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Influence of dietary nitrate supplementation on local sweating and cutaneous vascular responses during exercise in a hot environment.

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Abstract:	Purpose: We investigated the influence of inorganic nitrate (NO3-) supplementation on local sweating and cutaneous vascular responses during exercise in hot conditions. Method: Eight healthy, young subjects were assigned in a randomized, double-blind, crossover design to receive NO3--rich beetroot (BR) juice (140 mL/day, containing ~8 mmol of NO3-) and NO3--depleted placebo (PL) juice (140 mL/day, containing ~0.003 mmol of NO3-) for 3 days. On day 3 of supplementation, subjects cycled at an intensity corresponding to 55% of o2max for 30 minutes in hot conditions (30C, 50% relative humidity). Chest and forearm sweat rate (SR) and skin blood flow (SkBF), were measured continuously. Cutaneous vascular conductance (CVC) was calculated by SkBF/mean arterial pressure (MAP). Results: Prior to exercise, plasma NO3- (21 ± 6 and 581 ± 161 M) and nitrite (NO2-, 87 ± 28 and 336 ± 156 nM) concentrations were higher after BR compared to PL supplementation (P ≤ 0.011, n=6). Oesophageal, mean skin, and mean body temperatures during exercise were not different between conditions. In addition, BR supplementation did not affect SR, SkBF, and CVC during exercise. A lower MAP was found after 30 minutes of exercise following BR supplementation (112 ± 6 and 103 ± 6 mmHg for PL and BR, respectively, P = 0.021).

	Conclusion: These results suggest that inorganic NO3- supplementation, which increases the potential for O2-independent NO production, does not affect local sweating and cutaneous vascular responses, but attenuates blood pressure in young healthy subjects exercising in a hot environment.
Response to Reviewers:	see attachment

Responses to the Reviewers' Comments

We sincerely appreciate the reviewers' constructive comments that have allowed us to improve our manuscript. We noticed that a similar study which investigated the effect of beetroot supplementation on thermoregulatory and cardiovascular responses was recently published in European Journal of Applied Physiology (Kent et al. Effect of dietary nitrate supplementation on thermoregulatory and cardiovascular responses to submaximal cycling in the heat, Eur J Appl Physiol 118:657-668, 2018). Given that their findings strength our current study, we have decided to add this information to the discussion for blood pressure regulation (P10, L313-320). In addition, based on the comment 4 from reviewer #2, we presented CVC as absolute values (AU/mmHg) but not as % of baseline in the revised manuscript. Thus, the results obtained from core temperature thresholds and slopes for CVC were somewhat changed in the revised manuscript. The absolute CVC analysis revealed that there was no influence of beetroot juice supplementation on the thresholds and slopes for CVC during exercise (P20, Table 3) which is more consistent to general findings in the present study.

Reviewer #1

Comment 1 This is very nice study with strong physiological background. Although no effect of beetroot juice on heat loss responses, this study provides important information to advance our understanding of how oral intake of beetroot juice can modulate heat loss. Paper is well organized and concise. I have some minor comments specifically for discussion and interpretation of authors results. The authors do not necessarily reflect all of my comments in the manuscript, as some of comments are just my thoughts.
We sincerely appreciate the positive comment.
Comment 2 Although the authors rational is based on peripheral mechanisms, since taking beetroot can influence both central and peripheral mechanisms, is it possible that central increase in NO modulates for example thermoregulatory center thereby modulating efferent signaling to thermoeffectors? My understanding is that cardiovascular response can be influenced by central NO bioavailability based on animal studies.
This reviewer raised an important point. While the precise influence of beetroot juice supplementation on the central thermoregulatory mechanisms is unknown in the present study, it is traditionally considered that the shift of core temperature threshold for heat loss responses would reflect the activity of thermoregulatory center in the brain (e.g., Nadel et al. JAP 37:515-520, 1974). Given that we did not find any differences in the core temperature thresholds for sweating and CVC responses in the revised analysis based on absolute CVC (Table 3), it could be assumed that the beetroot juice supplementation does not affect the central thermoregulatory mechanisms in the present study.
Comment 3 Did authors measure respiratory variables such as VO2 during exercise? I think beetroot juice can lower VO2 during exercise (increased muscle efficiency), which may affect heat production? ultimately affect rate of increase in core temperature??
As the reviewer suggested, there might be a possibility that beetroot juice supplementation lowered VO2 during exercise; however, we did not measure VO2 in the present study. A recent study reported that beetroot juice supplementation lowers VO2 but elevates rectal temperature during exercise in hot condition (Kuennen et al. EJAP 2015). Therefore, it is unknown and

difficult to reveal how beetroot juice supplementation affected heat generation in the present study.

Comment 4
P4, L119
> "following the ingestion of 8 mmol NO3- following ingestions" fix the text as repeating "following ingestion" twice.

As the reviewer noted, we have revised the manuscript as shown below.

P4, line 118
"….systolic blood pressure following the ingestion of 8 mmol NO_3 ~~following ingestions~~ (Breese et al. …."

Comment 5
P9, "We further assumed that NO3- and NO2 - in sweat appearing onto the skin would be reduced to NO, and…" this is interesting idea, perhaps future study evaluating forearm immersion to NO3- or NO2-rich water would increase sweating and cutaneous vasodilation is warranted.

We agree with the reviewer's comment. Indeed, it has been reported that the topical application of a prodrug that generate NO can penetrate skin and induce cutaneous vasodilation (Vercelino et al. J Mater Sci: Mater Med, 2013). It would be interesting to investigate if this is true for sweating at rest and during exercise.

Comment 6
As for the potential mechanisms underpinning no effect of beetroot juice on sweating, it may be that NO3- or NO2- does not pass through vessels, or does not enter into sweat gland such that no change in NO bioavailability in sweat glands. Perhaps measuring NO3- or NO2- in sweat can answer this possibility. Please consider adding this point.

As the reviewer pointed out, we do not know if the NO3- and/or NO2- actually arrived at the sweat glands in the present study. We have addressed this point in the discussion in the revised manuscript as shown below.

P11, Line 338-342
"*Limitations*
There were several limitations in the present study. Firstly, while we observed increases in plasma NO_3^- and NO_2^- concentrations, it was unclear whether NO_3^- and NO_2^- delivery to sweat glands, and by extension the potential for NO synthesis, was increased in the present study. Future research should assess sweat NO_3^- and NO_2^- concentrations to verify or refute this possibility."

Comment 7
P9, L288 regarding individual variation, did the authors see some individuals show some improvement in sweating or cutaneous vasodilation??? I guess the authors did some analysis with VO2max, then perhaps better to briefly discuss in term of VO2max..

As the reviewer suggested, there were some subjects who showed increased heat loss responses after beetroot juice supplementation compared with placebo trial during exercise. However, we are unable to clarify if these effects were due to supplementation or between-day or between-site variation. We have analyzed individual data set as a function of VO2max while we did not see results that could be explained meaningfully. Thus, we decided not to revise this

point in the revised manuscript.

Comment 8
Can the authors discuss why blood pressure lowing effect occurred at the end of exercise only??

This comment indicates an important point. We observed a trend for the reduction of MAP at 15 min (P = 0.093) and 20 min (P = 0.060) during exercise. Thus, while the precise reason is unknown, it is assumed that the exercising time is an important factor for the attenuation of MAP during exercise in hot condition. We expect that the lowering blood pressure at the end of exercise might be associated with the fall of blood pH and PO_2 that potentiate the reduction of NO_2^- to NO. However, given that we could not assess this possibility in the present study, future study is needed to confirm this possibility. We have revised the manuscript to address this point in the revised manuscript as shown below.

P8, line 231-237, Results
"A supplementation × time interaction effect was observed for MAP (P = 0.035, η_p^2 = 0.265, 1-β = 0.782, Fig. 1). Post hoc analysis revealed that the BR supplementation lowered MAP during exercise, which attained significance after 30 min of exercise (112 ± 6 and 103 ± 6 mmHg for PL and BR, respectively, P = 0.021, η_p^2 = 0.559, 1-β = 0.724, CI_{95} = -15 to -2 mmHg) but not 15 min (110 ± 2 and 105 ± 4 mmHg, respectively, P = 0.093, η_p^2 = 0.350, 1-β = 0.389, CI_{95} = -11 to 1 mmHg) or 20 min (110 ± 2 and 105 ± 4 mmHg, respectively, P = 0.060, η_p^2 = 0.418, 1-β = 0.489, CI_{95} = -11 to 0 mmHg) of exercise (Fig. 1)."

P11, line 330-334
"It is also interesting that we observed the reduction of MAP at the end of exercise only. It is expected that the lowering blood pressure at the end of exercise might be associated with the fall of blood pH and PO_2 that potentiate the reduction of NO_2^- to NO (Castello et al. 2006; Modin et al. 2001) while future investigation is needed to confirm this possibility."

Reviewer #2

Comment 1
In the current study, Amano et al. examined the effects of 3-day dietary nitrate supplementation with beetroot juice on local sweating and cutaneous vascular responses during exercise in the heat. The authors identified a modest reduction in mean arterial pressure at end-exercise following beetroot juice supplementation, but no effects on local sweating or cutaneous vascular conductance were evident at either chest or forearm skin sites. The data appear to be carefully collected and I generally agree with the conclusions drawn from the results. However I do have some concerns with the study in its current form that need to be addressed.

We appreciate for the reviewer's suggestion and comments. We have revised the manuscript based on the comments.

Comment 2
Although females in this study may have technically been tested within a given phase of the menstrual cycle, the 10-day gap between trials suggests that circulating estrogen levels may have been very different between experimental sessions for some females. For example, during the follicular phase estrogen levels are lowest within the first 5-7 days, then a sharp

increase in estrogen occurs between days 7-14 as ovulation approaches. Since estrogen has well-established effects on NO bioavailability and cutaneous vasodilator responses to heating it would have been more appropriate to test only males or to test females 1-month apart to avoid this potential limitation.

Thank you very much for suggesting this important point. As the reviewer suggested, there might be a possibility that the menstrual cycle was not well controlled for in the female participants and that potential sex differences affected the results in the present study. However, given the small number of participants for males (n=5) and female (n=3), it is difficult to compare the results between males and females to interpret the data set meaningfully. Thus, we have decided to address this issue as a limitation in the revised manuscript as shown below.

P11, line 345-350
"Finally, while we tried to conduct female experiments in the same phase of menstrual cycle, there remained a possibility that the circulating sex hormone levels differed between the trials since we did not measure blood sex hormones concentrations in the present study. Given that the sex hormone levels might affect local cutaneous blood flow response through NO dependent mechanism (Charkoudian et al. 1999), this point is worthy of future study."

Comment 3
How was sample size determined for this study? Previous work demonstrating fairly modest effects of beetroot juice on CVC during local and whole body heating used similar sample sizes, however, in these studies CVC data were expressed as %maximum, which improves measurement precision considerably over absolute or %baseline values. Given the current data are presented as absolute SKBF (AU) and CVC normalized to %baseline, the relatively poor between-site and between-day reliability for these approaches necessitates a larger sample size to make meaningful inferences about the cutaneous vasodilator response, unless the anticipated effect size is large. This issue needs to be clearly addressed by the authors.

We determined the sample size based on the mean and SD of a previous study reported an increase in CVC%max in response to local heating following beetroot juice supplementation (Keen et al. Microvas Res 98:48-53, 2014) which suggested a minimal sample size of n=4 with 80% power and $\alpha = 0.05$. Thus we thought that the sample size n=8 would be adequate in the present study, however, we did not consider the method to normalize CVC for the sample size calculation. As the reviewer suggested, this issue may limit the findings in the present study. Thus, we decided to address a limitation about the reliability and the power of the CVC results as shown below.

P11, line 342-345
"Secondly, given that we did not normalize CVC as % of maximum vasodilation as has previously been conducted (Keen et al. 2014; Levitt et al. 2015), the potential inter-day and inter-site variations in cutaneous vascular response might have influenced the reliability of CVC in the present study."

Comment 4
Beyond the issue of reliability, expressing CVC as %baseline has produced some confusing results here. In figure 2, SKBF is higher on the chest compared to the forearm during exercise. If the authors had presented absolute CVC (AU/mmHg), blood pressure would have been accounted for and the same general trend between forearm and chest sites during exercise would have been evident. However, with the data converted to %baseline CVC, it now appears that conductance on the chest is lower than the forearm for each condition, which does not accurately reflect what is going on at both skin sites. Since CVC is typically very low during

rest under normothermic conditions, modest changes can have a large impact on the results when normalizing to this value, especially when a large vasodilatory response occurs. Even though baseline CVC is very low for both skin sites, it is still higher on average for the chest compared to the forearm. This means that for the same absolute CVC value during exercise, the response will appear much smaller when even a minor increase in baseline CVC occurs. Since measurement reliability is also an issue here, I would highly recommend reporting absolute CVC values over %baseline when normalizing to maximum is not an option.

The reviewer suggested an important point. As suggested, the expression of CVC would have an important impact in the present study and thus we decided to present absolute CVC instead of CVC of %BL in the revised manuscript. As the reviewer suggested, general trend between CVC and SkBF became similar indicating higher CVC on the chest than that of forearm. We have revised the manuscript based on absolute CVC throughout. The changes in discussion was shown as below.

P9-10, line 294-304
"It has recently been reported that NO_3^- supplementation increased CVC during passive heating (Levitt et al. 2015). These authors also reported that the increased CVC was due to a reduction in MAP during normothermic resting and passive hyperthermic conditions, whilst the SkBF per se was not influenced by the supplementation (Levitt et al. 2015). We did not observe measurable differences in CVC between conditions (Fig. 2) despite a reduction in MAP during exercise (Fig. 1). Given that the CVC was not measurably impacted by BR supplementation in the present study (Fig. 2), despite a reduction in mean arterial pressure during exercise (Fig. 1), it appears that BR supplementation has a distinct influence on cutaneous vascular response between whole body passive heating and exercise. However, the mechanisms for the disparate effects of BR supplementation on cutaneous blood flow during exercise and rest in hyperthermic conditions are unknown and therefore warrants further research."

Comment 5
1.It is not always clear if the p-values being reported are for main effects or for individual comparisons. Please clarify this in the abstract and results.

Thank you very much for suggesting this point. Based on the reviewer's comment, we have improved the abstract and results to clarify wht the specified P values relate to.

Comment 6
2. Please be consistent with reporting of p-values throughout the manuscript. In some cases raw p-values are reported and in other cases $P<0.05$ is used.

Based on the reviewer's comment, we have revised the manuscript to specify the actual P values.

Comment 7
Please include confidence intervals for effect size estimates.

Based on the reviewer's comment, we have included the confidence intervals to explain the results as shown below.

P7-8, line 220-257
"**RESULTS**

Plasma nitrate and nitrite concentrations

Compared with PL, three days BR juice supplementation increased resting plasma NO_3^- [P = 0.000, d = 4.916, 1-β =1.000, 95% confidence interval for mean difference (CI_{95}) = 390 to 729 μM] and NO_2^- (P = 0.011, d = 2.222, 1-β =1.000, CI_{95} = 88 to 410 μM, Table 1).

Cardiovascular, thermal, and perceived parameters

There were no differences in HR (P = 0.262, d = 0.190, 1-β = 0.110, CI_{95} = -1 to 5 beats/min) and MAP (P = 0.173, d = 0.416, 1-β = 0.344, CI_{95} = -9 to 2 mmHg) at rest between PL and BR supplementations (Table 2). Resting T_{es} (P = 0.069, d = 0.667, 1-β = 0.704, CI_{95} = -0.01 to 0.23 °C), T_b (P = 0.051, d = 0.118, 1-β = 0.635, CI_{95} = 0 to 0.20 °C), and T_{sk} (P = 0.616, d = 0.526, 1-β = 0.504, CI_{95} = -0.23 to 0.36 °C) were not different in BR compared with PL (Table 2). A supplementation × time interaction effect was observed for MAP (P = 0.035, η_p^2 = 0.265, 1-β = 0.782, Fig. 1). Post hoc analysis revealed that the BR supplementation lowered MAP during exercise, which attained significance after 30 min of exercise (112 ± 6 and 103 ± 6 mmHg for PL and BR, respectively, P = 0.021, η_p^2 = 0.559, 1-β = 0.724, CI_{95} = -15 to -2 mmHg) but not 15 min (110 ± 2 and 105 ± 4 mmHg, respectively, P = 0.093, η_p^2 = 0.350, 1-β = 0.389, CI_{95} = -11 to 1 mmHg) or 20 min (110 ± 2 and 105 ± 4 mmHg, respectively, P = 0.060, η_p^2 = 0.418, 1-β = 0.489, CI_{95} = -11 to 0 mmHg) of exercise (Fig. 1). The attenuation of MAP in BR relative to PL at 30 min of exercise was related to the levels of $\dot{V}o_{2max}$ such that individuals with smaller $\dot{V}o_{2max}$ showed a larger attenuation of MAP (P = 0.048, R^2 = 0.50). Neither a main effect of supplementation (all $P \geq 0.129$, all $\eta_p^2 \leq 0.298$, all 1-β ≤ 0.319) nor an interaction (all $P \geq 0.069$, all $\eta_p^2 \leq 0.312$, all 1-β ≤ 0.529) was observed for HR, T_{es}, T_{sk}, T_b, and RPE during exercise (Fig. 1).

Sweating and cutaneous vascular responses

Neither a main effect of supplementation (P = 0.164, η_p^2 = 0.256, 1-β = 0.270) nor an interaction effect (all $P \geq 0.121$, all $\eta_p^2 \leq 0.250$, all 1-β ≤ 0.437) was observed in SR during exercise (Fig. 2). Similarly, there were no main effects of supplementation (all $P \geq 0.114$, all $\eta_p^2 \leq 0.318$, all 1-β ≤ 0.346) and skin region (all $P \geq 0.089$, all $\eta_p^2 \leq 0.358$, all 1-β ≤ 0.401) or these interaction effect (all $P \geq 0.135$, all $\eta_p^2 \leq 0.289$, all 1-β ≤ 0.309) for T_{es} and T_b thresholds and slopes for SR (Table 3). A higher SkBF and CVC on the chest compared to the forearm was observed as indicated by a significant main effect of skin region during exercise (SkBF; P = 0.008, η_p^2 = 0.660, 1-β = 0.883, CI_{95} = 0.116 to 0.530 AU, CVC; P = 0.012, η_p^2 = 0.619, 1-β = 0.823, CI_{95} = 0.001 to 0.012 AU/mmHg, Fig. 2). The BR supplementation and regional difference did not affect T_{es} and T_b thresholds and slopes for CVC such that there were no main effects of supplementation (all $P \geq 0.087$, all $\eta_p^2 \leq 0.360$, all 1-β ≤ 0.403) and skin region (all $P \geq 0.079$, all $\eta_p^2 \leq 0.377$, all 1-β ≤ 0.427) or these interaction effect (all $P \geq 0.305$, all $\eta_p^2 \leq 0.149$, all 1-β ≤ 0.161) for T_{es} and T_b thresholds and slopes for CVC (Table 3)."

Comment 8

Please address spelling mistakes throughout the manuscript.

We have double-checked the manuscript for the spelling mistakes.

Reviewer #3

Comment 1

The study is on an interesting and relevant topic, with the potential mechanistic linkage between nitrate supplementation and blood flow/sweating clearly laid out in the Introduction.

The Methods is very sound in terms of research design, the Results are clearly presented, and the Discussion puts the results and also the existing literature clearly into context. Very nicely done.
We appreciate the very positive comment for this manuscript.

Influence of dietary nitrate supplementation on local sweating and cutaneous vascular responses during exercise in a hot environment

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Running head: Beetroot juice and heat loss responses during exercise

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ABSTRACT

Purpose: We investigated the influence of inorganic nitrate (NO_3^-) supplementation on local sweating and cutaneous vascular responses during exercise in hot conditions. **Method:** Eight healthy, young subjects were assigned in a randomized, double-blind, crossover design to receive NO_3^--rich beetroot (BR) juice (140 mL/day, containing ~8 mmol of NO_3^-) and NO_3^--depleted placebo (PL) juice (140 mL/day, containing ~0.003 mmol of NO_3^-) for 3 days. On day 3 of supplementation, subjects cycled at an intensity corresponding to 55% of $\dot{V}o_{2max}$ for 30 minutes in hot conditions (30°C, 50% relative humidity). Chest and forearm sweat rate (SR) and skin blood flow (SkBF), were measured continuously. Cutaneous vascular conductance (CVC) was calculated by SkBF/mean arterial pressure (MAP). **Results:** Prior to exercise, plasma NO_3^- (21 ± 6 and 581 ± 161 μM) and nitrite (NO_2^-, 87 ± 28 and 336 ± 156 nM) concentrations were higher after BR compared to PL supplementation ($P \leq 0.011$, n=6). Oesophageal, mean skin, and mean body temperatures during exercise were not different between conditions. In addition, BR supplementation did not affect SR, SkBF, and CVC during exercise. A lower MAP was found after 30 minutes of exercise following BR supplementation (112 ± 6 and 103 ± 6 mmHg for PL and BR, respectively, $P = 0.021$). **Conclusion:** These results suggest that inorganic NO_3^- supplementation, which increases the potential for O_2-independent NO production, does not affect local sweating and cutaneous vascular responses, but attenuates blood pressure in young healthy subjects exercising in a hot environment.

KEYWORDS: Nitric oxide synthesis, thermoregulation, heat loss response, sweat glands

ABBREVIATIONS: ANOVA, analysis of variance; d, Cohen's d; CVC, cutaneous vascular conductance; HR, heart rate; $\dot{V}o_{2max}$, maximal oxygen uptake; MAP, mean arterial blood pressure; T_b, mean body temperature; T_{sk}, mean skin temperature; NO_3^-, nitrate; NO, nitric oxide; NOS, nitric oxide synthase; NO_2^-, nitrite; T_{es}, oesophageal temperature; η_p^2, partial eta-squared; RPE, rating of perceived exertion; T_{re}, rectal temperature; SkBF, skin blood flow; SD, standard deviation; SR, sweat rate

INTRODUCTION

Sweating and cutaneous vasodilation are vital physiological functions that dissipate heat from the body during exercise. Previous studies suggest that nitric oxide (NO) is an important signalling molecule for modulating sweat rate (SR) and cutaneous blood flow in humans (Stapleton et al. 2014; Welch et al. 2009; Kellogg et al. 1998; McNamara et al. 2014; Wilkins et al. 2003; Fujii et al. 2016). There are two pathways for NO generation in humans. The most recognized is the enzymatic NO synthase (NOS) pathway, which catalyses the oxidation of L-arginine to NO and L-citrulline (Moncada and Higgs 1991). More recently, it has been shown that NO can be produced O_2-independently through the stepwise reduction of inorganic nitrate (NO_3^-) to nitrite (NO_2^-) and subsequently NO (i.e. $NO_3^- \rightarrow NO_2^- \rightarrow NO$ pathway) (Lundberg et al. 2008). The importance of NOS-derived NO on physiological responses that promote heat loss is already well defined, as evidenced by a lower SR and cutaneous vasodilation during exercise or passive heat stress following inhibition of skin NOS activity (Welch et al. 2009; Kellogg et al. 1998; Wilkins et al. 2003; Stapleton et al. 2014; Fujii et al. 2016; Amano et al. 2017a). On the other hand, the influence of the $NO_3^- \rightarrow NO_2^- \rightarrow NO$ pathway on heat loss responses during exercise has not been fully investigated.

Following ingestion, NO_3^- is absorbed and concentrated by the salivary glands for delivery to the oral cavity for second pass metabolism (Spiegelhalder et al. 1976). Here, oral microflora catalyses the reduction of NO_3^- to NO_2^- (Duncan et al. 1995). Ingested NO_2^- is subsequently reduced to NO and other reactive nitrogen species in the acidic pH of the stomach (Benjamin et al. 1994). It is also clear that a portion of the ingested NO_2^- passes into the systemic circulation, as evidenced by a dose-dependent increase in venous plasma [NO_2^-] after oral NO_3^- ingestion (Kapil et al. 2010; Wylie et al. 2013a). As this circulating NO_2^- arrives at the skin microvasculature, the ensuing fall in P_{O_2} (Kerger et al. 1995) would be conducive to the reduction of NO_2^- to NO (Castello et al. 2006) and might promote increases in NO-mediated cutaneous vasodilation (Kellogg et al. 1998; Fujii et al. 2016; Wilkins et al. 2003; Shastry et al. 1998; McNamara et al. 2014). It is also possible for circulating NO_2^- to pass into the eccrine sweat glands (Weller et al. 1996). Subsequently, NO_2^- might be reduced to NO, a reaction that would be facilitated by the acidic pH present in eccrine sweat (Morimoto and Johnson 1967). In addition, NO_3^- secreted in sweat might undergo reduction to NO_2^- when exposed to dermal NO_3^- reductases with this NO_2^- undergoing subsequent reduction to NO within the acidic

conditions of the skin (Burry et al. 2001; Weller et al. 1996). This dermal NO then has the potential to diffuse through the skin to promote vasodilation (Vercelino et al. 2013). Therefore, NO_3^- supplementation has the potential to augment sweating and cutaneous vascular responses via NO-mediated signalling during exercise.

In contrast to the postulate that NO_3^- supplementation has the potential to augment SR, it has recently been reported that dietary NO_3^- supplementation does not affect whole body sweat loss (indirectly inferred from changes in body mass) during submaximal treadmill walking in hot conditions (Kuennen et al. 2015). However, it is important to note the large inter-regional differences in local SR and skin blood flow (SkBF) previously reported across human skin (Havenith et al. 2008; Smith and Havenith 2011; Taylor and Machado-Moreira 2013; Kuno 1956; Hertzman and Randall 1948). Since higher SkBF would deliver more NO_2^- to the sweat gland, NO_3^- supplementation might be particularly effective at augmenting local SR at skin sites where blood flow is high (e.g. torso) compared to skin sites where blood flow is low (e.g. extremes) (Hertzman and Randall 1948). It has been reported that NO_3^- supplementation can increase cutaneous vasodilation to local heating (Keen et al. 2014) and whole body passive heat stress (Levitt et al. 2015). However, since disparate mechanisms underlie cutaneous blood flow regulation at rest and during exercise (McNamara et al. 2014; Fujii et al. 2016) and since the influence of NO_3^- supplementation on regional SkBF has not been investigated, further research is required to explore whether the greater cutaneous blood flow after NO_3^- supplementation is also manifest during exercise, and whether these effects might be site-specific.

The purpose of the present study was to investigate the influence of NO_3^--rich beetroot juice (BR) supplementation on local sweating and cutaneous vascular responses during exercise in a hot environment. We hypothesized that BR supplementation would augment local sweating and cutaneous vasodilation on the chest to a greater extent than on the forearm during exercise in a hot condition.

MATERIALS AND METHODS

Ethical approval

Each participant was informed of the purpose and procedures of the study prior to providing written informed consent. This study was approved by the Human Subjects Committee of the

Graduate School of Human Development and Environment, Kobe University (Kobe, Japan), and conformed to the standards set forth in the latest revision of the Declaration of Helsinki.

Participants

Five males and three females participated in the present study (mean ± SD age: 24 ± 4 years, height: 1.70 ± 0.09 m, and mass: 62.7 ± 10.3 kg, maximum oxygen uptake, $\dot{V}o_{2max}$: 43 ± 6 ml/kg/min). Participants were healthy and active and were excluded if they had history of hypertension, heart disease, diabetes, autonomic disorders or smoking. All participants were not currently taking prescription medication. None of the females were using oral contraceptives and all participated in the experimental testing sessions either during the self-reported follicular or luteal phases without crossing phases. All experiments were conducted between the month of June and August.

Dietary intervention

Participants were randomly assigned in a crossover, double-blind design to receive 3 days of dietary supplementation with NO_3^--rich beetroot juice (BR) (140 mL/day; ~ 8 mmol NO_3^-; Beet It, James White Drinks, Ipswich, UK) or NO_3^--depleted BR as a placebo (PL; 140 mL/day; 0.0034 mmol NO_3^-; Beet It, James White Drinks, Ipswich, UK). The dose of BR administered was based on a previous dose-response study reporting an increase in plasma NO_3^- and NO_2^- concentration and peak reduction in systolic blood pressure following the ingestion of 8 mmol NO_3^- (Breese et al. 2017; Cermak et al. 2012; Lansley et al. 2011; Kuennen et al. 2015). The NO_3^--depleted BR placebo beverage was identical in color, taste, smell and texture to the experimental NO_3^- -rich BR beverage. The PL beverage was created by passage of the juice, before pasteurization, through a column containing Purolite A520E ion exchange resin, which selectively removes NO_3^- ions. Four participants began with the BR condition, and the other four participants began with the PL condition. The subjects were instructed to consume the beverages (70 mL in the morning and afternoon) on days 1-2 of the supplementation period. On day 3, the subjects were instructed to consume the beverages over a 10-min period, 2 h prior to the start of the exercise test (see below), based on recent evidence that plasma [NO_2^-] peaks at approximately 2-2.5 h post-administration of BR containing 8.4 mmol NO_3^- (Wylie et al. 2013b). A 7-day washout period separated each supplementation period. Throughout the study, participants were asked to refrain from consumption of green leafy vegetables (e.g. Spinach), processed meats (e.g. Bacon), and Japanese traditional foods (e.g. Seaweed,

Sayaingen beans, Chin gin cai) which are high in NO_3^-(Sobko et al. 2010). Since the oral bacteria are integrated for reducing NO_3^- to NO_2^- in vivo (Govoni et al. 2008), participants were also asked to refrain the use of mouthwash.

Exercise protocol

After arrival at the laboratory on experimental days, venous blood samples were drawn from an antecubital vein in a seated position in an air-conditioned room (~27 °C) from 6 of 8 subjects who consented to venipuncture. All exercise trials were performed in an environmental chamber (SR-3000; Nagano Science, Osaka, Japan) maintained at an ambient temperature of 30°C and relative humidity of 50% with minimal air movement. Upon entering the chamber, participants rested in the semi-supine position for a minimum of 60 minutes while instruments were attached. After recording the baseline data for 5 minutes, participants started cycling at an exercise intensity of 55% of maximum oxygen uptake ($\dot{V}o_{2max}$) for 30 minutes.

Measurements

Oesophageal temperature (T_{es}) was measured continuously using a thermocouple temperature probe (Inui Engineering, Higashi Osaka, Japan). The tip of the probe was covered by silicon and inserted at a distance of one-fourth of the participant's standing height from the external nares past the nostril and into the esophagus. Skin temperatures were measured at six skin sites using the same thermocouples attached with surgical tape. Mean skin temperature (T_{sk}) was calculated using 6 skin temperatures weighted to the regional proportions determined as follows: forehead 7%, abdomen 35%, forearm 14%, hand 5%, lower leg 13%, and foot 7% (Mitchell and Wyndham 1969). The mean body temperature (T_b) was calculated as $0.8 \times T_{es} + 0.2 \times T_{sk}$ (Stolwijk and Hardy 1966).

Local SR was measured continuously on left ventral forearm (centre of the forearm) and chest (under the left clavicle) using a ventilated plastic capsule (3.14 cm^2) that was attached to the skin using collodion. Anhydrous nitrogen gas was passed through each capsule over the skin surface at a rate of 0.7 $L \cdot min^{-1}$. Water content from the effluent air was measured using a capacitance hygrometer (HMP50; Vaisala, Helsinki, Finland). An index of local SkBF on the forearm and chest were measured continuously using laser-Doppler velocimetry (ALF21; Advance, Tokyo, Japan) located adjacent to the ventilated capsule. Cutaneous vascular conductance (CVC) was calculated from the ratio of SkBF to mean arterial blood pressure

(MAP). All temperature, SR and SkBF data were recorded at 1-s intervals using a data logger (MX100; Yokogawa, Tokyo, Japan) and simultaneously displayed (MX100 standard software; Yokogawa, Tokyo, Japan) and recorded. Heart rate (HR) and MAP were continuously measured from left middle finger using the Finometer system (Finometer; Finapres Medical Systems, Amsterdam, The Netherlands). Standardized calibration was conducted before each trial. Ratings of perceived exertion (RPE) was measured every 5 minutes based on Borg 6-20 scale (Borg 1970).

Venous blood samples (~4 ml) were drawn into lithium-heparin tubes (7.5 ml Monovette Lithium Heparin, Sarstedt, Leicester, UK), which have very low levels of NO_2^- and NO_3^-. Within 3 min of collection, the samples were centrifuged at 2700 g and 4°C for 10 min. Plasma was extracted and immediately frozen at -80°C for later analysis of NO_2^- and NO_3^- using a modification of the chemiluminescence technique (Bateman et al. 2002). All glassware, utensils, and surfaces were rinsed with deionized water to remove residual NO_2^- and NO_3^- prior to analysis. Following defrosting at room temperature, the NO_2^- of the undiluted (non-deproteinized) plasma was determined by its reduction to NO in the presence of glacial acetic acid and 4% (w/v) aqueous NaI. The spectral emission of electronically excited nitrogen dioxide, produced from the reaction of NO with ozone, was detected by a thermoelectrically cooled, red-sensitive photomultiplier tube housed in a Sievers gas-phase chemiluminescence nitric oxide analyzer (Sievers NOA 280i. Analytix Ltd, Durham, UK). The NO_2^- concentration was determined by plotting signal (mV) area against a calibration plot of 100 nM to 1 µM sodium nitrite. Before determination of NO_3^-, samples were deproteinized using zinc sulfate ($ZnSO_4$)/sodium hydroxide (NaOH) precipitation. Aqueous $ZnSO_4$ [300 µl 5% (w/v)] and 500 µl 0.18 M NaOH were added to 100 µl of sample and vortexed for 30 s before being left to stand at room temperature for 15 min. Thereafter, samples were centrifuged at 4,000 rpm for 5 min, and the supernatant was removed for subsequent analysis. The NO_3^- concentration of the deproteinized plasma sample was determined by its reduction to NO in the presence of 0.8% (w/v) vanadium trichloride in 1 M HCl. The production of NO was detected using the chemiluminescence nitric oxide analyzer, as described above.

Data and statistical analyses

Variables were averaged for 5 minutes at pre-exercise baseline and for every 1 minute during exercise. SR and CVC were plotted against the changes in T_{es} (ΔT_{es}) and T_b (ΔT_b) during

exercise to assess the core temperature threshold and slope for inducing the responses. Segmented regression analysis was used to determine the core temperature onset thresholds and slopes of local SR and cutaneous vasodilation at each skin site (Cheuvront et al. 2009). The slopes were defined based on the linear portion of the changes in SR and CVC before and after the appearance of the onset thresholds during the exercise.

Baseline data in the BR and PL conditions were compared using a paired Student's t-test. T_{es} and T_b thresholds and slopes for SR and CVC between BR and PL were compared using two way-repeated measures ANOVAs (condition × skin region). HR, MAP, T_{es}, T_{sk}, and RPE during exercise were compared using two way-repeated measures ANOVAs (condition × time) with comparisons of baseline and each 5 minutes of exercise (baseline, 5, 10, 15, 20, 25, and 30 minutes). Three way-repeated measures ANOVAs were performed (condition × time × skin region) for SR and CVC during exercise. A Greenhouse-Geisser correction was applied if the assumption of sphericity was been violated. A Bonferroni correction was applied to control for the multiple comparisons. When an influence of BR supplementation was observed, a linear regression analysis was performed to determine the relationship between $\dot{V}o_{2max}$ and the variables (see results). The effect size of each ANOVA was calculated and reported as partial eta-squared values (η_p^2) and that of each t-test was calculated and reported as Cohen's d (d). Data are presented as mean ± SD, and statistical significance was set at $P < 0.05$. All statistical analyses were performed using a statistical package (SPSS) version 24.0.

RESULTS

Plasma nitrate and nitrite concentrations

Compared with PL, three days BR juice supplementation increased resting plasma NO_3^- [P = 0.000, d = 4.916, 1-β =1.000, 95% confidence interval for mean difference (CI_{95}) = 390 to 729 μM] and NO_2^- (P = 0.011, d = 2.222, 1-β =1.000, CI_{95} = 88 to 410 μM, Table 1).

Cardiovascular, thermal, and perceived parameters

There were no differences in HR (P = 0.262, d = 0.190, 1-β = 0.110, CI_{95} = -1 to 5 beats/min) and MAP (P = 0.173, d = 0.416, 1-β = 0.344, CI_{95} = -9 to 2 mmHg) at rest between PL and BR supplementations (Table 2). Resting T_{es} (P = 0.069, d = 0.667, 1-β = 0.704, CI_{95} = -0.01 to 0.23 °C), T_b (P = 0.051, d = 0.118, 1-β = 0.635, CI_{95} = 0 to 0.20 °C), and T_{sk} (P = 0.616, d =

0.526, 1-β = 0.504, CI_{95} = -0.23 to 0.36 °C) were not different in BR compared with PL (Table 2). A supplementation × time interaction effect was observed for MAP (P = 0.035, η_p^2 = 0.265, 1-β = 0.782, Fig. 1). Post hoc analysis revealed that the BR supplementation lowered MAP during exercise, which attained significance after 30 min of exercise (112 ± 6 and 103 ± 6 mmHg for PL and BR, respectively, P = 0.021, η_p^2 = 0.559, 1-β = 0.724, CI_{95} = -15 to -2 mmHg) but not 15 min (110 ± 2 and 105 ± 4 mmHg, respectively, P = 0.093, η_p^2 = 0.350, 1-β = 0.389, CI_{95} = -11 to 1 mmHg) or 20 min (110 ± 2 and 105 ± 4 mmHg, respectively, P = 0.060, η_p^2 = 0.418, 1-β = 0.489, CI_{95} = -11 to 0 mmHg) of exercise (Fig. 1). The attenuation of MAP in BR relative to PL at 30 min of exercise was related to the levels of $\dot{V}o_{2max}$ such that individuals with smaller $\dot{V}o_{2max}$ showed a larger attenuation of MAP (P = 0.048, R^2 = 0.50). Neither a main effect of supplementation (all $P \geq 0.129$, all $\eta_p^2 \leq 0.298$, all 1-β ≤ 0.319) nor an interaction (all $P \geq 0.069$, all $\eta_p^2 \leq 0.312$, all 1-β ≤ 0.529) was observed for HR, T_{es}, T_{sk}, T_b, and RPE during exercise (Fig. 1).

Sweating and cutaneous vascular responses

Neither a main effect of supplementation (P = 0.164, η_p^2 = 0.256, 1-β = 0.270) nor an interaction effect (all $P \geq 0.121$, all $\eta_p^2 \leq 0.250$, all 1-β ≤ 0.437) was observed in SR during exercise (Fig. 2). Similarly, there were no main effects of supplementation (all $P \geq 0.114$, all $\eta_p^2 \leq 0.318$, all 1-β ≤ 0.346) and skin region (all $P \geq 0.089$, all $\eta_p^2 \leq 0.358$, all 1-β ≤ 0.401) or these interaction effect (all $P \geq 0.135$, all $\eta_p^2 \leq 0.289$, all 1-β ≤ 0.309) for T_{es} and T_b thresholds and slopes for SR (Table 3). A higher SkBF and CVC on the chest compared to the forearm was observed as indicated by a significant main effect of skin region during exercise (SkBF; P = 0.008, η_p^2 = 0.660, 1-β = 0.883, CI_{95} = 0.116 to 0.530 AU, CVC; P = 0.012, η_p^2 = 0.619, 1-β = 0.823, CI_{95} = 0.001 to 0.012 AU/mmHg, Fig. 2). The BR supplementation and regional difference did not affect T_{es} and T_b thresholds and slopes for CVC such that there were no main effects of supplementation (all $P \geq 0.087$, all $\eta_p^2 \leq 0.360$, all 1-β ≤ 0.403) and skin region (all $P \geq 0.079$, all $\eta_p^2 \leq 0.377$, all 1-β ≤ 0.427) or these interaction effect (all $P \geq 0.305$, all $\eta_p^2 \leq 0.149$, all 1-β ≤ 0.161) for T_{es} and T_b thresholds and slopes for CVC (Table 3).

DISCUSSSION

Contrary to our hypothesis, BR supplementation did not affect local SR and cutaneous vascular responses on the chest or forearm during exercise in hot conditions. On the other hand, we observed a lowered end-exercise blood pressure following BR supplementation during exercise

in hot conditions. These results suggest that NO_3^--rich BR juice supplementation is not likely to influence local sweating and cutaneous vascular responses, but can lower systemic blood pressure during exercise in a hot environment.

Previous studies have reported a fundamental role for NO in the regulation of sweating during exercise, as evidenced by a reduction in SR when NOS activity was inhibited at the skin (Welch et al. 2009; Fujii et al. 2016; Fujii et al. 2015). In the present study, plasma NO_3^- and NO_2^- were significantly increased by BR supplementation (Table 1), implying an increased potential for O_2-independent NO production (Lundberg et al. 2008). We reasoned that BR supplementation would increase NO_2^- delivery to sweat glands where cutaneous blood flow was higher, thereby promoting an enhanced sweat response mediated by NO (Welch et al. 2009; Fujii et al. 2016; Fujii et al. 2015). We further assumed that NO_3^- and NO_2^- in sweat secreted onto the skin would be reduced to NO, and hence may have diffused through the skin to increase SkBF (Vercelino et al. 2013). However, the BR-induced increase in plasma NO_3^- and NO_2^- did not affect local SR on either the forearm or chest (Fig. 2). In addition, slopes describing the relationship between sweating response on the chest and forearm against the increase in core temperature were not affected by BR supplementation (Table 3). Therefore, contrary to the previously reported influence of NOS-dependent NO production on sweat regulation (Welch et al. 2009; Fujii et al. 2016; Fujii et al. 2015), it appears that augmenting the $NO_3^- \rightarrow NO_2^- \rightarrow NO$ pathway does not modify the sweat response during exercise in the heat, at least following short-term BR administration (3 days) employed herein. Given that NO_2^--derived NO production is potentiated within hypoxic and acidic tissues (Lundberg et al. 2008), there remains the possibility that exogenous NO_3^--supplementation may modulate sweating in hot environments at simulated altitude or during high- intensity exercise when NOS-dependent sweating is abolished (Fujii et al. 2014) as well as in exercising older individuals (Stapleton et al. 2014). In addition, given that NOS-dependent sweating is highly variable between individuals (Amano et al. 2017a; Amano et al. 2017b), it is conceivable that enhancing the $NO_3^- \rightarrow NO_2^- \rightarrow NO$ pathway may benefit some (but not all) individuals via an improved sweating response when exercising in the heat. Therefore, further studies are required to elucidate the precise influence of inorganic NO_3^- treatment on sweating during exercise.

It has recently been reported that NO_3^- supplementation increased CVC during passive heating (Levitt et al. 2015). These authors also reported that the increased CVC was due to a reduction

in MAP during normothermic resting and passive hyperthermic conditions, whilst the SkBF per se was not influenced by the supplementation (Levitt et al. 2015). We did not observe measurable differences in CVC between conditions (Fig. 2) despite a reduction in MAP during exercise (Fig. 1). Given that the CVC was not measurably impacted by BR supplementation in the present study (Fig. 2), despite a reduction in mean arterial pressure during exercise (Fig. 1), it appears that BR supplementation has a distinct influence on cutaneous vascular response between whole body passive heating and exercise. However, the mechanisms for the disparate effects of BR supplementation on cutaneous blood flow during exercise and rest in hyperthermic conditions are unknown and therefore warrants further research.

Numerous studies have reported a reduction in blood pressure at rest (Bailey et al. 2009; Keen et al. 2014; Levitt et al. 2015; Larsen et al. 2006; Sobko et al. 2010; Wylie et al. 2013a; Lee et al. 2015) and during exercise (Lee et al. 2015; Bond Jr et al. 2013) following NO_3^- supplementation. Whilst we did not observe a reduction in blood pressure at rest with NO_3^- treatment (Table 2), this lack of effect has also been reported in some previous studies following NO_3^- supplementation (Cermak et al. 2012; Larsen et al. 2010; Gilchrist et al. 2013). In contrast to previous studies that reported an influence of NO_3^- supplementation on blood pressure within thermoneutral ambient conditions, it is noteworthy that we reported a lowering of blood pressure with BR during exercise in a hot environment. On the other hand, a very recent study reported that BR supplementation does not alter blood pressure during exercise in trained cyclists ($\dot{V}o_{2max}$, 68 ml/kg/min) in hot conditions (Kent et al. 2018). Given that our participants were comparatively less trained ($\dot{V}o_{2max}$, 43 ml/kg/min) to those assessed in the study by Kent et al. (2018), it is possible that aerobic fitness accounted for the inter-study disparity in blood pressure following BR supplementation during exercise in hot conditions. To support this observation, we found that individuals with lower aerobic fitness manifest a larger attenuation of blood pressure during exercise in a hot environment. Notwithstanding this novel observation, given that unstable and falling blood pressure can signal cardiovascular failure during exercise in the heat (Rowell 1974), our data suggest that ingesting NO_3^--rich BR prior to exercising in the heat should be implemented with caution, particularly since its effect on exercise performance in a hot environment is currently controversial (Kent et al. 2017; McQuillan et al. 2017). While the potential ergogenic effects of BR supplementation appear to be inversely related to aerobic fitness (Porcelli et al. 2015) and is recommended to enhance endurance performance in recreationally-active individuals in thermoneutral conditions (Jones 2014), BR supplement should be used with caution in hot environments to limit the potential for the

development of excessive hypotension. It is also interesting that we observed the reduction of MAP at the end of exercise only. It is expected that the lowering blood pressure at the end of exercise might be associated with the fall of blood pH and PO_2 that potentiate the reduction of NO_2^- to NO (Castello et al. 2006; Modin et al. 2001) while future investigation is needed to confirm this possibility. Clearly, further studies are required to elucidate the impact and safety of the blood pressure lowering effects of BR supplementation during exercise in hot conditions.

Limitations

There were several limitations in the present study. Firstly, while we observed increases in plasma NO_3^- and NO_2^- concentrations, it was unclear whether NO_3^- and NO_2^- delivery to sweat glands, and by extension the potential for NO synthesis, was increased in the present study. Future research should assess sweat NO_3^- and NO_2^- concentrations to verify or refute this possibility. Secondly, given that we did not normalize CVC as % of maximum vasodilation as has previously been conducted (Keen et al. 2014; Levitt et al. 2015), the potential inter-day and inter-site variations in cutaneous vascular response might have influenced the reliability of CVC in the present study. Finally, while we tried to conduct female experiments in the same phase of menstrual cycle, there remained a possibility that the circulating sex hormone levels differed between the trials since we did not measure blood sex hormones concentrations in the present study. Given that the sex hormone levels might affect local cutaneous blood flow response through NO dependent mechanism (Charkoudian et al. 1999), this point is worthy of future study.

In summary, we showed that three days of BR juice supplementation, which increased plasma NO_3^- and NO_2^-, had no influence on sweating and cutaneous vascular responses at multiple skin sites during exercise in a hot condition among healthy young adults. However, BR juice supplementation lowered mean arterial blood pressure whilst exercising in the heat. Further research is required to assess the risk-reward weighting of this hypotensive effect during exercise in a hot environment.

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CONFLICTS OF INTEREST

None.

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FIGURE LEGENDS

Figure 1. Heart rate (HR), mean arterial blood pressure (MAP), oesophageal temperature (T_{es}), mean skin temperature (T_{sk}), mean body temperature (T_b), and ratings of perceived exertion (RPE) during exercise in PL and BR conditions. # indicates a significant difference between conditions at a given time point ($P = 0.021$).

Figure 2. Sweat rate (SR), skin blood flow (SkBF), and cutaneous vascular conductance (CVC) on forearm and chest during exercise in PL and BR conditions.

Table 1. Plasma nitrate and nitrite concentrations.

	PL	BR
NO_3^- (μM)	21 (6)	581 (161) *
NO_2^- (nM)	87 (28)	336 (156) *

The values given are the means (SD). NO_3^-, nitrate; NO_2^-, nitrite. *Significantly higher than that of PL ($P \leq 0.011$).

Table 2. Physiological variables at rest.

	PL	BR
HR (beats/min)	63 (11)	65 (10)
MAP(mmHg)	89 (8)	85 (11)
T_{es} (°C)	36.87 (0.12)	36.98 (0.20)
T_{sk} (°C)	34.42 (0.58)	34.48 (0.42)
T_b (°C)	36.38 (0.18)	36.48 (0.20)

The values given are the means (SD). HR, heart rate; MAP, mean arterial blood pressure; T_{es}, oesophageal temperature; T_{sk}, mean skin temperature; T_b, mean body temperature.

Table 3. Oesophageal and mean body temperatures thresholds and slopes for sweating and cutaneous vasodilation during exercise.

		SR		CVC	
		PL	BR	PL	BR
Forearm					
T_{es}	Threshold (°C)	36.98 (0.21)	37.07 (0.24)	37.06 (0.16)	37.16 (0.25)
	ΔThreshold (°C)	0.11 (0.16)	0.09 (0.12)	0.19 (0.18)	0.18 (0.16)
	slopes (mg/cm^2 /min/°C)	1.27 (0.46)	1.43 (0.45)	-	-
	slopes (AU/mmHg/°C)	-	-	0.0150 (0.0052)	0.0215 (0.0117)
T_b	Threshold (°C)	36.41 (0.21)	36.50 (0.22)	36.49 (0.14)	36.56 (0.21)
	ΔThreshold (°C)	0.03 (0.09)	0.01 (0.04)	0.11 (0.16)	0.08 (0.08)
	slopes (mg/cm^2 /min/°C)	1.73 (0.70)	1.92 (0.76)	-	-
	slopes (AU/mmHg /°C)	-	-	0.0222 (0.0082)	0.0260 (0.0142)
Chest					
T_{es}	Threshold (°C)	37.01 (0.21)	37.10 (0.27)	37.04 (0.15)	37.15 (0.24)
	ΔThreshold (°C)	0.14 (0.15)	0.12 (0.14)	0.17 (0.13)	0.17 (0.10)
	slopes (mg/cm^2 /min/°C)	1.58 (0.61)	2.09 (1.33)	-	-
	slopes (AU/mmHg/°C)	-	-	0.0180 (0.0062)	0.0247 (0.0146)
T_b	Threshold (°C)	36.42 (0.21)	36.52 (0.23)	36.44 (0.20)	36.56 (0.21)
	ΔThreshold (°C)	0.04 (0.09)	0.04 (0.06)	0.06 (0.08)	0.09 (0.07)
	slopes (mg/cm^2 /min/°C)	2.15 (1.13)	2.42 (1.04)	-	-
	slopes (AU/mmHg /°C)	-	-	0.0273 (0.0082)	0.0288 (0.0144)

The values given are the means (SD). T_{es}, oesophageal temperature; T_b, mean body temperature; SR, sweat rate; CVC, cutaneous vascular conductance.

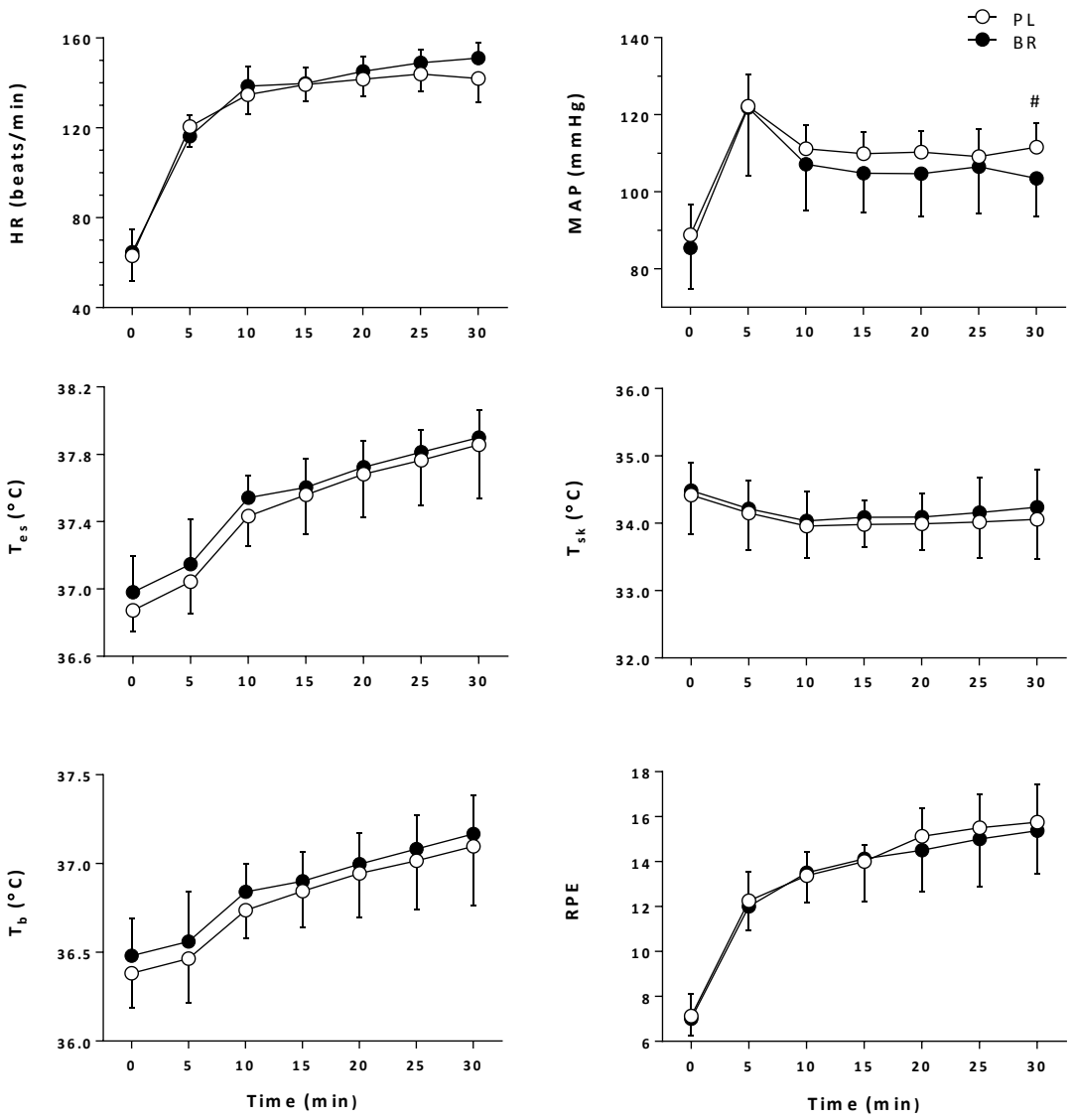

Fig. 1

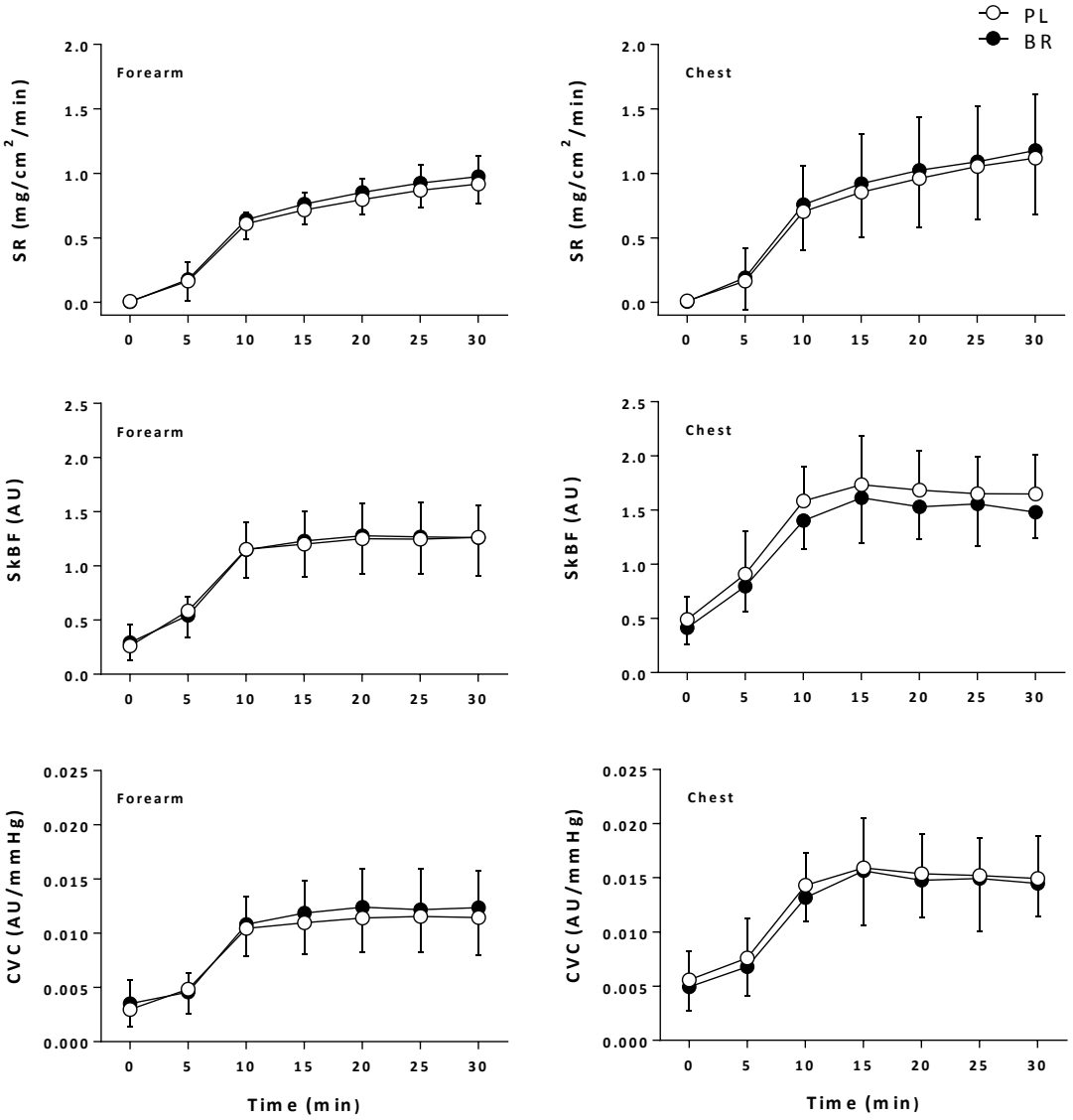

Fig. 2

AUTHOR CONTRIBUTIONS

Conception and design of research was undertaken by TA, DO, BB, and NK, data collection and analyses was undertaken by TA, DO, and BB, the manuscript was drafted by TA, DO, BB, and SB and all authors (TA, DO, BB, SB, SK, and NK) contributed to data interpretation, editing and revision of manuscript, and approved the final version.