocol. Participants then completed 10 min of squats at 0.2 Hz (5 min at start and end), and 70 min of calisthenics. Thus, the total "exercise protocol" was 90-min, consisting of the seated rest period, squats, and calisthenics. Respiratory gases, thermal, cardiac, and perceived strain was recorded throughout. The squat procedure was intended to provide insight into cerebrovascular autoregulation following orthostatically stressful exercise. Due to technical problems maintaining cerebrovascular measures throughout trials in the heat, these data are not of sufficient quality to present.

Participants then left the environmental chamber and sat in a temperate environment (∼26°C, 27% RH) for 10 min before capillary blood samples were obtained, and lay supine for another 10 min before BP was measured and another venous blood sample was drawn (both supine). Participants voided their bladder, toweled dry and had semi-nude body mass measured. Participants were then provided with 250 mL of yogurt (37 g carbohydrate, 9 g protein, 4 g fat and 90 mg sodium) and 500 mL of fruit juice (1:1 fruit juice and water mix, 25 g carbohydrate, 1 g protein, and ∼820 mg sodium) to consume as soon as possible, and return to their normal hydration level *ad libitum* thereafter (same juice/salt/water mix available throughout recovery). This standardized meal was chosen due to the importance of immediate protein (De Feo et al., 1992) and sodium (Luetkemeier et al., 1997) supplementation on outcome measures.

Participants returned to the laboratory 2, 3, 5, and 24 h after completing the exercise protocol for recovery measures. Participants provided a urine sample, and semi-nude body mass measurement, followed by BP measurement, venous blood (both

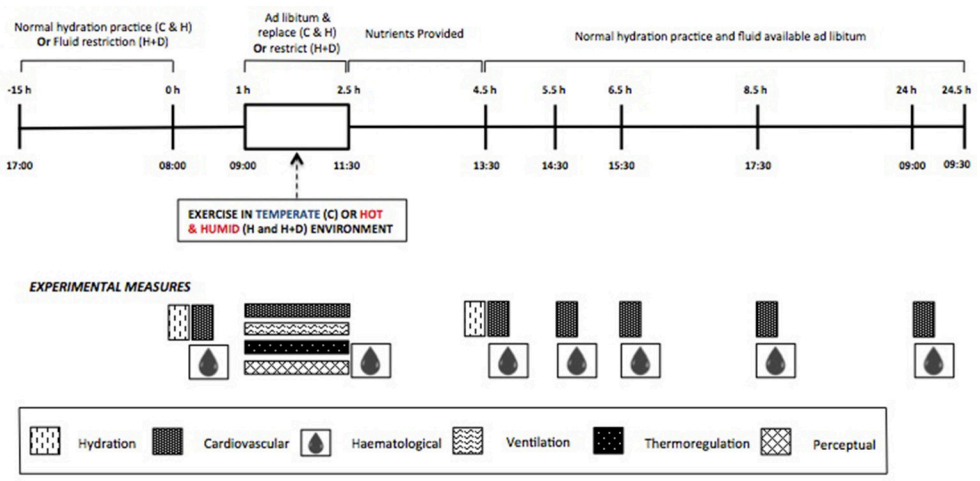

FIGURE 1 | Schematic representation of standardizations and experimental measurements in the three exercise conditions (C, Control; H, Heat; H+D, Heat and dehydration).

supine after 10-min rest), and capillary blood samples after 10 min of seated rest (**Figure 1**).

Exercise Protocol

The exercise protocol was intended to represent a routine suitable for individuals unable to complete more traditional exercise forms. The composition and progression of exercises was designed specifically for this study, with particular emphasis on a whole-body stress and repeated changes in posture (i.e., orthostatically stressful). A detailed description of the calisthenics protocol is provided in Supplementary Data Sheet 1 (https://www.youtube.com/watch?v=FxMdPDUk8Fo) and **Supplementary Figure 1**. To summarize, 28 body-weight resistance movements (i.e., calisthenics), and yoga postures (herein referred to collectively as "exercises") were completed for 70 min. The protocol consisted of ∼35 min of standing exercises (7 exercises at start, 5 exercises at the end), ∼15 min of supine exercises (7 exercises), ∼15 min of exercises on hands and knees (6 exercises), and ∼5 min of prone exercises (3 exercises).

Measurements

Semi-nude body mass was measured to a resolution of 20 g using calibrated electronic scales (Wedderburn, Dunedin, New Zealand) before and after each exercise condition, and at 15-min intervals during the two hydrated exercise conditions (i.e., CON and HEAT). Specific gravity of urine (U_{SG}) was measured using a hand-held refractometer (Atago, Tokyo, Japan) before and after exercise.

Blood pressure was measured using a stethoscope and sphygmomanometer, by the same researcher (AA) on all occasions, and reported as the mean of 3 readings over ∼5 min. Heart rate was measured from the R-R interval of ventricular depolarisation, and recorded at 1-min intervals via telemetry to a wristwatch (Model RS400, Polar, Finland). Core temperature was measured using a rectal thermistor (400 series; Mallinckrodt Inc. USA) inserted 10 cm beyond the external anal sphincter. Skin temperature was measured using thermistors attached to the thigh, chest, back, and bicep using adhesive tape. Core and skin temperatures were recorded at 1-min intervals (DaqPRO 5300, Omega, USA.). Ratings of perceived exertion (RPE, ranging from 6 to 20; Borg, 1982), thermal sensation (ranging from 1 to 13), and thermal discomfort (ranging from 1 to 5; both extended from Gagge et al., 1967) were obtained using 15-, 13-, and 5-point scales respectively. Expired oxygen (O_2) and carbon dioxide (CO_2) concentrations and volumes were determined using open-circuit spirometry (Cosmed, CosmedSrl, Rome, Italy) to calculate oxygen consumption ($\dot{V}O_2$) and partial pressure of end-tidal carbon dioxide ($P_{ET}CO_2$). Gas analysers were calibrated before a participant's exercise session.

Arterialised capillary blood samples (75 μL) were obtained from the finger, in triplicate. Hemoglobin (Hb) concentrations were determined using reflectance photometry (OSM3; Radiometer, Copenhagen, Denmark). Hematocrit (Hct) was measured from the remaining portion (∼50 μL) of each sample using a custom-made Vernier caliper reader after centrifugation (Thermo IEC, MicroCL 17, radius 8.5 cm) for 10 min at 855 g.

Venous blood samples (18 mL) were drawn without stasis from an antecubital vein. Whole-blood samples were centrifuged immediately at 4°C for 10 min, and plasma was separated and stored at −80°C for subsequent batch analysis. Plasma osmolality (P_{OSM}) was measured using a three-point calibrated (100, 290, and 1,000 mOsmol·kg^{-1}) dew-point temperature depression method, in duplicate (triplicate if ≥2 mOsmol·kg^{-1} difference; Model 5,520, Vapro osmometer, Wescore, Logan, UT, USA). Plasma aldosterone (P_{ALD}) and erythropoietin (P_{EPO}) were measured using enzyme-linked immunosorbent assay (ELISA) commercially available kits (Abnova, Taiwan; and Abcam, Australia, respectively). All samples for a given participant were analyzed within the same assay, and handled by the same researcher (AA or RK).

Plasma aldosterone concentrations were determined from calibration standards (0–1,000 pg·mL^{-1}) and control samples, using a standard curve derived from the log transformation of the optical density of each sample. The curve was generated using commercially available software (GraphPad Prism, Version 6, GraphPad Software, CA, USA). The intra-assay coefficient of variation for P_{ALD} concentration, derived from the calibration standards, was 14.4%. Plasma erythropoietin sample concentrations were determined from calibration standards (0–100 mIU·mL^{-1}) and control samples, using an applied four-parameter algorithm fit using a free internet-based ELISA analysis program (elisaanalysis.com). The intra-assay coefficient of variation for P_{EPO} concentration, derived from the calibration standards, was 5.3%.

Data Analysis

Calculations and Derivations

All measurements are $n = 10$ unless stated otherwise. To describe the effect of the dehydration protocol (i.e., vs. normal hydration/euhydration), an unstressed baseline was estimated by averaging CON and HEAT baseline measurements, against which variables are compared over time where relevant (i.e., ΔBM, ΔBP, and ΔPV).

Area-weighted T_{SKIN} was calculated according to International Organisation for Standardization (1992) from the following formula:

$$T_{SKIN} = ((0.175 * T_{CHEST}) + (0.175 * T_{BACK}) + (0.19 * T_{BICEP}) + (0.39 * T_{THIGH}))/0.93$$

Changes in PV, associated with the exercise conditions, were calculated from changes in Hb and Hct according to the equation of Strauss et al. (1952):

$$\%\Delta PV = 100\,[(Hb_0/Hb_t)^*((1 - Hct_t)/(1 - Hct_0))] - 100$$

where t and 0 subscripts denote measurements at time t and baseline (unstressed), respectively. Hb is in g·100 mL^{-1} and Hct is a fraction.

Statistics

Variables were individually analyzed longitudinally using linear mixed effect model analysis. Where appropriate, models incorporated up to seven repeated measurements (i.e., average hydrated, baseline, immediately post-exercise, 2-, 3-, 5-, and 24-h post-exercise) across three separate conditions (CON,

HEAT, HEAT+DEHY). The order of condition completion was incorporated as a fixed effect, while participant was modeled as a random effect. Homogeneity of variances was assessed visually via plotting of residuals vs. model-fitted values, and formally with Levene's test, across all combinations of factors in the model. Linearity and approximate normal distribution of residuals were assessed via visual inspection of histograms and Q-Q plots of model and individual residuals, and formally with Shapiro-Wilk test. Akaike' Information Criteria (AIC) was used to determine covariance structure of model errors (following significant Levene's test), random-effect structure, and incorporation of fixed effects. In the presence of non-significant (via AIC) condition:time interaction, and thus non-inclusion in the model, "NS" is reported. Multiple comparisons were made using the least squares means contracts derived from the mixed models, and adjustments for multiple testing made with the Hochberg-Bonferroni method. In line with the study aims and hypotheses being focused on delineating the addition of discrete stressors, only comparisons between conditions of interest (i.e., HEAT vs. CON, and HEAT+DEHY vs. HEAT) were examined.

Associations between mechanistically related variables were explored using standard least squares regressions. The relations between the variables are expressed as the Pearson's correlation coefficient (denoted as r) and corresponding p-values. The magnitude of correlation effect was qualitatively assessed from corresponding according to Hopkins et al. (2009); trivial ($r < 0.1$), small ($0.1 < r < 0.3$), moderate ($0.3 < r < 0.5$), large/strong ($0.5 < r < 0.7$), and very large/strong ($0.7 < r < 0.9$).

The mixed-model analysis was performed using R (R Development Core Team, 2008), and correlations were performed on raw values using GraphPad Prism (Prism Version 7.00, GraphPad Software, California, USA). Descriptive statistics in text are reported as raw means ± SD, whereas comparisons of interest are reported as least-squares means with corresponding 95% confidence limits [lower limit, upper limit]. A $p < 0.05$ was considered statistically significant *a priori*. To aid in interpretation, ANOVA-derived main effects and interactions are provided, and (where appropriate) only *post-hoc* p-values are presented in corresponding table or text.

RESULTS

Physical activity, diet, and menstrual cycle logs were reviewed prior to starting each of the second and third exercise conditions, and hydration compliance was checked verbally at this time. Prior to the two euhydrated conditions (CON and HEAT), if participants rated their thirst to be other than "not thirsty" (i.e., 1 on a 9-point validated Thirst Scale; Riebe et al., 1997), they ingested additional water *ad libitum*. All participants completed the 90-min exercise protocol.

Hydration

Body Mass Changes (Δ BM)

When compared against an average hydrated body mass (72.2 ± 11.8 kg), the overnight fluid restriction in HEAT+DEHY resulted in 1.1 ± 0.8% BM reduction (71.3 ± 11.6 kg), with a further 1.8 ± 0.8% loss by the end of exercise, leading to a net loss of 2.9 ± 1.2% (70.0 ± 11.0 kg). Net mass losses were 0.5 ± 0.6% in CON (71.8 ± 11.8 kg), and 0.1 ± 0.6% in HEAT (71.9 ± 11.3 kg; $n = 9$; $p = 0.527$ vs. CON).

Urine Specific Gravity (U_{SG})

Baseline U_{SG} was similar between HEAT (1.007 ± 0.005) and CON (1.008 ± 0.006, $p = 0.800$), but was 0.019 higher in HEAT+DEHY (1.026 ± 0.005) than in HEAT (95% CL: [0.014, 0.023]; $p < 0.001$). Across exercise, U_{SG} was unchanged in CON ($p = 0.155$) and HEAT ($p = 0.289$), but tended to increase in HEAT+DEHY ($p = 0.075$). As such, post-exercise U_{SG} was similar between HEAT (1.005 ± 0.005) and CON (1.005 ± 0.004; $p = 0.962$), but 0.024 higher in HEAT+DEHY (1.029 ± 0.004) than in HEAT ([0.019, 0.028]; $p < 0.001$).

Plasma Osmolality (P_{OSM}; Figure 2)

Baseline P_{OSM} was similar between HEAT and CON ($p = 0.243$), and HEAT and HEAT+DEHY ($p = 0.111$; **Figure 2**). Across exercise, P_{OSM} decreased by 4 and 5 mOsm·kg^{-1} in CON and HEAT, respectively, but increased by a further 4 mOsm·kg^{-1} in HEAT+DEHY (all $p \leq 0.014$). As such, post-exercise P_{OSM} was lower in HEAT than in CON (mean difference: −4 mOsm·kg^{-1}; [−6, −1]; $p = 0.002$), and higher in HEAT+DEHY than in HEAT (13 mOsm·kg^{-1}; [10, 15]; $p < 0.001$). Across the first 2 h of recovery, P_{OSM} remained stable in CON ($p = 0.249$) and HEAT ($p = 0.128$), but decreased by 5 mOsm·kg^{-1} in HEAT+DEHY ($p = 0.001$), remaining 4 mOsm·kg^{-1} higher in HEAT+DEHY than in HEAT at 2 h ([0, 9]; $p = 0.046$).

Thermal (Table 1)

Average core temperature (T_{CORE}) was 0.4°C higher with the addition of heat stress, and an additional 0.2°C higher with dehydration (**Table 1**). Peak T_{CORE} was 0.7°C higher with the addition of heat stress, and 0.3°C higher with dehydration (**Table 1**). Mean skin temperature (T_{SKIN}) was 5.0°C warmer with the addition of heat stress, but not additionally higher with dehydration (**Table 1**). Peak T_{SKIN} however was 5.4°C warmer with the addition of heat stress, and an additional 0.2°C warmer with dehydration (**Table 1**).

Heart Rate (Table 1)

Average HR was 37% higher with the addition of heat stress to exercise (i.e., CON vs. HEAT), and an additional 10% higher with dehydration (i.e., HEAT vs. HEAT+DEHY; **Table 1**). Similarly, peak HR was 41% higher with heat stress and 11% higher with dehydration (**Table 1**).

Perceptual Responses (Table 1)

Participants perceived exercise to be additively harder with the imposition of heat stress and dehydration (**Table 1**). They also reported higher ratings of thermal sensation with the addition of heat stress, but not additionally with dehydration (**Table 1**), and an additively higher thermal discomfort with heat stress and dehydration (**Table 1**).

Respiratory (Table 2)

Mean oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) were similar between conditions (**Table 2**), as were changes from baseline (all $p \geq 0.312$; **Table 2**).

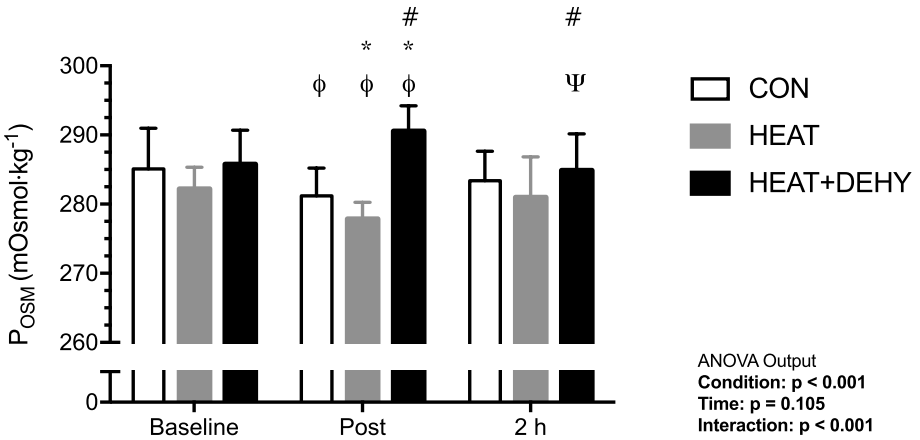

FIGURE 2 | The additive effects of heat stress and hydration status on plasma osmolality (P_{OSM}) before, immediately after, and 2 h after 90-min exercise in CON (all $n = 10$), HEAT ($n = 9$, 9, and 8 resp.), and HEAT + DEHY ($n = 9$, 9, and 10 resp.). Statistical significance (ANOVA Output) is illustrated. Data presented are group mean ± SD. $^{\Phi}$Denotes significantly different ($p < 0.05$) to baseline. $^{\Psi}$Denotes significantly different to post-exercise measurement. *Denotes significantly different than CON. $^{\#}$Denotes significantly different to HEAT.

TABLE 1 | The additive effects of heat stress and hydration status on the average and peak thermal, cardiac and perceptual strain during the 90-min exercise condition, with corresponding statistical significance (ANOVA Output) and comparisons of interest.

	Exercise condition			*P*-value	*H–C*	*H+D–H*
	C	*H*	*H+D*		*Mean diff; [95% CL]; p-value*	
THERMAL						
Average T_{CORE} (°C)	37.2 ± 0.3	37.6 ± 0.3	37.7 ± 0.3	<0.001	0.4; [0.2, 0.6]; <0.001	0.2; [0.0, 0.4]; = 0.059
Peak T_{CORE} (°C)	37.3 ± 0.2	38.0 ± 0.3	38.3 ± 0.3	<0.001	0.7; [0.5, 0.8]; <0.001	0.3; [0.2, 0.5]; <0.001
Average T_{SKIN} (°C)	31.6 ± 0.6	36.7 ± 0.3	36.8 ± 0.2	<0.001	5.0; [4.5, 5.5]; <0.001	0.2; [−0.1, 0.4]; = 0.328
Peak T_{SKIN} (°C)	31.8 ± 0.6	37.2 ± 0.3	37.4 ± 0.2	<0.001	5.4; [4.9, 5.8]; <0.001	0.2; [0.0, 3.3]; = 0.025
CARDIAC						
Average HR (b·min^{-1})	86 ± 9	115 ± 13	126 ± 12	<0.001	31; [27, 35]; <0.001	11; [6, 15]; <0.001
Peak HR (b·min^{-1})	100 ± 9	139 ± 19	154 ± 14	<0.001	40; [28, 51]; <0.001	15; [3, 27]; = 0.011
PERCEPTIONS						
Peak RPE (AU)	9.6 ± 2.4	13.0 ± 2.5	15.3 ± 1.6	<0.001	2.5; [1.5, 3.6]; <0.001	3.2; [2.2, 4.2]; <0.001
Peak TS (AU)	6.9 ± 0.6	10.8 ± 1.7	12.0 ± 0.9	<0.001	3.9; [2.4, 5.3]; <0.001	1.2; [−0.5, 2.9]; = 0.176
Peak TD (AU)	1.0 ± 0.0	3.1 ± 1.2	4.3 ± 0.9	<0.001	2.1; [1.2, 2.9]; <0.001	1.2; [0.3, 2.0]; = 0.006

Data presented are descriptive mean ± SD. All n = 10. Model estimates of differences between conditions of interest are presented as mean difference, (95% confidence limits), and corresponding p values (post-hoc). C, CON; H, HEAT; H+D, HEAT+DEHY. H–C, independent effect of heat. H+D–H, independent effect of dehydration. HR, heart rate. T_{CORE}, core (rectal) temperature. T_{SKIN}, mean skin temperature. RPE, Rating of perceived exertion; TS, Thermal sensation; TD, Thermal discomfort.

Whereas, mean ventilation ($n = 8$) and its component factors (frequency and tidal volume) increased 51% more with the addition of heat stress, but not additionally with dehydration (**Table 2**). Mean end-tidal carbon dioxide pressure ($P_{ET}CO_2$; **Table 2**) were therefore lower, by 9%, with the addition of heat stress, but not additionally with dehydration (**Table 2**). By end exercise, the reduction in $P_{ET}CO_2$ was 74% larger with heat stress, but also not additionally with dehydration (**Table 2**).

Blood Pressure (BP; Table 3 and Figure 3)

Unstressed baseline sBP, dBP and MAP were 108 ± 6, 69 ± 7, and 82 ± 5 mm Hg, respectively. Diastolic blood pressure (dBP) did not change significantly, whereas systolic (sBP) and mean arterial pressure (MAP) showed reductions as described below (**Table 3**, **Figure 3**). Individual responses are provided in **Supplementary Figure 2** for reference.

In response to exercise, sBP remained unchanged in CON (all $p \geq 0.990$ vs. baseline), and in HEAT ($p \geq 0.345$) except tending to be reduced at 24-h after exercise (by 5 mm Hg [0, 11]; $p = 0.077$). Whereas, sBP was elevated by 6 mm Hg at baseline in HEAT+DEHY ([0, 11]; $p = 0.033$ vs. unstressed baseline), but 7 mm Hg lower immediately post-exercise ([2, 13]; $p = 0.002$), then unaffected at 2–5 h (all $p \geq 0.494$), and reduced again by 5 mm Hg at 24 h ([0, 11]; $p = 0.049$). The MAP was unchanged from baseline in CON and HEAT at all time points ($p \geq 0.452$), but in HEAT+DEHY it progressed

TABLE 2 | The additive effects of heat stress and hydration status on the average and change from baseline in respiratory strain during the 90-min exercise condition, with corresponding statistical significance (ANOVA Output) and comparisons of interest.

	Exercise condition			*P*-value	*H–C*	*H+D–H*
	C	*H*	*H+D*		*Mean diff; [95% CL]; p-value*	
RESPIRATORY						
Average $\dot{V}O_2$ (mL·min^{-1})	757 ± 177	754 ± 170	764 ± 177	0.847		
Δ $\dot{V}O_2$ (mL·min^{-1})	323 ± 146	343 ± 146	370 ± 143	0.032	32; [−31, 94]; = 0.409	36; [−25, 97]; = 0.312
Average $\dot{V}CO_2$ (mL·min^{-1})	638 ± 180	667 ± 181	669 ± 180	0.236		
Δ $\dot{V}CO_2$ (mL·min^{-1})	266 ± 119	289 ± 129	280 ± 107	0.943		
Average $P_{ET}CO_2$ (mm Hg)	36 ± 3	32 ± 4	30 ± 4	<0.001	−3; [−1, −6]; = 0.013	−2; [−5, 0]; = 0.104
Δ $P_{ET}CO_2$ (mm Hg)	−4 ± 1	−7 ± 3	−11 ± 6	0.001	3; [0, 5]; = 0.020	4; [−1, 10]; = 0.154
Average $\dot{V}_E$ (L·min^{-1})	18 ± 4	20 ± 3	21 ± 4	0.045	2.1; [−0.9, 5.1]; = 0.182	0.9; [−2.2, 3.9]; = 0.741
Δ $\dot{V}_E$ (L·min^{-1})	7 ± 5	11 ± 7	12 ± 10	0.018	3; [0, 5]; = 0.019	−1; [−3, 2]; = 0.629
Average B_F (breaths·min^{-1})	21 ± 4	21 ± 4	22 ± 4	0.765		
Δ B_F (breaths·min^{-1})	9 ± 3	8 ± 4	9 ± 3	<0.001	3.3; [1.1, 5.5]; = 0.005	0.03; [−2.6, 2.0]; = 0.943
Average $\dot{V}_T$ (L·min^{-1})	0.9 ± 0.2	0.9 ± 0.2	1.0 ± 0.2	0.112		
Δ $\dot{V}_T$ (L·min^{-1})	0.6 ± 0.4	0.7 ± 0.5	0.6 ± 0.3	0.641		

Data presented are descriptive mean ± SD. Model estimates of differences between conditions of interest are presented as mean difference, (95% confidence limits), and corresponding p-values (post-hoc). C, CON; H, HEAT; H+D, HEAT+DEHY. H–C, independent effect of heat. H+D–H, independent effect of dehydration. $\dot{V}O_2$, oxygen consumption. $\dot{V}CO_2$, carbon dioxide production. $P_{ET}CO_2$, end-tidal carbon dioxide. $\dot{V}_E$, ventilation (n = 8). B_F, breathing frequency (n = 8). $\dot{V}_T$, tidal volume (n = 8).

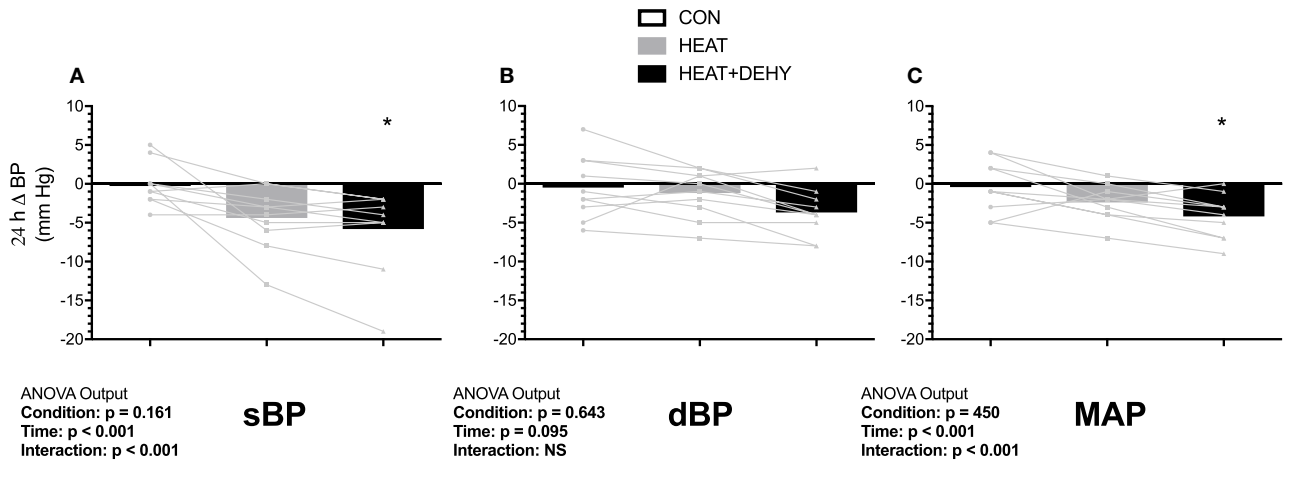

FIGURE 3 | The additive effects of heat stress and hydration status on the 24-h change in systolic (sBP, **A**), diastolic (dBP, **B**) and mean arterial blood pressure (MAP, **C**) after the 90-min exercise condition. Statistical significance (ANOVA Output) is illustrated. Data presented are group mean with individual values overlaid. *Denotes significantly different ($p < 0.05$) than CON.

from being equivalent at baseline ($p = 0.206$), to a 5 mm Hg reduction at end exercise ([1, 9]; $p = 0.005$), equivalent from 2 to 5 h ($p \geq 0.828$), and reduced again by 4 mm Hg at 24 h ([0, 8]; $p = 0.020$).

When comparing between stressors at a given time, the addition of heat affected neither sBP nor MAP at end exercise ($p \geq 0.753$ vs. CON), whereas the addition of dehydration reduced both; sBP by 7 mm Hg ([2, 11]; $p < 0.001$ vs. HEAT) and MAP by 6 mm Hg ([3, 9]; $p < 0.001$). The BP were thereafter unaffected by the addition of either stressor in the first 5 h of recovery (all $p \geq 0.219$), whereas by 24 h, sBP tended to be reduced following HEAT (by 4 mm Hg; [0, 8]; $p = 0.061$), comparably to HEAT+DEHY ($p = 0.837$ vs. HEAT; **Figure 3**). At 24 h, MAP was similar in HEAT and CON ($p = 0.218$), and in HEAT+DEHY ($p = 0.398$ vs. HEAT).

Association between Stress-induced Strain and Post-exercise Hypotension (PEH; Figure 4)

The association between stress-induced strain and 24-h change in MAP were explored to examine whether the addition of individual stressors (i.e., exercise, heat, and dehydration) provided a further stimulus for adaptation, by virtue of each inducing greater strain. When data from the three conditions were pooled for correlational analysis, the strain during exercise was strongly associated with the magnitude of PEH at 24 h (**Figure 4**).

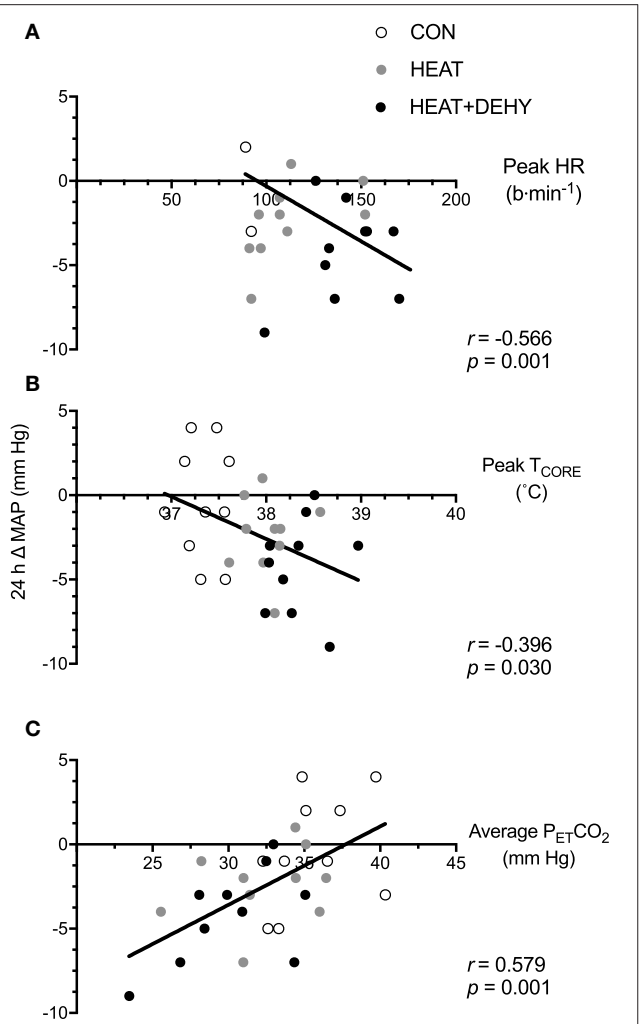

FIGURE 4 | The relation between different forms of stress-induced strain and the post-exercise hypotensive (PEH) response at 24 h. Illustrated are the contributions of the cardiovascular strain (**A**; peak HR in exercise), thermoregulatory strain (**B**; peak T_{CORE} in exercise), and an indicator of respiratory strain (**C**; average end-tidal carbon dioxide ($P_{ET}CO_2$) in exercise) to the 24-h change in MAP.

Plasma Volume (PV; Figure 5)

As per the analysis of BP, time effects were examined against an unstressed baseline (i.e., mean of both euhydrated conditions), whereas effects of heat and dehydration at a given time were examined by comparing their respective differences from this common baseline.

Plasma volume (PV) was reduced at baseline by 3% in HEAT+DEHY ([1, 6]; $p = 0.016$). Compared to baseline, PV was significantly reduced post-exercise in HEAT ($p = 0.007$), but not significantly in CON or DEHY (both $p \geq 0.401$). This reduction was larger with heat ([0, 10]; $p = 0.072$), but not additionally with dehydration ([−1, 2]; $p = 0.959$). By 2 h after exercise PV recovered such that it was no different to baseline in CON ($p = 0.114$), but higher than baseline in HEAT ($p = 0.049$) and DEHY ($p < 0.001$). Plasma volume then remained elevated throughout recovery in all conditions (all $p \leq 0.017$), and to a similar extent from 2 to 5 h after exercise (all $p \geq 0.420$; **Figure 5**). However, at 24 h the PV expansion was similar between HEAT and CON ([−1, 5]; p = 0.335) but 4% larger in HEAT+DEHY ([1, 8]; $p = 0.005$ vs. HEAT).

Association between Stress-Induced Strain and Plasma Volume Expansion (PVE; Figure 6), and between PEH and PVE (Figure 7)

The association between indices of strain during exercise and expansion of PV by 24 h after exercise was explored to examine whether the addition of individual stressors (i.e., exercise, heat, and dehydration) provided a further stimulus for adaptation, by virtue of causing cumulative strain. Furthermore, the association between PEH and PVE (both at 24 h) was explored to examine potential mechanisms of exercise-induced hypervolemia (**Figure 7**). Cumulative strain was strongly associated with the magnitude of PVE at 24 h (**Figure 6**). Similarly, PVE at 24 h was strongly predictive of PEH at 24 h (**Figure 7**).

Hormones

Aldosterone (ALD; Figure 8A)

Aldosterone concentrations ($n = 8$) were similar between conditions at baseline (all $p \geq 0.145$). Aldosterone was unchanged across exercise and at the measured (2 h) recovery point in CON (all $p \geq 0.472$), whereas it increased by 41% after exercise in HEAT ([9, 73]; $p = 0.010$), and by 82% in HEAT+DEHY ([56, 108]; $p < 0.001$; **Figure 8A**). When comparing conditions, post-exercise ALD concentration tended to be 28% higher by virtue of heat stress ([2, 58]; $p = 0.071$), and 62% higher again by adding dehydration ([39, 86]; $p < 0.001$). These effects were no longer evident at 2 h of recovery ($p \geq 0.163$ between conditions).

Erythropoietin (EPO; Figure 8B)

Erythropoietin concentrations did not change significantly with exercise in CON ($n = 8$), HEAT ($n = 5$), or DEHY ($n = 6$; **Figure 8B**).

Aldosterone-Mediated PVE (Figure 9)

The association between cardiovascular (Δ MAP; **Figure 9A**) and thermal strain (peak T_{CORE}; **Figure 9B**) and ALD concentration were examined to identify potential thresholds and mechanisms of the ALD response. In turn, the relation between the ALD response and PVE was examined to investigate potential mechanisms for exercise-induced hypervolemia (**Figure 9C**). Larger reductions in MAP and larger rises in T_{CORE} across exercise were strongly associated with post-exercise ALD concentrations, which was strongly predictive of PVE at 24 h.

DISCUSSION

This is the first study, to our knowledge, to investigate the additive roles of low-intensity, orthostatically-stressful exercise, heat, and dehydration on early cardiovascular outcomes. The main novel findings were that: (i) the addition of these separate stressors combine to augment short-term (24 h)

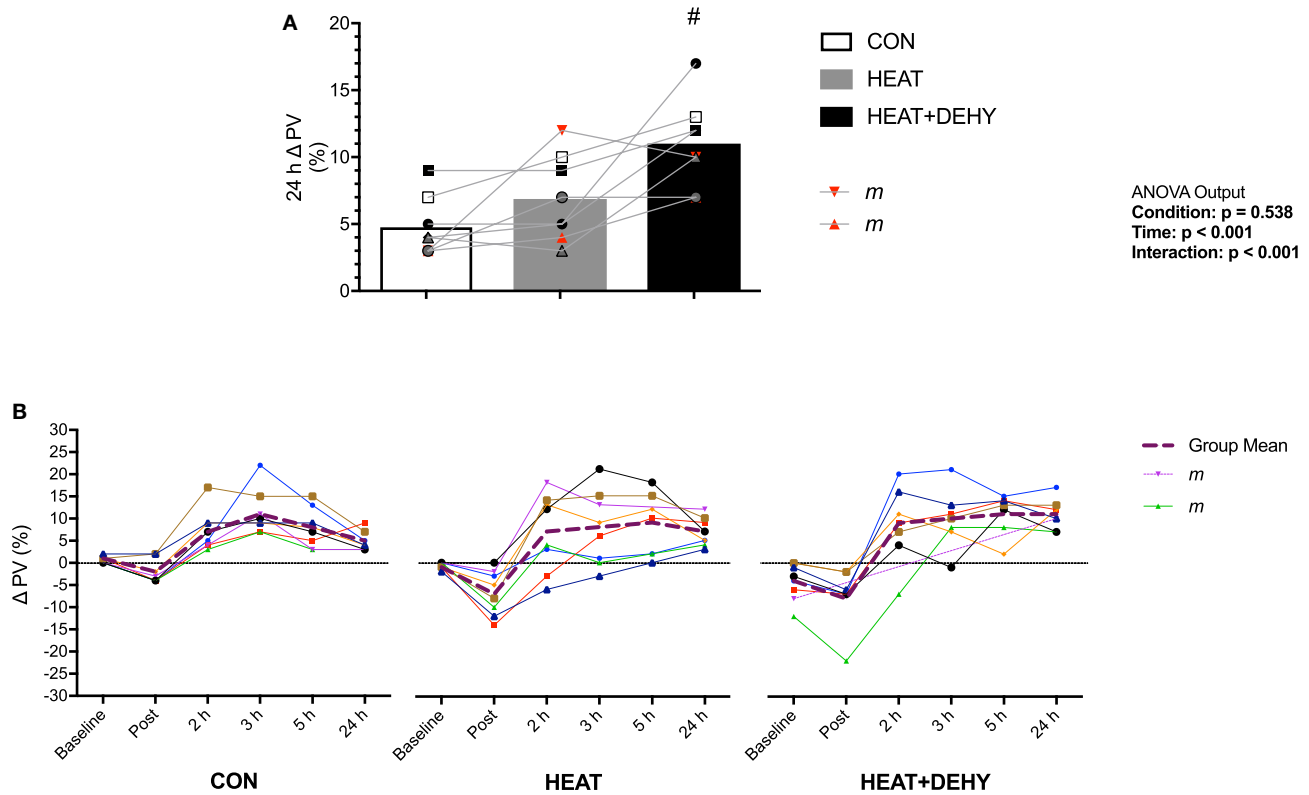

FIGURE 5 | The additive effects of heat stress and hydration status on the change in plasma volume at 24 h after exercise (**A**; $n = 8$), and the pattern of response **(B)** vs. the average hydrated measure immediately after, 2, 3, 5, and 24 h after exercise in CON (all $n = 8$), HEAT ($n = 8$, 8, 8, 8, 7, and 8 resp.), and HEAT+DEHY ($n = 8$, 7, 7, 7, 7, and 8 resp.). Statistical significance (ANOVA Output) is illustrated. Data presented are group mean with individual values overlaid. Two male participants ("*m*") are highlighted for reference. #Denotes significantly ($p < 0.05$) different to HEAT.

cardiovascular and hematological outcomes, particularly PEH and PVE; (ii) stress-induced strain, particularly the magnitude of T_{CORE} elevation, closely predicts these outcomes; and (iii) within the first 24 h, PEH at least partly facilitates PVE at 24 h. Acute outcomes, such as PEH and PVE are important in their own right, but must also be considered in the context as a precursor for short- and long-term cardiovascular and hematological adaptations. Collectively, these findings indicate that manipulating the strain elicited during an exercise/conditioning bout could facilitate short-term (24 h) outcomes that may aid in exercise performance or cardiovascular health.

Post-exercise Hypotension

Low-intensity exercise provides a widely accessible means of meeting physical activity guidelines, but falls below the threshold required to meaningfully impact some important cardiovascular health parameters. In the current study, low-intensity exercise alone did not elicit a significant drop in BP at 24 h (**Figure 3**). This supports findings of Forjaz et al. (2004), who found no reduction in MAP 22 h after normotensive individuals cycled for 45 min at 30% $\dot{V}O_{2\ MAX}$, but significant reductions in BP after 50 and 75% $\dot{V}_{2\ MAX}$. Similarly, Wallace et al. (1999) demonstrated no PEH 22 h after intermittent walking (50% $\dot{V}O_{2\ MAX}$) in normotensive individuals. Such data indicate that low-intensity exercise, particularly in normotensive young individuals, may be insufficient to elicit acute BP responses. Acute resistance exercise may also be insufficient in this respect (O'Connor et al., 1993), or more pronounced with large muscle mass exercise and those with hypertension (Casonatto et al., 2016). Even with resistance exercise (60% 1RM) eliciting sufficient stimulus for acute PEH, this may not eventuate over a longer period (i.e., 12 week; Moraes et al., 2012). Thus, higher intensity exercise, or a conditioning stimulus that provides a larger acute perturbation may be required to acquire the long-term (chronic) anti-hypertensive effects of exercise (Quinn, 2000; Thompson et al., 2001; Liu et al., 2012).

Adding heat stress to low-intensity exercise did not consistently affect BP. Elevated core and skin temperatures associated with exogenous heat stress (**Table 1**) were expected to elicit a greater degree of exercise-induced sympathetic activity (Ray and Gracey, 1997), and subsequent post-exercise residual systemic vasodilation, and thus greater baroreflex resetting and associated sympatho-inhibition (Halliwill et al., 2013). The combined stressors could conceivably also have larger transient effect on oxidative stress and the ensuing balance of oxidative stimuli and anti-oxidative capacity. We hypothesized that this would ultimately manifest in larger PEH at 24 h. Whilst

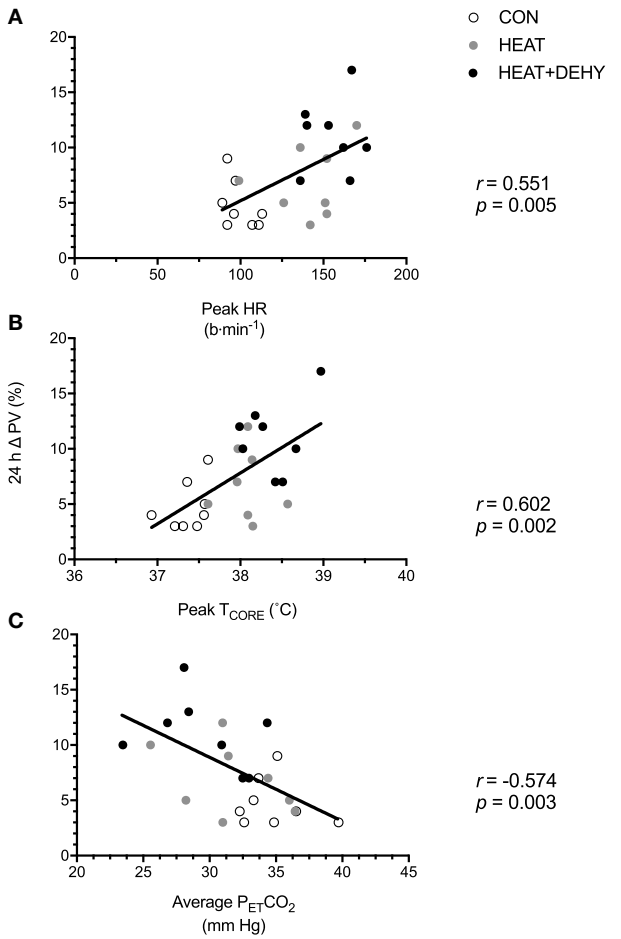

FIGURE 6 | The relation between different forms of stress-induced strain and the post-exercise hypervolemic response (PVE; $n = 8$). Illustrated are the contributions of the cardiovascular strain (**A**; peak HR in exercise), thermoregulatory strain (**B**; peak T_{CORE} in exercise), and an indicator of respiratory strain (**C**; average $P_{ET}CO_2$ in exercise) to the 24-h change in PV.

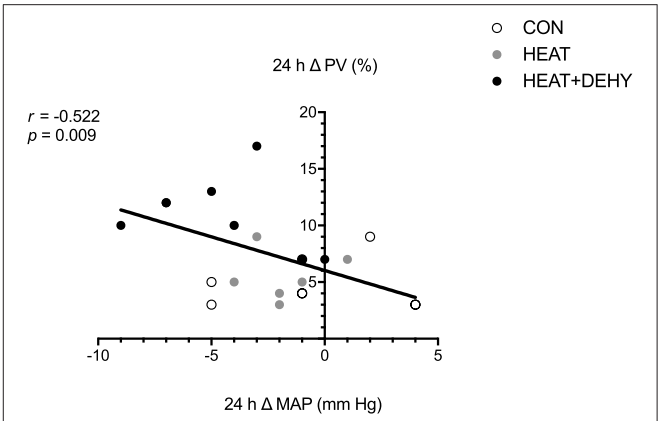

FIGURE 7 | The relation between the post-exercise hypotensive response (24-h change in MAP), and the hypervolemic response (24-h change in PV; $n = 8$).

systolic pressure tended to be reduced at 24 h after exercise in the heat ($p = 0.077$), diastolic and mean arterial pressures were not (**Figure 3**). Post-exercise hypotension is a complex and multifactorial response involving both peripheral and central mechanisms (Halliwill et al., 2013; Graham et al., 2016). Halliwill et al. (2013) suggest that the situational occurrence of hyperthermia may modulate the hypotensive response, however the additional thermal strain incurred in the present study did not have measurable effect. Elevated T_{CORE} in recovery may (Kenny and Niedre, 2002) or may not (Jones et al., 2007) affect immediate PEH (≤30 min after exercise), but this is likely modulated by the preceding exercise intensity (Kenny and Niedre, 2002; Kenny et al., 2006; Jones et al., 2007). Due to the self-determined intensity of the calisthenics, participants may have attenuated the intensity of contractions in the face of a higher cardiovascular and thermal strain, and thus provided insufficient additional stimulus for PEH. While self-pacing is ecologically valid, it may be instructive to use matched paced exercise to provide a more complete mechanistic understanding.

Blood pressure was significantly reduced at 24 h only when modest (3% BM; P_{OSM} ~290 mOsmol·kg^{-1}) dehydration was additively imposed on heat stress and low-intensity exercise (**Figure 3**). Furthermore, every participant demonstrated a reduction in MAP at 24 h when exposed to this combination of stressors (**Figure 3**), and to a meaningful extent, of 4 mm Hg (Chobanian et al., 2003). Acute dehydration augments physiological strain in multiple systems (Montain and Coyle, 1992b; Sawka et al., 1992; Gonzalez-Alonso, 1998; Gonzalez-Alonso et al., 1999), particularly via exacerbated tissue temperatures and cardiovascular strain in exercise (Montain and Coyle, 1992b; Sawka et al., 1992). This exacerbated strain facilitated PEH in the current study, presumably due at least in part to the thermal and cardiovascular strain elicited in response to this combination of stressors (**Figure 4**). The strain profile may therefore be as important as the work done, in determining the acute hypotensive response to exercise (Jones et al., 2007). If so, varied methods of modulating the strain induced by exercise (e.g., transient dehydration, ischemia, gravitational) may provide feasible means of eliciting (specific) short- and longer-term adaptations when not accessible via traditional and well-justified means (esp. moderate- and higher-intensity exercise).

Unfortunately, hydration status is sparsely reported in studies investigating PEH (Endo et al., 2012), likely due its complex association with muscular activity, heat production, and thermoregulatory responses. Fluid intake has been shown to abolish PEH in healthy sedentary males in a temperate environment (Endo et al., 2012), and in trained males in a hot (42°C and 20% RH) environment (Gagnon et al., 2012). Both these aforementioned studies demonstrated PEH in the 60–90 min after moderate exercise (1 h at 60% HRR, and 2 h at 120W, respectively) and modest dehydration (~1 and 2.5%, respectively). Further, the magnitude of PEH in early recovery was comparable to that of the current study (−5 mm Hg immediately after exercise; **Table 3**), but those studies did not report BP for the following day. Alterations in cardiac baroreflex sensitivity may partly explain the observed early-phase PEH in all three studies, as prevention of dehydration

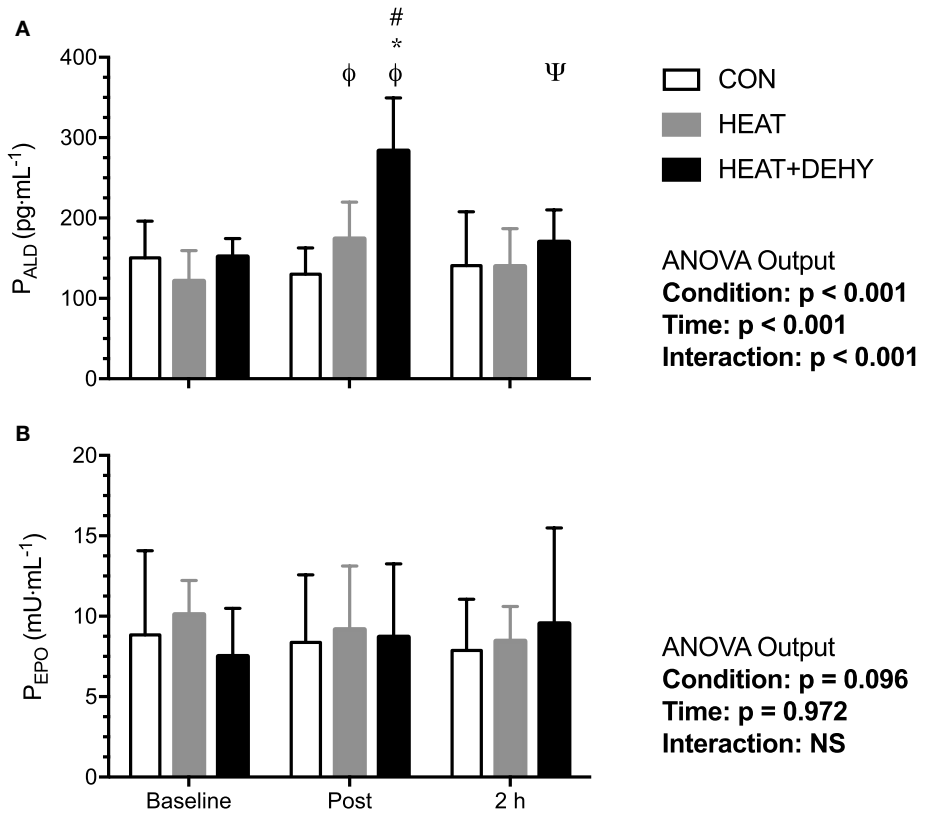

FIGURE 8 | The effect of heat stress and hydration status on plasma aldosterone concentration (P_{ALD}, all $n = 8$; **A**) and erythropoietin concentration (P_{EPO}; **B**) before, immediately after, and 2-h after the 90-min exercise in CON (all $n = 8$), HEAT (all $n = 5$), and HEAT+DEHY (all $n = 6$) condition. Statistical significance (ANOVA Output) is illustrated. Data presented are mean ± SD. $^{\Phi}$Denotes significantly different ($p < 0.05$) to baseline. $^{\Psi}$Denotes significantly different to immediately post-exercise measurement. *Denotes significantly different than CON. $^{\#}$Denotes significantly different to HEAT.

(by saline infusion or drinking) during exercise minimizes PEH and abolishes reductions to cardiac baroreflex sensitivity (Charkoudian et al., 2003). Furthermore, when coupled with higher tissue temperatures (**Table 1** and **Figure 4**), and the subsequent cutaneous vasodilation, dehydration may facilitate larger PEH via both inhibition of central vasoconstrictor output and sustained post-exercise vasodilation of active muscle beds (i.e., via central and peripheral mechanisms; Halliwill et al., 2013).

Hypervolemia

Plasma volume was elevated by 5% at 24 h with calisthenics alone (**Figure 5**). This magnitude of PVE was unexpected considering its similarity to that obtained from higher-intensity exercise models (Gillen et al., 1994; Yang et al., 1998; Nagashima et al., 1999, 2000), and the fact that these participants were regularly physically active. The dynamic and orthostatically stressful nature of this exercise may therefore partly explain the observed PVE. Rapid PV reductions during exercise are attributed partly to increased capillary hydrostatic pressure and muscle tonicity (Lundvall et al., 1972), muscular activity *per se* (Sjogaard and Saltin, 1982), and a more upright posture (Nagashima et al., 2000). Thus, sustained isometric contractions, adjustments in posture, and dynamic movement sequences using most muscle groups in the current exercise protocol may facilitate a modest PVE despite its relatively low intensity. Plasma volume changes in the current study must also be considered in the context of potential limitations. First, the 10-min rest period prior to capillary sampling was insufficient to allow full stabilization, but was consistent for each individual between conditions, thus no systematic bias is expected. Second, we cannot discount possible confounding by incomplete washout between our conditions (Gillen et al., 1991), but any such effects would also lead to under- rather than over-estimation of hypervolemic effects of exercise or the additional stressors.

Fluid-regulatory hormonal responses to exercise are important mediators of PVE following exercise and heat exposure, and are influenced by exercise intensity and posture (Francesconi et al., 1983a; Ray et al., 1990; Gillen et al., 1991). The 5% increase in PV in CON was not accompanied by an increase in aldosterone (ALD; **Figure 8A**). Elevated ALD following exercise is involved in renal reabsorption of sodium and coupled reabsorption of water and subsequent PVE (Wade et al., 1985). Longer duration submaximal exercise elicits larger increases in ALD than does maximal exercise (Costill et al., 1976; Wade et al., 1987) but may have a threshold dependence (∼60% $\dot{V}O_{2MAX}$; Tanaka et al., 1986). The current study is limited in its assessment

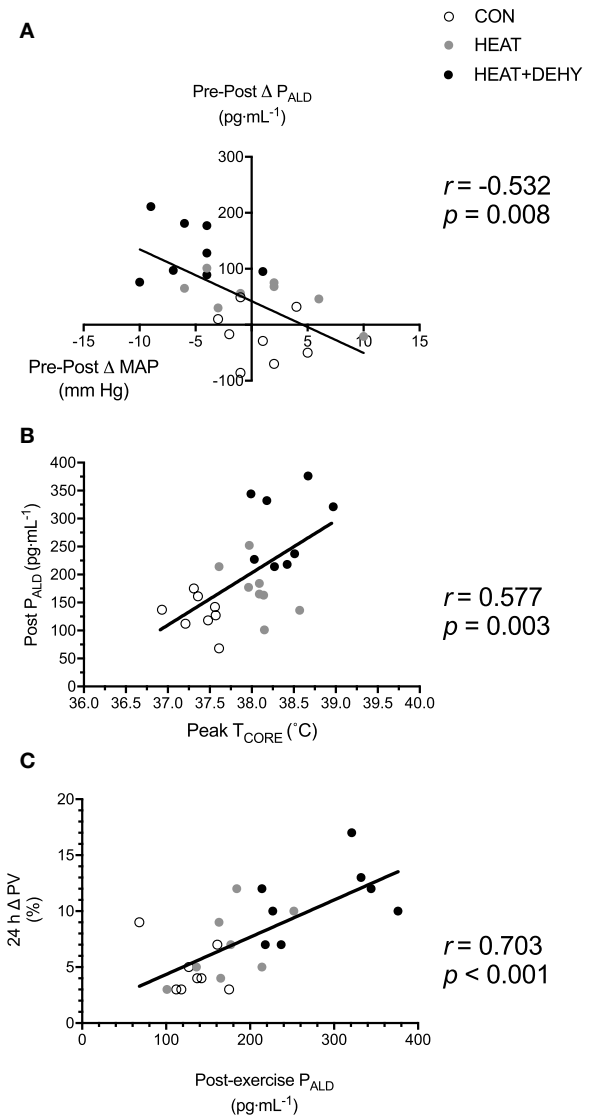

FIGURE 9 | Potential mechanisms of increased aldosterone concentration and its role in mediating the post-exercise hypervolemic response. Illustrated is the relation between the change in MAP immediately after exercise **(A)**, and peak T_{CORE} in exercise **(B)**, and P_{ALD} immediately after exercise (n = 8); and the relation between post-exercise P_{ALD} concentration and the 24-h change in PV **(C)**.

of aldosterone-mediated effects on 24-h PVE given the lack of plasma osmolality (P_{OSM}) measurements at this time point. Subsequent research should further determine the mechanisms of hypervolemia in response to different strain profiles utilizing a comprehensive assessment of body fluid regulation. Thus, the PVE in CON may therefore have been mediated by mechanisms other than those measured in the current study, including increased content of albumin due to increased lymphatic return, synthesis and decreased transcapillary escape (Gillen et al., 1991; Haskell et al., 1997; Yang et al., 1998).

Heat stress did not additively contribute to PVE (at 24 h) following low-intensity exercise (**Figure 5A**), despite increasing PV loss during exercise (**Figure 5B**). Exercise intensity (Nadel et al., 1979) and environmental temperature (Nadel et al., 1979; Maw et al., 1998) affect the PV loss during exercise, such that PV losses are larger in higher ambient temperatures regardless of hydration state (Kenefick et al., 2014). A more pronounced rise in T_{CORE} (**Table 1**), and concomitant cutaneous vasodilation was expected to elevate capillary hydrostatic pressure, promoting net filtration and therefore augment the PV losses (Starling, 1896; Saltin, 1964; Senay, 1975). However, this larger loss during exercise was not compensated for by a larger rebound hypervolemia. Environmental conditions are also known to affect ALD secretion (Davies et al., 1981), with the increase in T_{CORE} modulating this effect (Moller et al., 1989; Auernhammer and Strasburger, 1995; Wright et al., 2010). Despite a ∼40% increase in ALD concentration in HEAT, this did not additively effect PVE to measurable extent; possibly due to the modest thermal strain (∼38°C) coupled with the low intensity of exercise.

The combination of exercise, heat stress and dehydration resulted in the largest PVE (**Figure 5**). Dehydration can impair endurance exercise performance, due in part to increased tissue temperatures and cardiovascular strain (Sawka et al., 1992, 2011; Montain and Coyle, 1992a). These factors are also critically involved in acute hypervolemia (see Figure 2 in Akerman et al., 2016), however the obligatory mechanisms (if any) are largely unclear. Skin temperature was equivalently high in HEAT and HEAT+DEHY (**Table 1**), thereby rendering it as being unlikely in the mediation of PVE. These findings support those of Armstrong et al. (1997) and Gonzalez-Alonso et al. (1997) and indicate that hyperthermia and dehydration equally but not additively affect T_{SKIN} in these conditions. These findings thereby also support the case for T_{CORE} as a primary and more important modulator of PVE (Maw et al., 2000).

Fluid regulatory hormones are sensitive to changes in body water content and very likely contributed to the observed PVE in HEAT+DEHY. Hypohydration elicits larger increases in plasma renin activity (PRA) and ALD during light exercise in hot environments (Francesconi et al., 1983b; Maresh et al., 2004), whereas such exercise in a temperate environment is insufficient to stimulate these hormones (Finberg et al., 1974; Francesconi et al., 1983b). Furthermore, Francesconi et al. (1983b) also demonstrate an additive effect of heat stress on low-intensity exercise, and more-so with dehydration. The additive effect of dehydration in the current study was also a similar magnitude as Francesconi et al. (1983b), with dehydration approximately doubling the effect of heat alone on ALD concentration. Finberg et al. (1974) also found an association between PRA and cardiovascular strain in light exercise, which were attenuated when euhydration was maintained. Unlike Finberg et al. (1974), the increase in ALD in the current study was associated with the degree of thermal as well as cardiovascular strain experienced (**Figure 9**). It therefore appears that whilst ALD concentration, and its mediating effect on PVE (**Figure 9**; Nagashima et al., 1999) may be related most closely with thermal strain (i.e., T_{CORE}), other mechanisms of PVE may also contribute.

Heat stress and physical activity act additively to vasoconstrict the splanchnic and renal beds (Rowell et al., 1968). The combination of these stressors may therefore collective facilitate

TABLE 3 | The additive effects of heat stress and hydration status on arterial blood pressure before and after the 90-min exercise condition.

	Baseline			Post-exercise			2-h post-exercise			3-h post-exercise			5-h post-exercise			24-h post-exercise		
	C	*H*	*H+D*	*C*	*H*	*H+D*	*C*	*H*	*H+D*	*C*	*H*	*H+D*	*C*	*H*	*H+D*	*C*	*H*	*H+D*
sBP (mm Hg)	108 ± 6	107 ± 6	113 ± 6	108 ± 7	99 ± 5	108 ± 7	108 ± 7	109 ± 9	111 ± 8	107 ± 6	106 ± 7	107 ± 8	106 ± 7	105 ± 5	105 ± 7	107 ± 7	103 ± 5	102 ± 6
dBP (mm Hg)	68 ± 7	69 ± 8	71 ± 8	69 ± 7	70 ± 8	65 ± 8	69 ± 5	70 ± 6	70 ± 8	69 ± 5	67 ± 6	69 ± 5	68 ± 6	69 ± 7	68 ± 6	68 ± 6	67 ± 6	65 ± 6
MAP (mm Hg)	81 ± 5	82 ± 6	85 ± 6	82 ± 5	83 ± 7	77 ± 6	82 ± 3	83 ± 5	83 ± 6	81 ± 4	80 ± 5	81 ± 5	81 ± 5	81 ± 5	81 ± 4	81 ± 5	79 ± 5	77 ± 5

Data presented are group mean ± SD. All n = 10. C, CON; H, HEAT; H+D, HEAT+DEHY. sBP, systolic blood pressure; dBP, diastolic blood pressure; MAP, mean arterial blood pressure. For statistical significance see ***Figure 3****.*

the erythropoietic response to exercise (Sawka et al., 2000), but whether this occurs acutely has yet to be determined. We therefore hypothesized that the three stressors examined here would attenuate splanchnic and renal blood flow and might thus promote an erythropoietic response. However, plasma erythropoietin (EPO) was unchanged in all conditions (**Figure 8B**). The stimulus may therefore have been insufficient to reduce renal PO_2, the primary stimulus for EPO release. Plasma volume expansion without an accompanying erythropoeitic stimulation may be beneficial in untrained people, but its functional relevance in highly endurance-trained people is less clear (Hopper et al., 1988). Whereas, PVE in conjunction with red cell volume (RCV) expansion is functionally relevant in trained individuals (Heinicke et al., 2001; Lundby et al., 2016). It is therefore valuable to know what exercise-related stimuli would expand RCV. Elevated EPO concentrations are observed in response to reductions in central venous pressure (CVP; Gunga et al., 1996a), thermal stimuli (Gunga et al., 1996b), and hypovolemia (Szygula et al., 1995), independent of hypoxia (Kirsch et al., 2005). Yet, EPO was not elevated by any of the stimuli used in this study, so their erythropoietic potential remains unclear.

PEH and PVE

The causal relation between PEH and subsequent PVE is complex and not fully understood. In the current study, the two phenomena were strongly associated, but also showed some independence, as has been observed previously (Holtzhausen and Noakes, 1995; Graham et al., 2016). For instance, PVE was consistently demonstrated in CON and HEAT despite no significant change in MAP. Whilst CVP unloading, and thus some aspect of post-exercise BP reduction must arise as a stimulus for PVE (Nagashima et al., 1999; Hayes et al., 2000), evidently PVE can also occur via other mechanisms.

Arterial pressure is generated by cardiac output and systemic vascular resistance. For PVE to occur with a simultaneous reduction in arterial BP, then presumably arterial or arteriolar resistance must decrease, even if the PVE is accommodated mostly within less resistive venous vessels. With the combination of all three stressors, a rise in cardiac output and T_{SKIN} would facilitate higher skin blood flow and volume, and concomitant reduction in CVP. Atrial natriuretic peptide (ANP) release would be suppressed in these circumstances, thereby enhancing renin-angiotensin-aldosterone system (RAAS) activity. Considering that ALD is slow to act (Collins, 1966; Morel and Doucet, 1986; Nagashima et al., 2001), ANP suppression may also contribute to the rise in PV. Following a rise in PV, any ANP-mediated anti-diuresis may be inefficient in the face of a maximally stimulated RAAS (Altenkirch et al., 1990). However, the vasodilatory effects of ANP (Faber et al., 1988) may conceivably contribute to the occurrence of PEH, thus promoting a reduction in arterial pressure without an efficient anti-diuretic response. Histamine-mediated post-exercise vasodilation (Lockwood et al., 2005; McCord et al., 2006; Barrett-O'Keefe et al., 2013) and alterations in baroreflex control (Halliwill et al., 1996) appear to primarily facilitate PEH independent of changes in BV (Gillen et al., 1994). Post-exercise hypotension is therefore a complex

phenomenon involving peripheral and central mechanisms, and its interaction with vascular volumes in still incompletely understood. Hypotheses have postulated to explain apparent links between arterial pressure, volume expansion and sympatho-vagal balance (e.g., Yun et al., 2005) but they are still tentative and warrant further examination.

Perspectives and Summary

The results of the current study indicate the importance and cumulative role of discrete stressors (low-intensity exercise, heat, dehydration) in stimulating acute cardiovascular outcomes. Of note, PEH at 24 h after exercise was affected primarily by the addition of dehydration, with the largest response (and all participants responding) when all stressors were combined, and the largest strain produced. Furthermore, PVE at 24 h after exercise was also largest when exposed to all stressors. The exacerbated strain, particularly thermal, facilitated an increase in ALD, which mediated the rise in PV and was associated with the magnitude of PEH. The current study provided associations (i.e., non-causal) between additive stressors and outcome measures. Future research should investigate dose-response relations between mediating factors of acute PEH and PVE, particularly the occurrence of incremental hyperthermia and these outcomes. The same acute outcomes may also be possible without the imposition of dehydration, but instead increasing T_{CORE} by other means (e.g., more intense exercise or thermal stress). Whilst this is a reasonable assumption, not only does dehydration exacerbate the thermal strain of exercise in the heat (with no/low airflow at least), its independent effects may also provide a conditioning stimulus as detailed above. However, the role of dehydration in the context of acute conditioning is rarely considered. Future research should aim to identify the means by which dehydration may aid (or hinder) adaptation to exercise, rather than its well-established effects on performance acutely. In particular, the additive roles of individual stressors should be examined in a more extensive assessment; including (i) other known cardiovascular (CVP, SV, $\dot{Q}$, TPR) and fluid regulatory (AVP, ANP, ALB) modulators of PEH and PVE, and (ii) the recovery kinetics of body fluid dynamics (across minutes to days), as these are seldom considered (Luttrell and Halliwill, 2015).

Acute periods of high strain, or the resultant outcomes may also be unwarranted in particular individuals. Acute PEH, whilst beneficial to most individuals, would be unwarranted in individuals with hypotension or postural orthostatic tachycardia syndrome (POTS). Similarly, dehydration is unlikely to be recommended for clinical populations, as recurrent dehydration (Clark et al., 2016), or chronic stimulation of fluid- and sodium-conserving hormones may contribute to disease progression (Thornton, 2010). However, in those undergoing conditioning periods (e.g., exercise training), the exacerbated strain may (Garrett et al., 2014) or may not (Neal et al., 2016) elicit larger changes in short-term cardiovascular and hematological adaptive processes. Equally, high levels of hypohydration may also interfere with muscle metabolic control, protein synthesis and hypothalamic adaptations to heat acclimation (Schliess et al., 2006; Schwimmer et al., 2006). Thus, whilst periods of sustained hypohydration are likely unwarranted in certain clinical populations, it is currently unclear whether transient mild dehydration (and the strain therein) plays a substantial role in the process of exercise-associated adaptation (Akerman et al., 2016).

In conclusion, acute cardiovascular and hematological outcomes following exercise therefore appear to be (at least partly) determined by the magnitude of strain induced in the conditioning stimulus. The addition of multiple independent stressors to a low-intensity exercise may exacerbate the strain, and thus the potential adaptive stimulus. This added strain might be useful when exercise performance *per se* is not the primary outcome; in those unable to complete traditional exercise modes (e.g., elderly or injured); are anticipating a novel acute stress (e.g., before a heat wave or surgery); or avoiding intense exercise (e.g., tapering phase before competition for athletes). Future research should investigate whether this complementary response is evident in other modes and intensities of exercise, and what facilitates such adaptive responses.

AUTHOR CONTRIBUTIONS

AA, SL, and JC contributed to the conception and design of the study, acquisition, analysis and interpretation of the data, drafted, revised and approved the final version of the manuscript. RK contributed to analyzing the data (ELISA analysis) and reviewed and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2017.00756/full#supplementary-material

Supplementary Figure 1 | Example progression (from left to right, starting top left corner) of exercises during the 70-min callisthenics period of the exercise protocol.

Supplementary Figure 2 | The effect of heat stress and hydration status on the systolic (sBP; **A**), diastolic (dBP, **B**) and mean arterial blood pressure (MAP, **C**) response to the 90-min exercise condition, and in recovery. Data presented are group mean with individual values overlaid. m denotes male participant. For statistical significance please refer to **Figure 5** in the Manuscript.

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Hypoxic Air Inhalation and Ischemia Interventions Both Elicit Preconditioning Which Attenuate Subsequent Cellular Stress *In vivo* Following Blood Flow Occlusion and Reperfusion

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Ischemic preconditioning (IPC) is valid technique which elicits reductions in femoral blood flow occlusion mediated reperfusion stress (oxidative stress, Hsp gene transcripts) within the systemic blood circulation and/or skeletal muscle. It is unknown whether systemic hypoxia, evoked by hypoxic preconditioning (HPC) has efficacy in priming the heat shock protein (Hsp) system thus reducing reperfusion stress following blood flow occlusion, in the same manner as IPC. The comparison between IPC and HPC being relevant as a preconditioning strategy prior to orthopedic surgery. In an independent group design, 18 healthy men were exposed to 40 min of (1) passive whole-body HPC ($FiO_2 = 0.143$; no ischemia. $N = 6$), (2) IPC ($FiO_2 = 0.209$; four bouts of 5 min ischemia and 5 min reperfusion. $n = 6$), or (3) rest ($FiO_2 = 0.209$; no ischemia. $n = 6$). The interventions were administered 1 h prior to 30 min of tourniquet derived femoral blood flow occlusion and were followed by 2 h subsequent reperfusion. Systemic blood samples were taken pre- and post-intervention. Systemic blood and gastrocnemius skeletal muscle samples were obtained pre-, 15 min post- (15PoT) and 120 min (120PoT) post-tourniquet deflation. To determine the cellular stress response gastrocnemius and leukocyte Hsp72 mRNA and Hsp32 mRNA gene transcripts were determined by RT-qPCR. The plasma oxidative stress response (protein carbonyl, reduced glutathione/oxidized glutathione ratio) was measured utilizing commercially available kits. In comparison to control, at 15PoT a significant difference in gastrocnemius Hsp72 mRNA was seen in HPC (−1.93-fold; $p = 0.007$) and IPC (−1.97-fold; $p = 0.006$). No significant differences were observed in gastrocnemius Hsp32 and Hsp72 mRNA, leukocyte Hsp72 and Hsp32 mRNA, or oxidative stress markers ($p > 0.05$) between HPC and IPC. HPC provided

near identical amelioration of blood flow occlusion mediated gastrocnemius stress response (Hsp72 mRNA), compared to an established IPC protocol. This was seen independent of changes in systemic oxidative stress, which likely explains the absence of change in Hsp32 mRNA transcripts within leukocytes and the gastrocnemius. Both the established IPC and novel HPC interventions facilitate a priming of the skeletal muscle, but not leukocyte, Hsp system prior to femoral blood flow occlusion. This response demonstrates a localized tissue specific adaptation which may ameliorate reperfusion stress.

Keywords: ischemia, oxidative stress, heat shock proteins, hypoxic preconditioning, ischemic preconditioning, knee surgery

INTRODUCTION

Tourniquets are used in several surgical procedures (Fitzgibbons et al., 2012). Relative to total knee replacement (TKR) surgery their use facilitates a near bloodless field, improving visualization of crucial structures, and accelerating the surgical procedure (Smith and Hing, 2010; Estebe et al., 2011). However, the benefits of tourniquet application are not without negative post-surgical side-effects e.g., delayed wound healing, vascular injury, muscular damage, and greater post-operative pain (Estebe et al., 2011; Fitzgibbons et al., 2012). Tourniquet mediated side-effects are not restricted to solely tissue damage (via direct tourniquet tissue compression), but also through ischemic reperfusion (IR) mediated metabolic disruption (Fitzgibbons et al., 2012). Reperfusion of occluded tissue (i.e., the leg after TKR specific tourniquet use) may lead to ischemic injury with activated leukocytes increasing reactive oxygen species (ROS) and/or free-radical formation, consequently resulting in transient elevations in oxidative stress (OS, Grace, 1994). Although ROS are essential for normal cellular signaling (Ray et al., 2012) and demonstrate an association with hormesis (Radak et al., 2008), a rapid increase in ROS can disrupt redox balance, resulting in OS induced protein denaturation and lipid oxidation (Halliwell and Chirico, 1993; Hawkins and Davies, 2001).

Reduced glutathione (GSH) is the most abundant endogenous antioxidant and can acquiesce increases in OS via its oxidation (e.g., cysteine originated reducing equivalent donation of $H^+ + e^-$ to unstable molecules, such as, ROS; i.e., ROS scavenging) to oxidized glutathione (GSSG). The GSH:GSSG ratio can indicate cellular redox balance, with increases in the latter indicative of increased OS and a pro-oxidant state (Fisher-Wellman and Bloomer, 2009). During knee prosthesis implantation surgery, following ∼85 min tourniquet application (thigh), both local (the occluded leg), and systemic (the arm) blood borne free radical generators hypoxanthine and xanthine oxidase increased, whilst GSH:GSSG only demonstrated significant pro-oxidant values locally, all of which were seen 5 min post-reperfusion (Karg et al., 1997). Elevated OS following IR of tissues is associated with inflammation and impaired wound healing (Soneja et al., 2005), and postulated to induce greater post-operative pain (Orban et al., 2006), whilst acquiescing OS reduces oxidant mediated apoptosis (Primeau et al., 2002) and post-operative pain (Waikakul et al., 1999). Interestingly, short non-lethal cycles of IR [ischemic preconditioning (IPC)] can prime the intended tissue and bestow protection for future IR stress (Murry et al., 1986; Saita et al., 2002). This method of IR has been shown to reduce knee surgery mediated increases in OS following tourniquet use when compared to controls (Koca et al., 2011). Furthermore, IPC *in vivo* has been shown as effective in reducing post-operative knee surgery pain and/or corresponding length of hospital stay (Memtsoudis et al., 2010).

Inhaled hypoxic air preconditioning (HPC) can infer cellular tolerance to subsequent hypoxia-mediated OS *in vivo* (Taylor et al., 2012), inducing similar protective effects to those conveyed by IPC (Bushell et al., 2002a,b; Berger et al., 2010; Samaja and Milano, 2015; Verges et al., 2015; Chacaroun et al., 2017), with the simplicity of the intervention (i.e., inhaling hypoxic air) advantageous compared to IPC. At present HPC, unlike IPC, has not been utilized prior to TKR to alleviate IR stress *in vivo*, despite similar protective effects. Characterization of occlusion mediated blood and muscle IR stress responses are heavily biased to OS markers. Given cells can initiate protective mechanisms during OS complimentary to GSH reduction, particularly increasing transcription and translation of a highly conserved cytoprotective family of proteins, known as heat shock proteins (HSPs) (Kalmar and Greensmith, 2009; Morton et al., 2009b), characterization of both OS and HSP responses to TKR like occlusion IR stress appears mechanistically warranted. Indeed, *in vivo* HPC mediated increases in basal HSP72 and hemeoxygenase-1 (HSP32) were both associated with resisting subsequent hypoxia/reoxygenation (HReox, Samaja and Milano, 2015) induced OS upon systemic HReox; attributed to restoring the function of OS mediated denatured proteins (principally HSP72, Taylor et al., 2010a, 2012) and degradation of ROS-producing heme molecules (principally HSP32, Gozzelino et al., 2010; Taylor et al., 2012). Readers are directed to multiple reviews

Abbreviations: CON, control experimental condition; HSP, heat shock protein; Hsp, heat shock protein gene transcript; HPC, hypoxic preconditioning; HPC_I, hypoxic preconditioning experimental condition; IPC, ischemic preconditioning; IPC_I, ischemic preconditioning experimental condition; IR, ischemic reperfusion; HReox, hypoxia/reoxygenation; RT-qPCR, one-step reverse transcription quantitative polymerase chain reaction; OS, oxidative stress; OS, oxidized glutathione; PC, protein carbonyl; ROS, reactive oxygen species; GSH, reduced glutathione; TKR, total knee replacement surgery.

for a detailed overview of the transcription factors and functional roles of HSP72 and HSP32 (Kregel, 2002; Morton et al., 2009b; Gozzelino et al., 2010; Henstridge et al., 2014).

Contextually, the cost of TKR surgery is ∼ $7,500 (Dakin et al., 2012), with the majority of this cost associated with long duration post-operative patient hospitalization (Smith et al., 2008). IPC has some efficacy for positively influencing variables associated with post-operative length of stay, however its administration typically occurs within the operating theater, and thus is not economically viable regarding time (theater availability is finite and often pressurized, operating list congestion, etc.). Therefore, a pre-operative (ideally on the ward) easily administrable preconditioning intervention (i.e., HPC) with positive physiological effects akin to IPC would be advantageous clinically (improved surgical outcome) and financially (reduced post-operative length of stay without increased time within theater). Logistically, HPC may be facilitated by supplementary low O_2 gas inhalation in an equivalent manner to that of supplementary O_2 or medical Nitrous oxide (Berkowitz et al., 1976; Greif et al., 2000), or via inhalation of nitrogen rich gas administered through small portable generators utilized in athletic training (Millet et al., 2010) or by creating rooms of low O_2 concentration via nitrogen gas (Mekjavic et al., 2016; Simpson et al., 2016). Given acute exercise elicits significant OS across populations and modalities (Fatouros et al., 2004; Vincent et al., 2005; Hillman et al., 2011; Taylor et al., 2016), with this increase impacting muscle force production (Powers and Jackson, 2008), the benefits of HPC and IPC as a preconditioning strategies to ameliorate the cellular disruptions to contractile function in this paradigm also warrant further investigation. Physical activity (Debevec et al., 2017) and exercise training reduce OS (Miyazaki et al., 2001; Vinetti et al., 2015), with training induced elevations in intramuscular HSP content (Liu et al., 1999; Morton et al., 2009a) a potential pathway for these benefits (Oksala et al., 2014). As such HPC (and IPC) may provide an alternative strategy for individuals undertaking exercise for health, or athletic performance to reduce the potentially negative impact of acute OS (Braakhuis and Hopkins, 2015; Vinetti et al., 2015).

Therefore, the aim of this study was to examine the effects of interventional HPC and IPC prior to subsequent TKR like tourniquet induced IR stress, in comparison to a control condition using an ecologically valid model. It is hypothesized that a bout of either HPC or IPC would prime the HSP system, providing resistance to the physiological stresses induced via tourniquet ischemia. Furthermore, tourniquet mediated stress would be monitored via associated redox markers (GSH/GSSG, protein oxidation) post-occlusion.

METHODS

Ethical Approval

The protocol was ethically approved by the University of Bedfordshire's Sport and Exercise Science Departmental Human Ethics Committee and all participants signed informed consent in accordance with the ethical standards outlined in the 1964 Declaration of Helsinki.

Participants and General Experimental Controls

Eighteen apparently healthy male participants (see **Table 1** for participant characteristics) volunteered and were subsequently randomly allocated to either control (CON), or an HPC intervention (HPC_I) or an IPC intervention (IPC_I). A standardized meal [cornflakes (50 g), milk (250 mL), and 1 l of water], as employed by others (Foster et al., 2016), was utilized within the experimental design. Relative to HSP and OS outcome variables, their between- and within-subject variation is established at rest (Fisher-Wellman and Bloomer, 2009; Sandström et al., 2009; Taylor et al., 2010b) and in response to stressors (Hillman et al., 2011; Lee et al., 2014; Peart et al., 2015). Accordingly an array of robust and previously employed experimental controls were incorporated within the study design to control for the confounding influences on HSP and/or OS responses of smoking (Anbarasi et al., 2006), caffeine (Whitham et al., 2006), glutamine (Wischmeyer et al., 2001; Zuhl et al., 2015), alcohol (Wu and Cederbaum, 2003), dietary consumption (Kuennen et al., 2011; Marshall et al., 2017), fluid intake (attainment of euhydration; Logan-Sprenger et al., 2015), generic supplementation (Pingitore et al., 2015), prior exercise (Lee et al., 2014), previous environmental (hypoxia and heat) exposures (Gibson et al., 2015b; Lee et al., 2016), and diurnal variation in basal HSP (Taylor et al., 2010a,b, 2011, 2012; Hillman et al., 2011; Costa et al., 2012). Apparent compliance was confirmed in 100% of participants and was monitored via a questionnaire prior to each experimental visit (e.g., CON, HPC_I, or IPC_I).

Tourniquet applications throughout all relevant experimental procedures were produced via a straight 10 cm wide tourniquet cuff (AET, Anetic Aid, Leeds, UK) positioned superiorly to cotton wool padding on the thigh of the right leg, with pressure maintained by means of an electronic tourniquet unit (AET, Anetic Aid, Leeds, UK). During TKR this tourniquet pressure facilitates a bloodless field while minimizing direct compression injury (Worland et al., 1997).

Experimental Design

Participants arrived to the laboratory at 08:30 in a fasted stated (from at least 00:00 to arrival) and consumed the standardized meal for breakfast (this meal was provided again at 13:30 for lunch) and had anthropometric data collected. Blood pressure was recorded at baseline using an aneroid sphygmomanometer. They then rested within the laboratory under standardized environmental conditions until 11:30. At 11:30 participants were positioned in an inclined supine position for the remainder of the experimental protocol. Subsequently, participants rested for 1 h (until 12:30) prior to their 40 min allocated preconditioning intervention (HPC_Ior IPC_I), with CON receiving an extended rest period.

HPC_I

HPC_I participants inhaled ∼14.3% O_2 (simulated altitude of 2,980 m above sea level) for 40 min (from 12:30 until 13:10) in normobaric pressure via an adjustable hypoxicator (Everest Summit II, The Altitude Centre, UK) which produced the necessary hypoxic load via O_2 filtration. Heart rate (HR;

TABLE 1 | Participant characteristic data.

Measure	CON ($n = 6$)			HPC_I ($n = 6$)			IPC_I ($n = 6$)		
	Mean	*SD*	Range	Mean	*SD*	Range	Mean	*SD*	Range
Age (years)	22.2	2.9	18–26	20.8	2.4	19–25	18.5*	0.6	18–19
Height (m)	1.83	0.06	1.75–1.92	1.77	0.10	1.67–1.93	1.79	0.04	1.73–1.83
Mass (kg)	80.4	12.4	62.8–93.4	73.5	8.7	61.9–86.6	76.7	7.8	64.8–87.4
Lean mass (%)	85.8	3.5	79.3–88.5	84.9	5.3	78.9–91.4	86.5	3.0	81.5–90.1
Fat mass (%)	14.2	3.5	11.5–20.7	15.3	5.5	8.6–22.1	13.5	3.0	9.9–18.5
Thigh circumference (cm)	44.7	2.6	40–47	42.8	2.1	40–46	43.3	2.4	39–46
Systolic blood pressure (mmHg)	124.0	3.0	120–129	125.8	1.9	123–129	125.5	2.1	123–129
Diastolic blood pressure (mmHg)	76.2	7.0	65–83	75.0	6.0	65–81	79.7	8.0	70–92

** Significant difference vs. CON ($p < 0.05$).*

$b.min^{-1}$) and peripheral oxyhaemoglobin saturation (SpO_2; %) were measured every 5 min via finger pulse oximetry (Onyx® II 9550, Nonin Medical, USA) throughout the HPC_I intervention.

IPC_I

IPC_I received four cycles of 5 min ischemia and 5 min reperfusion (total of 40 min from 12:30 until 13:10) at 100 mmHg above the participant's systolic pressure on their right leg in line with previous research (Koca et al., 2011).

Upon cessation of the preconditioning intervention (HPC_I or IPC_I) or CON (13:10), participants rested again for 55 min (14:05) prior to their right leg being elevated at 45° for 5 min (14:05–14:10), immediately followed by 30 min (14:10–14:40) tourniquet application, and subsequent 2 h (14:40–16:40) period of reperfusion (**Figure 1**).

Sample size calculations were determined via G.Power 3.1, (Universität Dusseldorf, Germany) (Faul et al., 2009) using data describing changes in Hsp72 mRNA from a publication external to our group (Mestre-Alfaro et al., 2012). For a two tailed test with an alpha of 0.05 and power of 0.80, it was calculated that six participants were required to find an Hsp72 mRNA increase of 3.8-fold significant. This sample size is ≥others in the field (Puntschart et al., 1996; Febbraio and Koukoulas, 2000; Fehrenbach and Northoff, 2001; Fehrenbach et al., 2003b; Liu et al., 2004; Mee et al., 2016).

Blood Sampling

Preparation for Initial Storage

Venous blood was drawn from an antecubital vein into three separate 4 mL Vacuette tubes (Vacuette®, Grenier Bio-One, UK) treated with either K_3EDTA (Hsp), sodium citrate (glutathione) or lithium heparin [protein carbonyl (PC)] with all tubes filled to capacity. Blood samples were obtained (see **Figure 1**) at basal and immediately post-intervention (PoI), whilst also immediately pre-tourniquet application (PrT), 15 min post-tourniquet removal (15PoT), and 120 min post-tourniquet removal (120PoT) (**Figure 1**).

Leukocyte isolation for HSP analysis

Blood treated with K_3EDTA underwent leukocyte isolation utilizing a previously validated technique (Tuttle et al., 2015; Gibson et al., 2015a,b; Mee et al., 2016). Briefly, 1 mL of K_3EDTA blood was added to 1:10 red blood lysis solution (Miltenyi Biotec, UK) and allowed to incubate at room temperature for 15 min, prior to isolation via centrifugation at 400 × g for 5 min at 4°C. Supernatant was removed and the remaining pellet was washed twice in 2 mL of PBS solution (Fisher Scientific, UK) at 400 × g for 5 min at 4°C. The pellet was suspended in 1 mL of PBS and separated equally into two 1.5 mL RNase free microtubes (ThermoFisher Scientific, UK) then centrifuged at 17,000 × g for 5 min at 4°C. The remaining supernatant was aspirated prior to the pellet being completely re-suspended in 200 μL of TRIzol reagent (Sigma Aldrich, Dorset, UK) and stored at −80°C for subsequent RNA extraction.

Glutathione blood samples

Two milliliters of sodium citrate treated blood was immediately added to 8 mL of freshly prepared 5% metaphosphoric acid (Sigma Aldrich, Dorset, UK) and left to incubate on ice for 15 min prior to centrifugation at 12,000 × g for 15 min at 4°C. The clarified supernatant was collected and stored at −80°C until future analysis for total glutathione and GSSG utilizing commercially available kits [Glutathione (Total) Detection Kit, ADI-900-160, Enzo Life Sciences, Exeter, UK].

Protein carbonyl blood samples

A full 4 mL lithium heparinized blood tube was immediately centrifuged at 900 × g for 10 min at 4°C before the plasma was collected and stored at −80°C for future determination of PC concentration utilizing commercially available kits (Protein Carbonyl Colorimetric Assay Kit, 10005020, Caymen Chemical Company, Michigan, USA).

Blood Oxidative Stress Markers

Whole-Blood Glutathione

To determine the concentration of total glutathione, previously obtained supernatant (50 μL) was diluted to 1:40 with assay buffer solution and transferred to a 96-well plate in accordance with the manufacturer's instructions. A standard curve was created through serially diluting 50 μL GSSG standard and 50 μL of assay buffer solution (100–12.5 pmol). A 150 μL mixture of DTNB (5,5′-dithio-bis-2-nitrobenzoic acid) and

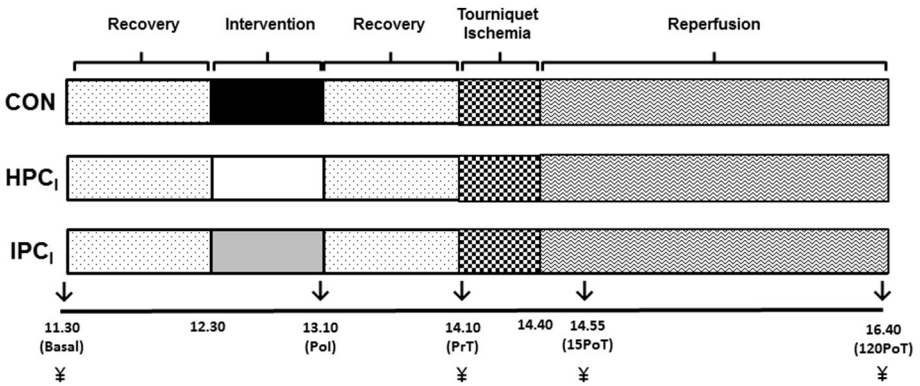

FIGURE 1 | Experimental overview blood samples (↓) were obtained at basal, immediately post-intervention (Pol), immediately pre-tourniquet application (PrT), 15 min post-tourniquet removal (15PoT), and 120 min post-tourniquet removal (120PoT) with gastrocnemius tissue (¥) collected at PrT, 15PoT, and 120 PoT.

10 μL glutathione reductase was added to all wells to produce TNB (5-thio-2-nitrobenzoic acid) and immediately assessed via a microplate reader (Sunrise™, Tecan, Reading, UK) at an absorbance of 405 nm every minute for 10 min. For determination of GSSG, the method outlined above was replicated with the addition of samples first being treated with 1 μL of 2 M 4-Vinylpyridine (Sigma Aldrich, Dorset, UK) to block any free thiols from cycling the reaction. Four microliters of 2 M 4-Vinylpyridine was added to 200 μL of GSSG standard to produce a standard curve. Samples and standards were incubated for 1 h and analyses were identical to the protocol for total glutathione. GSH was calculated via subtraction of GSSG concentrations from total glutathione and a final GSH/GSSG ratio was computed. All standards and samples were run in triplicate and an average was taken. The intra- and inter-coefficient of variance for the assay kits were 3.4 and 3.6%, respectively, in line with previous research (Taylor et al., 2012).

Protein Carbonyl

Plasma (200 μL) was added to 800 μL of 2,4-dinitrophenylhydrazine acting as the sample tube whilst 200 μL of plasma was added to 800 μL of 2.5 M hydrochloric acid to serve as the control tube. All tubes were required to incubate in the dark for 1 h at room temperature with a brief vortex every 15 min. One milliliter of 20% trichloroacetic acid was added to each tube, briefly vortexed and incubated on ice for 5 min prior to centrifugation at 10,000 × g for 10 min at 4°C. This was followed by a 10% trichloroacetic acid wash, incubation on ice for 5 min and centrifuged at 10,000 × g for 10 min at 4°C. Supernatant was discarded and the pellet suspended in a 1:1 ethanol/ethyl acetate wash before undergoing a thorough vortex and centrifugation at 10,000 × g for 10 min at 4°C. This was repeated twice more before the pellet was re-suspended in 500 μL of guanidine hydrochloride and centrifuged at 10,000 × g for 10 min at 4°C. An aliquot of 220 μL of both sample and control was added to a 96-well plate and the absorbance was measured at 360 nm using a microplate reader (Sunrise™, Tecan, Reading, UK). Calculation of PC concentration was determined following the manufacturer's instructions. All samples and standards were analyzed in duplicate. The intra and inter-assay coefficient of variance are 4.7 and 8.5%, respectively.

Muscle Biopsies

Muscle Biopsy Technique and Preparation for Initial Storage

All biopsies were taken by medically qualified Orthopedic Surgeons, with full UK General Medical Council registration. Muscle biopsies were obtained using a previously validated and HSP specific *in vivo* technique (Morton et al., 2006, 2007, 2008, 2009a) applied to the lateral head of the gastrocnemius of the right leg. Biopsies were taken 3 cm apart in a proximal to distal fashion, along an anatomically located muscle mid belly plane under local anesthetic (2% lidocaine hydrochloride). The fascia of the muscle was specifically avoided (Trappe et al., 2013). Disposable manually primed biopsy needle guns were utilized (12 × 16, Disposable Monopty Core Biopsy Instrument, Bard Biopsy Systems, USA). Samples collected (20–30 mg) were immediately frozen in liquid nitrogen (−196°C) and stored at −80°C for later analysis. Serial biopsies separated by 3 cm have been previously demonstrated not to provoke stress proteins in residual tissue (Khassaf et al., 2001). Muscle was obtained at PrT, 15PoT, and 120PoT (**Figures 1**, **3**).

Muscle samples were later ground under liquid nitrogen to remove non-muscle (i.e., adipose, connective) tissue prior to homogenization with a sonicator (T10 Basic, IKA, ThermoFisher Scientific, Loughborough, UK) on ice in 1 mL TRIzol reagent, followed by a 10 min incubation period on ice, in preparation for RNA extraction.

RNA Extraction Blood and Muscle Samples

RNA was extracted utilizing a previously validated (Chomczynski and Sacchi, 1987) technique that has been utilized specifically for HSP assessment *in vivo* (Tuttle et al., 2015; Gibson et al., 2015a,b; Mee et al., 2016). Briefly, chloroform (Sigma Aldrich, Dorset, UK) was added to (200 μL for muscle; 40 μL for leukocytes) samples suspended in TRIzol reagent, then vortexed and left to incubate on ice for 10 min prior to centrifugation at 17,000 × *g* for 15 min at 4°C. The aqueous phase was carefully aspirated

and equal volume of ice-cold 2-propanol (Sigma Aldrich, Dorset, UK) was added before a 15 min incubation period on ice and subsequent centrifugation at 17,000 × g for 15 min at 4°C. The supernatant was removed and the sample was washed with (1 mL for muscle; 100 μL for blood) ice-cold 75% ethanol (Sigma Aldrich, Dorset, UK) ahead of centrifugation at 5,400 × g for 8 min at 4°C. Two additional ethanol washes were performed. Remaining ethanol was aspirated and the pellet was allowed to air dry for 15 min prior to the addition of 50 μL of RNA storage solution (Invitrogen, Paisley, UK). RNA quantity and quality were assessed optically at a density of 260 nm and ratios of 260/280 and 260/230, respectively, utilizing spectrophotometry (Nanodrop 2000c, ThermoFisher Scientific, Loughborough, UK). Only samples with a 260:280 ratio of between 1.9 and 2.15 were carried forward for reverse transcription and PCR amplification detailed below.

One-Step Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Primers (**Table 2**) were designed using primer design software (Primer Quest and Oligoanalyzer-Integrated DNA technologies) and have been recently utilized elsewhere (Tuttle et al., 2015; Gibson et al., 2015a,b; Mee et al., 2016). During primer design, in line with (Tuttle et al., 2015), sequence homology searches were performed against the GenBank database to ensure the primers matched the gene of interest. Primers were designed to span exon-intron boundaries and avoided three or more guanine-cytosine bases within the last five bases at the 3' end of primer to avoid nonspecific binding. Further searches were performed to ensure primers did not contain secondary structures and intermolecular or intramolecular interactions (hairpins, self-dimer, and cross dimers), which can inhibit product amplification.

Relative Hsp mRNA expression was then quantified using RT-qPCR adhering to the method adopted by Tuttle et al., (2015). Reactions (20 μL) containing 10 μL of SYBR Green RT-PCR Mastermix (Quantifast SYBR Green kit; Qiagen, Manchester, UK), 0.15 μL of forward primer, 0.15 μL of reverse primer, 0.2 μL of reverse transcription mix (Quantifast RT Mix, Qiagen), and 9.5 μL sample (70 ng RNA/μL) were prepared using the Qiagility automated pipetting system (Qiagen). Each reaction was amplified in a thermal cycler (Rotorgene Q, Qiagen) and involved reverse transcription lasting 10 min at 50°C and a transcriptase inactivation and initial denaturation phase lasting 5 min at 95°C. The PCR reaction then followed with a denaturation step lasting 10 s at 95°C and a primer annealing and extension stage lasting 30 s at 60°C repeated for 40 cycles. Fluorescence was measured following each cycle as a result of the incorporation of SYBR Green dye into the amplified PCR product. Melt curves (50–95°C; Ramp protocol, 5-s stages) were analyzed for each reaction to ensure only the single gene of interest was amplified.

The relative quantification of mRNA expression for each sample was assessed by determining the ratio between the cycle threshold (CT)-value of the target mRNA and the CT-values for β2-microglobulin. Fold change in relative mRNA expression was calculated using the 2-ΔΔCT method (Schmittgen and Livak, 2008).

Statistical Analyses

All data was analyzed using the statistical software package IBM SPSS version 19.0 (SPSS Inc., Chicago IL, USA). Prior to any performance of inferential statistics, descriptive tables and graphical methods (Q–Q plots and scatter plots) were utilized to check for statistical assumptions with all data presented deemed to be normally distributed. A one-way ANOVA was used to assess for statistical differences between participants' anthropometric data. One-way repeated measures ANOVA was utilized to establish significant differences between hemoglobin saturation and HR during the hypoxic intervention period. Sphericity was assumed for all repeated measures analysis. Linear mixed models were used to identify significant group × time interactions in the remaining dependent variables across all groups. In the event of a significant F ratio for both linear mixed models and one-way repeated measures ANOVA, the *post-hoc* test Sidak was used to locate significant pairs. The most suitable covariant model was decided using the difference in −2 restricted log likelihood figures and the number of parameters of the two models tested against the χ^2 critical statistic (Field, 2013). Furthermore, residuals were checked for normality and homogeneity of variance using Q-Q plots and scatter plots, respectively, and were considered plausible for all dependant variables. Statistical significance was assumed at $p < 0.05$. Finally, Cohen's effect sizes for independent means were calculated utilizing the formula outlined by Cohen and were established as: small ($d = 0.2$), medium ($d = 0.5$), and large ($d = 0.8$) effects (Cohen, 1992).

RESULTS

A significant difference was observed in age [$F_{(2, 15)} = 4.36$, $p = 0.032$] between IPC_I and CON ($p = 0.032$). No other significant differences ($p \geq 0.34$) were noted in participant demographics (**Table 1**).

A significant main effect displayed a decrease in hemoglobin saturation [$F_{(8, 40)} = 17.331$, $p < 0.001$] between baseline and all subsequent time points ($p < 0.05$) in the HPC_I intervention. However, there was no significant main effect [$F_{(8, 40)} = 1.130$, $p = 0.365$] in HR noted by the same exposure (**Figure 2**).

Significant group × time interaction effects ($F = 3.058$, $p = 0.048$) were observed in muscle Hsp72 relative gene expression. There was an increase between time-points PrT and 15PoT (95% CI −3.771, −0.124; $p = 0.035$) in CON displaying a large effect size (1.44). Also, between PrT and 120PoT, a pronounced 116% increase (95% CI −3.779, −0.400; $p = 0.014$) was noted in IPC_I producing a large ES (1.59). Furthermore, when compared to CON at 15PoT muscle Hsp72 was lower in HPC_I (95% CI 0.634, 3.934; $p = 0.007$) and IPC_I (95% CI 0.675, 4.114; $p = 0.006$), both demonstrating large effect sizes (1.90 and 2.19, respectively) (**Figure 3**).

There were no significant ($p > 0.05$) group × time interaction effects for gastrocnemius Hsp32 ($F = 0.147$, $p = 0.961$) (**Figure 3**) leukocyte Hsp72 ($F = 1.195$, $p = 0.347$), leukocyte Hsp32 ($F = 1.406$, $p = 0.244$), PC ($F = 0.681$, $p = 0.707$), or GSH/GSSG ($F = 1.959$, $p = 0.105$) (see **Table 3**).

TABLE 2 | Primer sequences used in One-step reverse transcription quantitative polymerase chain reaction.

Target gene	Primer sequence (5′-3′)	Reference sequence no.	Amplicon length (bp)	GC% content
B_2 microglobulin	Forward: CCGTGTGAACCATGTGACT	NM_004048	19	52.63
	Reverse: TGCGGCATCTTCAAACCT		18	50.00
HSP 72	Forward: CGCAACGTGCTCATCTTTGA	NM_005345	20	50.00
	Reverse: TCGCTTGTTCTGGCTGATGT		20	50.00
HSP32	Forward: CAGCAACAAAGTGCAAGAT	NM_002133	19	42.11
	Reverse: CTGAGTGTAAGGACCCATC		19	52.63

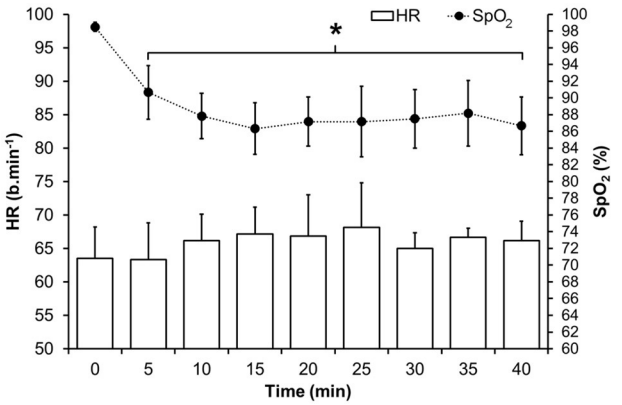

FIGURE 2 | Mean (*SD*) HR and oxyhaemoglobin saturation during the hypoxic intervention in HPC_I. *Indicates significant difference ($p < 0.05$) vs. baseline values.

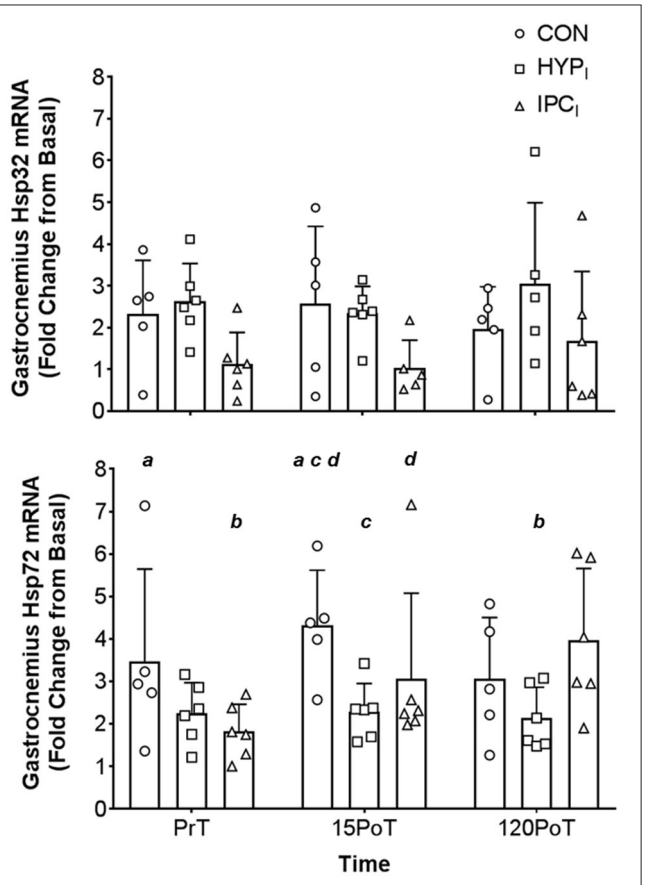

FIGURE 3 | Mean (*SD*) gastrocnemius Hsp32 mRNA **(top)** and Hsp72 mRNA **(bottom)** expression. Gastrocnemius samples were obtained from CON ($n = 5$), HPC_I ($n = 6$), and IPC_I ($n = 6$) immediately pre-tourniquet application (PrT), 15 min post-tourniquet removal (15PoT), and 120 min post-tourniquet removal (120PoT). All experimental samples were expressed relative to a laboratory control gastrocnemius sample (from Basal). Letters (a, b, c, d) denote significant differences between corresponding letter ($p < 0.05$).

DISCUSSION

In agreement with the presented hypothesis it is a novel experimental finding that both IPC_I and HPC_I primed the localized (gastrocnemius) HSP system producing a blunted response in Hsp72 mRNA post (15PoT) 30 min TKR like femoral blood flow occlusion (termed simply occlusion from this point forward) compared to CON (**Figure 3**). However, changes in local Hsp32 were not seen (**Figure 3**), contrary to the stated hypothesis, this lack of response may be indicative of minimal intramuscular OS within the gastrocnemius following 30 min of occlusion. This amelioration in Hsp72 response (15PoT) was seen without systemic changes in OS (PC and GSH:GSSG; **Table 3**) and leukocyte Hsp32, in agreement with the stated hypothesis.

HSPs are up-regulated in response to a variety of stressors relevant to IR and HReox mediated *in vivo* stress, including OS, hypoxia, and ischemia (Kalmar and Greensmith, 2009; Morton et al., 2009b; Gibson et al., 2017). Elevated localized Hsp72 mRNA in CON at 15PoT (+84 ± 78%) indicates that the occlusion stressor was sufficient to potentiate initiation of the heat shock response (Theodorakis et al., 1999; Noble et al., 2008), whilst amelioration of this response (indicative of early phase IPC tissue protection; Bushell et al., 2002a,b; Loukogeorgakis et al., 2005) was seen when occlusion was preceded by IPC_I (+19 ± 35%) or (HPC_I +7 ± 33%; **Figure 3**). This change in Hsp72 mRNA within CON was not confounded by the larger basal gene expression of one participant in this group at this time point. Amelioration of Hsp72 mRNA is not novel regarding efficacy of IPC to blunt the respective gene response post-reperfusion of occluded distal lower limb skeletal muscle (rat tibialis anterior) (Bushell et al., 2002a,b) however, blunting of

TABLE 3 | Mean (*SD*) circulatory markers of redox disturbance and stress protein expression.

Measure	Basal			Pol			PrT			15PoT			120PoT		
	CON	HPC_I	IPC_I	CON	HPC_I	IPC_I	CON	HPC_I	IPC_I	CON	HPC_I	IPC_I	CON	HPC_I	IPC_I
Leukocyte Hsp72 (relative fold change from basal)	1.46 (0.42)	1.29 (0.39)	1.72 (0.54)	1.46 (0.55)	1.23 (0.44)	1.31 (0.17)	1.43 (0.41)	1.45 (0.36)	1.31 (0.35)	1.44 (0.43)	1.57 (0.31)	1.33 (0.14)	1.23 (0.44)	1.35 (0.27)	1.35 (0.21)
Leukocyte Hsp32 (relative fold change from basal)	1.34 (0.43)	1.08 (0.24)	1.47 (0.55)	1.09 (0.33)	1.14 (0.24)	1.23 (0.40)	0.86 (0.14)	1.10 (0.28)	1.29 (0.29)	0.86 (0.28)	1.24 (0.27)	1.17 (0.33)	1.04 (0.45)	1.02 (0.41)	1.08 (0.38)
Protein Carbonyl ($nmol{\cdot}mL^{-1}$)	0.56 (0.14)	0.56 (0.19)	0.69 (0.24)	0.54 (0.16)	0.42 (0.13)	0.61 (0.13)	0.64 (0.13)	0.58 (0.08)	0.69 (0.21)	0.65 (0.18)	0.60 (0.24)	0.56 (0.15)	0.65 (0.32)	0.69 (0.15)	0.63 (0.21)
Reduced/oxidized glutathione ratio	23.0 (9.1)	31.4 (12.2)	22.7 (8.6)	24.6 (9.6)	29.3 (5.4)	28.9 (7.4)	27.1 (11.6)	23.9 (7.3)	18.6 (7.6)	27.6 (10.7)	20.5 (3.6)	23.1 (8.7)	23.0 (7.7)	22.4 (2.2)	20.5 (9.6)

Measurements were evaluated at basal, immediately post-intervention (Pol), immediately pre-tourniquet application (PrT), 15 min post-tourniquet removal (15PoT), and 120 min post-tourniquet removal (120PoT).

the Hsp72 mRNA stress response *in vivo* via HPC_I within the presented paradigm is. It is ecologically relevant that the blunted response in $HPC_I$is contained within a time frame whereby surgery may be performed i.e., up to 120PoTwith the time course of responses comparable to those observed in similar Hsp72 mRNA experiments (Febbraio et al., 2002; Tuttle et al., 2017). Mechanistically, this intervention mediated reduction in Hsp72 mRNA cannot be attributed to systemic reduction in OS (PC or GSH:GSSG; see **Table 3**) nor increased degradation of ROS-producing heme molecules by Hsp32 mRNA, as these outcome variables were unchanged within and between all conditions and time points (**Table 3**, **Figure 3**). However, the absence of localized measures of OS precludes inferences relative to a local to systemic difference in OS (although others have shown such a difference post knee surgery which utilized a tourniquet; Karg et al., 1997) mediating the local to systemic differential Hsp72 mRNA response at 15PoT. Given the high affinity for changes in OS to induce a HSP32 and Hsp32 mRNA heat shock response (Gozzelino et al., 2010; Taylor et al., 2012), and the absence of changes in Hsp32 mRNA locally (and systemically), it is likely local Hsp72 mRNA amelioration via the interventions at 15PoT is mechanistically distinct from OS—at least within the occlusion paradigm utilized within the present design.

IPC has been cited to diminish circulatory redox disturbances associated with tourniquet-induced IR stress following TKR surgery (Koca et al., 2011). This is in disagreement with the current study, whereby it was noted that systemic OS markers remained unchanged within and between conditions at all times points when assessing lipid peroxidation (malondialdehyde concentrations) via measurement of thiobarbituric acid-reactive substances (Koca et al., 2011). This particular method of assessing malondialdehyde lacks specificity (Powers et al., 2010b), as such, the changes noted may be due to methodical inaccuracies rather than experimental effect. Additionally, systemic OS may not be a reliable surrogate marker for an intracellular response as aforementioned. It is however plausible that the 30 min occlusion utilized by the present study did not induce sufficient stress to observe changes in systemic OS markers and leukocyte Hsp mRNA (**Table 3**). This occlusion duration is lower than that commonly used during TKR surgery (mean ± *SD*; 79.9 ± 12.7 min; Cheng et al., 2003). Therefore, it is expected that the OS would be greater during surgery thus the occlusion stressor in the present study is not completely externally valid. However, our pilot testing revealed a longer occlusion period was not tolerable *in vivo* by non-anesthetized humans thus precluding its implementation. In an exercise setting, variants of our IPC (de Groot et al., 2010; Bailey et al., 2012; Cruz et al., 2015; Kido et al., 2015; James et al., 2016; Sabino-Carvalho et al., 2016), and HPC (Lee et al., 2014; Turner et al., 2016; Chacaroun et al., 2017), have been implemented to induce positive physiological responses. These data reflecting positive responses in clinical paradigms (Landry et al., 1982; Tomai et al., 1999; Otani, 2008; Mateika et al., 2014; Verges et al., 2015; Baillieul et al., 2017). Our data present a novel contribution to the area demonstrating the potential facilitative role HSPs have in response to OS.

Early phase IPC protection is temporally aligned to a 1–2 h window (occlusion occurred within this window) post IPC,

whilst late phase IPC protection commences ∼24 h post IPC and has a window of effect between ∼24 and ∼72 h post IPC. This late phase protection is dependent on the induction of protective proteins (Loukogeorgakis et al., 2005) including HSP72 (Bushell et al., 2002a,b; Marber et al., 1993, 1995). HSP72 is thought to refold sub-lethally damaged proteins and diminish their interactions with viable proteins during repeated IR bouts (Marber et al., 1993), consequently conveying cellular protection. The cumulative IR signal in IPC_I [i.e., five distinct IR stressor bouts; intervention (four IR cycles) and occlusion (one 30 min IR cycle)] compared to HPC_I [occlusion (one 30 min IR cycle) preceded by interventional HReox] is greater. Therefore, intervention activated IR preconditioning specific biological processes may have been exacerbated (particularly IR mediated cyclical increases in bradykinin and adenosine; Loukogeorgakis et al., 2005) by the subsequent occlusion IR stress and may underpin the enhanced gastrocnemius Hsp72 mRNA transcription seen at 120PoT in IPC_I, a response absent in HPC_I. This could be an early transcriptional level initiation of late phase IPC, within HPC_I, to which HSP72 protein translation is central (Loukogeorgakis et al., 2005). Therefore, this enhanced Hsp72 mRNA transcription at 120PoT could be interpreted as a priming mechanism for late phase IPC HSP72 translation within IPC_I, a response absent within HPC due to a lack of sufficient IR signal. In support of this postulation it has been eloquently shown elsewhere that remote limb IPC within humans results in cumulative up-regulation of peptides, with the magnitude of increase dependent on the number of discrete IR bouts (Hepponstall et al., 2012). Future research should examine the accumulative and temporally ordered effects (in line with recent attempts within a rat model; Kocman et al., 2015) of IPC (IR) and HPC (HReox) conveyed preconditioning *in vivo* within humans. A specific emphasis should be placed on adenosine, bradykinin, extracellular signaling proteins (ERK1/2, AKT; Winter et al., 2016) and relevant gene transcription and translation relative to the temporally ordered protective effects (i.e., early and late phase IPC tissue protection; Loukogeorgakis et al., 2005). The lack of such an approach is a limitation of the present study.

Prior research has shown that both acute hypoxic exposure (Taylor et al., 2010a) and IPC (Konstantinov et al., 2004) can stimulate leukocyte HSP72, which is in contrary to the data presented here (**Table 3**). Indeed in support of an association between OS and HSP responses antioxidant elevations are associated with leukocyte Hsp72 reductions (i.e., a lesser necessity for HSP72 transcription), thus OS necessitating HSP transcription could increase Hsp72 in leukocytes (Simar et al., 2012). Likewise antioxidant supplementation (500 $IU.day^{-1}$ RRR-a-tocopherol for 8 days) attenuates the change in leukocyte Hsp72 mRNA after exhaustive exercise (Niess et al., 2002). The extended 75 min hypoxic exposure (approximately the same 14.3% O_2 as used within the present study) utilized previously (Taylor et al., 2010a), would have induced a more substantial OS stimulus for the induction of Hsp72, compared to the 40 min exposure utilized within HPC_I in the present study. Given the very nature of IPC, and the lack of a change in circulating markers of OS, it is possible that the leukocytes were unable to experience the necessary fluctuations in OS that occurred locally within the skeletal muscle undergoing biopsy. As such it may be an experimental artifact associated with IPC alone that is responsible for the lack of Hsp72 mRNA responses in leukocytes rather than a mechanistic one. Utilizing a multi-gender sample and gene array analysis to assess Hsp72 (Konstantinov et al., 2004), with the former known to influence stress mediated changes in HSP (Paroo et al., 2002; Morton et al., 2009a) and the latter known to both over and underestimate gene transcript response (Feldman et al., 2002), may also explain some of the disparity in Hsp72 mRNA results.

Given that systemic OS was unchanged within and between all conditions (in line with previous data; Karg et al., 1997), it is likely that this absence of the potent OS stimuli (particularly ROS producing free-heme molecules) underpinned the lack of leukocyte Hsp32 mRNA response (Fehrenbach et al., 2003a) (**Table 3**). Although local OS markers were not obtained, absence of muscle Hsp32 mRNA change suggests only minimal (if any) disturbances in OS locally. To determine whether the 30 min occlusion was sufficiently stressful to induce intramuscular OS, future work should measure markers of this within the target tissue. To the authors' knowledge measurement of leukocyte and skeletal muscle Hsp32 mRNA within the presented *in vivo* occlusion paradigm is novel, with previous rodent model data lacking external validity to the presented TKR specific rationale (particularly time course). Rodent model data indicates that with five cycles of IPC (compared to the present study), muscle Hsp32 protein displays a 2-fold increase with glutathione remaining unchanged, albeit only assessed 48 h post IPC (Badhwar et al., 2004). The stable Hsp32 mRNA-values could be a fiber type specific response, with type I muscle fibers shown to readily express HSP32 in comparison to a blunted response in type II fibers (Vesely et al., 1999). The lateral head of the gastrocnemius consists of equal proportions of both fiber types (Edgerton et al., 1975). Therefore, the response observed in the present study may only be proportional to the percentage of type II fibers in the biopsied muscle. A lack of fiber type characterization within the gastrocnemius samples within the present study is an experimental limitation that should be addressed by future research designs.

HPC has been shown to blunt IR mediated tissue damage in animal models (Beguin et al., 2005; Berger et al., 2010), however, to the author's knowledge, no previous studies have performed HPC in humans prior to limb-tourniquet application and subsequent IR stress. HPC is thought to confer cellular protection through similar mechanisms to IPC, essentially hormesis from appropriate protein accumulation (the precise mechanism/stimuli for such accumulation are not robustly described *in vivo*). The present data provides provisional evidence that *in vivo* HPC prior to IR stress provides similar reductions in localized cellular stress as an established IPC model. Interestingly, IPC has preliminary *in vivo* evidence supporting its use to reduce post-operative pain following TKR surgery (Memtsoudis et al., 2010), although some equivocal evidence is also present (Memtsoudis et al., 2014). Practically, IPC involves close monitoring to ensure correct ischemic periods (typically within theater), yet the HPC_I protocol used here would not require this human resource as the inhalation of hypoxia is

continuous, thus potentially allowing HPC to take place during surgical preparation (perhaps on the ward utilizing methods described earlier). It should be noted that previous work has utilized intermittent hypoxia as a preconditioning strategy, future experimental designs should make comparisons between continuous and hypoxic interventions as previously utilized (albeit with differing clinical applications) in animals (Beguin et al., 2005) and humans (Lyamina et al., 2011; Chacaroun et al., 2017), to determine which elicits the most desirable preconditioning responses. From a practical perspective an intermittent approach may be less practical to administer in clinical setting if using the current equipment i.e., it would require manually switching between hypoxic and normoxic air. However, further experimentation is required to demonstrate whether localized reductions in cellular stress (Hsp72 mRNA) from HPC_I (as also seen in IPC_I) can convey the same positive effects (reduced pain, accelerated wound healing, reduced length of hospital stay, as raised within the Introduction and Discussion sections) attributed to IPC, within externally valid models related to TKR like knee surgery which utilizes a tourniquet. Indeed, concerns could be raised to the safety of exposing elderly patients (population most likely to receive TKR surgery for example) to acute hypoxia. However, research has demonstrated that an elderly population with a high prevalence of cardiovascular disease tolerated hypobaric hypoxic remarkably well (Levine et al., 1997). Given that exercise and heating initiates increases in Hsp72 mRNA (Fehrenbach et al., 2001; Gibson et al., 2015a,b), in a temperature dependent manner (Gibson et al., 2016), passive heating [which has also been shown to increase Hsp72 mRNA; Horowitz et al., 1997; Maloyan et al., 1999] may also provide a viable preconditioning strategy that may be applied on the ward prior to surgery. In addition to the application presented (i.e., reperfusion) both HPC and IPC appear to initiate cellular responses (HSP increases) which are likely beneficial in across stimuli e.g., exercise, hypoxia, heat, though this is yet to be experimentally elucidated.

Although adequately powered it is important that the present data and postulations relative to the use of HPC are viewed relative to this sample size and preliminary nature of the experimental findings/design. It has been previously observed there can be high variability between participants with regards to basal and stress-mediated values of these measures, this variation based upon individual differences in physiological profile (Fehrenbach et al., 2000a,b, 2003a; Bruce et al., 2003) which require consideration relative to the presented data and discussion of the application of HPC vs. IPC prior to surgery. These reasons may explain the high basal Hsp72 mRNA content in one CON group participant in the present study. As such further work with populations displaying more diverse phenotypes are warranted. In additions to experimental limitations acknowledged throughout the discussion, given many antioxidants are procured naturally from the diet, all with varying half-lives, this could have influenced OS related outcome variables within the present study (Powers et al., 2010a). Although every effort was made to control this (standardized morning and afternoon meal), it is extremely challenging to control participants' diet over a long period of time, while ensuring continued participation. Therefore, the lack of change in OS markers could have partly been due to dietary variation.

In summary, it can be seen that a bout of HPC_I primed the HSP system thus bestowing localized cellular protection to tourniquet IR mediated stress induced via an ecologically valid TKR model. Furthermore, HPC_I provided similar levels of cellular protection to IPC_I, thus providing a novel framework for the use of HPC_I to convey cellular protection in light of a subsequent IR related stressor.

AUTHOR CONTRIBUTIONS

JB participated in the study conception, methodological optimization, data collection, sample and statistical analysis and manuscript drafting and revisions. OG participated in the statistical analysis and manuscript drafting and revisions. JT aided in data collection, sample and statistical analysis and manuscript revision. BC assisted with statistical analysis and manuscript revision. OP, JP, SG, CK, NG, FR, ZO, AS, and SN contributed to the study conception and optimization of muscle sample collection. LT participated in study conception, optimization of muscle sample collection, manuscript drafting and revision. All authors have read and approved the final manuscript.

AVAILABILITY OF DATA AND MATERIALS

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

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REVIEW
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Heat Acclimation-Mediated Cross-Tolerance: Origins in within-Life Epigenetics?

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The primary outcome of heat acclimation is increased thermotolerance, which stems from enhancement of innate cytoprotective pathways. These pathways produce "ON CALL" molecules that can combat stressors to which the body has never been exposed, via cross-tolerance mechanisms (heat acclimation-mediated cross-tolerance—HACT). The foundation of HACT lies in the sharing of generic stress signaling, combined with tissue/organ- specific protective responses. HACT becomes apparent when acclimatory homeostasis is achieved, lasts for several weeks, and has a memory. HACT differs from other forms of temporal protective mechanisms activated by exposure to lower "doses" of the stressor, which induce adaptation to higher "doses" of the same/different stressor; e.g., preconditioning, hormesis. These terms have been adopted by biochemists, toxicologists, and physiologists to describe the rapid cellular strategies ensuring homeostasis. HACT employs two major protective avenues: constitutive injury attenuation and abrupt post-insult release of help signals enhanced by acclimation. To date, the injury-attenuating features seen in all organs studied include fast-responding, enlarged cytoprotective reserves with HSPs, anti-oxidative, anti-apoptotic molecules, and HIF-1α nuclear and mitochondrial target gene products. Using cardiac ischemia and brain hypoxia models as a guide to the broader framework of phenotypic plasticity, HACT is enabled by a metabolic shift induced by HIF-1α and there are less injuries caused by Ca^{+2} overload, via channel or complex-protein remodeling, or decreased channel abundance. Epigenetic markers such as post-translational histone modification and altered levels of chromatin modifiers during acclimation and its decline suggest that dynamic epigenetic mechanisms controlling gene expression induce HACT and acclimation memory, to enable the rapid return of the protected phenotype. In this review the link between *in vivo* physiological evidence and the associated cellular and molecular mechanisms leading to HACT and its difference from short-acting cross-tolerance strategies will be discussed.

Keywords: heat acclimation, heat acclimation-mediated cross-tolerance, epigenetic mechanisms of gene expression, HSP72, HIF-1α, attenuated Ca^{2+} overload injuries

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INTRODUCTION

Research into the important topic of acclimation/acclimatization to an adverse environment began in the late Nineteenth century, in the wake of colonialization, and the necessity to adapt to harsh occupational environments in hot tropical countries. The concept was the subject of medical and scientific debates. Based on the experimental physiological evidence existing at the time, the

interactions between stressor(s) and the corresponding integrative response(s) were termed, "cross-adaptation" and/or "cross-tolerance" (Adolpf, 1964; Fregly, 1996), even though the exposures occurred over varying lengths of time, and no differentiation was made between simultaneous or sequential exposures to the co-adaptagents.

This lack of clarity in definition enabled multiple interpretations of the relevant terms. For example, some authors use "cross-acclimation" when physiological strain is attenuated (Lee et al., 2016) while others employ "cross-tolerance" when improved cellular protection is observed (Selye and Bajusz, 1961; Ely et al., 2014). Another phenomenon included within the concept of cross-tolerance, formerly termed "cross-resistance" [originally defined by Selye in 1961 (Selye and Bajusz, 1961)], is now known as "preconditioning" (Murry et al., 1986) or "hormesis", entailing adaptation to a high, even lethal dose of a stressor, following exposure to a lower dose of that stressor (Calabrese, 2016). To further complicate matters, physiologists use "preconditioning", while its synonym, "hormesis," was adopted by biochemists and toxicologists. Both describe *a rapidly evoked* cellular strategy that ensures homeostasis, in contrast to acclimation-induced cross-tolerance, which, takes place after acclimation homeostasis has been achieved (**Figure 1**).

Advances in our understanding of cellular, molecular and epigenetic mechanisms over the last century, have enabled researchers to fine-tune the meanings of these terms. Accordingly, this mini-review aims to clarify the differences between acclimation and environmentally induced cross-tolerance and other modes of cross-tolerance, using as models heat acclimation-mediated cross-tolerance in rodents and in humans. To do so, the link between *in vivo* physiological evidence and the associated cellular and molecular mechanisms will be discussed, initially regarding specific stress-protected targets, and then within the broader framework of acclimatory phenotype plasticity.

HEAT ACCLIMATION-MEDIATED CROSS-TOLERANCE VS. PRECONDITIONING-INDUCED CROSS-TOLERANCE

Heat acclimation (HA) is a reversible, within-lifetime phenotypic adaptation to high ambient temperatures. The primary physiological signature of HA is increased heat endurance via prolongation of the operating thermoregulatory span of physiological mechanisms, combined with improved thermotolerance associated with enhanced molecular cytoprotective mechanisms (Horowitz, 2014, 2016). An important feature of the HA phenotype is the acquired cytoprotection to novel stressors (i.e., stressors to which the body has never been exposed) via cross-tolerance mechanisms (so-called heat acclimation-mediated cross-tolerance, or HACT). This cytoprotection stems from the enhancement of innate cytoprotective pathways that also confer cellular thermotolerance. Based on a sharing principle, these cytoprotective pathways produce "on call" molecules to counteract the consequences of other forms of stress. Gene chip experiments and bioinformatic analyses have collectively

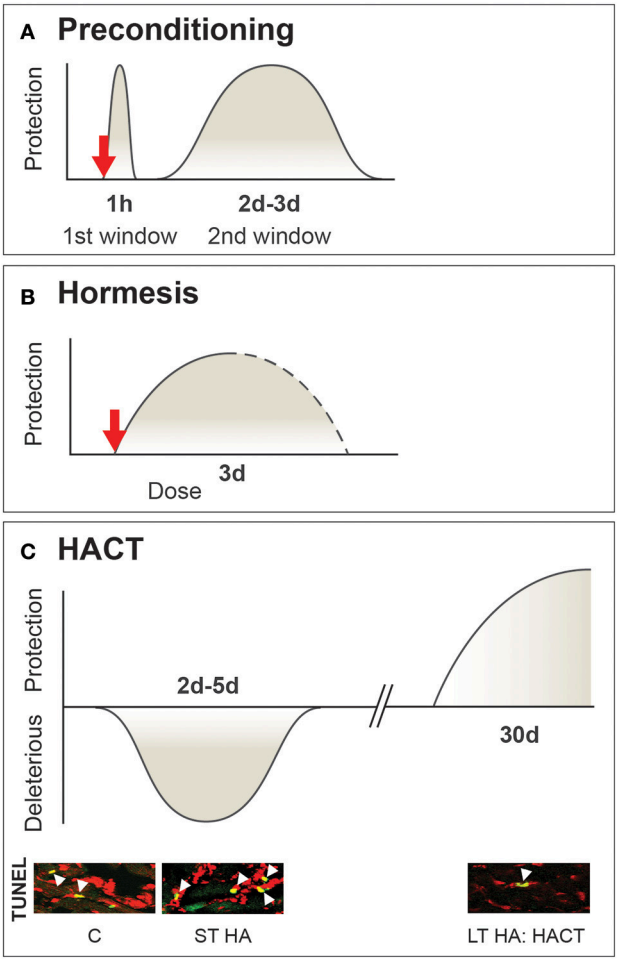

FIGURE 1 | Multifaceted timeframe and mechanisms of β adrenergic signaling cross-tolerance While preconditioning and hormesis are transient short acting cross-tolerance strategies activated upon perturbations to defense cellular homeostasis, HACT takes place only when acclimatory homeostasis has been achieved. **(A)** Preconditioning, with sub-lethal stress induces two windows of protection. The first involves adenosine receptors, K_{ATP} mitochondrial channels, and salvage kinases. The second (delayed) window of protection is associated with transcriptional and translational processes involving cytoprotective molecules. **(B)** Hormesis a dose-dependent stress response initiated upon stress exposure (i.e., toxins only). **(C)** HACT, established following the bi-phasic HA acclimation process, initially signified by enhanced neural activity and triggered molecular processes to maintain DNA integrity. These processes are replaced by the enhancement of innate cytoprotective features. HA temperature is the upper point of the temperature neutral zone (TNZ) of the species. Based on (Horowitz, 1998; Yellon and Downey, 2003; Bolli, 2007; Calabrese et al., 2007).

Abbreviations: HA, heat acclimation; HACT, heat acclimation mediated cross-tolerance; HAEX, heat acclimation combined with exercise training; EX, exercise training; COX, cytochrome c oxidase; HIF-1α, hypoxia inducible transcription factor 1α; HSP, heat shock protein (number denotes molecular weight); NMDA, N-methyl-D-aspartate; ROS, reactive oxygen species.

demonstrated that HACT is conferred by sharing generic signaling pathways involved in the stress response, with tissue-specific protective pathways (Horowitz et al., 2004). Evidence from animals and humans confirms its effectivity in cardio- and neuroprotection under conditions of hypoxic, hyperoxic, ischemic and traumatic brain injury (TBI; Horowitz, 2014).

HACT (as well as acclimation-induced cross-tolerance in general) differs from the cross-tolerance that occurs following classical preconditioning [analogous to the heat shock response (HSR)], a two-window transient effect (**Figure 1A**) evoked by short exposures to sublethal stress, thus protecting the subject from an otherwise fatal exposure to a second stress, either similar or different in nature from the original conditioning stress (Bolli, 2007). The first window of protection, involving salvage kinases and adenosine receptors, is rapid, and lasts for ∼1 h. The second window of protection appears after 24 h, lasts ∼48 h, and relies on transcriptional activation of cytoprotective pathways (Das and Das, 2008).

In contrast, HACT develops slowly during exposure to the acclimating stressors, and is shaped by the nature of the acclimation, as it occurs. HA is a biphasic process, with an initial, transient phase, during which intensive neural stimulation controls physiological processes to alleviate increased body temperature, while molecular pathways are recruited to maintain DNA integrity. This phase, known as "short-term HA," takes place over ∼5 days, and constitutes the "on" switch, which triggers the lengthier processes necessary to achieve acclimatory homeostasis (∼3–4 weeks in sedentary rats; Horowitz, 2014). HACT only becomes apparent after the longer second phase, as demonstrated in rodents by Assayag et al. (2010) in their discussion of HA-mediated cardioprotection in cardiac mitochondria, and in the frontal cortex and hippocampus, shown by Yacobi et al. (2014) in studies of HA-mediated neuroprotection. Acclimation in humans displays short and long phases, as well, e.g., Wyndham et al. (1976); however, HA protocols in humans include exercise training last ∼2 weeks and no data are as yet available to assess whether the cellular acclimatory homeostasis stage has been completely achieved following that period of time.

Exceptional features of HACT are that it lasts for ∼2–3 weeks, depending on the specific feature studied after the HA session (Cohen, 2002), and is memorized. In the rat HA-reacclimation model, 2 days of re-acclimation subsequent to 1 month of HA, and 1 or 2 months of exposure to normothermic ambient temperatures, are sufficient to restore a protected phenotype (Tetievsky et al., 2008). The transient phase of short-term acclimation which, via autonomic control, compensates for thermoregulatory effector-cellular perturbations, only aggravates the response to novel cellular stress, as exemplified in **Figure 1C** by the enhanced apoptosis in ischemic/reperfused or heat-stressed hearts, on short term HA. However, it doesn't abolish but enhances physiological thermoregulatory responses; i.e., the evaporative cooling or peripheral vasodilation controlled by the central autonomic thermoregulatory centers. This short-term HA impact was confirmed for both passive acclimation (e.g., in rats, which are capable of enhancing evaporation and enduring heat stress longer than rats undergoing long-term HA (Horowitz et al., 1983) and active acclimation (namely, HA and exercise training), described in Garrett et al. for humans enhancing their exercise capacity due to improved cardiovascular performance, following short-term heat acclimation (Garrett et al., 2012).

To date, accumulating experimental evidence and bioinformatic analyses of the HA transcriptome have established that, irrespective of the organ studied, HACT employs a two-tier protective response: (i) constitutive injury attenuation; and (ii) the abrupt, post-insult release of help signals that are enhanced in the acclimated phenotype. Moreover, recent experimental evidence regarding "injury attenuation" (Kodesh et al., 2011; Yacobi et al., 2014) demonstrates the involvement of groups of mechanisms that operate according to similar principles.

HACT: CONSTITUTIVE INJURY ATTENUATION—A LESSON FROM THE ISCHEMIC HEART

Cytoprotection and Heat Shock Proteins (HSP)

Constitutive enhancement of cytoprotective networks enables recruitment of help signals to maintain cellular homeostasis, without the need for *de novo* induction of cytoprotective elements. Chronologically, the first publications regarding acclimatory plasticity leading to constitutive cytoprotection were a series of studies by Maloyan et al. (Maloyan et al., 1999; Maloyan and Horowitz, 2002), demonstrating profound augmentation of HSP72 reserves in rat hearts, following long-term heat acclimation. Sustained low-plasma thyroxine levels, characteristic of acclimatory homeostasis, play a pivotal role in alterations in the density and affinity of the adrenergic receptors and, in turn, lead to altered responsiveness of the HA phenotype to sympathetic signaling, which includes HSP72 induction as well (Maloyan and Horowitz, 2002). Collectively, in the HA phenotype, sustained low thyroxine levels diminish thyroxine's effects on the β-adrenergic pathway, leading to depressed *hsp72* transcription (Maloyan and Horowitz, 2002). Activation of this branch of the thyroxine pathway (namely down regulation of HSP72 transcription, in contrast to enhancement of β adrenergic induced HSP72 transcription at high thyroxine levels) constitutes one explanation for the abolishment of cardioprotection, when HA is conducted with a β adrenergic blockade. This pharmacological tool provides unequivocal proof of the importance of HSP70s to the HACT response against ischemia. However, accumulating evidence implies that anti-oxidative and anti-apoptotic pathways associated with HSP70s are part of their functional performance via downstream pathways—e.g., anti-apoptosis—that inhibit the activation of caspase-9 or caspase-3 by preventing apoptosome formation or cytochrome C release through binding to the apoptosome (Saleh et al., 2000), mitochondrial protein importation (Schulz et al., 2015) or anti-oxidation (Chong et al., 2013).

A particularly interesting aspect of HACT lies in the outcome of concomitant exposure to a second adaptagent, with opposing demands, during HA. If HACT indeed occurs, it constitutes

the outcome of additive or cross-over effects. For example, HA and exercise training (HAEX), are usually employed in studies of human HA. Aerobic exercise training boosts muscle performance; consequently, biochemical adaptations enhance aerobic and anaerobic energy metabolism to meet the need for greater ATP generation. These alterations seem to be in conflict with critical adaptive features of HA, such as a decreased basal metabolic rate. Proceeding with the example of HACT against ischemia, **Figure 2** shows, in rat hearts, that despite adaptive conflicts between heat and exercise, similar cardioprotection, as determined by infarct size, was achieved in the HA alone, in HA combined with swimming training (HAEX), and in normothermic swimming-trained (EX) groups (Levi E MSc, The Hebrew University 2002 and (Levy et al., 1997). The heat-treated rats maintained higher ATP levels in their ischemic cardiac muscles than that of the non-acclimated animals (Levi et al., 1993; Levy et al., 1997; **Figure 2A**); however, we have no unequivocal experimental evidence that under ischemic conditions, the impact of HA is central to the cardioprotection seen in the acclimated/trained phenotype.

The HA phenotype (passive heat exposure as a singular adaptagent), however, displays a variety of specific features that can be cardioprotective, in and of themselves. Thus, a deeper understanding of the roles played by environmental heat vs. metabolic heat load (achieved by training) in the protected phenotype may enable us to determine the origins of the protective features in the aforementioned experimental groups. Here, we are referring to decreased Ca^{2+} sensitivity (and, in turn, protection from Ca^{2+} overload) seen in both the HA and HAEX, but not in the EX groups (**Figure 2B**; Cohen et al., 2007; Kodesh et al., 2011). These findings suggest that the consequences of prolonged heat exposure predominate in this case, at least (see ***Phenotypic plasticity underlying HACT—a broad perspective,*** below, for further discussion).

In a review by Powers et al. (2014), the authors stated that in exercise training cross-tolerance, the mitochondrial phenotype is central to exercise-induced cardioprotection "through increased expression of beneficial antioxidant proteins and decreased expression of proteins with potentially deleterious functions". Our bioinformatic data imply that equally enhanced redox buffer systems are implicated in adaptive responses in HA, EX, and HAEX hearts (Kodesh et al., 2011). Currently, however, data from our models are insufficient to attribute a central protective role to these buffer systems.

Pollak et al. (1998) in a novel study of "translational medicine" on human patients, were the first to demonstrate that HACT, arising out of concomitant exposure to a second adaptagent with opposing demands during HA, is beneficial for humans with coronary artery disease. The impact of HA with modest exercise training on patients undergoing coronary artery bypass surgery was evaluated, using transesophageal echocardiography in conjunction with simultaneous hemodynamic monitoring immediately after the surgery. Findings demonstrated that HA protected chamber elasticity and improved diastolic function in the heat-acclimated patients, vs. increased post-surgery stiffness developed in the non-acclimated cohort (control).

Cytoprotection-HIF-1α (Hypoxia Inducible Transcription Factor-1α)

Observations of the HA-protected rat cardiophenotype indicate: (i) enhanced glycolytic capacity, though at a slower rate; (ii) larger pre-ischemic endogenous glycogen stores; and (iii) upregulation of the transcript phosphofructokinase 2 (PFK2), a rate-limiting glycolytic enzyme in the normoxic HA rat heart (Eynan et al., 2002). The regulation of these components by HIF-1α (Semenza, 2011) led us to hypothesize that HIF-1α, the master regulator of oxygen homeostasis, is also an important mediator of HACT. This hypothesis was confirmed by Maloyan et al. (2005), and Shein et al. (2005), who were the first to show that HA induces constitutive upregulation of HIF-1α, independent of O_2 levels in the rat heart and mouse brain subjected to cardiac ischemia and TBI, respectively.

HIF-1α exerts its transcriptional activation as the heterodimer HIF-1, the product of HIF-1α with the constitutive HIF-1β. Its role in cardio-HACT and neuro-HACT was confirmed by instituting a HIF-1α dimerization blockade either prior to ischemia in the heart, or prior to recovery from traumatic brain injury. In the rat, a HIF-1α dimerization blockade was also conducted during the entire 1 month HA session. We have shown that a continuous HIF-1α dimerization blockade is needed to attenuate HACT, even though no changes in acclimatory levels of HSP72 were measured (Alexander-Shani et al., 2017). his attenuation adds metabolic aspects to HACT. In the HA hearts, inhibition of HSP72 induction by β adrenergic blockade during acclimation, abolished HACT (Maloyan and Horowitz, 2002). A β adrenergic blockade was also shown to decrease HIF-1α induction (Li et al., 2015). In retrospect, the experimental series in which the magnitude of HACT was measured (e.g., infarct size) during blockade of HIF-1α dimerization and HSP72 augmentation, serve as examples of the integrated beneficial effects needed to achieve HACT. More recently, experiments involving HIF-1α mitochondrial target genes demonstrated the positive contribution of HIF-1α to HACT by shifting pyruvic acid away from the mitochondria to enhance glycolysis (Alexander-Shani et al., 2017). The beneficial effects of HA in experimental animal models will be further discussed below (see "*Phenotypic plasticity underlying HACT—a broad perspective*").

CELLULAR CYTOPROTECTION AND HACT—CONFIRMATION OF THE ROLE PLAYED BY HSP AND HIF-1α IN HUMANS UNDERGOING HEAT EXERCISE AND HYPOXIA ACCLIMATION

In humans, Yamada et al. demonstrated HA-induced expression of HSP70 during exercise (Yamada et al., 2007). Consequently, by using the *hsp70* transcript as an indicator of strain, Lee et al. (2016) and Gibson et al. (2015) demonstrated HACT to hypoxia, also in humans. Notably, the acclimation protocols used by Gibson et al. (2015); Lee et al. (2016) included both heat and exercise, thereby examining the contribution of HA to HACT

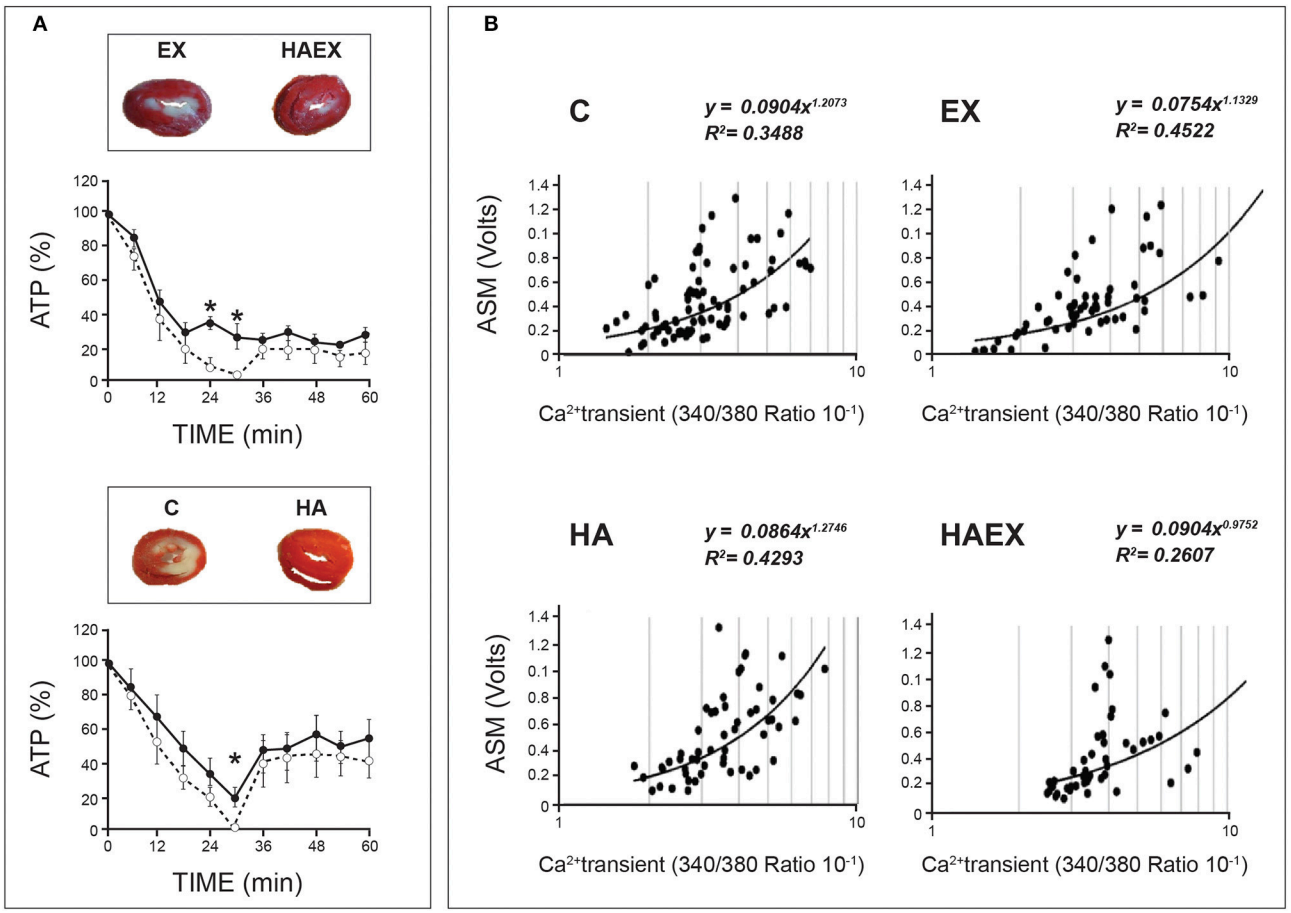

FIGURE 2 | Demonstration of heat acclimatory features linked to HACT in rat hearts. Rats were acclimated either to passive heat acclimation (HA) or to HA and swimming training (HAEX). **(A)** Phospho-NMR monitoring of %ATP in rat hearts subjected to global ischemia/reperfusion (I/R) insults. Groups exposed to heat maintained significant ATP levels following 30 min of global ischemia (vs. non-acclimated rats' heart). Solid lines: Heat-exposed groups (HA & HAEX). Broken lines: Non-acclimated controls (C & EX). Insets: Heart slices post 30 min ischemia/40 min reperfusion. **(B)** Ca^{2+} sensitivity of isolated cardiomyocytes expressed by contractile response (ASM), vs. calcium transients. Data from the two heat-exposed groups yielded a rightward shift of the curves, suggesting decreased Ca^{2+} sensitivity. HA, Heat acclimation. HAEX, Heat acclimation and swimming training. EX, Swimming training alone. C, Control (Non-acclimated). Adapted from (Levi et al., 1993; Levy et al., 1997; Kodesh et al., 2011). Courtesy of the American Physiological Soc.

with co-adaptagents with conflicting/competitive demands, when adjusting to environmental demands. Supporting evidence of the diverse roles played by HIF-1α in HACT is derived from Lee et al. (2016), who compared the impact of HA and exercise to that of hypoxia acclimation and exercise, and demonstrated that HA elevates extracellular levels of HIF-1α, thus confirming in humans that HA induces HIF-1α under normoxic conditions. Likewise, the authors demonstrated that HA improves performance under hypoxic conditions, and that HA increases cellular and systemic physiological tolerance to exercise under conditions of moderate hypoxia. Moreover, the authors showed that HA improves performance during hypoxia in a manner similar to that achieved by hypoxic acclimation. As only extracellular HIF-1α was measured, the specific role of HIF-1α in HACT cannot be assessed.

HACT: ABRUPT POST-INSULT RELEASE OF HELP SIGNALS

Unfortunately, the role of abrupt post-insult release of help signals has been less studied; hence, it will only be briefly discussed here. Empirical data were collected from ischemic heart and mouse brain models. In the heart, a noteworthy finding that emerged was the earlier activation threshold (upregulation) of *hsp72* and the glutathione-*S*-transferase P subunit (*GST-P*-antioxidant) to ischemic insult, and the downregulation of the *bcl-2* death promotor (BAD) to heat stress, after heat acclimation (Horowitz et al., 2004). Of prime interest was the finding of enhanced help signals evoked following traumatic head injury in the mouse brain, demonstrating angiotensin-2 (AT2) receptor induced neurogenesis, and acute activation of the AKT-HIF-1a cascade (Akt, HIF-1, GLUT1, VEGF, NGF, BDNF, and Erk1/2).

For further information, readers are referred to Umschweif et al. (2014).

PHENOTYPIC PLASTICITY UNDERLYING HACT: A BROAD PERSPECTIVE

Unequivocal evidence underscores the important role played by HSP72 and HIF-1α in HACT against impairment in oxygen homeostasis, possibly only because of elevated constitutive levels, which mitigate the need for immediate *de novo* induction upon stress. However, HA also exerts a global protective effect that is implemented in the featured HACT. Here, the impact of (i) the HIF-1α metabolic switch, and (ii) how HA induces processes to cope with the deleterious effects of Ca^{2+}, both studied extensively in our laboratory, will be discussed as prototypes.

HIF-1α is an O_2-regulated subunit; however, HA upregulates HIF-1α under normoxic conditions. An important adaptive strategy of the HA phenotype to decreased oxygen availability stems from its reliance upon enhanced glycolysis at a slower rate (vs. normothermic animals; Eynan et al., 2002), balancing pH with greater cytosolic ATP production and decreased mitochondrial ROS production. The engine that sets this adaptation in motion is the HIF-1α-mediated elevation of pyruvate dehydrogenase kinase 1 (PDK1) and, in turn, pyruvate dehydrogenase (PDH) activity, shifting pyruvic acid away from the electron transport machinery (Alexander-Shani et al., 2017). Activation of glycolytic enzymes is thus enhanced, while mitochondrial ROS production is diminished, due to decreased aerobic respiration. Canaana (2003) and Horowitz et al. (2006) demonstrated this outcome in HA rat cardiomyocytes, while measuring mitochondrial ROS production during heat stress and anoxia in the presence and absence of complex I-III inhibitors. Furthermore, the performance of the respiratory complex I, II and IV of HA mitochondria following ischemia/reperfusion insult was enhanced, compared to non-acclimated mitochondria (Assayag et al., 2010). In tandem with this important metabolic adaptive response is the HIF-1α-controlled reprogramming of two COX4 isoforms (components of Complex IV), elevating levels of the COX4.2 hypoxic-resistant unit (Alexander-Shani et al., 2017). Notably, the metabolic shift described above was first identified as altitude-induced adaptation in humans (Papandreou et al., 2006).

Experimental data suggests that remodeling of the COX4 isoform ratio is only one example of the principle involving remodeling of the subunit ratio of protein complexes, leading to functional changes and performance as protective mechanisms in HACT, conditioned to "attenuate injury". Both the heart and brain use this principle to alter Ca^{2+} management, and to reduce the deleterious effects of Ca^{2+} overload. In rats, the cardiac heat-acclimated phenotype demonstrates reduced Ca^{2+} sensitivity; namely, more Ca^{2+} is needed to induce cardiac muscle contraction (Cohen et al., 2007; Kodesh et al., 2011). In cardiac mitochondria, serving also as a Ca^{2+} sink, Ca^{2+} levels increase in these organelles without affecting their performance (Assayag et al., 2012). The frontal cortex and hippocampus display decreased normoxic NMDA density, with an even greater decrease noted during hypoxic stress. Upon exposure to hypoxic/ischemic stress, a massive glutamate discharge is seen, causing calcium overload and oxidative stress. The combination of fewer NMDA receptors in the acclimated phenotype, coupled with an increase in the NMDA receptor subunit ratio favoring decreased Ca^{2+} permeability, is likely to reduce Ca^{2+} overload, and attenuate its deleterious effects in the stressed brain (Yacobi et al., 2014).

The unifying principle underlying changes in Ca^{2+} management in these two organs entails remodeling of the Ca^{2+} channels. In the heart, the gating subunit of the L-type channel (Kodesh et al., 2011), and in the NMDA (Yacobi et al., 2014), an elevation of the GluN2B/GluN2A NMDA receptor subunit ratio (to ratios >1), together with a significant increase in the GluA2 subunit of the AMPA receptors, lead to decreased opening permeability of the channels which, in turn, limits Ca^{2+} flux during stress. For a scheme describing highlighted changes leading to HACT, see **Figure 3**, lower panel.

HACT: TRANSLATIONAL ASPECTS TO HUMANS' HEALTH

The beneficial effects of heat acclimation have been used for human benefit long ago. But while HA protocols were initially developed to reduce the physiological strain caused by hot environments, the cellular and molecular processes underlying HA were consolidated only recently. Consolidating the cellular and molecular mechanisms of acclimation led to the emergence of recent investigations/publications demonstrating that HA can be exploited for human benefit beyond its evolutionary function of "heat tolerance". In humans, Lee et al. (2016) demonstrated improvements in hypoxic exercise tolerance after 10 days of HA; Brunt et al. (2016a,b) showed decreased vascular stiffness following 8 weeks of prolonged thermal therapy. Interestingly, Pollak et al. (1998) reported decreased cardiac chamber stiffness immediately post-coronary bypass surgery, undertaken after 2–3 weeks of acclimation in early-summer desert temperatures. We can only now, provide partial explanation to these finding. The accumulated data raise questions regarding the time required for HACT. In rodents, for example, HACT is achieved following 3–4 weeks of HA. Are time differences in the induction of HACT the result of species variation? Considering HA in humans, it is important to bear in mind that for practical reasons, we examine physiological acclimation, rather than molecular processes. The physiological processes occur within a shorter timeframe than those required to achieve cellular and molecular homeostasis, and are controlled by dynamic neural processes. With the few available molecular cytoprotective responses (Gibson et al., 2015, 2016; Lee et al., 2016), and cellular processes (e.g., muscles' contractility, Racinais et al., 2017) it is likely that acclimatory features are similar in humans and rodents. Our data suggest that acclimation constitutes a gradually progressive process. It is likely that in the short-range human protocols (∼2 weeks), full acclimatory homeostasis cannot be achieved. Molecular acclimatory homeostasis, on the other hand, entails epigenetic processes of gene transcription and may thereby

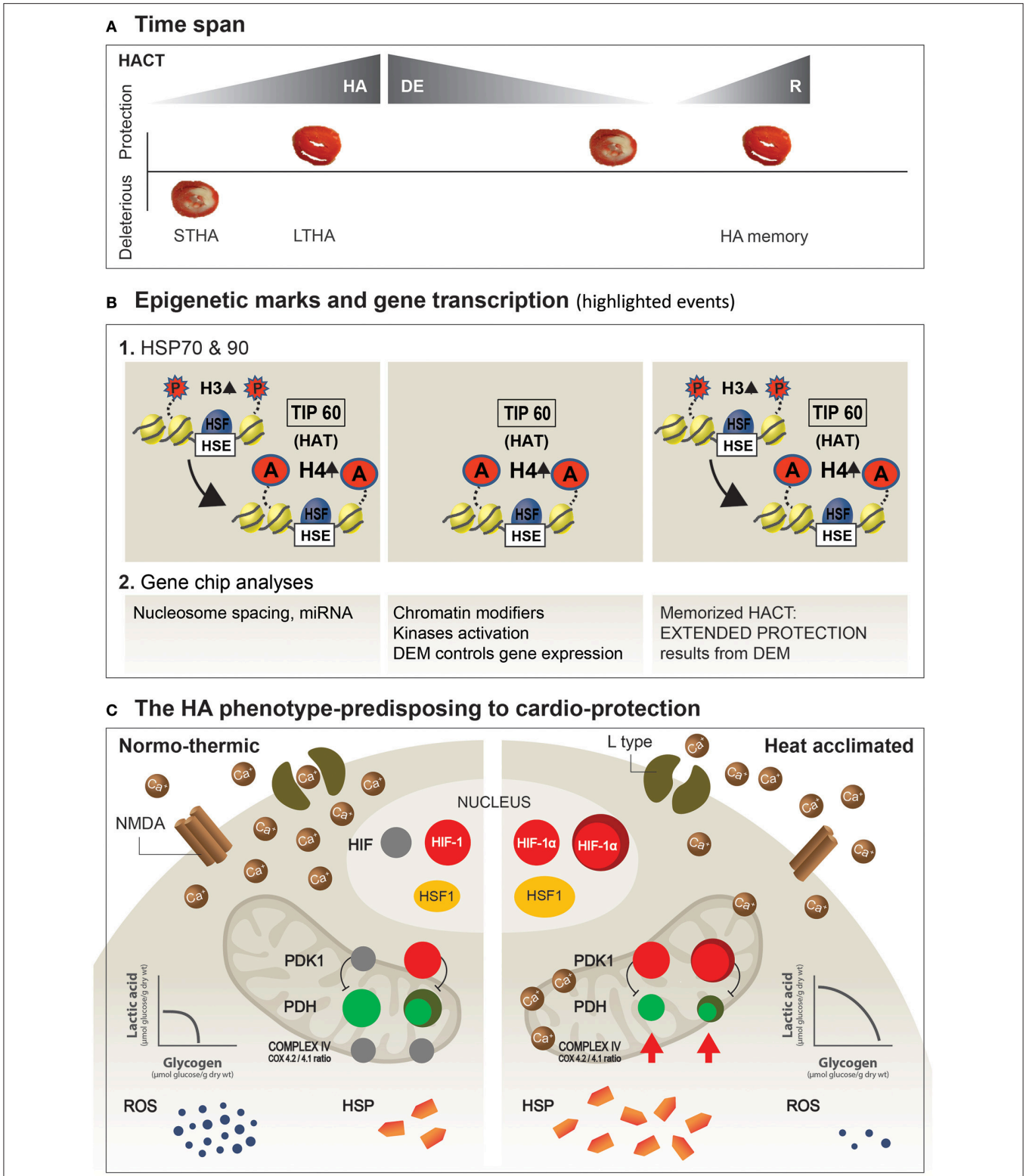

FIGURE 3 | Suggested dynamics of epigenetic machinery and highlighted mechanisms shaping the HA-protected phenotype. **(A)** HA and HA memory time span. Ischemic/reperfused heart slices illustrate protective and deleterious responses. **(B)** Post-translational modifications in histones (histone H3-P; histone H4-Act) underlie heat acclimation-mediated cytoprotective memory. The onset of acclimation triggers histone H3-P, tagging on LTHA, to H4 acetylation at the HSP promotor (Cheung et al., 2000). Long-term histone H4 acetylation promotes a constitutive open chromatin at the HSP promotor and constitutive HSF1 binding long after the

(Continued)

FIGURE 3 | Continued
initial HA, as well as during re-HA (For a detailed explanation, see in "Future perspectives" above). **(C)** Protective features developed in the HA phenotype.The illustrated features are those described in the main text. Left side: Normothermic heart. Right side: HA heart. Increased expression of HSF1 leads to augmented constitutive cellular HSP levels, while constitutive HIF-1α marks the metabolic switch, which upregulates mitochondrial PDK1. In turn, phosphorylation of ser(232) in the PDHE1α subunit of the PDH complex shifts pyruvic acid away from the mitochondria, to enhance glycolysis and to decrease ROS produced by the mitochondria. An additional, important aspect of HA is the "principle" of receptor remodeling, or abundance of Receptors/ion channels on the cell membrane (i.e., heart, brain) as a mechanism to attenuate the deleterious effects of Ca^{2+} overload. STHA, Short-term heat acclimation; LTHA, Long-term heat acclimation; DEM, Dynamic epigenetic machinery (Based on Eynan et al., 2002; Horowitz et al., 2004; Tetievsky and Horowitz, 2010; Yacobi et al., 2014; Alexander-Shani et al., 2017).

enable extension of the therapeutic windows discussed below ("Future perspectives").

FUTURE PERSPECTIVES: DO EPIGENETIC MECHANISMS PLAY A ROLE?

A hallmark of HACT is the extended time for which the animal is protected: the effects of cross-tolerance last ∼3 weeks post-acclimation. HACT is characterized by a unique ability to be memorized and reinstated, even after 1 or even 2 months of decline. A short acclimating stimulus of only 2 days (vs. 30 days for initial acclimatory homeostasis) was required for the return of HACT (Tetievsky et al., 2008).

Accordingly, epigenetics attracted our attention. Using *hsp72* and *hsp90* in a rat heart model for studying post-translational histone modifications (H3 phosphorylation, H4 acetylation), Tetievsky et al. (Tetievsky and Horowitz, 2010) demonstrated that the memorized HACT stems from within-life epigenetic mechanisms controlling gene expression.

Moreover, bioinformatic analyses of the acclimated, de-acclimated, and re-acclimated transcriptomes provided evidence that epigenetic processes exist on a continuum, occurring during acclimation and de-acclimation, that are likely to enhance both the protected HA phenotype, and the rapid re-induction of HACT. Among the processes involved are: (i) changes in the linker histones participating in nucleosome spacing, transcription factor accessibility and, in turn, gene expression control during (ii) de-acclimation, a molecular set-up whereby constitutive histone H4 acetylation by the histone-acetylase Tip60 enables HSF1 (heat shock transcription factor 1) binding to the heat shock element, thus predisposing to maintain HSP72 proteostasis, and a return of HACT (Tetievsky et al., 2008, 2014; Tetievsky and Horowitz, 2010). Notably, there exists a dichotomy between the transcriptome of the reinstated, protected, and HA phenotypes, while the physiological phenotype is similar in both phases. Central signaling pathways are most active during the transition from normothermic to acclimated temperatures, perhaps implying that ambient temperature has an impact on HACT.

Corroborating our postulation of the importance of epigenetics in cross-tolerance is the review by Khoury et al. (2016) on the recapitulation of evolutionary metabolic plasticity by means of epigenetic mechanisms. Pharmacological preconditioning by administering resveratrol, an activator of the epigenetic enzyme Sirtuin1 (SIRT1) resulted in a cross-tolerance lasting ∼14 days, by "mimicking" the depressed metabolic state and enhanced hypoxic tolerance, an evolutionary adaptive feature among hypoxic-tolerant species (Storey, 2015). Thompson et al. (2013) demonstrated bimodal activity of the histone deacetylase SIRT1 in ischemic brain protection, seen as either hypermethylation and gene suppression, or hypomethylation and gene activation.

Gidday (2015), in a review published in Frontiers in Neurology (hypotheses and theory articles) entitled, "Extending injury- and disease-resistant CNS phenotypes by repetitive epigenetic conditioning," discusses the idea of broadening ischemic tolerance in the brain by "extension of the period over which adaptive epigenetic changes persist, as well as its memory" and suggests that "it is epigenetics that deserves attention as the fundamental mechanism responsible for the long-lasting responses to repetitive conditioning stimuli". Horowitz's lab (Tetievsky et al., 2008, 2014; Tetievsky and Horowitz, 2010) provided experimental physiological and epigenetic evidence in a rat heart that a within-life epigenetic mechanism induces memorized HACT. Empirical evidence points toward constitutive histone H4 acetylation on the HSP72 promotor, and upregulation of this cytoprotective transcript following long-term acclimation when HA homeostasis has been achieved. Histone H4 acetylation is absent following short periods of acclimation (Tetievsky and Horowitz, 2010). However, histone H4 acetylation is maintained throughout acclimation, acclimation decline, and re-acclimation, suggesting an "epigenetics" imprinting of the long-, but not short-term stressful episode (**Figure 3B**).

In sum, the dynamic epigenetic phenomenon not only induces long-lasting HACT, but enables preservation of its physiological beneficial features in a dormant manner. A rapid, short acclimation stimulus re-establishes the physiological, protected HA phenotype. The transcriptional machinery, however, is a continuum. HACT has beneficial roles in health and disease and is already employed in thermotherapy. Yet, our knowledge of the epigenetic within-life dynamic mechanisms involved, as well as how long they persist, is still in its infancy.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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frontiers in Physiology

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The Hsp72 and Hsp90α mRNA Responses to Hot Downhill Running Are Reduced Following a Prior Bout of Hot Downhill Running, and Occur Concurrently within Leukocytes and the Vastus Lateralis

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The leukocyte heat shock response (HSR) is used to determine individual's thermotolerance. The HSR and thermotolerance are enhanced following interventions such as preconditioning and/or acclimation/acclimatization. However, it is unclear whether the leukocyte HSR is an appropriate surrogate for the HSR in other tissues implicated within the pathophysiology of exertional heat illnesses (e.g., skeletal muscle), and whether an acute preconditioning strategy (e.g., downhill running) can improve subsequent thermotolerance. Physically active, non-heat acclimated participants were split into two groups to investigate the benefits of hot downhill running as preconditioning strategy. A hot preconditioning group (HPC; $n = 6$) completed two trials ($HPC1_{HOTDOWN}$ and $HPC2_{HOTDOWN}$) of 30 min running at lactate threshold (LT) on −10% gradient in 30°C and 50% relative humidity (RH) separated by 7 d. A temperate preconditioning group (TPC; $n = 5$) completed 30 min running at LT on a −1% gradient in 20°C and 50% ($TPC1_{TEMPFLAT}$) and 7 d later completed 30 min running at LT on −10% gradient in 30°C and 50% RH ($TPC2_{HOTDOWN}$). Venous blood samples and muscle biopsies (vastus lateralis; VL) were obtained before, immediately after, 3, 24, and 48 h after each trial. Leukocyte and VL Hsp72, Hsp90α, and Grp78 mRNA relative expression was determined via RT-QPCR. Attenuated leukocyte and VL Hsp72 (2.8 to 1.8 fold and 5.9 to 2.4 fold; $p < 0.05$) and Hsp90α mRNA (2.9 to 2.4 fold and 5.2 to 2.4 fold; $p < 0.05$)

responses accompanied reductions ($p < 0.05$) in physiological strain [exercising rectal temperature (−0.3°C) and perceived muscle soreness (∼ −14%)] during $HPC2_{HOTDOWN}$ compared to $HPC1_{HOTDOWN}$ (i.e., a preconditioning effect). Both VL and leukocyte Hsp72 and Hsp90α mRNA increased ($p < 0.05$) simultaneously following downhill runs and demonstrated a strong relationship ($p < 0.01$) of similar magnitudes with one another. Hot downhill running is an effective preconditioning strategy which ameliorates physiological strain, soreness and Hsp72 and Hsp90α mRNA responses to a subsequent bout. Leukocyte and VL analyses are appropriate tissues to infer the extent to which the HSR has been augmented.

Keywords: downhill running, heat shock response, heat stress, heat tolerance, preconditioning, cross tolerance, thermotolerance

INTRODUCTION

Preconditioning of an individual using environmental stressors, with the intent of ameliorating physiological and cellular stress in extreme conditions has applications for athletic, military and occupational populations (Taylor et al., 2012; Lee et al., 2014). One pathway for preconditioning these populations is the initiation of the heat shock response (HSR) which is characterized by induction of heat shock proteins (Hung et al., 2005; Madden et al., 2008; Taylor et al., 2012). The leukocyte HSR, principally heat shock protein 72 (HSP72; protein and mRNA) is used to indicate the extent of cellular heat acclimation (Amorim et al., 2015), and identify individuals at risk of exertional heat illnesses within athletic, military and occupational settings (Moran et al., 2006; Marshall et al., 2007; Ruell et al., 2007). This is primarily due to the role of Hsp72 mRNA and HSP72 as markers of the cellular stress response and thermotolerance [attenuated cellular stress response suggests a greater likelihood of cellular survival (Kampinga et al., 1995; Theodorakis et al., 1999)] in response to isolated, combined, and cross-environmental stressors (Gibson et al., 2017). Ideally the assessment of thermotolerance would take place in skeletal muscle due to its important role in locomotion and exertional heat illness pathophysiology (Sawka et al., 2011). Unfortunately, obtaining multiple muscle biopsies prior to relocation to a hot environment is not always viable for ethical, performance, cost, comfort and medical reasons (MacInnis et al., 2017). Leukocytes are a desirable tissue site for determining thermotolerance given the relative ease by which they can be collected, and because leukocytes, as circulating cells, are exposed to both systemic signals and to signals of the perfused tissues (Sonna et al., 2007). As such Hsp72 mRNA from leukocytes has been utilized as a surrogate to skeletal muscle samples with inferences made from changes in circulating intracellular sites across many exercise, heat, and nutritional experiments whereby the cellular stress response and thermotolerance are augmented (Fehrenbach et al., 2000a,b, 2001; Niess et al., 2002; Connolly et al., 2004; Marshall et al., 2007; Selkirk et al., 2009; Gibson et al., 2015a,c; Tuttle et al., 2015; Mee et al., 2016). Consequently, determining whether the HSR occurs concurrently within both tissues (leukocytes and the vastus lateralis; VL) following an acute stressor (initial experimental trial), and whether this response is attenuated in both tissues following a second trial (i.e., following preconditioning), requires elucidation to assess the viability of the leukocyte HSR to represent the skeletal muscle HSR.

The Hsp72 mRNA response is particularly pertinent during this acute stress response because HSP72 protein concentrations (due to translational inhibition) may not necessary directly represent the magnitude of the cellular stress response, particularly during the early stages of adaptation to stress (Paulsen et al., 2007) and within heat intolerant individuals (Moran et al., 2006). The differential kinetics of the Hsp72 response in the VL [typically delayed, peak between 24 h and 7d; (Morton et al., 2006; Tupling et al., 2007)] compared to leukocyte subsets [0–24 h (Fehrenbach et al., 2000a; Oehler et al., 2001)] suggests the leukocyte Hsp72 mRNA specific response which peaks within 0–3 h (Fehrenbach and Northoff, 2001; Neubauer et al., 2014), is more practical (shorter sampling time course required) for assessing the cellular stress response in the VL for comparative purposes. In addition to Hsp72 mRNA, Hsp90α mRNA is of interest due to its important role within restoration of proteostasis (Kourtis and Tavernarakis, 2011; van Oosten-Hawle et al., 2013), regulation of the transmission of signaling cascades (Taipale et al., 2010), recovery of global protein synthesis (Duncan, 2005) and regulation of cellular repair (Erlejman et al., 2014). Additionally it is unknown if the physiological signals e.g., increases in systemic temperature (Gibson et al., 2016), which elicit increases in leukocyte Hsp72 and Hsp90α mRNA transcription to damaging (Tuttle et al., 2015), and non-damaging exercise-heat stress (Gibson et al., 2015c), are as relevant in skeletal muscle. The current study also sought to investigate the gene transcript response of another HSP, glucose regulated protein 78 mRNA (Grp78 mRNA) given its ability to indicate when the unfolded protein response ends (Ron and

Abbreviations: CT, cycling threshold; DOMS, Delayed onset muscle soreness; Grp78, Glucose regulated protein 78; HOT, Hot testing conditions; HPC, Hot preconditioning group; Hsp, Heat shock protein (number indicates molecular weight); HSF-1, Heat Shock factor-1; HSR, Heat shock response; LT, Lactate threshold; mRNA, Messenger RNA; PBS, Phosphate-buffered saline; QT, Quadriceps tenderness; RH, Relative humidity; RNA, Ribonucleic acid; RPE, Rating of perceived exertion; RT-QPCR, Reverse transcription quantitative polymerase chain reaction; TEMP, Temperate testing conditions; TPC, Temperate preconditioning group; TS, Thermal sensation; UOsm, Urine Osmolality; VL, Vastus lateralis; $\dot{V}O_2$, Oxygen uptake; $\dot{V}O_{2max}$, Maximal oxygen uptake.

Walter, 2007). Importantly Grp78 mRNA may also act as a biomarker of thermotolerance within heat intolerant individuals where Heat Shock factor-1 (HSF-1) signaling and Hsp72 and Hsp90α mRNA transcription are attenuated (McMillan et al., 1998). However, it is currently unclear if previous *in vitro* observations demonstrating the role of Hsp72 and Hsp90α mRNA in the cellular stress response (Heldens et al., 2011) occur within human leukocytes and skeletal muscle (VL) *in vivo* (i.e., following exercise, and exercise and heat related stressors).

Experimental aims were to determine whether a prior bout of hot downhill running [eliciting large changes in exercising rectal temperature (T_{re}) and delayed onset muscle soreness (DOMS)], when compared to a temperate flat run, could provide a preconditioning effect relative to attenuation of the VL Hsp responses (Hsp72, Hsp90α, and Grp78 mRNA) during a subsequent trial of hot downhill running 7 d later. The second experimental aim was to determine whether this response occurred concurrently within leukocytes and the VL. It was hypothesized that a prior bout of hot downhill running would attenuate both the VL and leukocyte Hsp72 and Hsp90 mRNA responses during a second trial, and that a significant relationship between the VL and leukocyte Hsp72 and Hsp90 mRNA responses following the first trial would exist.

METHODS

Ethical Approval

The protocol was approved by the University of Bedfordshire's Sport Science and Physical Activity Departmental Human Ethics Committee and all participants signed informed consent in accordance with the ethical standards outlined in the 1964 Declaration of Helsinki.

Participants

Demographic variables were recorded for 11 male Caucasian participants (see **Table 1**) who were non-smokers and were not heat acclimated (experimental trials completed between January and March, within the UK; average temperatures 1.5°C–8.1°C). Body mass (kg) and height (cm) were measured with a single set of mechanical scales (Weylux Marsden 424 London, UK) and a stadiometer (Harpenden HAR- 98.602, Crymych, UK) respectively. Body composition was measured using air displacement plethysmology (Bod Pod 2000A, Cranlea, UK). The lactate threshold (LT) and maximum oxygen uptake ($\dot{V}O_{2max}$) were determined using a graded treadmill test (Winter et al., 2007). This test consisted of 6–8 incremental 3 min stages at a 1% gradient. Participants started running at 8–9 $km.h^{-1}$ and running velocity was increased by 1 $km.h^{-1}$ per stage until exhaustion. Fingertip capillary blood samples (40 μL) were taken at rest and the end of each 3 min stage to determine blood lactate concentrations (B[La]). Blood lactate concentrations were plotted against running velocity to determine LT which was defined as the first sustained B[La] increase above baseline. Pulmonary gas exchange was measured breath by breath using an online gas analysis system (Cortex Metalyser 3B, Biophysik, Leipzig, Germany) to determine changes in oxygen uptake ($\dot{V}O_2$) with the highest $\dot{V}O_2$ attained over a 30 s period accepted as $\dot{V}O_{2max}$.

TABLE 1 | Participant demographic characteristics.

	Temperate preconditioning group (TPC; $n = 5$)	Heat preconditioning group (HPC; $n = 6$)	Group sig ($p < 0.05$)
Age (Years)	20.4 ± 2.8	21.7 ± 2.3	0.426
Height (cm)	177 ± 7	180 ± 10	0.593
Body Weight (kg)	75.2 ± 18.1	76.1 ± 12.3	0.931
	50.8 ± 6.9	52.8 ± 5.0	0.587
% Lean mass	88.3 ± 11.5	86.8 ± 4.8	0.777
% Body Fat	11.7 ± 11.5	13.2 ± 4.8	0.777

Values are expressed as mean ± SD. $mL.kg.min^{-1}$ (milliliters per kilogram per minute), $\dot{V}O_{2max}$ (maximum oxygen uptake).

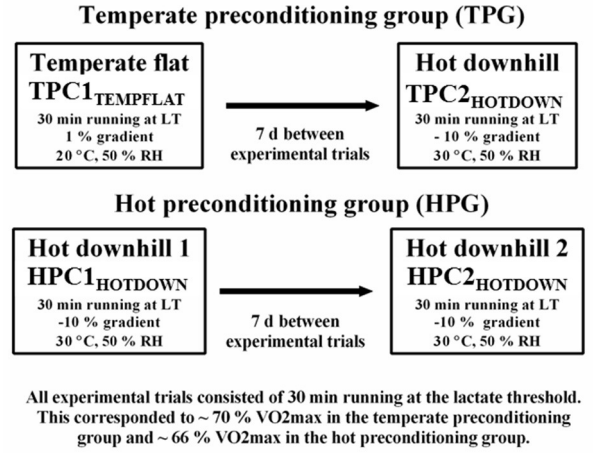

FIGURE 1 | Schematic representation of the experimental design. $\dot{V}O_{2max}$ (maximum oxygen uptake).

Sample size calculations of Hsp72 mRNA were determined via G.Power 3.1, (Universität Dusseldorf, Germany; Faul et al., 2009) using data from a previous paper (Mestre-Alfaro et al., 2012). For a two tailed test with an alpha of 0.05 and power of 0.8, Six participants were required to find an Hsp72 mRNA increase of 3.8-fold significant. This sample size is ≥ others in the field (Puntschart et al., 1996; Febbraio and Koukoulas, 2000; Fehrenbach and Northoff, 2001; Fehrenbach et al., 2003; Liu et al., 2004; Mee et al., 2016).

Experimental Design

Participants were split into two experimental groups (see **Figure 1**). The temperate (TPC; five participants) and HOT (HPC; six participants) preconditioning groups (conditions) both featured two exercise trials separated by 7 d:

TPC Exercise trial (1): Temperate flat ($TPC1_{TEMPFLAT}$) which involved 30 min running at the LT on a 1% gradient in 20°C, 50% RH. *TPC Exercise trial (2):* 7 d post $TPC1_{TEMPFLAT}$, hot downhill

($TPC2_{HOTDOWN}$) which involved 30 min downhill running at the LT on a −10% gradient in 30°C, 50% RH.

HPC Exercise trial (1): Hot downhill ($HPC1_{HOTDOWN}$) which involved 30 min downhill running at the LT on a −10% gradient in 30°C, 50% RH. *HPC Exercise trial (2):* 7 d post $HPC1_{HOTDOWN}$, hot downhill 2 ($HPC2_{HOTDOWN}$) which involved 30 min downhill running at the LT on a −10% gradient in 30°C, 50% RH.

Previous work from our research group has demonstrated that the leukocyte Hsp72 and Hsp90α mRNA responses are larger following exercise in hot compared to temperate environments (Gibson et al., 2015c, 2016), and following downhill compared to flat running (Tuttle et al., 2015). It is known that downhill running is an effective whole body preconditioning strategy (Shima et al., 2008; Touchberry et al., 2012; Isanejad et al., 2015), consequently, an acute preconditioning trial featuring both stressors (hot environmental conditions and downhill running; hot downhill running) was selected in the current experimental design to maximize stimuli to initiate the HSR and subsequent cellular preconditioning. This was compared to a temperate flat trial (flat running in a temperate environment) where no change in leukocyte Hsp72 and Hsp90α mRNA has been previously observed (Tuttle et al., 2015) and thus no preconditioning effect was hypothesized to occur. A 7 d period between trials was selected to ensure any spontaneous preconditioning effect from exercise stress on core temperature (Barnett and Maughan, 1993) and leukocyte HSP72 (Fehrenbach et al., 2001; Lee et al., 2014), had returned to baseline following $TPC1_{TEMPFLAT}$.

All experimental trials were completed at the running velocity which elicited the LT to minimize differences in metabolic strain between experimental trials (Baldwin et al., 2000). However, environmental temperature mediated differences still remained as relative exercise intensity is higher at the same velocity during exercise in hot environments (Lorenzo et al., 2011). All experimental trials were completed at the same time of day to minimize the influence of diurnal and circadian variations on exercise performance (Drust et al., 2005). Confounding variables were controlled for via abstinence prior to testing and throughout the testing period (see brackets for duration). These confounding variables were caffeine and alcohol (72 h), non-steroidal anti-inflammatory medications [48 h (Nielsen and Webster, 1987; Van Wijck et al., 2012)], dietary supplementation (vitamins, ergogenic aids; 30 d), exercise [7 d (Morton et al., 2006)], thermal stressors [3 months (Gibson et al., 2014)] and hypoxic and hyperbaric stressors [3 months (Taylor et al., 2010a, 2011, 2012)]. A questionnaire was administered prior to each experimental trial to determine adherence to the aforementioned experimental control measures with apparent adherence 100% in all participants.

Participants were instructed to drink 500 mL of water 2 h before each experimental trial as per the ACSM position stand (Sawka et al., 2007). Hydration status was assessed via urine osmolality (UOsm) using a handheld digital refractometer (Osmocheck, Vitech Scientific Ltd, Horsham, UK) before any pre exercise measures were obtained and immediately after exercise. All participants were euhydrated [UOsm was <600 mOsmols.kg.H_20 (Hillman et al., 2011, 2013)] prior to all experimental conditions and remained euhydrated during each experimental trial despite UOsm increasing (Time; $F = 63.7$, $p < 0.001$) immediately post exercise compared to basal.

MOLECULAR PHYSIOLOGY MEASURES

Blood Sampling and Leukocyte Isolation

Venous blood was obtained from the antecubital vein into a 6 mL EDTA tube immediately before (basal), immediately post, 3 h post, 24 h post, and 48 h post exercise. Using an adaptation of a previously validated method (Taylor et al., 2010b), 500 μL of venous blood was pipetted into 10 mL of 1 in 10 red blood cell lysis solution (10X Red Blood Cell Lysis Solution, Miltenyi Biotech, UK). Samples were incubated for 15 min at room temperature and then isolated via centrifugation at 400G for 5 min and washed twice in 2 mL phosphate-buffered saline (PBS) at 400 G for 5 min. The pellet was suspended in 1 mL of PBS, pipetted into a 1.5 mL RNase free microtube and then centrifuged at 17 000 G for 5 min at 4°C. The remaining supernatant was aspirated prior to the pellet being completely re-suspended in 200 μL of TRIzol reagent (Sigma Aldrich, Dorset, UK) and stored at −80°C for subsequent RNA extraction.

Muscle Biopsies

All biopsies were taken by medically qualified Orthopedic Surgeons, with full UK General Medical Council registration. Muscle Biopsies were obtained using a previously validated and HSP specific *in vivo* technique (Morton et al., 2006, 2007, 2008, 2009) applied to the lateral portion of the vastus lateralis. Biopsies were taken 3 cm apart in a proximal to distal fashion, under local anesthetic (2% lidocaine hydrochloride). The fascia of the muscle was specifically avoided (Trappe et al., 2013). Disposable manually primed biopsy needle guns were utilized (12 × 16, Disposable Monopty Core Biopsy Instrument, Bard Biopsy Systems, USA). Samples collected (20–30 mg) were immediately frozen in liquid nitrogen (−196°C) and stored at −80°C for later analysis. Serial biopsies were separated by 3 cm to ensure muscle damage from previous incisions did not influence the Hsp72, Hsp90α, and Grp78 mRNA responses (Khassaf et al., 2001).

Biopsy samples were later ground under liquid nitrogen to remove surrounding tissue (i.e., adipose, and connective tissue) prior to homogenization with a sonicator (T10 Basic, IKA, Thermo Fisher Scientific, Loughborough, UK) on ice in 1 mL TRIzol reagent followed by a 10 min incubation period on ice, in preparation for RNA extraction.

RNA Extraction

The TRIzol method was used to extract RNA from the biopsy samples and the leukocytes in accordance with manufacturer instructions (Invitrogen, Life Technologies, Carlsbad, USA). Quantity was determined at an optical density of 260 nm while quality was determined via the 260/280 and 260/230 ratios using a nanodrop spectrophotometer

TABLE 2 | Primer sequences.

Gene	NCBI Accession No.	Primer	Sequence (5′ → 3′)	Amplicon length
β2-Microglobulin (β2-M)	NM_004048	Forward	CCGTGTGAACCATGTGACT	91
		Reverse	TGCGGCATCTTCAAACCT	
Grp78	NM_005347	Forward	TGGAGGTGGGCAAACAAAGACA	154
		Reverse	TGCTTGGCGTTGGGCATCATTA	
Hsp72	NM_005345	Forward	CGCAACGTGCTCATCTTTGA	198
		Reverse	TCGCTTGTTCTGGCTGATGT	
Hsp90α (variant 1 & variant 2)	NM_001017963 & NM_005348	Forward	AAACTGCGCTCCTGTCTTCT	180
		Reverse	TGCGTGATGTGTCGTCATCT	

3′ (3 primer end), 5′ (5 primer end), Grp78 (Glucose regulated protein 78), Hsp72 (Heat shock protein 72), Hsp90α (Heat shock protein 90 α).

(Nanodrop 2000c, Thermo Scientific). Only samples with a 260:280 ratio of between 1.9 and 2.15 were carried forward for reverse transcription and PCR amplification detailed below.

One Step Reverse Transcription Quantitative Polymerase Chain Reaction (RT-QPCR)

Primers (see **Table 2**) were designed using primer design software (Primer Quest and Oligoanalyzer—Integrated DNA technologies). During primer design sequence homology searches were performed against the Genbank database to ensure the primers matched the gene of interest. Primers were designed to span exon-intron boundaries and avoided three or more GC bases within the last 5 bases at the 3′ end of primer to avoid non-specific binding. Further searches were performed to ensure primers did not contain secondary structures and inter or intra molecular interactions (hairpins, self-dimer and cross dimers), which can inhibit product amplification. Hsp72, Hsp90α and Grp78 relative mRNA expression was then quantified using RT-QPCR. 20 μL reactions containing 10 μL SYBR-Green RT-PCR Mastermix (Quantifast SYBRgreen Kit, Qiagen, Manchester, UK), 0.15 μL forward primer, 0.15 μL reverse primer, 0.2 μL reverse transcription mix (Quantifast RT Mix, Qiagen) and 9.5 μL sample (70 ng RNA/ μL) were prepared using the Qiagility automated pipetting system (Qiagen). Each reaction was amplified in a thermal cycler (Rotorgene Q, Qiagen) and involved reverse transcription lasting 10 min at 50°C and a transcriptase inactivation and initial denaturation phase lasting 5 min at 95°C. The PCR reaction then followed with a denaturation step lasting 10 s at 95°C and a primer annealing and extension stage lasting 30 s at 60°C repeated for 40 cycles. Fluorescence was measured following each cycle as a result of the incorporation of SYBR green dye into the amplified PCR product. Melt curves (50 to 95°C; Ramp protocol 5 s stages) were analyzed for each reaction to ensure only the single gene of interest was amplified.

The relative quantification of mRNA expression for each sample (Hsp72, Hsp90α, and Grp78) was assessed by determining the ratio between the cycling threshold (C_T) value of the target mRNA and the C_T values for β2-Microglobulin (β2-M) mRNA. Fold change in relative mRNA expression was calculated using the 2-$\Delta\Delta C_T$ method (Schmittgen and Livak, 2008). β2-Microglobulin was used as a housekeeping gene as it was stable between experimental trials and across time in both the VL and leukocytes, as previously observed following exercise (Mahoney et al., 2004, 2008; Tuttle et al., 2015). The coefficient of variation for β2-M mRNA, Hsp72 mRNA, Hsp90α mRNA and Grp78 mRNA were 0.55, 0.34, and 0.28% respectively.

Statistical Analysis

Central tendency and dispersion are reported as the mean and standard deviation for normally distributed data and as the median and interquartile range for non-normally distributed data. Inferential statistical analyses were completed using linear mixed models for repeated measures (IBM SPSS Statistics 19, Chicago, IL) with comparisons made for main effects, two way interactions (experimental trial × time) and three way interactions (group × experimental trial × time). The best fitting covariance structure was selected by minimizing the Hurvich and Tsai's criterion (Field, 2013). Changes in Hsp72, Hsp90α, and Grp78 mRNA are presented as fold change from basal in accordance with previous literature (Tuttle et al., 2015; Gibson et al., 2015c). Where significant F ratios for main and interaction effects occurred, *post-hoc* pairwise comparisons were made with Bonferroni adjusted *p*-values. Pearson's product correlation was performed between leukocyte and vastus lateralis Hsp72 mRNA and Hsp90α mRNA before, immediately after and 3 h after $TPC1_{TEMPFLAT}$ and $HPC1_{HOTDOWN}$. Pearson's product correlations were also performed between physiological variables T_{re} and HR, and leukocyte and VL Hsp72 mRNA and Hsp90α mRNA immediately and 3 h after the corresponding $TPC1_{TEMPFLAT}$ and $HPC1_{HOTDOWN}$. The mRNA responses to $TPC2_{HOTDOWN}$ and $HPC2_{HOTDOWN}$ were not included in the correlational analyses given the likelihood of the prior trials to be a confounding factor due to the hypothesized preconditioning effect i.e., increase gene transcription and therefore signal post translational events to increase basal HSP (Tuttle et al., 2015). Statistical significance was accepted at $p < 0.05$ (two tailed).

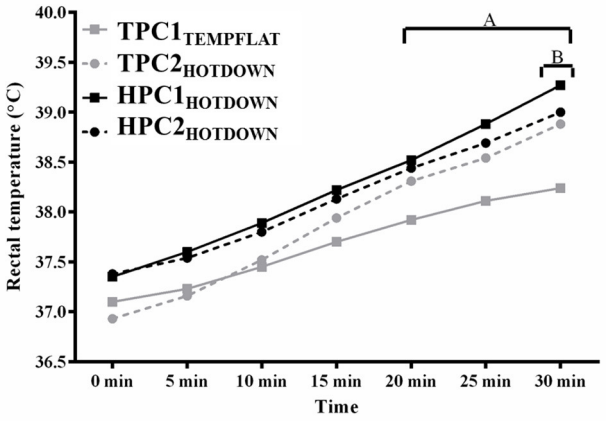

FIGURE 2 | Rectal temperature (T_{re}) at 0–30 min of exercise. A, T_{re} increased ($p < 0.005$) during $TPC2_{HOTDOWN}$ compared to $TPC1_{TEMPFLAT}$. B, T_{re} decreased ($p = 0.017$) during $HPC2_{HOTDOWN}$ compared to $HPC1_{HOTDOWN}$. Mean data presented. Error bars omitted to maintain clarity.

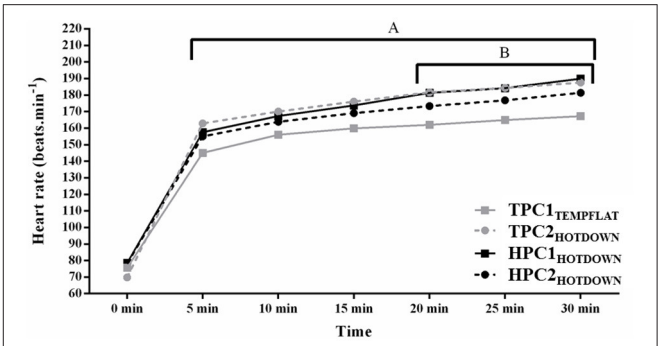

FIGURE 3 | Heart rate (HR) at 0–30 min of exercise. A, increased ($p < 0.010$) during $TPC2_{HOTDOWN}$ compared to $TPC1_{TEMPFLAT}$ at 5–30 min. B, increased ($p < 0.050$) during $HPC1_{HOTDOWN}$ increased compared to $TPC1_{TEMPFLAT}$ at 20–30 min. Mean data presented. Error bars omitted to maintain clarity.

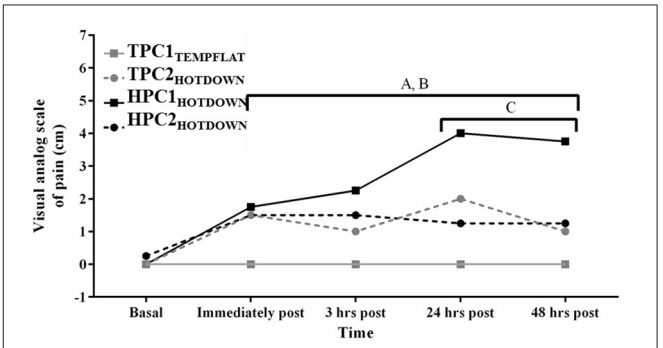

FIGURE 4 | Perceived muscle soreness measured via the Visual analog scale of pain (VAS) immediately before, immediately post, 3 h post, 24 h post, and 48 h post exercise. A, increased ($p < 0.005$) during $TPCHEAT_{DOWN}$ compared to $TPC1_{TEMPFLAT}$. B, increased ($p < 0.001$) during $HPC1_{HOTDOWN}$ compared to $TPC1_{TEMPFLAT}$. C, decreased ($p < 0.001$) during $HPC2_{HOTDOWN}$ compared to $HPC1_{HOTDOWN}$. Median data presented. Error bars omitted to maintain clarity.

RESULTS

Thermoregulatory Response

Exercising T_{re} (**Figure 2**) increased as main effect between 5 and 30 min ($p < 0.001$) compared to basal. Average exercising T_{re} was higher during the hot downhill running trials ($HPC1_{HOTDOWN}$; 38.3°C; $F = 14.3$, $p = 0.002$, and $TPC2_{HOTDOWN}$ (37.9°C; $F = 6.1$, $p = 0.017$) compared to the temperate flat trial ($TPC1_{TEMPFLAT}$; 37.7°C). Exercising T_{re} was greater during the hot downhill trials ($TPC2_{HOTDOWN}$; 20–30 min, $p < 0.05$, $HPC1_{HOTDOWN}$; 5–30 min, $p < 0.05$) compared to the temperate flat trial ($TPC1_{TEMPFLAT}$). Exercising T_{re} was also 0.3°C higher (39.3 ± 0.3°C compared to 39.0 ± 0.4°C) at 30 min during $HPC1_{HOTDOWN}$ compared to $HPC2_{HOTDOWN}$ ($F = 6.1$, $p = 0.017$).

Heart rate (**Figure 3**) was increased compared to basal between 5 and 30 min ($p < 0.001$). Average HR was higher during $TPC2_{HOTDOWN}$ (162 beats.min^{-1}) compared to $TPC1_{TEMPFLAT}$ (147 beats.min^{-1}; $F = 22.3$, $p = 0.001$). No difference in average HR was observed between $HPC1_{HOTDOWN}$ (161 beats.min^{-1}) and $TPC1_{TEMPFLAT}$ ($F = 3.3$, $P = 0.096$) or $HPC2_{HOTDOWN}$ (157 beats.min^{-1}; $F = 2.8$, $p = 0.128$). Heart rate was higher during the hot downhill trials ($TPC2_{HOTDOWN}$; 5–30 min, $p < 0.05$ and $HPC1_{HOTDOWN}$; 20–30 min, $p < 0.05$) compared to the temperate flat trial ($TPC1_{TEMPFLAT}$). A trend for HR to be reduced during $HPC2_{HOTDOWN}$ compared to $HPC1_{HOTDOWN}$ between 20 and 30 min (8 beats.min^{-1}; $\sim F = 3.8$, $p = \sim 0.069$) was observed.

Perceived muscle soreness (indicated by the VAS; **Figure 4**) was increased over time as a main effect immediately post to 48 h post exercise compared to basal ($p < 0.001$). Perceived muscle soreness also increased from basal between immediately post to 48 h post exercise following $TPC2_{HOTDOWN}$ and $HPC1_{HOTDOWN}$ ($p < 0.001$) and between immediately post—3 h post $HPC2_{HOTDOWN}$ ($p < 0.05$). Perceived muscle soreness was greater following the hot downhill running trials ($TPC2_{HOTDOWN}$ and $HPC1_{HOTDOWN}$) compared to the temperate flat running trial ($TPC1_{TEMPFLAT}$) immediately post, ($F = 7.2$, $p = 0.011$ and $F = 11.8$, $p = 0.002$), 3 h post ($F = 6.1$, $p = 0.019$ and $F = 9.1$, $p = 0.005$), 24 h post ($F = 12.2$, $p = 0.001$ and $F = 25.0$, $p < 0.001$) and 48 h post exercise ($F = 14.3$, $p = 0.001$ and $F = 30.4$, $p < 0.001$) respectively. Perceived muscle soreness was attenuated 24 and 48 h after the second hot downhill running trial ($HPC2_{HOTDOWN}$) compared to the first hot downhill trial ($HPC1_{HOTDOWN}$; $F = 12.6$, $p = 0.001$ and $F = 11.3$, $p = 0.002$, respectively) in the HPC.

Quadriceps tenderness (QT; **Table 3**) was increased as a main effect immediately post to 48 h post exercise ($p < 0.05$) compared to basal. No difference in QT was observed between experimental trials ($P > 0.05$).

Metabolic and Perceptual Responses

Compared to basal, blood lactate concentrations (**Table 3**) increased following the hot downhill running trials ($TPC2_{HOTDOWN}$; $F = 11.0$, $p = 0.006$, $HPC1_{HOTDOWN}$;

TABLE 3 | Physiological and perceptual responses.

		$TPC1_{TEMPFLAT}$	$TPC2_{HOTDOWN}$	$HPC1_{HOTDOWN}$	$HPC2_{HOTDOWN}$
B[La] ($mmol.l^{-1}$)	Basal	1.0 ± 0.3[A]	0.8 ± 0.1	0.6 ± 0.1	0.8 ± 0.2
	Immediately post	1.0 ± 0.3	1.8 ± 0.5*	1.6 ± 0.8*	1.1 ± 0.6*
Urine Osmolality (mOsmols.kg H_20)	Basal	250.0 ± 200.0	150.0 ± 40.0	165.0 ± 152.5	170.0 ± 185.0
	Immediately post	310.0 ± 60.0[B]	330.0 ± 100.0[B]	390.0 ± 267.5[B]	430.0 ± 305.0[B]
Quadriceps tenderness (% decrease in force required to elicit tenderness)	Basal	100 ± 0.0	98.9 ± 10.9	100 ± 0.0	101.4 ± 8.7
	Immediately post	87.8 ± 9.3*	79.3 ± 16.7*	85.3 ± 12.5*	87.0 ± 11.3*
	3 hrs post	86.6 ± 15.6*	82.3 ± 14.7*	88.6 ± 7.7*	92.3 ± 8.8*
	24 hrs post	88.9 ± 13.8*	79.9 ± 22.5*	73.1 ± 9.7*	85.3 ± 4.8*
	48 hrs post	93.6 ± 7.9*	89.0 ± 27.9*	82.8 ± 16.9*	99.3 ± 10.5*

$\dot{V}O_2$ ($ml.kg.min^{-1}$)	0 min	5 min	10 min	15 min	20 min	25 min	30 min
$TPC1_{TEMPFLAT}$	6.4 ± 1.8	34.3 ± 4.2*	35.1 ± 5.6*	36.2 ± 6.4*	36.1 ± 5.6*	35.9 ± 5.0*	36.0 ± 4.0*
$TPC2_{HOTDOWN}$	6.0 ± 1.0	34.2 ± 6.2*	35.1 ± 6.5*	36.1 ± 7.8*	36.7 ± 7.8*	36.1 ± 6.8*	36.2 ± 7.2*
$HPC1_{HOTDOWN}$	6.3 ± 1.5	32.0 ± 1.8*	32.6 ± 2.1*	34.4 ± 1.7*	35.6 ± 1.4*	36.7 ± 1.8*	37.2 ± 1.7*
$HPC2_{HOTDOWN}$	6.5 ± 0.8	32.0 ± 1.3*	33.4 ± 1.6*	33.6 ± 2.5*	35.4 ± 2.4*	36.0 ± 2.7*	36.4 ± 2.8*
RPE (Units)							
$TPC1_{TEMPFLAT}$	6.0 ± 0.0	10.0 ± 2.0	10.4 ± 0.8	11.8 ± 1.5	12.6 ± 1.5	13.2 ± 1.6	13.4 ± 1.6
$TPC2_{HOTDOWN}$	6.0 ± 0.0	11.4 ± 1.3[C]	13.4 ± 0.9[C]	14.4 ± 0.6[C]	15.4 ± 0.9[C]	16.0 ± 0.8[C]	17.3 ± 1.0[C]
$HPC1_{HOTDOWN}$	6.0 ± 0.0	11.5 ± 1.1[C]	13.2 ± 0.8[C]	14.2 ± 1.1[C]	15.3 ± 0.5[C]	16.5 ± 0.6[C]	17.5 ± 1.2[C]
$HPC2_{HOTDOWN}$	6.0 ± 0.0	11.8 ± 0.8	12.8 ± 0.8	13.7 ± 1.0	15.1 ± 1.0	16.0 ± 1.3	17.0 ± 1.3
TS (Units)							
$TPC1_{TEMPFLAT}$	4.0 ± 0.0	3.6 ± 1.1	4.4 ± 1.0	5.0 ± 0.9	5.4 ± 0.7	5.5 ± 0.6	5.5 ± 0.7
$TPC2_{HOTDOWN}$	4.0 ± 0.0	5.1 ± 0.7[C]	5.6 ± 0.7[C]	6.0 ± 0.7[C]	6.6 ± 0.7[C]	7.0 ± 0.4[C]	7.3 ± 0.3[C]
$HPC1_{HOTDOWN}$	4.0 ± 0.0	4.7 ± 0.5[C]	5.3 ± 0.4[C]	5.8 ± 0.5[C]	6.3 ± 0.4[C]	6.8 ± 0.4[C]	6.9 ± 0.4[C]
$HPC2_{HOTDOWN}$	4.0 ± 0.0	4.8 ± 0.4	5.5 ± 0.7	5.7 ± 0.7	6.3 ± 0.4	6.7 ± 0.4	6.8 ± 0.4

*Values are expressed as mean ± SD for quadriceps tenderness and $\dot{V}O_2$. Values are expressed as median ± IQR for B[La], RPE, TS and Urine Osmolality. *Increased compared to basal ($p < 0.05$).*

[A]Increased compared to $HPC1_{HOTDOWN}$.

[B]Increased from basal (main effect).

[C]Increased compared to $TPC1_{TEMPFLAT}$.

$F = 13.3$, $p = 0.003$ and $HPC2_{HOTDOWN}$; $F = 5.7$, $p = 0.035$), but not the temperate flat trial ($TPC1_{TEMPFLAT}$; $F = 0.0$, $p = 0.874$). Oxygen uptake ($\dot{V}O_2$) increased ($F = 236.0$, $p < 0.001$) over time as a main effect but there was no difference between experimental trials ($P < 0.05$). Participants exercised at an average % $\dot{V}O_{2max}$ of 70.2 ± 6.0% during the $TPC1_{TEMPFLAT}$ trial, 70.8 ± 6.9% during the $TPC2_{HOTDOWN}$ trial, 66.2 ± 6.0% during the $HPC1_{HOTDOWN}$ trial and 65.8 ± 8.4% during the $HPC2_{HOTDOWN}$ trial.

Both the rate of perceived exertion (RPE; **Table 3**) and thermal sensation (TS; **Table 3**) were greater during the hot downhill running trials ($TPC2_{HOTDOWN}$ and $HPC1_{HOTDOWN}$; $p < 0.05$) compared to the temperate flat trial ($TPC1_{TEMPFLAT}$). No difference in RPE or TS was observed between the $HPC1_{HOTDOWN}$ and $HPC2_{HOTDOWN}$ trials ($p > 0.05$).

Cellular Stress (Hsp mRNA) Response

The responses of Hsp72, Hsp90α, and Grp78 mRNA were assessed to determine their suitability as markers of the cellular stress response. Vastus lateralis Hsp72 mRNA (**Figure 5A**) increased as a main effect immediately post ($p < 0.001$) and 3 h post exercise ($p = 0.002$) compared to basal. Vastus lateralis Hsp72 mRNA increased immediately post exercise compared to basal in the hot downhill running trials ($TPC2_{HOTDOWN}$ and $HPC1_{HOTDOWN}$; $p < 0.001$). Vastus lateralis Hsp72 mRNA expression was greater immediately post $TPC2_{HOTDOWN}$ and $HPC1_{HOTDOWN}$ compared to the temperate flat trial ($TPC1_{TEMPFLAT}$; $F = 24.2$, $p < 0.001$ and $F = 9.2$, $p = 0.004$, respectively) and the second hot downhill trial in the hot preconditioning group ($HPC2_{HOTDOWN}$; $F = 9.7$, $p = 0.003$ and $F = 5.0$, $p = 0.028$, respectively). Vastus lateralis Hsp72 mRNA was also greater 3 h after $HPC1_{HOTDOWN}$ compared to $TPC1_{TEMPFLAT}$ ($F = 6.6$, $p = 0.013$).

Leukocyte Hsp72 mRNA expression (**Figure 5B**) increased as a main effect immediately post ($p < 0.001$) and 3 h post exercise ($p = 0.004$) compared to basal. Leukocyte Hsp72 mRNA expression was greater following $HPC1_{HOTDOWN}$ compared to $TPC1_{TEMPFLAT}$ ($F = 4.2$, $p = 0.049$) and $HPC2_{HOTDOWN}$ ($F = 10.2$, $p = 0.003$).

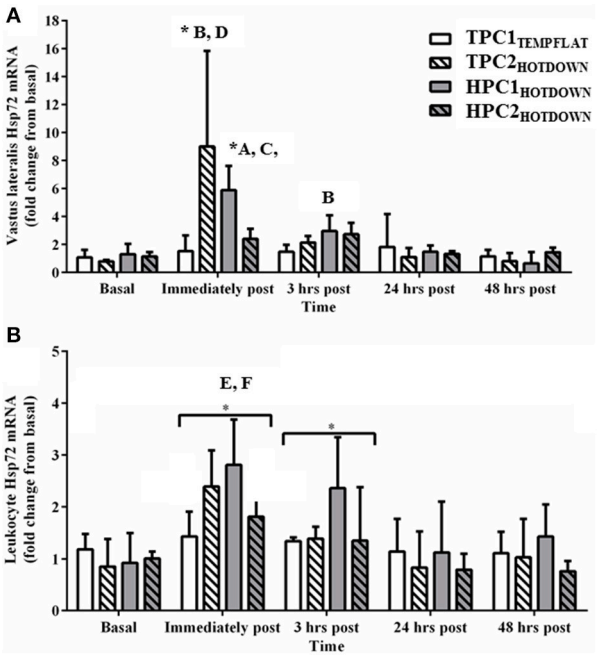

FIGURE 5 | Hsp72 mRNA response immediately before, immediately post, 3 h post, 24 h post and 48 h post exercise in the Vastus lateralis **(A)** and Leukocytes **(B)**. * Increased ($p < 0.001$) compared to basal. A, increased ($p = 0.020$) in $HPC1_{HOTDOWN}$ compared to $TPC1_{TEMPFLAT}$. B, increased ($p < 0.001$) in $TPC2_{HOTDOWN}$ compared to $TPC1_{TEMPFLAT}$. C, increased ($p = 0.028$) in $HPC1_{HOTDOWN}$ compared to $HPC2_{HOTDOWN}$. D, increased in $TPC2_{HOTDOWN}$ compared to $HPC2_{HOTDOWN}$. E, $HPC1_{HOTDOWN}$ increased ($p = 0.049$) compared to $HPC2_{HOTDOWN}$. F, $HPC1_{HOTDOWN}$ increased ($p = 0.003$) compared to $TPC1_{TEMPFLAT}$. Data presented as median ± interquartile range.

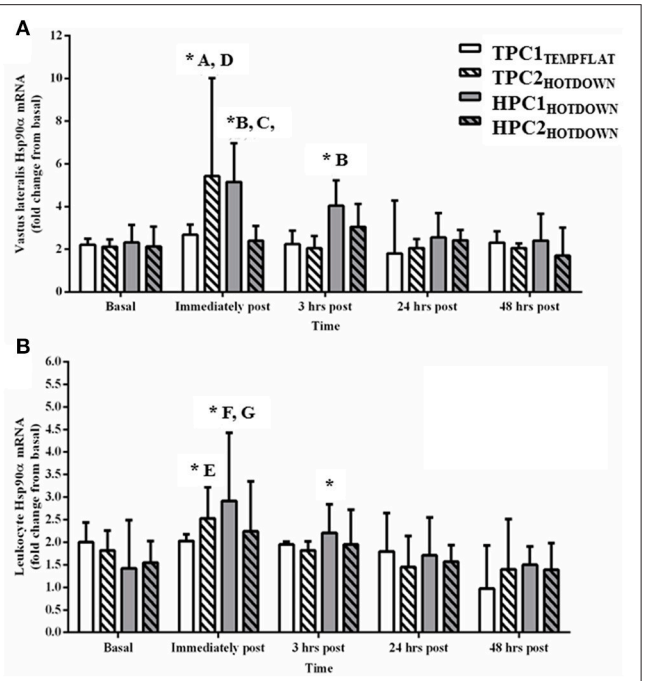

FIGURE 6 | Hsp90α mRNA response immediately before, immediately post, 3 h post, 24 h post, and 48 h post exercise in the Vastus lateralis **(A)** and Leukocytes **(B)**. * Increased ($p < 0.02$) compared to basal. A, increased ($p = 0.006$) during $TPC2_{HOTDOWN}$ compared to $TPC1_{TEMPFLAT}$. B, increased ($p < 0.001$) during $HPC1_{HOTDOWN}$ compared to $HPC2_{HOTDOWN}$. C, increased ($p < 0.05$) during $HPC1_{HOTDOWN}$ compared to $TPC1_{TEMPFLAT}$. D, increased ($p = 0.01$) during $TPC2_{HOTDOWN}$ compared to $HPC2_{HOTDOWN}$. E, ($p = 0.024$) during $TPC2_{HOTDOWN}$ compared to $TPC1_{TEMPFLAT}$. F, increased ($p = 0.030$) during $HPC1_{HOTDOWN}$ compared to $HPC2_{HOTDOWN}$. G, ($p = 0.002$) during $HPC1_{HOTDOWN}$ compared to $TPC1_{TEMPFLAT}$. Data presented as median ± interquartile range.

Vastus lateralis Hsp90α mRNA (**Figure 6A**) increased compared to basal following the hot downhill running trials $TPC2_{HOTDOWN}$ (immediately post exercise; $p < 0.001$) and $HPC1_{HOTDOWN}$ (immediately post; $p < 0.001$ and 3 h post; $p = 0.020$). Vastus lateralis Hsp90α mRNA expression was greater immediately post $TPC2_{HOTDOWN}$ compared to $TPC1_{TEMPFLAT}$ ($F = 8.4$, $p = 0.006$), and $HPC2_{HOTDOWN}$ ($F = 7.4$, $p = 0.010$). Vastus lateralis Hsp90α mRNA expression was also greater following $HPC1_{HOTDOWN}$ compared to $TPC1_{TEMPFLAT}$ (immediately post; $F = 4.3$, $p = 0.044$ and 3 h post; $F = 4.4$, $p = 0.043$) and $HPC2_{HOTDOWN}$ (immediately post; $F = 19.4$, $p < 0.001$).

Leukocyte Hsp90α mRNA expression increased as a main effect immediately post exercise compared to basal ($p < 0.001$). Leukocyte Hsp90α mRNA expression also increased following $TPC2_{HOTDOWN}$ (immediately post; $p = 0.024$) and $HPC1_{HOTDOWN}$ (immediately post; $p < 0.001$ and 3 h post; $p = 0.041$) compared to basal. Leukocyte Hsp90α mRNA expression was greater immediately after $TPC2_{HOTDOWN}$ compared to $TPC1_{TEMPFLAT}$ ($F = 5.3$, $p = 0.024$). Leukocyte Hsp90α mRNA expression was also greater immediately after $HPC1_{HOTDOWN}$ compared to $TPC1_{TEMPFLAT}$ ($F = 10.1$, $p = 0.002$) and $HPC2_{HOTDOWN}$ ($F = 4.9$, $p = 0.030$).

Vastus lateralis Grp78 mRNA (**Figure 7A**) increased as a main effect immediately post to 48 h post exercise compared to basal ($p < 0.002$). Vastus lateralis Grp78 mRNA also increased within the TPC immediately post ($p = 0.003$) and within the HPC at 3 h ($p < 0.001$) and 24 h post ($p < 0.001$). Vastus lateralis Grp78 mRNA increased compared to basal following the hot downhill running trials, $TPC2_{HOTDOWN}$ (immediately post; $p < 0.001$), $HPC1_{HOTDOWN}$ (3 and 24 h post; $p < 0.010$) and $HPC2_{HOTDOWN}$ (24 h post; $p = 0.003$), but did not change following the temperate flat trial ($TPC1_{TEMPFLAT}$; $p > 0.05$).

All main effects and interactions had no effect ($p > 0.05$) on leukocyte Grp78 mRNA (**Figure 7B**).

Relationship between mRNA Responses

A strong correlation was observed between vastus lateralis Hsp72 and Hsp90α mRNA expression ($r = 0.863$, $p < 0.001$; **Figure 8A**), and between leukocyte Hsp72 and Hsp90α mRNA expression ($r = 0.844$, $p < 0.001$; **Figure 8B**). Modest correlations were also observed between leukocyte Hsp72 mRNA and vastus lateralis Hsp72 mRNA ($r = 0.651$, $p < 0.001$; **Figure 8C**), and between leukocyte Hsp90α mRNA and vastus lateralis Hsp90α mRNA. ($r = 0.640$, $p < 0.001$; **Figure 8D**). Relationships between Hsp72

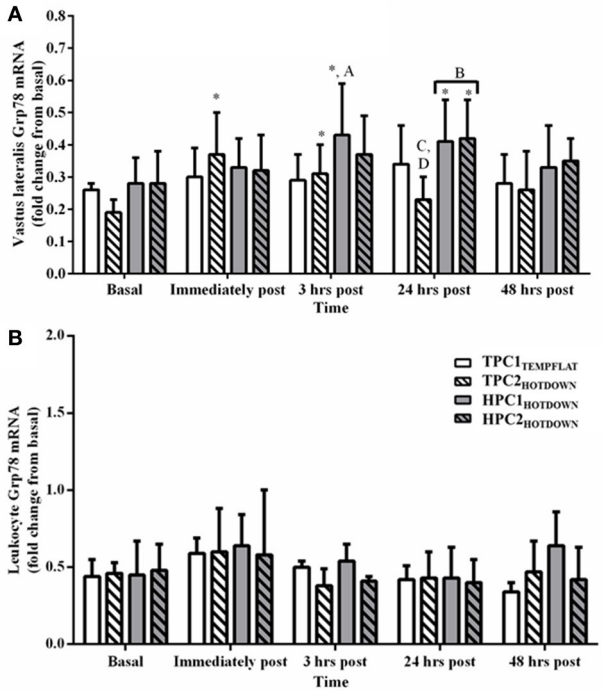

FIGURE 7 | Grp78 mRNA response immediately before, immediately post, 3 h post, 24 h post, and 48 h post exercise in the Vastus lateralis **(A)** and Leukocytes **(B)**. * Increased ($p < 0.01$) compared to basal. * Increased ($p < 0.01$) compared to basal. A, increased ($p = 0.031$) during $HPC1_{HOTDOWN}$ compared to $TPC1_{TEMPFLAT}$. B, increased ($p < 0.001$) during HPC compared to TPC. C, decreased ($p = 0.006$) during $TPC2_{HOTDOWN}$ compared to $HPC2_{HOTDOWN}$. D, decreased ($p = 0.01$) during $TPC2_{HOTDOWN}$ compared to $TPC1_{TEMPFLAT}$. Data presented as median ± interquartile range.

and Hsp90α mRNA, and Grp78 mRNA were not analyzed given the absence of a change in leukocyte Grp78 mRNA (**Figure 7B**).

A strong relationship was also observed between the peak T_{re} during $TPC1_{TEMPFLAT}$ and $HPC1_{HOTDOWN}$ and the immediately post measured leukocyte Hsp72 ($r = 0.665$, $p = 0.026$) and Hsp90α mRNA ($r = 0.708$, $p = 0.015$), and the 3 h measured leukocyte ($r = 0.786$, $p = 0.004$) and vastus lateralis ($r = 0.720$, $p = 0.013$) Hsp72 mRNA, and vastus lateralis Hsp90α mRNA ($r = 0.682$, $p = 0.021$). A strong relationship was also observed between peak heart rate during $TPC1_{TEMPFLAT}$ and $HPC1_{HOTDOWN}$, and leukocyte ($r = 0.739$, $p = 0.009$) and vastus lateralis ($r = 0.766$, $p = 0.006$) Hsp72 mRNA, and leukocyte ($r = 0.677$, $p = 0.022$) and vastus lateralis Hsp90α mRNA ($r = 0.746$, $p = 0.008$) at 3 h post exercise. No significant relationship was observed immediately post $TPC1_{TEMPFLAT}$ or $HPC1_{HOTDOWN}$.

DISCUSSION

The current study demonstrated that both VL and leukocyte Hsp72 and Hsp90α mRNA increases following the first trial of downhill running in a hot environment ($HPC1_{HOTDOWN}$) were attenuated concurrently with reductions in exercising T_{re} and DOMS during the second trial of downhill running in a hot environment ($HPC2_{HOTDOWN;}$ see **Figures 5**, **6**). This suggests that the cellular stress response (Hsp72 and Hsp90α mRNA) occurred simultaneously within both tissues (**Figure 8**) and likely contributed to the preconditioning effect. This was not demonstrated in GRP78 mRNA (**Figure 7**). The absence of change in GRP78 mRNA in leukocytes suggests this is not an appropriate tissue to determine changes in its expression levels. Therefore, the leukocyte Hsp72 and Hsp90α mRNA responses could potentially be a useful surrogate for the VL response. At a physiological level the attenuated T_{re} (**Figure 2**), HR (**Figure 3**) and VAS (**Figure 4**) responses to an equivalent downhill run following HPC demonstrates an acute preconditioning response was attained. This was not discernible in the TPC group whom demonstrated the known responses to downhill running under heat stress in comparison to level gradient running in temperate conditions i.e., increased T_{re} (**Figure 2**), HR (**Figure 3**) and VAS (**Figure 4**).

Cellular Stress Response and Surrogate Hsp mRNA Response

Increased Hsp72 mRNA transcription has frequently been demonstrated following exercise [leukocytes and VL (Walsh et al., 2001; Mestre-Alfaro et al., 2012)], muscle damaging exercise [VL (Vissing et al., 2009)] and exercise heat stress [leukocytes (Mestre-Alfaro et al., 2012)] within humans. However, there is less data available regarding the Hsp72 mRNA response being attenuated during repeated trials of muscle damaging exercise, or exercise heat stress as observed frequently during repeated trials of *in vitro* heat shock (Kiang et al., 1996; Theodorakis et al., 1999). Studies have previously only observed a blunted response following muscle damaging exercise in the VL (Paulsen et al., 2007) and exercise heat stress within leukocytes (Fehrenbach et al., 2001; Marshall et al., 2007). Within these studies reductions in thermal strain [exercising T_{re} (Fehrenbach et al., 2001; Marshall et al., 2007)] and muscle damage (Paulsen et al., 2007) during subsequent experimental trials were suggested to be responsible for the attenuated Hsp72 mRNA response observed. The current study also observed a reduction in thermal strain (T_{re} $-0.3^{\circ}C$) equivalent to that of various heat acclimation regimes (Gibson et al., 2015b; Tyler et al., 2016), and an attenuated perceived muscle soreness (24 h post = +12.2%, 48 h post = −16.5%) response that is indicative of muscle damage (Fridén et al., 1981) from near identical exercise trials [$HPC2_{HOTDOWN}$ compared to $HPC1_{HOTDOWN}$ (see **Figure 2** and **Table 3**)]. Together these responses indicate that downhill running models may be able to elicit a beneficial preconditioning effect (Dolci et al., 2015; Tuttle et al., 2015). Given that acute non-damaging exercise heat stress does not improve thermal responses to a greater extent than equivalent temperate condition exercise [**Figure 2**, (Lee et al., 2014)], the cellular responses to the eccentric muscle action of the damaging downhill running is important. The attenuated exercising T_{re} response could be suggestive of a reduction in relative exercise intensity and therefore potentially reduced requirement for ATP production (Febbraio et al., 1996), though no statistical difference in absolute intensity as

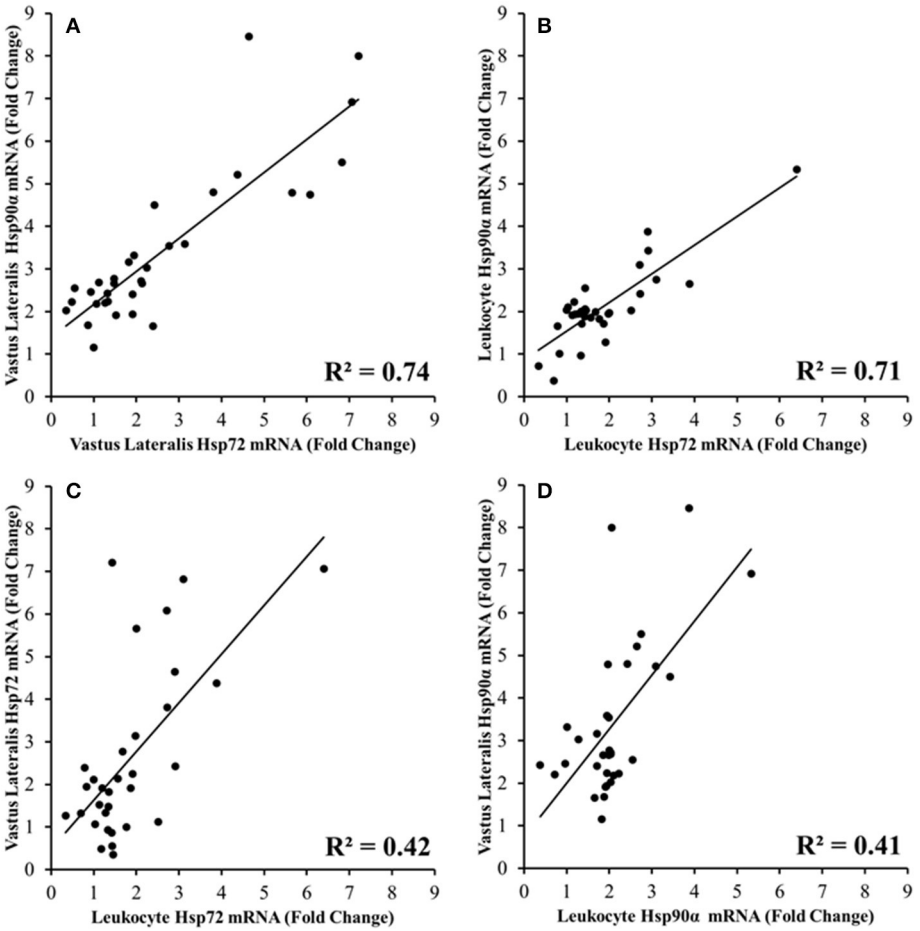

FIGURE 8 | Relationships between Hsp72 **(A,C)** and Hsp90α mRNA **(B,D)** responses in the Vastus Lateralis and Leukocytes immediately before, immediately post, 3 h post $TPC1_{TEMPFLAT}$ and $HPC1_{HOTDOWN}$ (all $p < 0.001$).

indicated by $\dot{V}O_2$ was observed (**Table 3**). Therefore, metabolic strain was likely reduced. Protein denaturation, the key cellular change associated with heat shock factor-1 (HSF-1) activation and Hsp72 and Hsp90α mRNA transcription, is temperature (Mestre-Alfaro et al., 2012), metabolic strain (Beckmann et al., 1992) and muscle damage (Michailidis et al., 2013) dependent. This suggests the observed attenuated thermal strain and muscle damage responses could be an important mechanism explaining the attenuated Hsp72 mRNA response in leukocytes ($HPC1_{HOTDOWN}$ = +207%; $HPC2_{HOTDOWN}$ = +79%) and VL ($HPC1_{HOTDOWN}$ = +353%; $HPC2_{HOTDOWN}$ = +109%) observed following the $HPC2_{HOTDOWN}$ trial, compared to $HPC2_{HOTDOWN}$ trial. Although, the expression of VL (Neubauer et al., 2014) and leukocyte (Moran et al., 2006) Hsp90α mRNA have previously been observed to increase following exercise and exercise heat stress, respectively, with equality of physiological stimuli i.e., T_{re} maintaining Hsp90α mRNA transcription (Gibson et al., 2015c), no studies have determined whether Hsp90α mRNA is attenuated during repeated trials of muscle damaging exercise. Consequently, the attenuated Hsp90α mRNA response in both leukocytes ($HPC1_{HOTDOWN}$ = +106%, $HPC2_{HOTDOWN}$ = +45%) and skeletal muscle ($HPC1_{HOTDOWN}$ = +122%, $HPC2_{HOTDOWN}$ = +113%) following reductions in physiological strain is a novel observation (see **Figure 6**). It is a novel finding that the relationship between Hsp72 and Hsp72 mRNA transcription is equivalent in the VL (**Figure 8A**, $R^2 = 0.74$), as has been previously shown in leukocytes [$R^2 = 0.77$ (Gibson et al., 2016)]. It has also been observed that the Hsp72 and Hsp72 mRNA transcription response is comparable following damaging exercise (**Figure 8B**, $R^2 = 0.71$), as it has previously in non-damaging exercise models (Gibson et al., 2016). Within leukocytes, it has been observed that Hsp72 mRNA transcription (Gibson et al., 2015a,c; Mee et al., 2016), and Hsp90α mRNA transcription (Gibson et al., 2015c) returns to baseline 24 h following non-damaging exercise heat stress (Moran et al., 2006). The heat shock factor-1 (HSF-1) transcription pathway likely highlights the mechanism between equality of increases in Hsp72 and Hsp90α mRNA as demonstrated in this experiment (**Figure 8**), and others utilizing a non-damaging model (Gibson et al., 2016) with the attenuated mRNA response in the $HPC2_{HOTDOWN}$ trial reflecting a reduction in the physiological

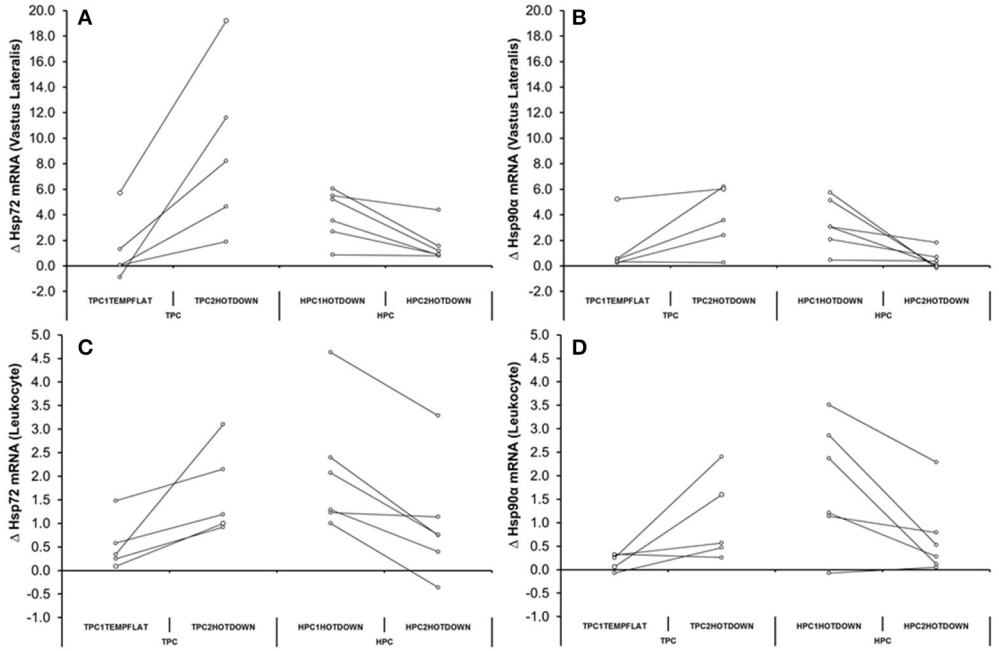

FIGURE 9 | Individual responses reflecting the change in mRNA (**A** = Hsp72 mRNA in Vastus Lateralis, **B** = Hsp90α mRNA in Vastus Lateralis, **C** = Hsp72 mRNA in Leukocytes, D_S Hsp90α mRNA in Leukocytes) from baseline to immediately post $TPC1_{TEMPFLAT}$ and $TPC2_{HOTDOWN}$ **(A,C)**, and $HPC1_{HOTDOWN}$ and $HPC2_{HOTDOWN}$ **(B,D)**.

stimuli as a result of the prior HPC for all participants (**Figure 9**).

HSP72 protein concentrations (due to translational inhibition) may not necessary directly represent the magnitude of the cellular stress response therefore the mRNA response has been proposed as more appropriate (Amorim et al., 2015; Gibson et al., 2015a; Lee et al., 2015). A reduction in the mRNA response is therefore representative of a gain in protein concentration (Marshall et al., 2007). The VL cellular adaptations associated with the repeated bout effect include a strengthened cytoskeleton [increased desmin concentrations (Feasson et al., 2002)] and elevated small HSP concentrations [αβ-crystallin and HSP27 (Paulsen et al., 2009)] and therefore, could be responsible for the attenuated Hsp72 and Hsp90α mRNA responses observed following $HPC2_{HOTDOWN}$. Optimization of transcriptional and translational processes (Touchberry et al., 2012) and elevated concentrations of anti-apoptotic (Horowitz, 2014) and antioxidant (Horowitz and Kodesh, 2010) proteins, which are implicated in enhanced thermotolerance, could also be responsible for the attenuated Hsp72 and Hsp90α mRNA responses observed following $HPC2_{HOTDOWN}$ within both the VL and leukocytes.

The current study observed for the first time that the leukocyte and VL Hsp72 and Hsp90α mRNA response occurs concurrently (**Figures 8C,D**). This novel data supports the notion that leukocytes are a desirable tissue site for determining the cellular stress response due to accessibility for analysis following exposure to both systemic signals and to signals of the perfused tissues (Sonna et al., 2007). Some caution should be raised as this experiment did not quantify the leukocyte infiltration to skeletal muscle, a known component of the intramuscular response which follows damaging exercise (Malm et al., 2004), though the time course and magnitude of this response are controversial (St. Pierre Schneider and Tiidus, 2007). A resolution to this issue within future experiments would be quantification of total mRNA (Sanders et al., 2014). As previously discussed the reduction in thermal and metabolic strain mediated within both leukocytes and the VL likely attenuated the increases in protein denaturation during $HPC2_{HOTDOWN}$ and thus could explain the attenuated Hsp72 and Hsp90α mRNA response observed in both tissues. Muscle damage mediated release of ligands [damage associated molecular patterns (DAMPs), circulating cell free DNA and extracellular HSPs (Neubauer et al., 2014)] from skeletal muscle could also explain the concurrent Hsp72 and Hsp90α mRNA responses via a toll like receptor mediated stress response within leukocytes, as previously observed following muscle damaging exercise (Fernandez-Gonzalo et al., 2012). Although elevations in these ligands may be exercise related (Neubauer et al., 2013), evidence for these ligands actually being released from skeletal muscle following exercise is limited. Consequently, the concurrent Hsp72 and Hsp90α mRNA responses are probably dependent on increases in thermal strain and metabolic strain within both leukocytes and the VL, and are unlikely to be muscle damage dependent.

Increases in VL Grp78 mRNA were observed following both $HPC1_{HOTDOWN}$ and $HPC2_{HOTDOWN}$ despite the observed reductions in exercising T_{re} and DOMS, which are associated with reduced protein denaturation, the key cellular change

regulating Grp78 mRNA transcription. Activation of the unfolded protein response also occurs when the endoplasmic reticulum protein load increases during cellular remodeling (Ron and Walter, 2007). Therefore, the Grp78 mRNA response may reflect the need to increase ER protein folding capacity to aid cellular adaptation (Ron and Walter, 2007). These observations combined with the absence of Grp78 mRNA increases within leukocytes suggest that Grp78 mRNA cannot be used as a marker of the cellular stress response, or thermotolerance, at least within the current experimental model.

Practical Applications and Future Directions

The results of this experiment highlight that an acute bout of downhill running in a hot environment is an effective preconditioning strategy to attenuate the increase in thermal strain experienced during a subsequent, equivalent exercise in hot conditions. Typically it is proposed that athletes, workers and the military should perform acclimation/acclimatization prior to traveling to unfamiliar, hot conditions (Racinais et al., 2015). An acute bout of downhill running in hot conditions i.e., whole body preconditioning may therefore be there an appropriate method to expediently elicit thermal protection i.e., a reduction in thermal strain prior to exercise in hot conditions. Given recent evidence of cross acclimation between stressors (Gibson et al., 2015c; Lee et al., 2016; White et al., 2016), it is also possible that this whole body preconditioning strategy will induce physiological and cellular adaptations which are beneficial in unfamiliar stressors e.g., hypoxia. These adaptations may become greater with repeated stress, i.e., repeated HPC, thus providing either a greater magnitude of cytoprotection, or a more prolonged post-HPC level of protection, or a combination of both. It is currently unknown how long the preconditioning effect elicited by $HPC1_{HOTDOWN}$ is retained beyond the 7 d duration we have observed. Without evidencing the decay in HSP72 and HSP90α content this is difficult to estimate, as such this remains an area for future investigation. Measurement of RNA/protein ratios may also aid understanding of the cytoprotective dynamics. The current study suggests that the leukocyte Hsp72 and Hsp90α mRNA responses could potentially be used as a surrogate measure of the HSR within skeletal muscle, at least within the current experimental model (preconditioning via downhill running in a hot environment). Consequently, the leukocyte Hsp72 and Hsp90α mRNA responses are potentially a relevant marker of individuals thermotolerance and thus could be useful for allocating appropriate athletic or occupational workloads without the potential reductions in performance and increased infection risk (within the biopsy incision) associated with skeletal muscle biopsies. The current experimental model utilized a combination of exercise heat stress and downhill running. Consequently, leukocyte Hsp72 mRNA and Hsp90α mRNA responses could be useful for suggesting thermotolerance within situations where exercise heat stress occur, such as military exercises or during athletic competition. Although the concurrent Hsp72 and Hsp90α mRNA responses are unlikely to be mechanistically linked exclusively to a muscle damage response, future work should set out to confirm whether this concurrent leukocyte and skeletal muscle response also occurs within a non-damaging exercise heat stress trial.

Summary and Conclusions

Hot downhill running is an effective preconditioning strategy which ameliorates physiological strain, muscle soreness and the cellular stress response (Hsp72 and Hsp90α mRNA transcription) to a subsequent bout of exercise-heat stress. This preconditioning strategy has applications for athletic, occupational and military populations. The current study suggests that Hsp72 and Hsp90α mRNA act as markers of the cellular stress response within both the VL and leukocytes. Consequently, the leukocyte Hsp72 mRNA and Hsp90α mRNA responses appear to be a surrogate measure of the cellular stress response in the VL. Accordingly, venepuncture to obtain circulating leukocytes provides a viable alternative to muscle sampling via biopsies to determine the cellular stress response to exercise-heat stress.

AUTHOR CONTRIBUTIONS

JT, PC, LT, and ML conception and design of research; JT, JB, DH, AJM, OP, CK, FR, and SA performed experiments; JT, BC, OG, PC, AWM, LT, and ML analyzed data; JT, BC, OG, PC, LT, and ML interpreted results of experiments; JT and OG prepared figures; JT drafted manuscript; JT, BC, OG, JB. DH, AJM, OP, CK, FR, SA, PC, AJM, AWM, LT, and ML edited and revised manuscript; JT, BC, OG, JB, DH, AJM, OP, CK, FR, SA, PC, AJM, AWM, LT, and ML approved the final version of manuscript.

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Repeated Excessive Exercise Attenuates the Anti-Inflammatory Effects of Exercise in Older Men

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Introduction/Purpose: A number of studies have investigated the effect of training with a moderate exercise dose (3–6 h/weekly) on the inflammatory profile in blood, and the data are inconsistent. Cross-sectional studies indicate a positive effect of physical activity level on inflammation levels and risk of metabolic disease. However, it is not clear whether this may be dose dependent and if very prolonged repeated exercise therefore may be beneficial for low-grade inflammation. Based on this we studied how excessive repeated prolonged exercise influenced low-grade inflammation and adipose tissue anti-inflammatory macrophage content in six older male recreationally trained cyclists. Low-grade inflammation and adipose tissue macrophage content were investigated in six older trained men (age: 61 ± 4 years; VO_{2peak}: 48 ± 2 mL kg^{-1} min^{-1}) following repeated prolonged exercise.

Methods: Cycling was performed daily for 14 days covering in total 2,706 km (1,681 miles). Maximal oxygen uptake (VO_{2peak}) was measured before and after the cycling. Duration and intensity of the exercise were determined from heart rates sampled during cycling. An adipose tissue biopsy from subcutaneous abdominal fat and a blood sample were obtained at rest in the overnight fasted state before and after the cycling. Anti-inflammatory adipose tissue macrophages (ATM) were immunohistochemically stained in cross sectional sections using a CD163 binding antibody. The ATM and adipocyte sizes were analyzed blindly.

Results: The cyclists exercised daily for 10 h and 31 ± 37 min and average intensity was 53 ± 1% of VO_{2peak}. Body weight remained unchanged and VO_{2peak} decreased by 6 ± 2% ($P = 0.04$). Plasma inflammatory cytokines, TNFα and IL-18 remained unchanged, as did hsCRP, but plasma IL-6 increased significantly. CD163 macrophage content remained unchanged, as did adipocyte cell size. The HbA1c was not significantly decreased, but there was a trend ($P < 0.07$) toward an increased insulin resistance as estimated by the Quicki Index.

Conclusion: The regular prolonged exercise did not influence abdominal adipose tissue inflammation, but the higher plasma IL-6 concentration concurrent with a trend toward higher insulin resistance and decreased VO_{2peak} implies that the excessive amount of exercise probably attenuated the possible potential anti-inflammatory effects of exercise.

Keywords: prolonged exercise, cytokines, skeletal muscle, macrophages, low-grade inflammation

INTRODUCTION

Physical inactivity and an unhealthy energy rich diet are often named as primary causes behind the worldwide increase in obesity and type 2 diabetes (WHO, 2016). Therefore, increasing daily physical activity is desirable and is beneficial for the individual as well as for the society. However, it is uncertain if extensive exercise is beneficial. Today there is an increasing number of people that participate in very prolonged exercise; marathons, long distance bike rides, triathlons, and even extreme ultra endurance exercise, yet there are several aspects of very prolonged exercise, where the mechanisms and health effects, positive and negative, are not well described.

Based on cross-sectional data higher physical activity levels are associated with reduced low-grade inflammation and thus lower susceptibility to obesity and type 2 diabetes (Abramson and Vaccarino, 2002; King et al., 2003; Schmidt et al., 2015). The available data from longitudinal studies are inconclusive, with studies showing that training and regular exercise attenuates low-grade inflammation (Larsen et al., 2001; Troseid et al., 2009; Thompson et al., 2010), but other studies, and in addition some very large Randomized Clinical Trials (RCT), have shown no effect on low-grade inflammation (Hammett et al., 2004; Beavers et al., 2013; Cooper et al., 2016). There is evidence that physical activity and weight loss combined attenuates low-grade inflammation (Beavers et al., 2013). Moreover, we previously demonstrated that 15 weeks lifestyle intervention consisting of a hypocaloric diet and physical activity in very obese individuals decreased low-grade inflammation and the density of inflammatory macrophages in abdominal adipose tissue concurrent with an improved insulin sensitivity and metabolic fitness (Bruun et al., 2006; Helge et al., 2011). Interestingly, a RCT in male overweight subjects demonstrated that 12 weeks controlled supervised endurance training with no weight loss did not influence low-grade inflammation, but did induce a higher abdominal adipose tissue anti-inflammatory CD163 macrophage content (Auerbach et al., 2013). The majority of the studies above have applied moderate exercise doses (3–6 h/weekly) and it is not clear whether very prolonged, repeated exercise will be beneficial for the inflammatory profile in blood and adipose tissue.

Based on this, we studied how excessive repeated prolonged exercise influenced low-grade inflammation and adipose tissue anti-inflammatory macrophage content in six older male recreationally trained cyclists. We hypothesized that repeated prolonged exercise without a weight loss would not influence plasma inflammatory markers, but would increase adipose tissue anti-inflammatory macrophage content.

MATERIALS AND METHODS

Study Design

The design of this study has been published in detail elsewhere (Rosenkilde et al., 2015; Morville et al., 2017). In brief, six male recreationally trained cyclists (61 ± 4 years, VO_{2peak} 48 ± 2 $mL \cdot kg^{-1} \cdot min^{-1}$) participated in the study. The subjects were all members of a bicycle club and most were active cycling 2–4 times per week in the months prior to departure. On their own accord, to win a bet (not initiated by any of the authors), the subjects organized a 14 days cycle trip with a total distance of 2,706 km (1,681 miles) from the Town Hall Square in Copenhagen, Denmark to North Cape in Norway, as far north as possible on the European continent (**Figure 1**; Rosenkilde et al., 2015). On this trip, the subjects' own support team handled all the practical logistics, providing transport of extra gear and maintenance of bikes and supplying, cooking, and preparing all food and beverages before, during, and after cycling. Two to five days before leaving and again 28–33 h after arrival at North Cape, the subjects were tested under standardized conditions in the morning after an overnight fast. All subjects were tested using the same equipment and protocols on both occasions. The sampling of blood and adipose tissue were done exactly as before the departure from Copenhagen and efforts were made to do the tests at the same time of day. In the day prior to the test day subjects were asked to refrain from participating in strenuous or prolonged exercise.

Ethical Approval

The subjects were informed about the possible risks and discomfort involved before written consent to participate was obtained. The study was performed according to the Declaration of Helsinki and was approved by the Science Ethical Committee of the Copenhagen Region (H3-2011-008) and registered at clinicaltrials.gov (NCT02353624).

Experimental Conditions

Subjects arrived in the morning overnight fasted and after a 15 min rest a venous blood sample was collected from an antecubital vein. After this, an adipose tissue biopsy was obtained with a Bergström needle with applied suction from subcutaneous adipose tissue 5–7 cm lateral to the umbilicus.

Following this an incremental exercise test applying increments of 40 watts per min until exhaustion in order to determine maximal oxygen uptake. Conventional criteria; a leveling off of VO_2 despite an increase in power output, a respiratory exchange ratio exceeding 1.15 and achievement of estimated maximal heart rate was used to control the test. The

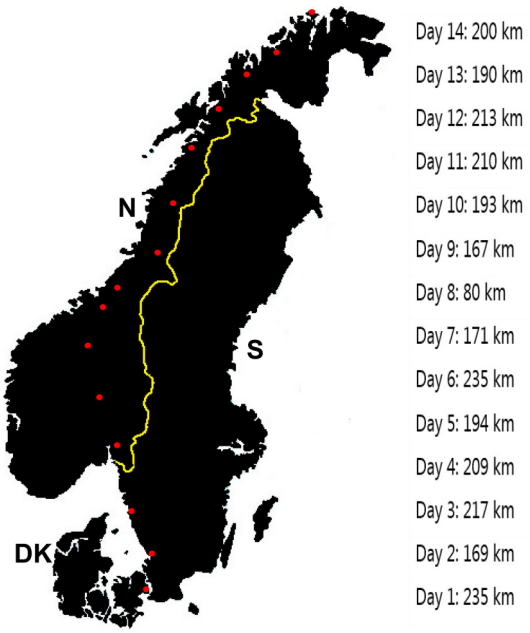

FIGURE 1 | Geographical depiction of the 2,706 km (1,681 miles) route from Copenhagen, Denmark, to North Cape, Norway, via Sweden. The red dots depict departure and arrival points of daily stages throughout the trip. Daily stage distances in kilometers are shown from days 1 to 14. DK, Denmark; N, Norway; S, Sweden. Used by permission (32).

pulmonary values during exercise were measured with an online system (CosMed, Quark b^2, Rome, Italy).

Analytical Procedures

Blood Analysis

Blood was transferred into tubes containing 0.3 mol·L^{-1} EDTA (10 μL·ml^{-1} blood) and immediately centrifuged at 4°C for 10 min at 23,000 g. The plasma was stored at –80°C until analysis. Analysis of plasma insulin and glucose has been described previously (Ara et al., 2011). The Quicki index was calculated as previously described (Katz et al., 2000). Blood HbA1c was analyzed on a DCA Vantage Analyzer (Siemens Healthcare, NY, USA). Plasma IL-6 and plasma TNF-α were measured with high-sensitivity ELISA kits from R&D Systems (Minneapolis, MN, USA). The plasma IL-18 was analyzed with HS ELISA (R&D Systems) and CRP using a conventional high-sensitivity assay on a Cobas Hitachi (Roche, Indianapolis, USA). The plasma adiponectin concentration was determined with a human radioassay kit (EMD Millipore. Billerica, MA, USA). The plasma leptin concentration was measured by a high-sensitivity human ELISA kit (Human leptin Immunoassay, R&D systems).

Adipose Tissue

The description of the adipose tissue fixation and analysis procedure is described in full detail in a prior publication (Auerbach et al., 2013). In brief, the adipose tissue was fixed in 2% Zamboni fixative and embedded in paraffin. At time of analysis the paraffin block was sectioned (5 μm thick) on a microtome (Reichter, Münich, Germany). To determine adipocyte area and the general morphology sections of subcutaneous adipose tissue were stained with Mayer's haematoxylin (S3309, Dako, Copenhagen, Denmark) and 2% eosin (1345, Merck, NJ, USA).

The macrophages were immunostained with a primary antibody against CD163 (diluted 1:200, Visionbiosystems Novocastra, Newcastle, UK) and visualized using Dako's REAL Envision Detection system (K5007, Dako, Copenhagen, Denmark) and 3,3′-diaminobenzidine as a chromogen. Sections with no primary antibody were included as control. The stained sections were scanned by means of a Panoramic slide scanner (Zeiss, Oberkochen, Germany). The quantification was done as described by Cancello et al. (2005) and further qualified by Auerbach et al. (2013). The CD163+ cells and the adipocytes were counted by an observer blinded for slide identity in eight randomly chosen areas (magnified × 20) within each slide, representing an area of 123 ± 7 adipocytes and excluding macrophages located in abundant stroma-vascular areas. The total number of CD163+ macrophages was expressed as a percentage of the total number of adipocytes counted.

The cross-sectional adipocyte area was determined using Adobe PhotoShop CS4 Extended Version 11.0.1 (Adobe Systems Incorporated, San Jose, California, USA). Areas were determined by contrasting differences in color (**Figure 1**). Background variations due to uneven illumination was compensated with the "high pass filter" function and the "threshold" function was applied to achieve a stronger contrast between adipocyte "content" and adipocyte "wall." Due to variation in the quality of the fixation, sectioning and staining a lower cut-off value at 10,000 pixels ≈ 633 μm^2 was used to ensure only adipocytes were quantified. The number of adipocytes quantified per subject was 123 ± 7.

Statistical Analysis

The statistics were calculated using Sigma Plot ver. 13.0 (Systat Software Inc., San Jose, USA). To assess changes before and after the 14 days of cycling a two-tailed paired *t*-test was used. Results are given as mean ± SEM., if not otherwise stated. In all cases, $P < 0.05$ was used as the level of significance in a two-tailed test.

RESULTS

Study Characteristics

The characteristics of the cycling trip have been published previously (32). In summary all six subjects completed the full distance of 2,706 km (1,681 miles) in 14 days with an average daily distance of 193 ± 10 km·day^{-1} (range: 167–235 km·day^{-1}). Daily exercise time was 10 h and 31 ± 37 min·day^{-1} with an exercise intensity of 53.1 ± 1.1% of VO_{2peak} and approximately 198 ± 58 min·day^{-1} exercise intensity above 60% of VO_{2peak} (Morville et al., 2017). After cycling, maximal oxygen uptake was reduced (3.71 ± 0.17 to 3.49 ± 0.20 L O_2/min and 48 ± 2 to 45 ± 2 mL O_2/min/kg, $P < 0.04$) (Morville et al., 2017). The peak workload attained during the VO_{2peak} test was not significantly lowered ($P = 0.22$) from before to after the 14 days 357 ± 31 and 333 ± 24 watt, respectively. As previously reported body weight remained unchanged (**Table 1**), but we observed a 2.2 ± 0.7 kg

TABLE 1 | Anthropometric and body composition data measured at rest overnight fasted before and after 14 days of daily prolonged cycling.

	Pre	Post	*t*-test
Age (years)	61.3 ± 8.4	–	–
Height (cm)	178 ± 8	–	–
Weight (kg)	77.4 ± 10.2	77.7 ± 10.6	NS
BMI (kg m^{-2})	24.5 ± 2.2	24.5 ± 2.0	NS
Waist (cm)	83.6 ± 8.1	84.4 ± 5.9	NS

The values are mean ± SEM.

loss of fat mass and 2.5 ± 0.6 kg gain of lean mass after 8 days of cycling using an isotope dilution technique (Rosenkilde et al., 2015).

Plasma glucose concentrations at rest remained unchanged and there was a trend ($P = 0.06$) toward higher plasma insulin after cycling (**Table 2**; Morville et al., 2017). The glycosylated hemoglobin was not significantly increased, probably due to the limited time of the experiment, but there was a trend ($P < 0.07$) toward an increase in the quantitative insulin sensitivity check index (QUICKI) (**Table 2**). Plasma hsCRP, TNFα, and IL-18 were not changed after the 14 days (**Table 2**), but there was a significantly higher plasma IL-6 concentration (**Table 2**). The plasma leptin and adiponectin concentrations at rest remained unchanged (**Table 2**; previously published Rosenkilde et al., 2015; Morville et al., 2017).

The abdominal adipose tissue content of CD163 macrophages were not influenced by the prolonged cycling and likewise the abdominal adipocyte size were also unchanged after the intervention (**Figure 2**).

TABLE 2 | Plasma glucose, insulin and Quicki-index as well as plasma cytokines, hsCRP and adiponectin and leptin measured at rest overnight fasted before and after 14 days of daily prolonged cycling in 6 older males.

	Pre	Post	*t*-test
Plasma glucose (mmol L^{-1})	5.9 ± 0.2	6.0 ± 0.3	NS
Plasma Insulin (pmol L^{-1})	16 ± 2	33 ± 2	($P < 0.07$)
HbA1c (mmol L^{-1})	5.9 ± 0.5	6.4 ± 0.4	NS
Quicki index	0.42 ± 0.01	0.38 ± 0.01	($P < 0.07$)
Hs CRP (mg dl^{-1})	0.19 ± 0.08	0.17 ± 0.05	NS
Plasma IL-6 (pg ml^{-1})	0.7 ± 0.1	1.1 ± 0.1	($P < 0.05$)
Plasma IL-18 (pg ml^{-1})	310 ± 44	321 ± 39	NS
Plasma TNFα (pg ml^{-1})	2.1 ± 0.8	2.2 ± 0.8	NS
Plasma leptin (pg ml^{-1})	1,257 ± 201	1,357 ± 177	NS
Plasma Adiponectin (μg ml^{-1})	27.6 ± 1.6	43.5 ± 13.1	NS

The values are mean ± SD. hsCRP, C-reactive protein; IL-6, interleukin 6; IL-18, Interleukin 18; TNFα, Tumor Necrosis Factor α. The values are mean ± SEM. Plasma insulin, glucose, adiponectin, and leptin have been previously published (Rosenkilde et al., 2015; Morville et al., 2017).

DISCUSSION

In the present study, the hypothesis was refuted, as the major finding was a slightly increased low-grade inflammation and an unchanged anti-inflammatory macrophage content in abdominal adipose tissue after 14 days of excessive exercise.

In line with prior studies we observed no exercise induced changes in plasma hsCRP, TNFα, and IL-18 (Auerbach et al., 2013), but we did see a modest increase in plasma IL-6 concentration and thus a slightly higher low-grade inflammation. Interestingly, there was a lower fat mass after the intervention (Rosenkilde et al., 2015), albeit body weight was unchanged, and if anything this should lead to lowered plasma inflammatory cytokine concentrations (Bruun et al., 2006, 2007; Beavers et al., 2013). There is evidence that cytokine release is positively correlated to adipocyte size (Coppack, 2001) and therefore the unchanged adipocyte cell size, as observed in our study, is consistent with the unchanged inflammatory cytokines, but obviously not the change in plasma IL-6. The plasma leptin and adiponectin concentrations were not changed (data previously reported Rosenkilde et al., 2015; Morville et al., 2017), and this is in line with the unchanged adipocyte cell size reported here. Although we did observe a small decrease in fat mass and a trend toward a minor increase in insulin resistance, as evaluated by the Quicki Index, this did not translate into decreased plasma leptin and adiponectin, probably due to both the short duration of our intervention and the low number of subjects. The mechanism behind the insulin resistance is not readily available as we previously observed both an increased muscle GLUT4 and HKII expression as well as unchanged muscle glycogen, triacylglycerol, and ceramide content (Morville et al., 2017). An increased plasma IL-6 concentration, albeit of a much larger magnitude, has previously been linked to increased lipolysis and fat oxidation at rest (Mathur and Pedersen, 2008), but clearly here, is in contrast to the very marked decrease in maximal fat oxidation and plasma FA concentration observed previously (Morville et al., 2017). Somewhat surprising peak oxygen uptake was reduced by 6% and citrate synthase in muscle, a mitochondrial marker, remained unchanged after the 14 days excessive exercise (Morville et al., 2017), which may indicate that the older males participating in this study were possibly at or just over the maximal tolerable sustainable repeated daily exercise load. It is thus possible that we could not confirm our hypothesis that low-grade inflammation would remain unchanged due to an "overload" of exercise. The mechanisms behind the anti-inflammatory effect of exercise are not fully elucidated in the literature, but includes a reduction in visceral fat, a reduction in expression of toll-like receptor on macrophages and monocytes, production of anti-inflammatory molecules from muscle and leukocytes and a phenotypic switching of macrophages toward the M2 anti-inflammatory state in adipose tissue (Flynn and McFarlin, 2006; Astrom et al., 2010; Goh et al., 2016).

The second part of our hypothesis was also refuted as we could not detect an increase in abdominal adipose tissue CD163 anti-inflammatory macrophage area and thus were not able to confirm Auerbach and colleagues findings of an increased abdominal adipose tissue CD163 macrophage content after 12 weeks endurance training in young overweight males (Auerbach et al., 2013). The available data in man is very limited, but Leggate and colleagues found a decrease in

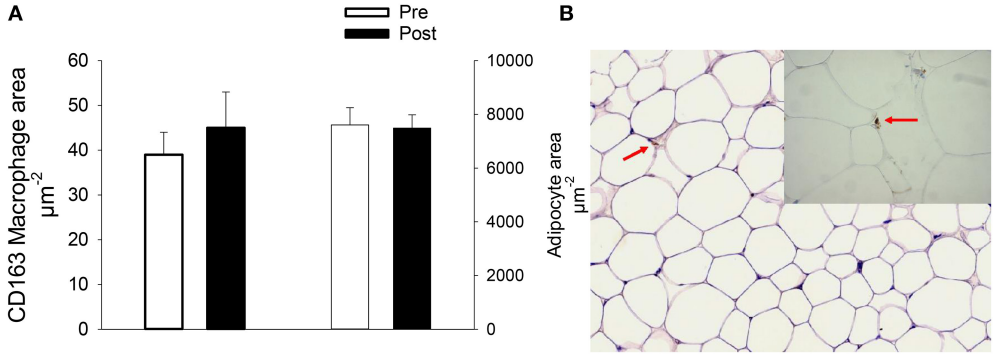

FIGURE 2 | CD163 macrophage content and adipocyte cell size in abdominal adipose tissue **(A)** and a representative picture of CD163 staining (Red arrows) in adipose tissue **(B)** in older male recreationally trained cyclist before and after 14 days repeated very prolonged exercise. The values are mean ± SEM.

plasma IL-6 and a trend toward a decrease in adiponectin in subcutaneous adipose tissue after 2 weeks high intensity intermittent training in young overweight and obese males implying that even shorter training may also mediate changes in adipose tissue (Leggate et al., 2012). In mice there is evidence, but not conclusive, that training will lead to a switch in macrophage phenotype from M1 (pro-inflammatory) toward M2 [anti-inflammatory (CD163)] (recently reviewed in Kawanishi et al., 2010; Oliveira et al., 2013; Linden et al., 2014; Goh et al., 2016). However, further studies in man are needed to elucidate the anti-inflammatory effects of very prolonged repeated exercise on adipose tissue including the different adipose tissue depots and the mechanisms that control and regulate this.

A limitation in this study was that we due to logistic reasons were not able to obtain blood and tissue samples during the 14 days and therefore the potential changes during the study cannot be distinguished.

In summary, regular prolonged exercise did not influence abdominal adipose tissue anti-inflammatory macrophage content, but the slightly increased low-grade inflammation, higher plasma IL-6 concentration, implies that the excessive amount of exercise in these older trained men probably attenuated the potential anti-inflammatory effects of exercise.

AUTHOR CONTRIBUTIONS

The study was performed at Xlab, Center for Healthy Aging, Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark. MR, JH conceived and designed the study. All authors collected samples, performed analyses and/or took part in data interpretation. RS and JH wrote the first draft of the manuscript, and all authors took part in critical revision for intellectual content of the manuscript. The final manuscript was presented to and approved by all authors, who agreed to be liable for all aspects of the study. All those who were eligible to be authors in this study were included as authors.

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Bed Rest and Hypoxic Exposure Affect Sleep Architecture and Breathing Stability

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Objective: Despite over 50 years of research on the physiological effects of sustained bed rest, data characterizing its effects on sleep macrostructure and breathing stability in humans are scarce. This study was conducted to determine the effects of continuous exposure to hypoxia and sustained best rest, both individually and combined, on nocturnal sleep and breathing stability.

Methods: Eleven participants completed three randomized, counter-balanced, 21-days trials of: (1) normoxic bed rest (NBR, P_IO_2 = 133.1 ± 0.3), (2) hypoxic ambulatory confinement (HAMB, P_IO_2 = 90.0 ± 0.4) and (3) hypoxic bed rest (HBR, P_IO_2 = 90.0 ± 0.4; ∼4,000 m equivalent altitude). Full objective polysomnography was performed at baseline, on Night 1 and Night 21 in each condition.

Results: In NBR Night 1, more time was spent in light sleep (10 ± 2%) compared to baseline (8 ± 2%; $p = 0.028$); Slow-wave sleep (SWS) was reduced from baseline in the hypoxic-only trial by 18% (HAMB Night 21, $p = 0.028$) and further reduced by 33% (HBR Night 1, $p = 0.010$), and 36% (HBR Night 21, $p = 0.008$) when combined with bed rest. The apnea-hypopnea index doubled from Night 1 to Night 21 in HBR (32–62 events·h^{-1}) and HAMB (31–59 events·h^{-1}; $p = 0.002$). Those who experienced greatest breathing instability from Night 1 to Night 21 (NBR) were correlated to unchanged or higher (+1%) night SpO_2 concentrations ($R^2 = 0.471$, $p = 0.020$).

Conclusion: Bed rest negatively affects sleep macrostructure, increases the apnea-hypopnea index, and worsens breathing stability, each independently exacerbated by continuous exposure to hypoxia.

Keywords: hypoxia, periodic breathing, high altitude, polysomnography, duty ratio

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Abbreviations: AHI, apnea-hypopnea index; AMS, acute mountain sickness; BDC, baseline data collection; F_IO_2, fraction of inspired O_2; HAMB, hypoxic ambulatory confinement condition; HAPE, high-altitude pulmonary edema; HBR, hypoxic bed rest condition; HR, heart rate; HVR, hypoxic ventilatory response; LLS, Lake Louise Score; N1, non-REM Stage 1 light sleep; N2, non-REM Stage 2 light sleep; N3, non-REM Stage 3 slow wave sleep; NBR, normoxic bed rest condition; NREM, on-REM sleep; PSG, polysomnography; REC, recovery day, out of condition; R, rapid eye movement sleep; SpO_2, peripheral oxyhemoglobin saturation; TST, total sleep time; WASO, wake after sleep onset.

INTRODUCTION

Sleep, defined as a periodic and reversible state of being (Berry et al., 2016), is critical for rest, repair and survival of a species (Hardin, 2009). Voluntary respiratory control becomes absent during sleep, and hypoxic and hypercapnic ventilatory drive is reduced compared to wakefulness (Douglas, 2005). Poor sleep is frequently reported when patients are confined to their bed for extended periods of time, e.g., in intensive care unit (ICU) settings, where sleep disturbances are reported in up to 50% of critically ill patients (Walder et al., 2007). Polysomnography has verified alterations in sleep architecture, decreased total sleep time, and sleep fragmentation in ICU patients (Buckle et al., 1992). These disruptions are often attributed to noise, interruptions to complete diagnostic tests, or other environmental factors (Freedman et al., 1999). Healthy subjects sleep-deprived 24–30-h can experience 17–24% decreases in ventilatory responsiveness to hypercapnia, and increased respiratory muscle fatigue (Chen and Tang, 1989). Evidence alludes to prolonged time spent in bed altering patients' ventilatory drive; sleep deprivation *per se* may play a role in ventilatory chemoreceptor mechanisms, although these findings are not universally supported (Spengler and Shea, 2000). A clinical example includes chronic obstructive pulmonary disease, which is a frequent cause of ICU admission (Faisy et al., 2016), however little is known about the respiratory pattern in patients experiencing prolonged mechanical ventilation in combination with prolonged bed rest. These patients usually remain in the ICU up to 30 days, although chronic alterations can be observed in as little as 3 weeks' duration (Chlan et al., 2015).

Determining whether bed rest and subsequent sleep disturbances lead to poor ventilatory control in ICU patients can be especially difficult since critical care patients often exhibit complicated co-morbidities. Confining otherwise healthy humans to bed rest is a relatively common experimental model in space life-sciences, used as a ground-based analog to induce similar physiological strain as experienced in microgravity (reviewed in Pavy-Le Traon et al., 2007). Yet despite over 50 years of bed-rest studies in humans, there is very little objectively-measured sleep data (Pavy-Le Traon et al., 2007). Of the few experimental studies investigating any sleep measure, many have not included baseline night recordings (Komada et al., 2006) or are confounded by experimental designs which manipulate circadian rhythm or light exposure (Monk et al., 1997), directly affecting sleep data. Thus, the effect of sustained bed rest on objective sleep parameters in healthy humans is not well known.

Sleep studies conducted in hypoxic environments have reported alterations in sleep architecture, including reductions in slow-wave sleep (SWS), more frequent arousals, and marked periodic breathing (reviewed in Ainslie et al., 2013). Ascent to high altitude in newcomers leads to unstable breathing during both wakefulness and sleep in up to 90% of those venturing above 5,000 m (Burgess et al., 2004), and this breathing instability can persist up to 12 months when living in hypoxic confinement (Tellez et al., 2014). The recurrent hypoxemia and hypercapnia as a consequence of the respiratory pauses in periodic breathing can have extensive adverse health consequences, including decreased neurocognitive function (sleepiness, mood changes, depression), and cardiovascular complications such as pulmonary hypertension, arrhythmias, ischemic heart disease, myocardial infarction, diabetes, and stroke (Peppard et al., 2000; Wolk et al., 2003; Arzt et al., 2005). These abnormal breathing patterns can lead to serious complications for the heart and brain, especially in patient populations who may suffer from temporary or chronic respiratory insufficiency and are thus both hypoxic and physically inactive.

It is important to note that disease-states or high-altitude exposures are not the only scenarios where humans may find themselves exposed to hypoxic conditions for long durations. Indeed, it is envisioned that future planetary habitats will maintain lower partial pressure of oxygen. Data on the interactions between hypoxic exposure and bed rest are scarce, although recent work has investigated the physiological effects of both stressors on body composition (Debevec et al., 2014b), psychological strain of confinement (Stavrou et al., 2015) and sleep architecture (Rojc et al., 2014) in short-term (10-days) exposures. Considering the clinical data on certain patient populations finds significant differences in breathing after 3 weeks, and also international space-science guidelines for moderate-duration bed rest studies, a 21-days exposure was chosen to mimic the conditions of prolonged immobility and hypoxia.

The purpose of this study was to determine the separate and combined effects of a 21-days duration bed rest and hypoxic exposure on nocturnal sleep macrostructure and respiratory outcomes. It was hypothesized that sleep would be more fragmented at the start of bed rest under normal (normoxic) conditions, but that hypoxia would induce significant, negative alterations in respiratory control compared to baseline recordings, and sleep quality and breathing stability issues would be exacerbated when both stimuli were combined.

MATERIALS AND METHODS

This sleep study was part of a larger investigation exploring the separate and combined effects of inactivity/unloading and hypoxia on several physiology systems. Specific data related to appetite (Debevec et al., 2016), insulin sensitivity (Simpson et al., 2016), bone health (Rittweger et al., 2016), hematology (Keramidas et al., 2016b), peak oxygen uptake (Keramidas et al., 2016a), and skeletal muscle miRNA expression (Rullman et al., 2016), are available elsewhere.

Study Participants

All procedures performed were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments. This study was approved by the Ministry of Health of the Republic of Slovenia and the National Committee for Medical Ethics, (#205/02/11). Informed consent was obtained from all individual participants prior to inclusion in the study.

All testing was performed at the Olympic Sports Centre Planica (Rateče, Slovenia) situated 940 m above sea level.

Sixty-five males were initially screened for inclusion into the study. Following preliminary testing, including a supervised, overnight familiarization weekend at the hypoxic facility, 14 participants were selected and invited to participate in the study. Eleven participants completed all testing trials and their details are reported herein. Exclusion criteria followed international standards for bed rest investigations (Standardization of bed rest study conditions, Version 1.5, August 2009). Participants were excluded if they reported: any underlying medical conditions, were smokers, were uncomfortable remaining within the hypoxic facility, or were unable to commit to completing three full trials. Thus, all participants were non-smokers, sea-level dwellers, with no history of high-altitude exposures within the past 4 months, no known cardiorespiratory, musculoskeletal, asthma, allergy, or circulatory disease.

In the final trial, two participants did not return because of employment; one participant was withdrawn due to gastrointestinal issues (unrelated to the bed rest procedure). Eleven of 14 participants completed all three trials and are included in all subsequent analyses (20–41 years). This sleep and respiration study was part of a larger investigation exploring the separate and combined effects of simulated low-gravity (bed rest) and hypoxic exposure on various aspects of human physiology.

Study Design and Hypoxic Environment

Participants entered the experimental trial in pairs. Each trial had a total duration of 32-days. Initial baseline testing was 7-days duration, during which participants engaged in a host of experimental procedures, including assessments of: body composition, aerobic fitness, bone mineral density, orthostatic tolerance, muscle physiology (isokinetic dynamometry, muscle biopsy), behavioral thermoregulation, sleep, and others. Baseline testing was immediately followed by a 21-days intervention (i.e., a medium-duration bed-rest protocol according to European Space Agency and National Aeronautics and Space Agency standards) in a randomized, counterbalanced, repeated-measures fashion (NBR, HAMB, or HBR), with a final 4-days recovery phase, during which all post-testing measurements were completed (**Figure 1**). Participants remained confined to the testing facility for the entire experimental session (thus the 32-days total confinement at the facility). When the participants were not in the 21-days intervention period, they were permitted short, supervised outings to a picnic area around the immediate building site. Due to the repeated-measures aspect of testing, each subject acted as their own control, with baseline testing constituting the normal "control" dataset when they were both normoxic and ambulatory. Subsequent experimental conditions were balanced to determine the effect of minimal activity (bed rest), or hypoxic exposure, or the effect of both stimuli combined. Specifically, the experimental conditions were: (1) normoxic bed rest (NBR, inspired partial pressure of oxygen, $P_IO_2 = 133.1 \pm 0.3$), (2) hypoxic ambulatory (HAMB, $P_IO_2 = 90.0 \pm 0.4$), and (3) hypoxic bed rest (HBR, $P_IO_2 = 90.0 \pm 0.4$, ∼4,000 m equivalent altitude). For safety reasons, and because the physical impact of remaining in such a hypoxic environment is very obvious, neither the subjects nor the researchers were blinded regarding their breathing gas. There was a 4-month wash-out period between trials to ensure adequate physiological and psychological recovery. The normobaric hypoxia was maintained by a vacuum pressure swing absorption system (b-Cat, Tiel, The Netherlands), described in detail elsewhere (Debevec et al., 2014a). Oxygen levels in the common area and individual rooms were continuously monitored with O_2 sensors (PGM-1100; Rae Systems, San Jose, CA).

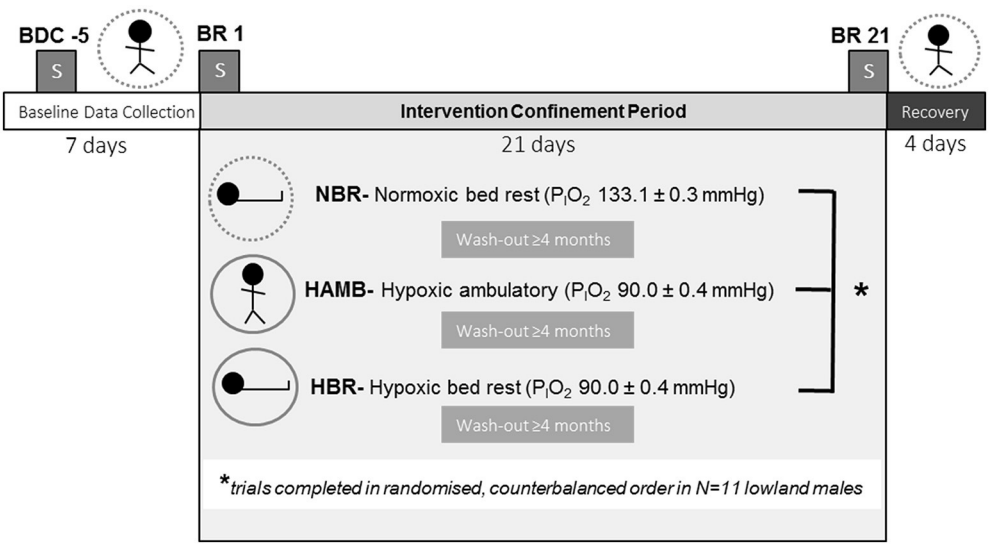

FIGURE 1 | Experimental protocol. BDC, baseline data collection; BR, bed rest; NBR, normoxic bed rest; HAMB, hypoxic ambulatory; HBR, hypoxic bed rest confinement conditions. Symbols for each experimental condition include: dashed circle (normobaric normoxia), or solid circle (normobaric hypoxia), and the subject either upright (ambulatory) or supine (bed rest). Blocked "S" symbol indicates on which night(s) sleep PSG measurements were conducted.

Daily Experimental Test Protocols

Participants were awakened at 7:00 and lights turned off at 23:00 each day throughout the experiment. Napping was prohibited. Resting heart rate and peripheral SpO_2 were measured each morning (short-range telemetry, iBody, Wahoo Fitness, Atlanta, USA, 3100 WristOx, Nonin Medicals, Minnesota, USA, respectively). Participants then completed the various tests they had scheduled for that day, or engaged in sedentary leisure activities (e.g., reading, watching movies, studying, visiting the other subjects) whilst waiting for the next examination. Participants filled in the self-assessment portion of the Lake Louise questionnaire score (LLS) at 17:00 each evening. Diagnoses of acute mountain sickness (AMS) were defined as: (1) LLS $\geq$3 and (2) presence of headache.

Participants were confined to a horizontal position during both NBR and HBR following established protocols (Pavy-Le Traon et al., 2007). All activities of daily living (e.g., eating, reading, and showering) were carried out in the horizontal position. Participants used one pillow for head support. They were allowed to drink water *ad libitum*, and were actively encouraged to drink at least 2 L per day. Five meals were served daily (breakfast, morning snack, lunch, afternoon snack and dinner), and always at the same time of day throughout each trial. Participants were encouraged to consume all food provided; they could opt to consume less, but they did not receive any additional food than the original prescribed amounts. A 14-day menu was used during the first trial and rotated throughout the trial duration. This same menu was then applied to the two subsequent trials. Thus, participants consumed identical meals, and on the same testing day(s), of each trial. The menu was designed using an in-house web-based application (OPKP, Jozef Stefan Institute, Ljubljana, Slovenia), consisting of targeted baseline macronutrient compositions of ~55% carbohydrate, ~30% fat and ~15% protein. Alcohol and caffeine were not included in the standardized diet. A detailed summary of daily nutritional protocols (including sample menus) are available elsewhere (Debevec et al., 2014a).

During HAMB, participants were encouraged to move about the common hypoxic living area (~200 m^2) and maintain an upright (standing) position. To mimic unconfined activity levels participants performed two structured, 30-min low-intensity exercise sessions each day, once in the morning and once in the afternoon. Exercise mode varied with each session (stepping, cycling or activity-based video games) to avoid monotony. During all sessions, HR and pulsed oximetry were monitored to ensure the individual's intensity was maintained within the targeted value (~123 $\pm$ 4 beats·min^{-1}), roughly corresponding to 50% of the participants' hypoxic-specific peak power output determined previously from a hypoxic graded exercise test (Debevec et al., 2014a). The graded test was performed before confinement on a cycle ergometer under hypoxic condition (F_IO_2 = 0.144) using 25 W·min^{-1} workload increments until task failure, defined as an inability to maintain cycling cadence >60 rotations per minute. Activity-based video games and table football were also provided to promote upright daily activity.

Night Polysomnography

Full ambulatory polysomnography (PSG, Nicolet One, Viasys, Healthcare, Neurocare, Madison, WI, USA) was performed using standard set-ups (Burgess et al., 2004; Ainslie et al., 2007; Rojc et al., 2014). This included PSG recordings of: electroencephalography (EEG), electro-oculography (EOG), chin and tibial surface electromyography (EMG), electrocardiography (ECG), nasal pressure (nasal pressure cannula), respiratory movements (chest and abdominal belts), and capillary oxyhaemoglobin saturation.

Continuous video surveillance during the night was implemented for subjects' safety, and to monitor subjects' movements. Testing occurred after subjects had spent at least two nights in the testing facility; during each trial, measurements were conducted on three occasions (1) baseline data collection day 3 (i.e., the "control" data), (2) Night 1, and (3) Night 21 of the intervention.

All recordings were visually scored and analyzed by a certified sleep physician from an accredited sleep laboratory, based on the American Academy of Sleep Medicine (AASM) manual for the scoring of sleep and associated events (Berry et al., 2016) (detailed below). Participants were excluded from the sleep study if it was discovered they had periodic limb movements (PLM index > 5/h), obstructive sleep apnea (apnea-hypopnea index, AHI >5), or any other sleep abnormalities.

Data Analysis and Statistical Measures

Duty ratio (DR) was calculated as a surrogate for loop gain (Edwards et al., 2008; Sands et al., 2011), using methods described elsewhere (Andrews et al., 2012). Briefly, apneas were scored as a drop in the peak signal excursion by $\geq$90% of pre-event baseline using the oro-nasal sensor for $\geq$10 s with associated absent inspiratory effort throughout the entire period of absent airflow, and hypopneas scored as a peak signal excursion drop by $\geq$30% of pre-event baseline using nasal pressure for $\geq$10 s with a $\geq$3% oxygen desaturation from pre-event baseline, unless the apneas or hypopneas occurred as a segment of continuous periodic breathing, in which case the entire duration of the respiratory event was taken. DR was calculated as mean time (in seconds) of hyperpnea/(hyperpnea + apnea) (Edwards et al., 2008; Andrews et al., 2012).

On all dependent variables, a 2-way repeated measures ANOVA was conducted with two between subjects' factors [time, (Baseline, Night 1, Night 21)] and condition (NBR, HBR, HAMB) and paired *t*-tests employed *post-hoc* (Bonferroni correction). Bivariate correlations were run between variables of interest; statistical analyses were limited to reduce the likelihood of Type I error, at an alpha level of 0.05. Data were analyzed using SPSS (v.20.0, IBM Statistics, Chicago, IL, USA) and expressed as means $\pm$ standard deviations in all text and figures.

RESULTS

General Environmental Adaptations

One subject experienced severe hypoxemia (SpO_2 < 75%) combined with dizziness and headache during the first hours of his HBR intervention. He was relocated to a separate room where

the simulated altitude was 3,000 m, during which the symptoms subsided. The following day, the simulated altitude was increased to 3,500 m, and finally on the third day to 4,000 m, with no further difficulties. This ascent profile was repeated for his HAMB condition. All sleep-breathing parameters for this subject were within one standard deviation of group means, thus his data are included in all further analyses.

For all subjects, daily morning SpO_2 was lower in HAMB (88 ± 1%) and HBR (88 ± 2%) compared to NBR (97 ± 0%) during the 21-days intervention ($p < 0.01$). There was a gradual recovery of SpO_2 following the first day in hypoxia, such that SpO_2 was higher in HBR from day 3–21 (89 ± 1%), and in HAMB from 16 to 21 (89 ± 0%) compared to the first day in normobaric hypoxia (85 ± 3%; $p < 0.05$). Supine morning HR was higher in HBR (74 ± 9 b·min^{-1}) than in NBR (60 ± 10 b·min^{-1}) from days 14 to 21 ($p < 0.05$). Average HR and SpO_2 responses to the low-intensity physical activity sessions performed during HAMB were 124 ± 9 b·min^{-1} and 87 ± 3%, respectively.

Sleep Architecture and Quality

Sleep architecture responses to all experimental conditions are displayed for one representative subject in **Figure 2**. In terms of mean group data, participants spent more time in N1 during NBR Night 1 compared to the baseline control night (Baseline: 8 ± 2, NBR Night 1: 10 ± 2%; $p = 0.028$). There were significant differences between NBR and HBR conditions (95% CI: −4.4 to −0.2%; $p = 0.028$), irrespective of time. After 21-days in hypoxia, subjects continued to experience a greater proportion of time spent in N1 (HAMB Night 21, 11 ± 4%, $p = 0.018$, HBR Night 21, 11 ± 3% $p = 0.031$, **Table 1**). After 21-days of bedrest (both NBR and HBR), the proportion of R was increased 17.8 ± 4.3% compared to the baseline control night (NBR Night 21 95% CI: 9.7–11.8% $p = 0.017$; HBR Night 21 95% CI: 8.1–13.3%, $p = 0.012$). Thus, sleep architecture was altered by bed rest alone, and the continuous bed rest plus hypoxic exposure combination.

Wake after sleep onset (WASO) scores were reduced (i.e., improved) from −32 to −5 min after 21-days in any condition ($p = 0.011$). *Post-hoc* testing reveled that these improvements were specifically attributed to ameliorated values in the HAMB condition, which decreased from 73 ± 43 min (HAMB Night 1) to 38 ± 19 min (HAMB Night 21; 95% CI: 8–62 min; $p = 0.015$). Other markers of sleep quality (e.g., number of awakenings, sleep latency, sleep efficiency) remained unchanged between the baseline control night and the other experimental conditions (NBR, HBR, and HAMB). There were no interactions between-conditions present for sleep quality variables.

Breathing Stability, AHI and Relationship to Night SpO_2

In both HAMB and HBR, AHI doubled in severity after 21-days of exposure (Night 1: 31 ± 32, Night 21: 60 ± 37 events·h^{-1}; $p = 0.002$; **Table 2**). DRs were 28% lower than baseline (i.e., more unstable) in both hypoxic exposure groups ($p < 0.001$), but were not different between-groups. Breathing stability significantly worsened from NBR Night 1 to NBR Night 21 (CI: −0.283 to −0.093; $p = 0.001$). Mean night SpO_2 values were affected

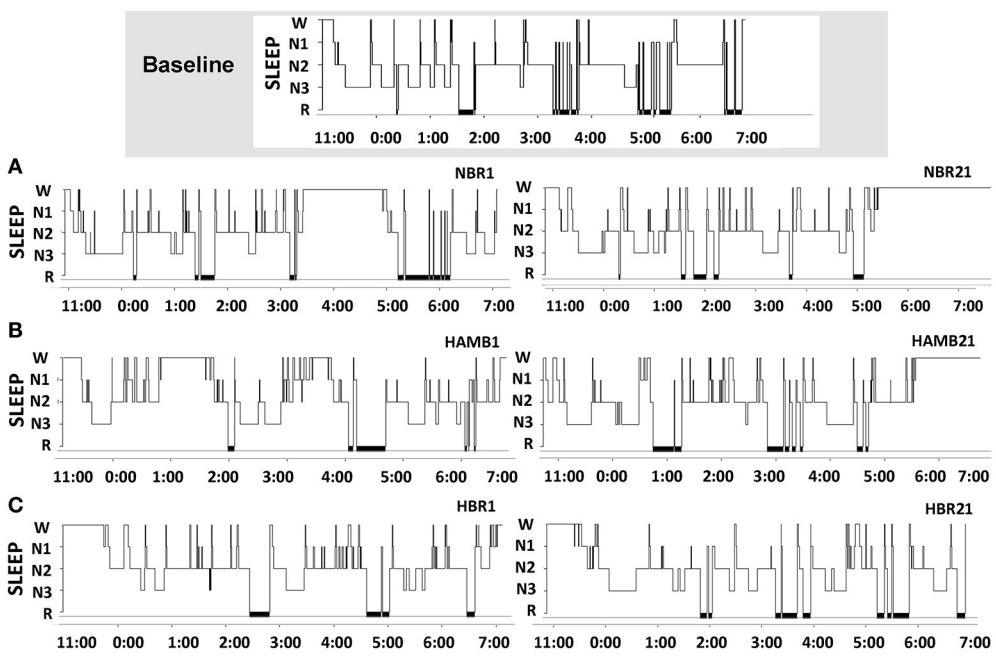

FIGURE 2 | Sleep architecture changes across the protocol in one representative subject for the baseline control night, then **(A)** normoxic bed rest night one (NBR1) and normoxic bed rest night 21 (NBR21), **(B)** hypoxic ambulatory night one (HAMB1) and hypoxic ambulatory night 21 (HAMB21), **(C)** hypoxic bed rest night one (HBR1) and hypoxic bed rest night 21 (HBR21). SLEEP, sleep stages (N1, N2, N3, R); x-axis denote time of night in 24-h clock (lights out 23:00, lights on 07:00).

TABLE 1 | Polysomnography sleep data for the baseline control night, Night 1 and Night 21 experimental trials, including the normoxic bed rest (NBR), hypoxic ambulatory (HAMB) and hypoxic bed rest (HBR) conditions.

Sleep Variables	Baseline	Night 1			Night 21		
		NBR	HAMB	HBR	NBR	HAMB	HBR
SLEEP ARCHITECTURE							
Total Sleep Time, h	7.3 ± 0.6	7.2 ± 0.7	7.3 ± 0.5	6.8 ± 0.8*	6.7 ± 0.8*	7.2 ± 0.4	6.8 ± 0.5*
	(6.3–7.8)	(5.7–8.1)	(6.0–7.8)	(4.8–7.8)	(5.5–7.6)	(6.6–7.7)	(5.7–7.3)
N1, %	7.7 ± 2.1	9.7 ± 2.4*	9.3 ± 4.0	11.8 ± 3.3*	8.1 ± 2.8†	10.8 ± 3.6*	10.6 ± 3.3*
	(5.0–12.0)	(7.0–14.0)	(5.0–15.0)	(9.0–21.0)	(4.0–13.0)	(6.0–19.0)	(6.0–17.0)
N2, %	52.3 ± 4.1	51.8 ± 4.6	51.5 ± 8.1	57.0 ± 3.4	49.3 ± 4.0*	50.0 ± 5.1	50.2 ± 5.2
	(46.0–59.0)	(43.0–58.0)	(38.0–68.0)	(52.0–64.0)	(43.0–57.0)	(43.0–60.0)	(41.0–59.0)
N3, %	22.2 ± 4.3	19.2 ± 5.4§	20.1 ± 6.8	17.1 ± 4.1*	19.1 ± 6.1§	18.2 ± 5.7*	16.5 ± 5.7*
	(17.0–31.0)	(10.0–30.0)	(8.0–30.0)	(12.0–25.0)	(9.0–28.0)	(9.0–26.0)	(8.0–27.0)
R, %	17.8 ± 4.3	19.4 ± 5.2§	17.5 ± 3.7	16.5 ± 5.0*	23.3 ± 6.1†	20.9 ± 5.2*	22.5 ± 4.0*†
	(11.0–26.0)	(10.0–28.0)	(11.0–24.0)	(11.0–29.0)	(13.0–32.0)	(10.0–29.0)	(15.0–28.0)
SLEEP QUALITY							
WASO, min	45.5 ± 42.6	51.5 ± 32.6	73.2 ± 43.4	41.0 ± 15.4	40.6 ± 31.6	38.2 ± 19.2†	42.6 ± 23.7
	(17.0–150.0)	(15.5–108.0)	(7.0–140.0)	(21.0–68.5)	(11.0–107.5)	(6.5–75.5)	(17.0–81.0)
Sleep Latency, min	33.1 ± 31.4	24.8 ± 18.5	16.7 ± 7.4	33.0 ± 15.5	30.7 ± 31.9	30.6 ± 26.8	33.4 ± 19.8
	(5.0–91.0)	(5.0–64.5)	(5.0–31.0)	(8.5–54.5)	(7.5–115.5)	(2.0–99.0)	(10.5–65.5)
REM Latency, min	85.7 ± 30.2	84.2 ± 40.1	111 ± 58	99.3 ± 42.7	76.1 ± 41.3	86.2 ± 38.3	90.0 ± 40.7
	(49.5–156.5)	(33.0–163.5)	(21.5–205.5)	(28.5–162.0)	(36.0–192.5)	(46.5–154.5)	(29.0–145.5)
Sleep Efficiency, %	83 ± 12	82 ± 6	78 ± 9	82 ± 9	79 ± 9	84 ± 7	83 ± 7
	(58–94)	(71–93)	(68–94)	(59–90)	(65–93)	(73–94)	(70–89)
Awakenings, #	26 ± 9	25 ± 4	28 ± 10	27 ± 7	22 ± 6	27 ± 8	26 ± 7
	(12–40)	(19–31)	(9–49)	(16–36)	(12–32)	(8–39)	(15–41)
Stage Shifts, #	109 ± 32	109 ± 9	109 ± 29	120 ± 21	92 ± 22	115 ± 26	112 ± 19
	(42–155)	(97–128)	(68–167)	(86–155)	(66–128)	(67–153)	(76–142)

**Significantly different from Baseline, † significantly different from Night 1 within-condition. §p-value between 0.05 and 0.08 compared to Baseline. Values are means ± standard deviations (p < 0.05). Low and high range values are denoted in brackets directly beneath mean scores.*

TABLE 2 | Sleep-breathing parameters from the Baseline control night, Night 1 and Night 21 of the experimental trials, including the normoxic bed rest (NBR), hypoxic ambulatory (HAMB) and hypoxic bed rest (HBR) conditions.

	Baseline	Night 1			Night 21		
		NBR	HAMB	HBR	NBR	HAMB	HBR
Incidence rate, %	64%	18%	100%	91%	82%	100%	100%
AHI, events.h^{-1}	2.1 ± 2.4	0.4 ± 0.8	30.9 ± 28.0*	31.5 ± 36.9*	4.6 ± 9.0	58.7 ± 36.6*†	61.8 ± 39.3*†
Hyperpnea length, s	20.8 ± 5.6	28.6 ± 4.7	14.0 ± 3.8§	15.6 ± 3.9§	24.7 ± 7.6	14.4 ± 3.1*‡	15.9 ± 3.6‡§
Apnea length, s	18.9 ± 4.9	18.1 ± 4.0	11.6 ± 1.8*	12.4 ± 1.3*	17.0 ± 3.4	11.9 ± 1.4*‡	12.2 ± 1.9*‡
Total cycle length (H+A)	39.6 ± 6.7	46.7 ± 8.7	25.6 ± 5.2*	28.0 ± 4.6*	41.6 ± 10.6†	26.3 ± 4.0*‡	28.0 ± 5.2*‡
Duty ratio (H/H+A)	0.972 ± 0.040	0.949 ± 0.113	0.687 ± 0.022*	0.719 ± 0.096*	0.761 ± 0.120*†	0.687 ± 0.022*	0.696 ± 0.018*

*Incidence Rate refers to whether a person demonstrated any classifiable apneas and/or hypopneas at any point during sleep in their night PSG recordings. This is not indicative of clinically-relevant AHI scores, only whether there were any officially classifiable respiratory events throughout the night *Significantly different from baseline, † different from Night 1 within-condition, ‡ different from NBR21 within that time-point. §p-value between 0.05 and 0.07 compared to baseline. Group-wise comparisons between NBR1 and the other time-points were not analyzed because of the lack of incidence rate within that condition (N = 2/11 only). Values are means ± standard deviations (p < 0.05).*

by hypoxic exposure, rebounding significantly on Night 21, but remaining a full ∼5% less than normoxic values (**Figure 3**). Notably, there were small, but significant decreases in NBR Night 21 compared to NBR Night 1 (95% CI: −1.76 to −0.04%; $p = 0.041$). Changes in AHI from Night 1 to Night 21 (**Figure 4**) were not correlated to DR or SpO_2 for any condition (**Table 3**), whereas changes in DR from NBR Night 1 to Night 21 were significantly correlated to the delta night SpO_2 change, i.e., the lower the DR, the higher SpO_2 night concentration ($R^2 = 0.4708$; $p = 0.020$).

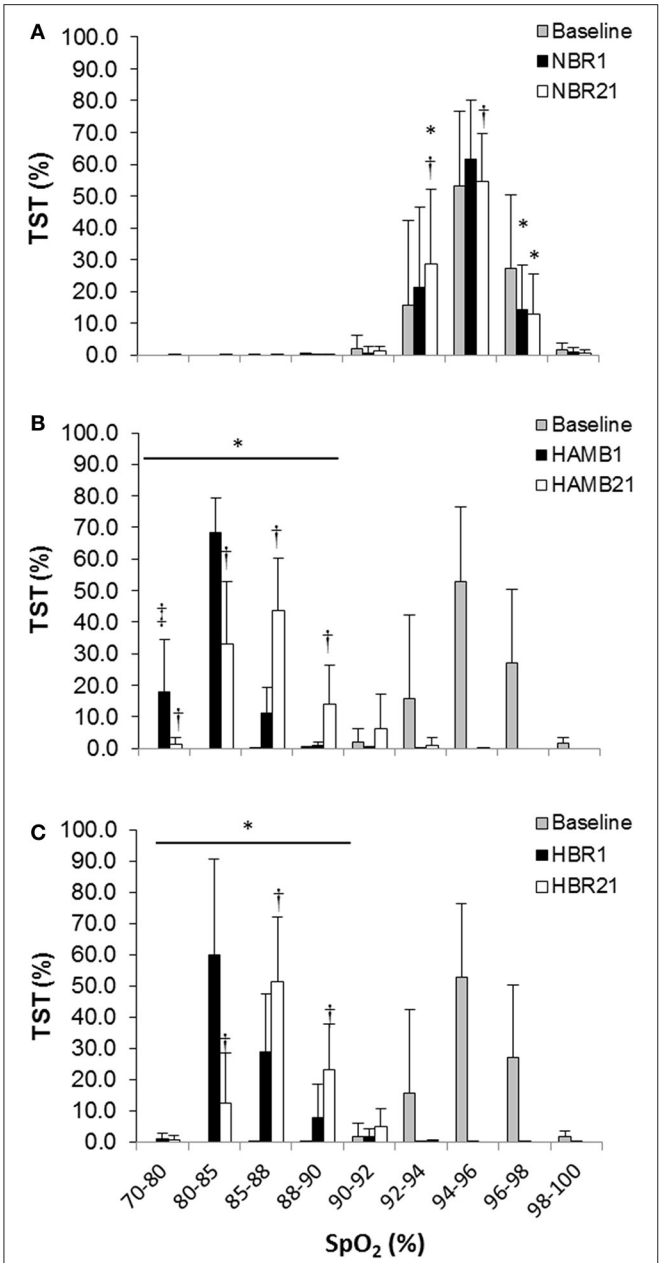

FIGURE 3 | Proportion of total sleep time (TST) spent at a given night peripheral oxyhemoglobin concentration (SpO_2) for **(A)** normoxic bed rest (NBR), **(B)** hypoxic ambulatory (HAMB) **(C)** hypoxic bed rest (HBR) conditions. Error bars depict standard deviations. (*) Significant difference from baseline (all concentrations in both hypoxic trials), (†) significant difference from Night 1, within-condition (‡) significant difference from all other conditions within-night at that saturation ($p < 0.05$).

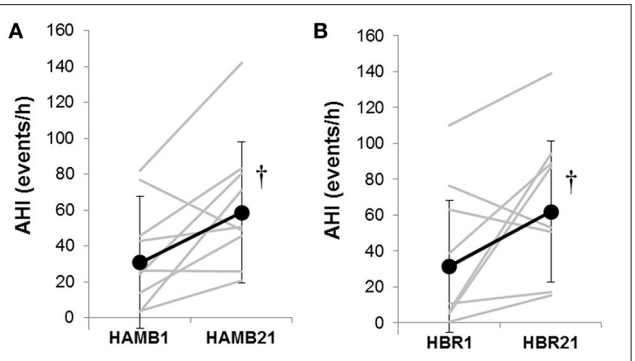

FIGURE 4 | The Apnea-Hypopnea Index (AHI) responses from Night 1 to Night 21 in **(A)** hypoxic ambulatory (HAMB) and **(B)** hypoxic bed rest (HBR) conditions. Individual data are denoted in gray lines; group means are denoted in black lines with closed circles and error bars depicting standard deviations. (†) significant increase from Night 1 in both hypoxic conditions ($p < 0.05$).

DISCUSSION

The present study demonstrates that breathing stability (characterized by the DR) is worsened during 21-days of normoxic bed rest (NBR Night 21), by acute exposure to hypoxia (HAMB Night 1), and by bed rest combined with hypoxia (HBR Night 1). Breathing responses during nocturnal sleep remain poor after 21-days of exposure, whether people remain recumbent or ambulatory. Sleep architecture is affected in all trials, with the most noticeable shifts occurring in the combined HBR condition. There appears to be a direct relationship between breathing stability and mean night SpO_2 when otherwise healthy adults are confined to bed, echoing published data that have reported ventilatory drive changes in patients (Schiffman et al., 1983; Chen and Tang, 1989), and lower hypoxic chemoresponsiveness in susceptible individuals at high altitude (Nespoulet et al., 2012).

The initial increase in ventilation during exposure to hypoxia (i.e., hypoxic ventilatory response; HVR) is highly variable (Hirshman et al., 1975). Some studies have stratified test populations into "susceptible" and "resistant" phenotypes when characterizing one's predisposition to suffering AMS and high-altitude pulmonary edema (HAPE) (Hohenhaus et al., 1995; Nespoulet et al., 2012). In one investigation, AMS susceptible patients' night SpO_2 concentrations were ∼5% lower than their non-susceptible counterparts, yet these patients experienced significantly fewer AHI events per hour (18 vs. 33 events per hour; $p = 0.038$) (Nespoulet et al., 2012). The present study observed a highly individualized response in breathing stability, especially after 21-days in bed rest, such that those who experienced the greatest change in breathing stability from Night 1 to Night 21 also maintained or increased night SpO_2 saturations. Greater oscillations in breathing stability may serve to circulate more overall blood flow to cerebral tissues, affecting ventilatory drive, and further optimizing night mean SpO_2 values (Ainslie et al., 2007, 2013). These findings are in-line with research conducted on normal, healthy subjects who develop periodic breathing at altitude, and in whom one usually finds that mean SpO_2 is higher than in individuals who do not develop periodic breathing (Ainslie et al., 2013). Of note, DRs are calculated based on the number of apneas and hyperpnea experienced. Thus, although we observed a significant decline in breathing stability in the NBR trial, these data are based on fewer absolute events per hour compared to in the hypoxic trials

TABLE 3 | Relationships of selected variable change scores from the baseline control night to Night 1, and from Night 1 to Night 21 of the experimental trials, including the normoxic bed rest (NBR), hypoxic ambulatory (HAMB) and hypoxic bed rest (HBR) conditions.

		Δ from Baseline to Night 1			Δ from Night 1 to Night 21		
		NBR	**HAMB**	**HBR**	**NBR**	**HAMB**	**HBR**
Δ DR vs. Δ AHI	R^2	0.198	0.119	0.201	0.017	0.011	0.012
	p-value	0.170	0.298	0.167	0.704	0.756	0.751
Δ AHI vs. Δ SpO_2	R^2	0.404*	0.123	0.309	0.168	0.111	0.209
	p-value	0.035*	0.290	0.076	0.210	0.317	0.158
Δ DR vs. Δ SpO_2	R^2	0.058	0.017	0.309	0.471*	0.009	0.233
	p-value	0.478	0.706	0.076	0.020*	0.776	0.133

*DR, duty ratio; AHI, apnea-hypopnea index; SpO_2, peripheral oxyhemoglobin saturation; NBR, normoxic bed rest; HBR, hypoxic bed rest; HAMB, hypoxic ambulatory condition. *Significant correlation ($p < 0.05$).*

(either with bed rest or ambulatory). Therefore, the significant decrease in DR and increased total cycle length observed in NBR may be more heavily weighted on less disturbances overall. The relationship between breathing stability and bed rest should continue to be explored in future research.

Respiration can vary markedly throughout each sleep stage, independent of environmental conditions. Sensitivity to high PCO_2 and low PO_2 is lowest in REM vs. NREM sleep (Douglas et al., 1982). Transitioning from wakefulness to N1 is characterized by significant decreases in minute ventilation, and generally attributed to variations in both respiratory rate and tidal volume (Kreiger, 1985). Thus, as NREM sleep progresses, hypoventilation, and a 3–7 mm Hg increase in arterial PCO_2, occurs as a result of: moderated central respiratory drive, upper respiratory muscle relaxation, and increased airway resistance(Kreiger, 1985). Minute ventilation decreases across N2 and N3 sleep, and is characterized by particularly high individual variability observed between subjects' HVR when transitioning between these sleep stages. Although the alterations in sleep architecture observed in the present study may be, predominantly, an after-effect of the frequent periodic breathing experienced from the continuous hypoxic stimulus, it is useful to note that spending more time in a given sleep stage may affect the HVR in its own right.

It is also important to note that sleep-breathing patterns observed during strict bed rest within a clinical population may be affected by additional whole-body challenges not considered in the present work (e.g., increased inflammatory responses, bone frailty, respiratory complications, or certain medications), in which healthy adults were studied. By contrast, this randomized, repeated-measures investigation sought to objectively measure the influence of sustained bed rest *per se*, and in combination with hypoxia, on sleep-breathing parameters, and accordingly, required the participants to adhere to very strict rules, including prohibiting daytime naps. Certainly, these standards would provide a different sleep experience than those of patients who may be bedridden, but are permitted to nap during the day, for example. Other research involving the combined effects of bed rest and hypoxic exposure have been conducted using shorter, 10-days protocols. In one study, Rojc et al. (2014) found that participants ($N = 10$ males) spent ∼40% less time in N1 sleep after 10 days in HBR compared to baseline recordings. There were near-universal incidences of periodic breathing after 10 days in the hypoxic trials (HBR Night 10: 100% HAMB Night 10: 80%), although no data were reported for AHI or breathing stability (DR, or other measure), nor were any data reported on whether the observed periodic breathing was related to any other dependent measure. However, it must also be acknowledged that for practical purposes, the present study did not include a 21-days normoxic ambulatory condition ("NAMB"). We considered that the baseline sleep measurement would be a valid assessment of the subjects' normoxic ambulatory responses under resting conditions, but without the confinement aspect. Thus, it cannot be ruled out that the values we might have obtained with an "NAMB" intervention may have differed significantly from baseline values. Consequently, it is conceivable that, at least to some extent, the effects we have attributed to bed rest *per se* or hypoxia *per se* may be confounded by other unidentified factors, like remaining in confinement.

In the current study, AHI doubled in severity in terms of events per hour, during the course of both hypoxic interventions (**Figure 4**). Data on progressive increases in AHI with partial or full acclimatization to high altitude have been reported in works that encompass hypoxic exposures from 14 days (at 5,050 m) (Andrews et al., 2012) up to 1 year exposure duration on the high Antarctic plateau (∼3,000 m altitude at equator) (Tellez et al., 2014). It has been suggested that instability in respiratory control may be dependent on altitude, (Andrews et al., 2012) not just exposure duration. That there were increases observed in DR and AHI during the course of the NBR intervention remains an avenue to be further explored. In terms of the stated hypotheses, this study did find that sleep architecture was negatively altered at the start of bed rest for both NBR and HBR compared to baseline. Hypoxia induced significant, negative alterations in respiratory control, and clinical indexes like the AHI in HAMB and HBR; however there were no appreciable differences in sleep quality variables found in any condition. Breathing stability issues were not systematically exacerbated when both stimuli were combined, i.e., the HBR condition was no worse than HAMB in terms of

AHI, DR or length of the breathing cycle. These sleep data have implications for clinical populations who are bedridden, military personnel stationed at altitude and humans on prolonged space expeditions in microgravity or on ground-based analog environments.

CONCLUSIONS

Breathing stability is worsened after bed rest, throughout hypoxic exposure, and when bed rest and hypoxic stimuli are combined. These data are clinically relevant for patients who may be hypoxic and inactive (e.g., chronic obstructive pulmonary disorder). The symptoms of these patients represent a frequent cause of ICU admissions. Sleep architecture is affected in all trials, with a greater time spent in light sleep, consistent in each of the three test conditions after prolonged exposures. These results are clinically important for their implications in applying and enforcing strict sleep hygiene rules in ICU units, as well as planning of further treatment options for ICU-induced sleep disorders.

AUTHOR CONTRIBUTIONS

OE, IM, and LD concept and research design; SM, DM, SK, and LD, performed experiments; SM, DM, SK, and LD analyzed data; SM and LD interpreted results; SM, DM, SK, and LD drafted manuscript; SM, DM, SK, OE, IM, and LD edited and revised manuscript; SM, DM, SK, OE, IM, and LD approved final version of manuscript.

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Effect of Permissive Dehydration on Induction and Decay of Heat Acclimation, and Temperate Exercise Performance

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Purpose: It has been suggested that dehydration is an independent stimulus for heat acclimation (HA), possibly through influencing fluid-regulation mechanisms and increasing plasma volume (PV) expansion. There is also some evidence that HA may be ergogenic in temperate conditions and that this may be linked to PV expansion. We investigated: (i) the influence of dehydration on the time-course of acquisition and decay of HA; (ii) whether dehydration augmented any ergogenic benefits in temperate conditions, particularly those related to PV expansion.

Methods: Eight males [VO_{2max}: 56.9(7.2) $mL{\cdot}kg^{-1}{\cdot}min^{-1}$] undertook two HA programmes (balanced cross-over design), once drinking to maintain euhydration (HA_{Eu}) and once with restricted fluid-intake (HA_{De}). Days 1, 6, 11, and 18 were 60 min exercise-heat stress tests [HST (40°C; 50% RH)], days 2–5 and 7–10 were 90 min, isothermal-strain (T_{re} ~ 38.5°C), exercise-heat sessions. Performance parameters [VO_{2max}, lactate threshold, efficiency, peak power output (PPO)] were determined pre and post HA by graded exercise test (22°C; 55%RH).

Results: During isothermal-strain sessions hypohydration was achieved in HA_{De} and euhydration maintained in HA_{Eu} [average body mass loss −2.71(0.82)% vs. −0.56(0.73)%, $P < 0.001$], but aldosterone concentration, power output, and cardiovascular strain were unaffected by dehydration. HA was evident on day 6 {reduced end-exercise T_{re} [−0.30(0.27)°C] and exercise heart rate [−12(15) $beats.min^{-1}$], increased PV [+7.2(6.4)%] and sweat-loss [+0.25(0.22) $L.h^{-1}$], $P < 0.05$} with some further adaptations on day 11 {further reduced end-exercise T_{re} [−0.25(0.19)°C] and exercise heart rate [−3(9) $beats.min^{-1}$], $P < 0.05$}. These adaptations were not notably affected by dehydration and were generally maintained 7-days post HA. Performance parameters were unchanged, apart from increased PPO (+16(20) W, irrespective of condition).

Conclusions: When thermal-strain is matched, permissive dehydration which induces a mild, transient, hypohydration does not affect the acquisition and decay of HA, or endurance performance parameters. Irrespective of hydration, trained individuals require >5 days to optimize HA.

Keywords: thermoregulation, fluid, acclimatization, hydration, hypohydration

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INTRODUCTION

The heat acclimated phenotype has been extensively described (e.g., Armstrong and Maresh, 1991; Périard et al., 2015) and is characterized by adaptations enabling an individual to better accommodate a given thermal-stressor. Typically, heat acclimation (HA) is acquired by frequently and repeatedly elevating both core (T_C) and skin (T_{Sk}) temperature (Regan et al., 1996) to a level challenging sudomotor and vasomotor thermoeffector responses for a sufficient duration (Fox et al., 1963). Although passive approaches have sometimes been employed (Beaudin et al., 2009), the increased thermal strain is often achieved through a combination of environmental heat-stress and increased metabolic heat-production through exercise (e.g., Lorenzo et al., 2010; Gibson et al., 2014, 2015; Keiser et al., 2015). More recently, it has been suggested that dehydration, the process of losing fluid and achieving a state of hypohydration (lower-than-normal body water volume), may also represent an important stimulus for facilitating HA (Garrett et al., 2012, 2014; Périard et al., 2015; Akerman et al., 2016), although this may be controversial (Horowitz et al., 1999; Schwimmer et al., 2006) and in contrast to traditional guidelines for maintaining fluid and electrolyte balance (Armstrong and Maresh, 1991; Bergeron et al., 2012).

Dehydration through combined exercise and heat-stress causes hyperosmotic hypovolemia, reducing thermoeffector function [lower sweating and skin blood flow (Sawka, 1992)], and increasing thermal, cardiovascular, and fluid-regulatory strain (Sawka, 1992; Kenefick et al., 2007). Whilst impaired thermoeffector activity might possibly be maladaptive in terms of sudomotor and vasomotor function, the resultant increased tissue-temperature is important; for T_Cs between 37.3°C and 38.5°C the magnitude of HA is proportional to the thermal forcing-function (Fox et al., 1963), although increasing T_C beyond 38.5°C may not confer any additional benefit (Gibson et al., 2014, 2015). Indeed, because dehydration and heat are often inter-linked in their causation and the strain they induce, demarcating their individual effects can be difficult (Akerman et al., 2016). Recent research employing an isothermal strain (target rectal temperature (T_{re}) = 38.5°C) HA programme suggests that dehydration can provide a thermally-independent adaptation stimulus (Garrett et al., 2014). Restricting fluid ingestion (permissive dehydration) during the five, daily, exercise-heat exposures (90 min·day^{-1}) increased plasma aldosterone concentration ($[aldo]_p$) over the HA programme, relative to euhydration; this correlated with an increased plasma volume (PV), while increased resting forearm perfusion and reduced exercise heart rate were also observed during a subsequent heat stress test (HST). The adaptations that appear to be most affected by permissive dehydration (e.g., PV expansion and cardiovascular stability) are among the most rapidly acquired during HA (∼4–5 days) and also the quickest to decay upon cessation of HA (Williams et al., 1967; Périard et al., 2015). It remains to be established whether permissive dehydration positively influences the adaptive responses to heat over the longer timescales (∼10 days) typically necessary to optimize HA (Périard et al., 2015), or whether permissive dehydration affects the retention of the heat acclimated phenotype following HA. Evidence from rodent studies indicates that severe (10% body mass loss) acute hypohydration can adversely affect the longer-term adaptive response to heat (Horowitz et al., 1999; Schwimmer et al., 2006), although the relevance of this work to humans repeatedly dehydrating to a milder hypohydration (<3% body mass loss) over the course of HA is unclear.

The ergogenic potential of HA under more temperate conditions is currently under debate (Minson and Cotter, 2016; Nybo and Lundby, 2016). Lorenzo et al. (2010) demonstrated improved exercise performance in a cool environment (13°C; 30% RH) following a 10-day exercise-heat acclimation programme (40°C; 30% RH) compared to the same training in the cool conditions, possibly related to PV expansion and its influence on VO_{2max} by a Frank-Starling effect. Studies also provide indication that HA elicits improvements in VO_{2max} (Sawka et al., 1985; Lorenzo et al., 2010), exercise economy (Sawka et al., 1983) and lactate threshold (Lorenzo et al., 2010) in temperate conditions; together these are key determinants of endurance performance (Joyner and Coyle, 2008). However, many of these studies have been criticized for inadequate control (Corbett et al., 2014) and this ergogenic effect has not been replicated in recent experiments employing more appropriate controls (Karlsen et al., 2015; Keiser et al., 2015). Moreover, the influence of PV expansion on VO_{2max} depends on the balance between increased cardiac output and the haemodilution effect on O_2-carrying capacity, which may be unfavorable in an already hypervolemic population. Recently, Keiser et al. (2015) showed no effect of PV expansion on VO_{2max} or exercise performance among a well-trained cohort, whether induced through HA, or by albumin-solution infusion, although there was considerable inter-individual variation. Given that dehydration may augment the hypervolemic aspect of HA (Garrett et al., 2014), understanding the resultant effects on VO_{2max} and exercise performance is important, particularly as these programmes are often used by athletes and individuals undertaking heavy physical work. Interestingly, there is some evidence of an ergogenic effect of short-term HA programmes with permissive dehydration amongst trained individuals in hot (Garrett et al., 2014) and temperate conditions (Neal et al., 2016), but these studies must be interpreted cautiously due to the lack of an appropriate comparison group.

Accordingly, the primary aim of this study was to investigate the influence of permissive dehydration on the time-course and magnitude of the acquisition and decay of HA over a short- and longer-term, using a matched thermal-strain HA programme. An ancillary aim was to investigate the ergogenic potential of HA and specifically to examine whether permissive dehydration augmented any ergogenic effects of HA, particularly those effects related to PV expansion.

METHODS

Participants

Eight trained male athletes participated in this study which was approved by the University's Ethics Committee [Mean(*SD*) age:

21(3) years; height: 1.81(0.05) m; mass: 77.31(4.88) kg; body fat: 10.0(3.5)%; $VO_{2max:}$ 56.9(7.2) $mL{\cdot}kg^{-1}{\cdot}min^{-1}$; peak power output (PPO): 338(46) W]. This sample size is consistent with previous work in this area that has identified between-conditions differences in key thermo-physiological indices (Garrett et al., 2014). Participants were all engaged in recreational endurance exercise (running, cycling, triathlon). All participants provided written informed consent.

Experimental Design

A within-participant, balanced cross-over design was employed, with participants undertaking both control [euhydrated heat acclimation (HA_{Eu})] and intervention [permissive dehydration (HA_{De})] HA programmes (target ambient conditions: 40°C; 50% RH). Each HA programme lasted 11-days and consisted of three bouts of exercise at a fixed external work rate [heat stress test (HST)], undertaken on day 1 (HST_{pre}), day 6 (HST_{mid}), and day 11 (HST_{post}), interspersed with eight isothermal heat strain exercise-heat exposures (ISO). The ISO approach was used to induce HA so as to avoid the potential for a dehydration-induced elevation in T_{re}, which would provide an additional thermal stimulus for adaptation and the HSTs enabled assessment of the induction of short- and longer-term adaptations. A temperate (target ambient conditions: 20°C; 55% RH) graded exercise test (GXT) was completed before (GXT_{pre}) and after (GXT_{post}) HA for assessment of performance parameters and thermoregulatory responses during temperate exercise. To obtain an index of decay the HST was repeated 1 week after the HA programme (HST_{decay}). HA programmes were identical apart from the fluid consumption during ISO, where a regimen was prescribed to either maintain hydration or facilitate dehydration. A minimum 3-month wash-out period was prescribed between HA programmes (see **Figure 1**). All testing was completed in the UK winter months (November-February) with an average ambient temperature of 2°C during the data collection periods. The average temperature in the 3 months preceding the data collection period was 8°C.

Experimental Procedures

Heat Stress Test

Participants cycled in the hot environment on a calibrated CompuTrainer cycle ergometer (RacerMate Inc., Seattle, Washington, USA) for 60 min at 35% of PPO reached in the GXT (described subsequently). 1.25 L of 3.6% carbohydrate solution (drink temperature 20°C) was ingested to replace fluid losses, divided into five equal boluses (0.25 L) and consumed immediately prior to commencing exercise and every 15 min thereafter. Convective cooling was provided at a rate of $3.5\ m{\cdot}s^{-1}$; this prevented most participants from reaching the T_{re} withdrawal criteria of 40°C, whilst maintaining an acceptably high mean skin temperature ($\overline{T}_{Sk}$) and allowing thermoeffector responses to be assessed.

Isothermal Heat Strain Sessions

During each ISO participants exercised in the hot environment on a calibrated CompuTrainer cycle ergometer (RacerMate Inc., Seattle, WA, USA), initially selecting a work rate eliciting a rating of perceived exertion (RPE; Borg, 1982) of 15. This was maintained until $T_{re} = 38.3$°C, at which point external power output was adjusted as appropriate to maintain the target T_{re} (38.5°C) and a small amount of convective cooling ($\sim$2–3 $m{\cdot}s^{-1}$) was used to facilitate the exercise component and provide some perceptual benefit whilst maintaining a high $\overline{T}_{Sk}$. During HA_{Eu} participants consumed 1.75 L of 3.6% carbohydrate-electrolyte fluid (Science In Sport, Nelson, UK) in 0.25 L boluses every 15 min (drink temperature 20°C), including immediately prior to and at the end of each ISO. After the exercise, participants were encouraged to drink *ad libitum* to ensure similar hydration for the following days. Permissive dehydration is defined as purposefully allowing a person to dehydrate through restricting fluid intake (Garrett et al., 2014); during HA_{De} no fluid consumption was permitted during each ISO, or for 10 min after. Thereafter, participants consumed 1.75 L of the aforementioned beverage and were subsequently encouraged to drink *ad libitum* to ensure adequate hydration on arrival the following day. The drinking regimens that we employed were used in a previous study where a clear separation of hydration state was achieved and an influence of permissive dehydration on (short-term) HA was demonstrated (Garrett et al., 2014).

Graded Exercise Test

All GXTs were performed on a Lode Excalibur cycle ergometer (Lode, Groningen, The Netherlands) in a temperate environment. Participants exercised for 20 min at 85 or 110 W, dependent upon the estimated fitness of the participant (fixed within-participant). Thereafter, work-rate was incremented by 25 W every 3 min until blood lactate concentration [Lac] was $\geq$4 $mmol{\cdot}L^{-1}$, following which, the participant was given a 5 min break before beginning cycling again at 100 W for 5 min. Work-rate was then increased $25\ W{\cdot}min^{-1}$ until volitional exhaustion. [Lac] was determined from fingertip capillary blood obtained at the end of each exercise stage (Biosen C-line, EKF Diagnostic, Cardiff, UK). Convective cooling was provided at a rate of $3.5\ m{\cdot}s^{-1}$.

General Procedures

Participants wore the same clothes (cycling shorts, shoes, socks) on each day, abstained from alcohol throughout the experimental period or caffeine for 12 h before exercise, consumed a similar diet before each test and drank 0.5 L of water 2 h before every attendance. Participants were instructed to maintain their normal high-intensity training (except 24 h before HSTs or GXTs) and replace an equivalent duration of low/moderate training with that completed in the laboratory to maintain usual training volume; this was reiterated throughout the study and verbally verified.

To ensure similar hydration before HSTs and to ascertain the extent to which participants were able to maintain hydration status across the course of each HA regime, urine osmolality was assessed from daily pre-exercise urine samples (Osmometer 3320, Advanced Instruments Inc., Norwood, MA, USA). This equipment was also used to determine plasma osmolality. Nude body mass (dry) was measured pre- and post- each test session (Industrial Electronic Weight Indicator, Model I10, Ohaus Corporation, Parsippany, NJ, USA); body mass changes were

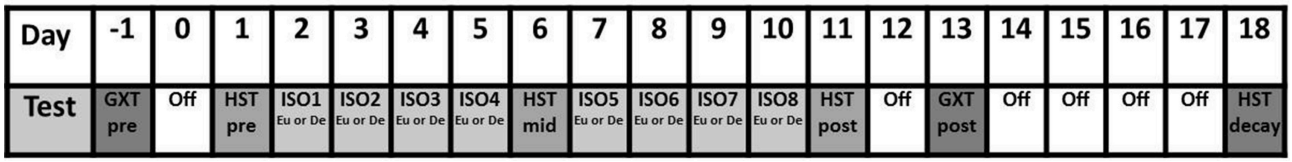

FIGURE 1 | Experimental protocol for examining the effect of hydration on the adaptive responses to exercise in the heat. GXT, Graded Exercise Test; HST, Heat Stress Test; ISO, Isothermal strain acclimation session; Eu, Euhydration; De, Dehydration.

used to determine whole-body sweat rate (SR), adjusted for fluid ingested. Ambient conditions were measured by a WBGT logger (Squirrel 1000, Grant Instruments, Cambridge, UK), T_{re} by a thermistor (Grant Instruments, Cambridge, UK) self-inserted 15 cm beyond the anal sphincter and cardiac frequency (f_C) by short range telemetry (Polar RS800, Polar Electro, Kempele, Finland). During HSTs and GXTs, skin temperature (T_{Sk}) was measured using thermistors on the chest, biceps, thigh and calf (Grant Instruments, Cambridge, UK) and local SR [upper-right back (Q-Sweat, WR Medical Electronics, Maplewood, MN, USA)] and forearm skin blood flow (MoorLAB, Moor Instruments, Devon, UK) were recorded. During HSTs expired gases (Douglas bag method), RPE (Borg, 1982), thermal sensation and thermal comfort (Zhang, 2003) were measured at 15 min intervals; a sample of sweat was collected using a custom patch constructed from Parafilm® (Bemis NA, Neenah, WI, USA) for determining sodium concentration [Na^+] by flame photometry (Corning 400, Essex, UK). During GXTs $VO_2$was measured breath-by-breath throughout (Quark B2, COSMED, Rome, Italy).

Immediately before and after HSTs and ISO1 a 10 mL venous blood samples was obtained (K2 EDTA blood collection tubes, Beckton Dickson & Company, Plymouth, UK) from the antecubital vein following 10 min of seated rest for the measurement of hemoglobin concentration [Hb] (201^+ HemoCue, Sweden) and haematocrit (Hct) (Hawksley, Lancing, UK). Whole blood samples were centrifuged (1500 g for 15 min at 4°C, Heraeus™ Multifuge™ 3 S-R, Thermo Electron Corporation, Germany) and the resultant plasma stored at −80°C for subsequent biochemical analyses using enzyme linked immunosorbent assays for $[aldo]_p$ (ELISA Kit #ADI-900-173, Enzo Life Sciences, Exeter, UK) and extracellular heat shock protein 70 concentration (e[HSP70])(Amp'd® HSP70 High Sensitivity ELISA Kit #ENZ-KIT-101, Enzo Life Sciences, Exeter, UK).

Data Analysis

Mean skin temperature was calculated according to Ramanathan (1964) and mean body temperature ($\overline{T}_b$) as the weighted mean of T_{re} and $\overline{T}_{Sk}$ according to Parsons (1993). For GXT data the lactate threshold was defined as the power output at [Lac] of 4 mmol·L^{-1}, gross mechanical efficiency (GME) was calculated at 185 W (highest work rate below lactate threshold achieved by all participants), and VO_2max was defined as the highest 15 s VO_2. Physiological strain index (PSI) was determined according to Moran et al. (1998) and plasma and blood volume shifts were determined according to Dill and Costill (1974). Metabolic heat production (MHP) was calculated according to ISO 8996 Malchaire (2004).

Statistical Analysis

Statistical analyses were undertaken using SPSS (IBM Version. 22, IBM, New York, NY, USA). Significance was set at $P \leq 0.05$; data are presented as mean(*SD*) unless otherwise stated. Following tests for normality, two-way repeated measures ANOVA was used to analyse the main effects, i.e., changes in responses over time and between condition (HA_{Eu} vs. HA_{De}), as well as the interaction effect (i.e., time × condition). The Greenhouse-Geisser statistic was employed to account for violations of sphericity; Bonferroni adjusted Students *t*-tests were used *post-hoc* for analysis of main and interaction effects. *Post-hoc* analysis of significant time effects for ISO sessions were made relative to ISO1 only, with alpha adjusted accordingly. The Wilcoxon sign ranked test was used to analyse ordinal (RPE) data. Relationships between the change in PPO and thermoregulatory parameters were assessed by Pearson's correlation coefficient.

RESULTS

Isothermal Heat Strain Sessions

Ambient conditions for ISO sessions were 39.3(0.5)°C, 56.2(5.1)% RH. All participants completed each ISO, in both conditions, with the daily exercise responses to each HA programme summarized in **Table 1**. A main effect for the influence of condition on mean session body weight loss indicted that hypohydration was achieved in HA_{De} and euhydration maintained in HA_{Eu} [body mass loss −2.71(0.82)% vs. −0.56(0.73)%, $P < 0.001$]. This effect was supported by the plasma osmolality changes within ISO1 whereby a significant condition ($P = 0.013$) and interaction effect were evident ($P = 0.016$), with *post-hoc* analysis indicating that plasma osmolality did not differ between conditions at baseline and was unchanged over HA_{Eu} [Pre = 290(4) vs. Post = 287(4) mOsmo·kg^{-1}], but increased over the course of the ISO session for HA_{De} [Pre = 293 (5) vs. Post = 297(7) mOsmo·kg^{-1}, $P = 0.006$]. Aldosterone concentration increased over ISO1 ($P = 0.001$), but the extent of any increase was not different between conditions and there was no interaction effect [HA_{Eu}Pre = 2651(2700) vs. Post = 5859(4044) pmol·L^{-1}; HA_{De} Pre = 2686(2496) vs. Post = 7741(4763) pmol·L^{-1}].

Over the course of each HA programme the time to reach the target T_{re} did not differ between conditions and the

TABLE 1 | Mean(*SD*) daily responses during 90 min isothermal strain heat acclimation sessions, with (HA_{De}) and without (HA_{Eu}) permissive dehydration ($n = 8$).

	ISO1		ISO2		ISO3		ISO4		ISO5		ISO6		ISO7		ISO8		*P*-value		
	HA_{Eu}	HA_{De}	HA_{Eu}	HA_{De}	HA_{Eu}	HA_{De}	HA_{Eu}	HA_{De}	HA_{Eu}	HA_{De}	HA_{Eu}	HA_{De}	HA_{Eu}	HA_{De}	HA_{Eu}	HA_{De}	Time	Condition	Interaction
Time to 38.5°C T_{re} (min)	29(5)	31(10)	31(6)	28(7)	31(8)	28(8)	31(5)	33(7)	32(8)	29(5)	37(12)	32(6)	39(15)	36(11)	34(11)	32(7)	0.018*	0.335	0.812
Average T_{re} (final 60 min) (°C)	38.68 (0.07)	38.65 (0.18)	38.56 (0.16)	38.62 (0.09)	38.60 (0.08)	38.59 (0.20)	38.60 (0.16)	38.59 (0.08)	38.58 (0.16)	38.60 (0.12)	38.50 (0.19)	38.56 (0.11)	38.43 (0.20)	38.48 (0.11)	38.56 (0.20)	38.57 (0.10)	0.063	0.684	0.899
Average fc (beats·min^{-1})	148 (10)	146 (13)	146 (8)	146 (11)	141 (10)	139 (10)	141 (9)	136 (7)	143 (9)	142 (9)	140 (7)	142 (9)	143 (11)	143 (10)	138 (8)	147 (10)	0.019 [3]	0.918	0.154
External work rate (W)	80 (19)	70 (22)	105 (19)	88 (20)	90 (22)	81 (25)	93 (18)	92 (19)	97 (26)	91 (17)	97 (28)	97 (19)	109 (28)	98 (16)	108 (29)	106 (18)	<0.001[8]	0.485	0.649
Pre-exercise mass (kg)	76.8 (4.7)	75.9 (4.8)	77.3 (4.3)	76.4 (5.1)	77.4 (4.7)	76.4 (5.2)	77.4 (4.7)	76.4 (5.3)	77.5 (5.0)	76.4 (5.1)	77.3 (5.0)	76.7 (5.1)	77.1 (4.5)	76.5 (5.0)	77.3 (4.3)	76.7 (4.7)	0.186	0.263	0.800
Whole-body SR (L·h^{-1})	1.21 (0.41)	1.18 (0.40)	1.33 (0.31)	1.27 (0.41)	1.33 (0.33)	1.25 (0.34)	1.43 (0.34)	1.29 (0.37)	1.49 (0.35)	1.42 (0.38)	1.48 (0.34)	1.46 (0.35)	1.58 (0.37)	1.56 (0.45)	1.60 (0.40)	1.57 (0.38)	<0.001[4–8]	0.229	0.066
Urine osmolality (mOsmo·kg^{-1})	329 (188)	487 (273)	277 (152)	408 (243)	325 (168)	432 (219)	420 (209)	304 (103)	294 (115)	415 (320)	348 (209)	404 (190)	337 (122)	335 (210)	249 (144)	292 (212)	0.649	0.287	0.442
Body mass loss (%)	−0.26 (0.81)	−2.35 (0.89)	−0.45 (0.69)	−2.51 (0.89)	−0.32 (0.68)	−2.46 (0.75)	−0.54 (0.70)	−2.56 (0.82)	−0.64 (0.72)	−2.80 (0.83)	−0.62 (0.71)	−2.88 (0.77)	−0.78 (0.78)	−3.04 (0.84)	−0.86 (0.82)	−3.09 (0.81)	<0.001 [5,7,8]	<0.001	0.756

*Significant difference = $P < 0.05$. Significant post-hoc time effects are relative to ISO1 only and denoted by superscripted letter ([2] = ISO1 vs. ISO2; [3] = ISO1 vs. ISO3; [4] = ISO1 vs. ISO4 etc.).*Post-hoc comparisons not significant relative to ISO1.*

T_{re}, rectal temperature; SR, sweat rate.

same elevated average T_{re} was maintained over the final 60 min of each session. Average power over the ISO sessions increased, but to a similar extent in both conditions; *post-hoc* analysis identified significant increases from the first day (ISO1) to the final day (ISO8). Conversely, f_C reduced over time, particularly at ISO3, but again, this did not differ between conditions. Whole-body SR was augmented with HA irrespective of condition, with *post-hoc* comparisons to the initial ISO session indicating that this occurred from ISO4 onwards. Participants managed to maintain a stable pre-exercise body mass and urine osmolality over the course of the intervention, in both conditions, despite an increased sweat rate and temporary hypohydration during HA_{De}.

Heat Acclimation

The ambient conditions [39.4(0.3)°C, 52.8(2.8)% RH] and the external work rate [Mean 122(14) W] were the same across all HSTs. The thermophysiological, metabolic, biochemical, and perceptual changes over the course of each HA programme, as measured during the HSTs, are summarized in Table 2 (Supplementary Material), with select thermophysiological adaptations shown in **Figure 2**. A number of main effects for time were identified, with *post-hoc* analysis showing that some HA was evident by HST_{mid}, as indicated by significantly reduced thermal strain at rest and during exercise, lower exercise cardiovascular strain, increased whole-body SR and increased blood volume and PV. However, improved thermal comfort and sensation and reduced PSI were only becoming evident at HST_{post} and there were further improvements in a number of thermal parameters from HST_{mid} to HST_{post}. These adaptations were well maintained during the decay period with no significant changes in any parameter from HST_{post} to HST_{decay}, with the exception of a reduced whole-body SR and RER, whereas MHP was reduced relative to HST_{pre} and suggests improved metabolic efficiency, given that external work rate was unchanged. Plasma aldosterone concentration was not assessed during HST_{decay} but a time effect was evident over the time points assessed ($P = 0.048$). Although the location of this effect could not be identified *post-hoc*, numerically, $[aldo]_p$ increased over the HA programme, but this did not differ between conditions and there was no interaction effect.

The only significant differences between HA conditions was for Δ blood volume, which was lower in HA_{De}, and also demonstrated a significant time × condition interaction. Although the location of any differences could not be located *post-hoc,* there was a trend for a between-conditions difference in HST_{decay} ($P = 0.06$). An interaction effect was also noted for Δ plasma volume, but again, the location of any differences could not be located *post-hoc*, although numerically, the greatest difference between conditions was also in the decay period.

Temperate Exercise

Ambient conditions for the GXT were 22.0(0.2)°C, 54.6(5)% RH. Both of the heat acclimation programmes reduced the thermophysiological burden under temperate conditions, as evidenced by a significant time effect (GXT_{pre} vs. GXT_{post}) for resting and exercise T_{re} and heart rate, end exercise $\overline{T}_b$ (all reduced), and skin blood flow (increased). The only significant condition effect was for RER, which was higher in HA_{Eu} than HA_{De}, but there were no significant interaction effects [see Table 3 (Supplementary Material)]. With regard to parameters related to endurance performance, there were no significant main effects for time or condition, or the time × condition interaction for VO_{2max}, lactate threshold or GME (see **Figure 3**). There was a significant main effect of time on PPO achieved during the GXT ($P = 0.033$), but the condition and interaction effects were not significant (see **Figure 3**) and the increase in PPO was not correlated with any of the improvements in thermoregulatory function. Likewise, maximum heart rate (f_{Cmax}) reached in the GXT was significantly reduced following HA [from 187(7)b·min^{-1} to 183(7) beats·min^{-1} in HA_{Eu} and from 189(10) to 181(9) beats·min^{-1} in HA_{De}, $P = 0.003$] but, the condition and interaction effects were, again, not significant.

DISCUSSION

The main findings of the present study were: (i) there was substantial evidence of adaptation to heat over both the short- and longer-term phases of the present study, but when thermal strain is matched, the time course and magnitude of the acquisition and decay of HA are largely unaffected by permissive dehydration, compared to maintaining euhydration; (ii) permissive dehydration did not notably influence the effect of HA on key parameters related to endurance performance (VO_{2max}, LT, GME) and although there was a small ergogenic effect [4.6(5.8)% increased PPO], this was not affected by the drinking regimen.

Our primary finding does not support the suggestion that dehydration provides an additional stimulus for the induction of HA (Garrett et al., 2012, 2014; Périard et al., 2015; Akerman et al., 2016). The data from the short-term phase are somewhat at odds with recent work indicating that dehydrating during 90 min daily exercise-heat stress within a 5-day isothermal HA programme facilitated some aspects of HA (Garrett et al., 2014), but the reason for these discrepant findings is unclear. Aerobic fitness reduces the strain induced by mild hypohydration (Merry et al., 2010) and aerobically fit individuals require a greater stimulus to challenge the fluid-regulatory processes than less fit individuals (Merry et al., 2008). However, the fitness of our participants [VO_{2max} 57(7) mL·kg^{-1}·min^{-1}; PPO 338(49) W)] was comparable to Garrett et al. (2014) [VO_{2max} 60(7) mL·kg^{-1}·min^{-1}; PPO 340(30) W] and greater hypohydration lacks ecological validity, could impair some training adaptations (Judelson et al., 2008) and in rodents at least, might impair aspects of the genomic (Schwimmer et al., 2006) and phenotypic (Horowitz et al., 1999) adaptation to heat. A more sustained stimulus might be required to optimize the rebound hypervolemic response (Akerman et al., 2016), but the drinking regimes were virtually identical and earlier, rather than later, carbohydrate-electrolyte fluid replacement is crucial for recovering PV following ∼3% body weight loss (Kovacs

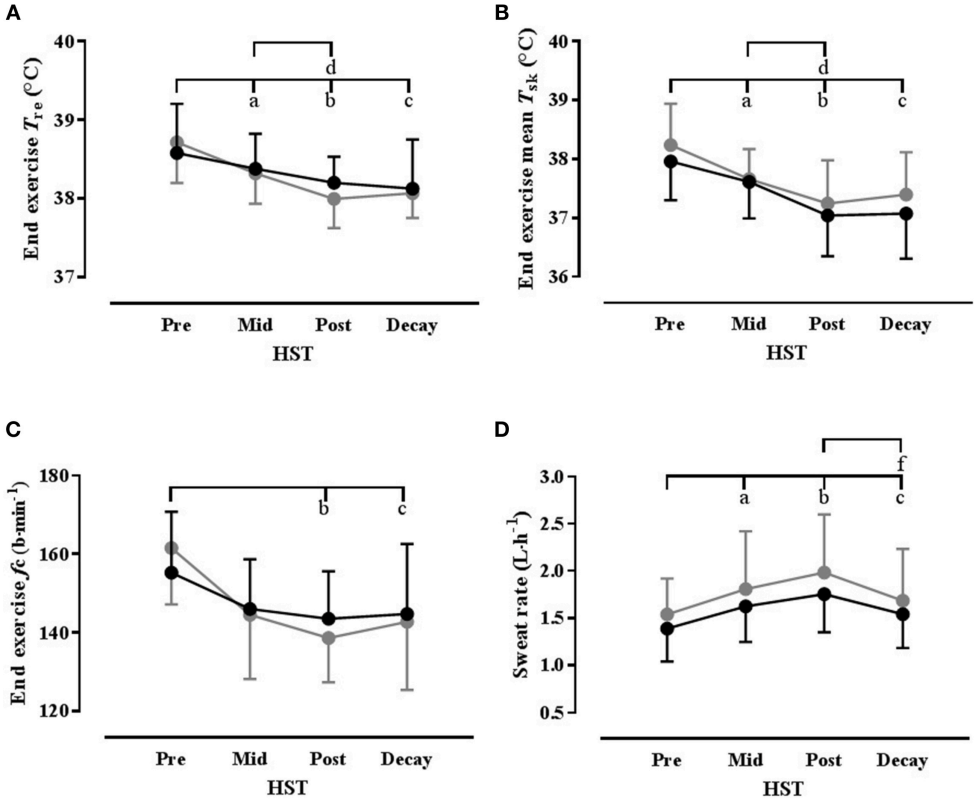

FIGURE 2 | Select thermophysiological variables showing time course of heat acclimation with (HA_{De}: black) and without (HA_{Eu}: gray) permissive dehydration as determined from standard heat stress tests (HST). Data are mean(*SD*) and $n = 8$ unless otherwise stated. **(A)** End exercise rectal temperature (T_{re}); **(B)** End exercise mean skin temperature ($\bar{T}_{Sk}$); **(C)** End exercise mean heart rate (f_C); **(D)** Mean HST whole-body sweat rate ($n = 7$). Significant *post-hoc* time effects ($P < 0.05$) are denoted by superscripted letter (^{a} = HST_{pre} vs. HST_{mid}; ^{b} = HST_{pre} vs. HST_{post}; ^{c} = HST_{pre} vs. HST_{decay}; ^{d} = HST_{mid}; vs. HST_{post}; ^{e} = HST_{mid}; vs. HST_{decay}; ^{f} = HST_{post} vs. HST_{decay}).

et al., 2002). Alternatively, because fluid consumption may need to exceed fluid losses by ∼50% to restore euhydration in a hypohydrated individual (Shirreffs and Maughan, 1998), the *ad libitum* intake of fluid, electrolyte and protein following the permissive dehydration may have been insufficient to enable any additional hypervolemic adaptation (Kay et al., 2005), but this is not supported by the stable daily baseline body mass and (euhydrated) urine osmolality and while there was some evidence for reduced blood volume change in HA_{De}, this appeared to be during the decay, rather than induction, phase.

A clear separation of hydration state was achieved; in HA_{Eu} body mass was maintained consistent with euhydration [−0.56(0.71)% body mass change]; in HA_{De} body mas was reduced [−2.71(0.82)% body mass change] to a degree consistent with hypohydration (Cheuvront et al., 2010; Cheuvront and Kenefick, 2014) and similar to previous studies employing a HA_{De}programme (−1.8 to −3.1% average body mass change (Garrett et al., 2012, 2014; Neal et al., 2016). Likewise, baseline plasma osmolality was within the normative range (Cheuvront et al., 2010) and was maintained in HA_{Eu}, but increased in HA_{De} to a level consistent with mild dehydration (Cheuvront et al., 2010), although this was not measured in all ISO sessions. Nevertheless, assuming a constant sweating rate, hypohydration (body mass change $>$ −2%) will only have been achieved for the final ∼23 min of each ISO and maintained for a further 10 min rest period before fluid consumption, which may have been insufficient to influence the fluid-regulatory mechanisms that are hypothesized to be integral to any effects on HA (Garrett et al., 2012, 2014; Périard et al., 2015; Akerman et al., 2016), although once dehydration is achieved $[aldo]_p$ does not appear to further increase with time (Kenefick et al., 2007). Nevertheless, the increased plasma osmolality in HA_{De} did not surpass the threshold 2% increase in osmolality that may be obligatory for compensatory renal water conservation (Cheuvront and Kenefick, 2014) and although $[aldo]_p$ was increased by the exercise-heat stress, this was not affected by permissive dehydration, at least within ISO1. Overall, given the substantial similarities in study-design, the reasons for differences between Garrett et al. (2014) and the short-term phase of the present study remain largely unclear. The lack of effect of permissive dehydration over a longer-term HA is, perhaps, less surprising given the modest degree of hypohydration induced,

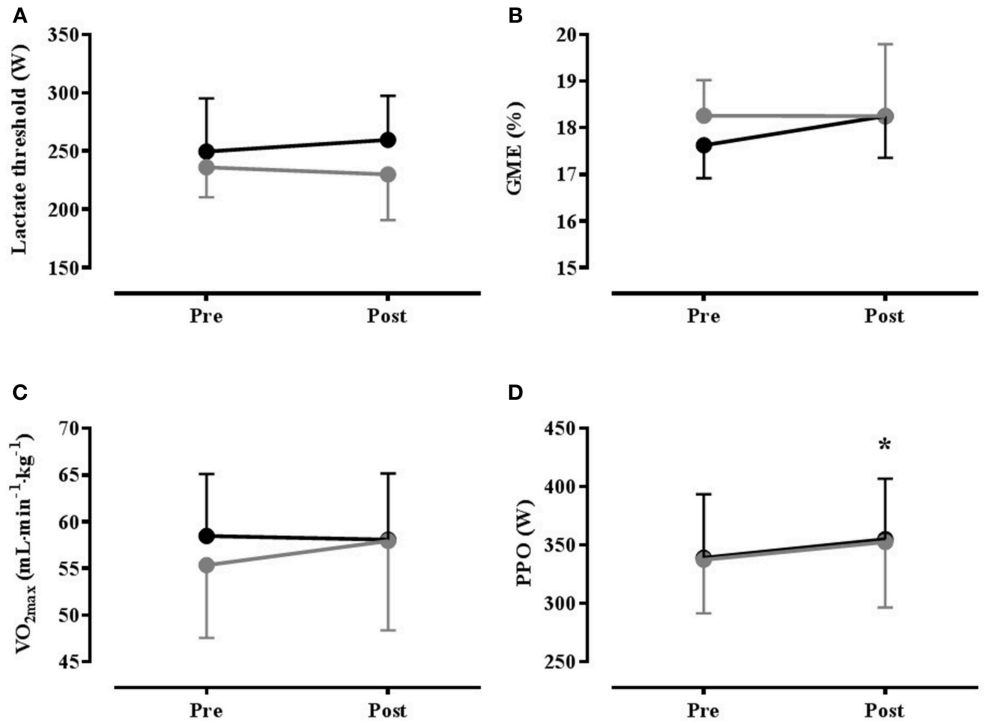

FIGURE 3 | Mean(*SD*) results from temperate (22°C, 55% RH) graded exercise test performed Pre- and Post- heat acclimation, with (HA_{De}: black) and without (HA_{Eu} gray) permissive dehydration ($n = 8$). (A) Lactate Threshold; **(B)** Gross Mechanical Efficiency (GME); **(C)** Maximal Oxygen Uptake (VO_{2max}); **(D)** Peak Power Output (PPO). * = Significant main effect for time ($P < 0.05$).

the minimal influence that this likely had on fluid-regulatory mechanisms (Cheuvront and Kenefick, 2014), the rapid time-course over which hematological and cardiovascular adaptations to heat manifest (Armstrong and Maresh, 1991; Périard et al., 2015), and the isothermal strain.

Because some aspects of HA develop rapidly (Périard et al., 2015), there has been considerable interest in short-term HA programmes (Garrett et al., 2012, 2014; Neal et al., 2016), particularly for trained individuals who are typically partially heat acclimated and may adapt more rapidly (Périard et al., 2015), as well as for logistical reasons. A recent meta-analysis suggests there is little difference in some aspects of HA over the short and longer-time scales that we studied (Tyler et al., 2016), although few of the studies included repeated measures on the same participants and most employed a controlled work-rate regimen (66%), rather than isothermal-exercise approach (11%), meaning that the adaptation stimulus would have reduced over time. In the present study, which employed an isothermal exercise-heat stress approach, significant hypervolemia, increased whole-body sweat rate and reductions in indices of thermal and cardiovascular strain were evident at $HST_{mid,}$ indicating that notable adaptation was achieved within this brief timescale, as others have also demonstrated (Garrett et al., 2012, 2014; Neal et al., 2016). For some indices, such as plasma volume expansion, exercise heart rate and whole body SR, there was no further significant change beyond HST_{mid}. In contrast, further reduction in thermal strain, including exercise T_{re}, $\overline{T}_{Sk,}$ and $\overline{T}_{b}$, was evident from HST_{mid} to $HST_{post,}$whereas reduced PSI and perceptual benefits (improved thermal comfort and sensation) did not manifest until HST_{post}. Taken together, this indicates that the heat acclimated phenotype was not fully developed by HST_{mid}. The temporal pattern of adaptation was broadly consistent with the general consensus regarding the time-course of human HA, particularly with respect to the rapid accrual of plasma volume and associated improvement in cardiovascular function (Armstrong and Maresh, 1991; Périard et al., 2015). In contrast, sudomotor adaptations are typically regarded as being slower to develop (Armstrong and Maresh, 1991; Périard et al., 2015), but in the present study whole body sweat rate was unchanged beyond HST_{mid}. However, the reducing sweat [Na^+] will have facilitated sweat evaporation and the progressive reductions in T_{re} and $\overline{T}_{b}$ observed in the HSTs would reduce the thermoafferent sudomotor drive. Moreover, our participants displayed high initial sweating rates, presumably as a consequence of frequent exposure to high endogenous thermal load through their habitual training; fitter individuals have smaller scope for adaptation, but tend to adapt more rapidly than less fit individuals (Périard et al., 2015) and pronounced sudomotor adaption has previously been documented with short-term HA (Neal et al., 2016). Resting $[aldo]_p$ also increased over the HA regimen, which is in keeping a recent meta-analysis indicating a small effect of HA on resting $[aldo]_p$,

(Tyler et al., 2016) but e[HSP70] was unchanged following HA. The e[HSP70] response was somewhat surprising since we repeatedly exceeded the proposed endogenous temperature threshold for e[HSP70] release (Gibson et al., 2014), although results from meta-analysis suggests that the effect of HA on e[HSP] is trivial, relative to intracellular [HSP] (Tyler et al., 2016) and basal values may be unchanged during HA (Magalhães et al., 2010). Moreover, the responses could have been blunted by the aerobic training habitually undertaken by our participants and the associated frequent elevations in $T_{C,}$which would likely render them partially heat acclimated.

The present study also sought to investigate the extent to which any adaptation to heat was maintained over a 7-day decay period, and whether this was affected by the fluid consumption regimen employed during the HA. Relative to the time-course of induction, the decay in adaptation following HA is poorly documented, but it is generally believed that the hematological and cardiovascular adaptions are among the quickest to decay (Williams et al., 1967; Périard et al., 2015); aspects of the adaptive response most likely to be affected by permissive dehydration (Garrett et al., 2014). Nevertheless, the multitude of approaches used for the induction and assessment of HA and use of limited sample sizes of varying fitness means that there is considerable variation within the published literature regarding the time course of decay of HA. For instance, Williams et al. (1967) reported that, among a group of South African miners who had undertaken a 16 day HA regimen in hot-humid conditions, adaptations in heart rate and mean sweat rate declined by ∼50% within 1 week, with a 25% loss in the adaptation in T_{re}. In contrast, Pandolf et al. (1977) showed little decline in heart rate or T_{re} in fit young men up to 18 days after a 9-day dry-heat acclimation regime and Weller et al. (2007) showed little decay in T_{re} or heart rate 12 days after completing a 14-day dry-heat acclimation regimen. Indeed, it has been suggested that the retention of HA benefits is superior in aerobically fit individuals and with acclimating to dry heat (Pandolf, 1998). The results of the present study are broadly in keeping with this assertion as there was no significant decay in most of the typical indices of physiological strain HA over the 7-day decay period; although SR and RER were diminished relative to HST_{post}, they remained above baseline values and no differences were evident between the drinking conditions. However, these assertions should be tempered by reduced metabolic heat production evident at HST_{post} (discussed subsequently), which occurred despite a fixed external work rate and would have reduced heat-loss requirements during the HST. Moreover, there was a trend for blood volume to decay to a greater extent with HA_{De}, but this did not notably influence indices of thermophysiological strain and should be interpreted cautiously given that it was under free-living conditions.

An ancillary aim of the present study was to investigate the ergogenic potential of HA and whether permissive dehydration augmented any ergogenic effects of HA. However, irrespective of drinking regimen, there was no effect of HA on VO_{2max}, LT, or GME, but given the similarity in the adaptive response to heat, the lack of between-groups differences is unsurprising. This finding is in contrast to a number of studies that have shown an effect of HA on these parameters (Sawka et al., 1983, 1985; Lorenzo et al., 2010), although these studies have often lacked adequate control and often a simple training effect cannot be excluded (Corbett et al., 2014). The possibility of a training effect was reduced in the present study by the recruitment of competitive athletes, although this may have diminished the adaptation potential due to a ceiling effect, whilst the perception based prescription of work rate during the ISO session and modest hypohydration resulted in similar cardiovascular strain and training stimulus in each group. Although pronounced PV expansion was evident in both drinking conditions, there was no evidence of any change in VO_{2max}. This is in contrast to Lorenzo et al. (2010), who demonstrated increased VO_{2max} concomitant with HA induced PV expansion, but is consistent with recent work showing no effect of HA induced PV expansion on VO_{2max} (Karlsen et al., 2015; Keiser et al., 2015). The reason for these equivocal findings is not entirely clear, although in Lorenzo et al. (2010) the relative intensity of training sessions in the heat was higher than for a control group undertaking training under cool conditions and the possibility of an additional training stimulus cannot be excluded. Cardiovascular strain was matched between control and experimental groups in Keiser et al. (2015), although it may have been higher in the experimental group of Karlsen et al. (2015). Alternatively, while the effect of PV expansion on VO_{2max} appears unfavorable at the population level for trained individuals, there appears to be substantial inter-individual variation (Keiser et al., 2015), possibly due to individuality in the balance between increased cardiac output and the haemodilution effect on O_2-carrying capacity. When pronounced inter-individual variation is combined with relatively small sample sizes, the data may not reflect population characteristics, although at the elite performance level these individual differences may be important.

Although our data from the HST indicate that the O_2 cost of exercise was diminished 1 week post exercise, this was not evident in the GME data obtained during the GXT. Because the improved economy was specific to performance in a hot environment it could simply represent the effect of reduced thermal strain. Alternatively, a move to a more efficient phenotype has been demonstrated in rodents undergoing prolonged HA (Kodesh et al., 2011); this could explain why this effect had not developed at HST_{mid} or HST_{post}. Results from a recent meta-analysis have also concluded that there may be a small effect of HA on GME during exercise in the heat (Tyler et al., 2016), but with the exception of studies lacking appropriate control (Sawka et al., 1983), there appears to be little evidence for an effect of HA on GME in humans under temperate conditions (Karlsen et al., 2015). Nevertheless, a small ergogenic effect was apparent as indicated by a 4.6% increase in PPO achieved at the end of the GXT, irrespective of drinking condition, but the mechanisms underpinning this ergogenic effect are unclear given the lack of change in VO_{2max}, LT and GME. The effect of ambient temperature on aerobic exercise is a continuum, with an exponential performance decline at temperatures above ∼10°C (Galloway and Maughan, 1997). Although it is clear that HA attenuates the performance decrement in hot environments, it has been hypothesized that the improved

thermoregulatory capability with HA should also attenuate the heat-related performance decrement evident under more temperate conditions (Corbett et al., 2014). Indeed significant reductions in thermal-strain were evident in the sub-maximal exercise preceding the GXT, but none of these changes were correlated with the performance improvement, and the T_{re} at exercise termination was similar pre vs. post HA, and below the levels associated with impaired performance. Alternatively, we cannot exclude a simple placebo or learning effect on PPO, as we did not include a sham treatment or temperate training group; the primary purpose of the present study was to examine the influence of hydration on HA and performance, rather than the effect of HA *per se*. This assertion is strengthened by our (unpublished) observation of a similar magnitude of improvement in PPO (6.0%) for 8 trained individuals following an identical protocol to the present study, but with all ISO session undertaken with exercise at a matched RPE, under cool conditions (13°C; 60% RH).

In summary, the present study is the first to examine the influence of dehydration on short- and longer-term HA and its subsequent decay, as well as the effect of a longer-term HA regimen with permissive dehydration on key endurance performance parameters. Our data demonstrate that, when thermal strain is matched, the time course and magnitude of the acquisition and decay of HA are largely unaffected by permissive dehydration, compared to maintaining euhydration. Furthermore, neither HA regimen affected VO_{2max}, LT, or GME. PPO was increased consistent with a small ergogenic effect of HA, but this was not affected by the drinking regimen and should be interpreted cautiously in the absence of a plausible mechanism. However, it is important to note that no notable negative effects of permissive dehydration were evident either, and traditional guidance to maintain hydration during HA (Armstrong and Maresh, 1991; Bergeron et al., 2012) may be unnecessary when trained individuals commence exercise in a euhydrated state, when thermal strain is matched, and where a transient mild hypohydration is induced.

AUTHOR CONTRIBUTIONS

RN, HM, MT, JY, and JC were involved in conceptual design, data collection, interpretation, and manuscript preparation. All authors approve the submission of this work and agree to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2016.00564/full#supplementary-material

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