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Skin blood flow responses to acetylcholine and local heating at rest and 60%V O2max, and associated nitric oxide contribution, in boys vs. girls

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Abstract

Purpose: To determine sex-related differences in the skin-blood-flow (SkBF) response to exercise, local heating, and acetylcholine (ACh) in children. Additionally, the contribution of nitric oxide (NO) was examined. **Methods***:* Forearm SkBF during local heating (44˚C), ACh iontophoresis, and exercise (30 min cycling, 60%$\dot{V}O_2$max) was assessed, using Laser-Doppler fluxmetry, in 12 boys and 12 girls (7–13 yrs old), with and without NO synthase inhibition, using N^{ω}-nitro-L-arginine methyl ester (L-NAME) iontophoresis. **Results**: Local-heating-induced and ACh-induced SkBF increase were not different between boys and girls (Local heating: 1445±900% and 1432±582% of baseline, , p=.57; ACh: 673±434% and 558±405% of baseline, respectively, p=0.18). Exercise-induced increase in SkBF was greater in boys than girls (528±290 and 374±192% of baseline, respectively, p=0.03). L-NAME blunted the SkBF response to ACh and during exercise (p<0.001), with no difference between sexes. S**ummary:** SkBF responses to ACh and local heat stimuli were similar in boys and girls, while the increase in SkBF during exercise was greater in boys. The apparent role of NO was not different between boys and girls. It is suggested that the greater SkBF response in the boys during exercise is related to greater relative heat production and dissipation needs during this exercise intensity. The response to body-size-related workload should be further examined.

Key words: Children, Cutaneous vascular conduction, Heat, Iontophoresis, Nitric oxide, Thermoregulation, Vasodilation

Running head: Skin blood flow response in boys and girls

Introduction

The skin blood flow (SkBF) response to local and to systemic heating, as well as to pharmacological stimuli is affected by factors such as age (6, 11, 13, 22, 32) and fitness level (23, 40). Sex-related differences in SkBF responses to such stimuli may help explain thermoregulatory differences, such as the sweating or skin temperature response, between males and females. Observations in adults are inconsistent, with some showing sex-related differences (1, 8, 16, 23, 37), while others do not (15, 18, 19, 47). Research on corresponding differences in children is limited to SkBF responses to local perturbations – pharmacological stimuli and blood flow occlusion (2, 30, 44), with no sex-related comparisons of the reflex SkBF response to a systemic stressor (i.e., exercise). Investigating sex-related differences in children's SkBF response would therefore contribute to understanding whether such sex-related differences, if any, already exist during childhood, or develop during growth and maturation.

In a recent study, we found no *age*-related differences in SkBF increase during exercise between boys and men (50, 51). However, females were not examined. Studies on sex-related differences in the SkBF response in children are limited to responses to ACh iontophoresis or post-occlusive reactive hyperemia, and their findings are inconsistent (2, 30, 38, 44). Using wavelet analysis, Baboshina recently demonstrated an increase tissue perfusion, as well as in its variability with age (8–20 yrs) at rest (at room temperature)(2). No differences were reported between males and females (2). An increase in post-occlusion reactive hyperemia with age (8–18 yrs) was also demonstrated (38, 44), but sex-related differences were only apparent in adolescents (12–18 yrs) (44). Lastly, following ACh iontophoresis, adolescent girls (11–14 yrs) exhibited a greater cutaneous vascular response compared with boys (30). Importantly, sex-related differences in SkBF response to exercise have not been examined in children.

ACh is a neurotransmitter which causes cutaneous vasodilation during exercise-induced heat stress. Local ACh delivery (e.g., via iontophoresis) also results in stimulation of cutaneous endothelium leading to vasodilation, which is partly mediated by nitric oxide (NO) along with prostaglandins and endothelial hyperpolarizing factors (6,10). Recent studies in adults on ACh-mediated vasodilation have presented contradictory results as to the effects of ACh iontophoresis in men and women, where Algotsson et al. (1) demonstrated a greater response in women, yet no sex-related differences were reported by Ferrell et al. (15). In children, only one study examined the effect of ACh iontophoresis, demonstrating a greater increase in SkBF in adolescent girls than in boys (30). Such studies can potentially provide insight into possible mechanistic differences in exercise-induced cutaneous vasodilation between boys and girls.

In adults, nitric oxide synthase (NOS) accounts for ~30–45% of the increase in SkBF during passive heat stress, as well as during exercise (27, 33, 45). To our knowledge, sex-related differences in the role of NOS in the SkBF response during exercise have not been examined in adults nor in children. Using spectral analysis of the SkBF response to local skin heating, Hodges et al. recently suggested that the endothelial activity might contribute more to cutaneous vasodilation in boys than in men (22). Indeed, we recently demonstrated a greater role of NO in cutaneous vasodilation during exercise in boys compared with men (51), but females were not examined. Sex-related differences in the NO contribution to vasodilation can provide insight into possible mechanistic differences in cutaneous vasodilation between males and females.

This study aimed to examine whether there are sex-related differences in the SkBF response to local heating, to local ACh administration (via iontophoresis), and to exercise among children. Additionally, the NO-inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME) was used

to elucidate the vasodilatory role NO plays in children, It was hypothesized that no significant sex-related differences will be found in response to any of the examined factors.

Methods

Participants

Twelve boys and 12 girls, aged 7–13yrs, were included in this study. Participants were healthy, recreationally active (≤2 h/wk of structured physical activity), not on any medications, non-smokers, and had no current or recent lower leg injuries (within the past 6 months). Their physical characteristics are provided in Table 1. There were no differences between groups in physical characteristics (except in body mass, surface-area-to-mass ratio, and % body fat; $p<0.05$), physical activity level, or somatic maturity (Table 1). Participants were classified as pubertal stage 1 (8 boys, 4 girls), stage 2 (3 boys, 7 girls) or stage 3 (1 boy, 1 girl).

Insert Table 1

The study was cleared by Brock University's Research Ethics Board (REB #17-045) and all participants and their parents/guardians signed an informed assent or consent form prior to experimental procedures.

Procedures and Protocols

Participants attended two testing sessions at the Brock University Applied Physiology Laboratory. All testing took place during the Winter or early Spring months. Thus, participants were not acclimatized.

Visit 1

Anthropometry and physical activity: Participants completed a medical screening questionnaire, an activity questionnaire (21) and a pubertal-stage self-assessment, according to

secondary sex characteristics (48). Body mass, standing and sitting height were assessed using standard methods (see Measurements, below), and body composition was estimated using skinfold thicknesses (46).

Exercise testing: Prior to familiarization with the equipment and exercise protocol, resting heart rate (HR) and blood pressure (BP) were recorded. Participants were then exercise-tested in three phases: submaximal, maximal and 'supramaximal', on a semi-recumbent cycle ergometer (Corival recumbent cycle ergometer, Lode, Netherlands). The submaximal phase consisted of 4 steady-state stages, each 4 min in duration. The workload progressively increased (by 10–20W/stage), while participants maintained a cadence of 60–80 rpm. These submaximal exercise stages were used to create a regression equation (power vs. $\dot{V}O_2$), used for the determination of the exercise intensity corresponding to 60% of the participant's $\dot{V}O_2$max. Next, participants completed progressive cycling to exhaustion for $\dot{V}O_2$max determination. The workload increased each minute (typically, by 10W) and cadence was maintained between 60–80 rpm. Lastly, following a 10 min rest, participants performed a 'supramaximal' exercise bout at 105% of the highest workload achieved during the progressive phase to ascertain that $\dot{V}O_2$max had indeed been achieved (3, 43). Oxygen consumption, HR, power, and rating of perceived exertion (RPE)(4) were recorded during exercise.

Visit 2

Visit 2 included ACh and L-NAME iontophoresis, followed by 30 min of submaximal exercise at the power-output corresponding to 60% $\dot{V}O_2$max, and then local heating of the forearm (Figure 1). All testing was performed in thermoneutral conditions (Ambient air temperature 23.5±1.6°C and relative humidity 32.5±11.9%).

Insert Figure 1

Iontophoresis: During the iontophoresis procedure and all subsequent SkBF measurements, participants sat in a comfortable position, with the left arm at approximately heart height. The iontophoresis probes were placed on the left dorsal forearm (avoiding superficial veins, cuts, bruises etc.) after hair was trimmed (if necessary) and the skin was cleaned with alcohol swabs. There were 4 measurement sites for SkBF and local skin temperature (see Measurements, below): one site with ACh iontophoresis, one site with ACh and L-NAME iontophoresis, one site with L-NAME iontophoresis only, and one site served as a control site. A pharmacological micro-iontophoresis system (MilliporeSigma Canada, Oakville, Ontario, Canada) was used to transdermally administer the designated pharmacological agent via a Perimed (PF383) drug-delivery electrode.

To examine the effect of ACh on SkBF, two laser-Doppler fluxmetry (LDF) probes were used, one at the site of ACh administration (200 µL of 2% solution; Sigma-Aldrich), and the other at the site where both, ACh and L-NAME were administered. In order to examine the effect of L-NAME during exercise, LDF was examined and compared during exercise using two probes: at the site where L-NAME was administered and at a control site (no iontophoresis). The probes were secured to the skin with 3M Transpore tape. The SkBF response was measured continuously and recorded every 5 min. The last 30 s of each 5 min interval were used for analysis. Participants were asked to abstain from any movement of the left arm or any large body movements. Steady and normal breathing was encouraged to limit any movement artifacts.

L-NAME, a non-specific NOS inhibitor was used to locally inhibit the cutaneous NO-mediated vasodilatory effect, by preventing the synthesis of NO (28). Iontophoresis of 200 µL, 2% L-NAME solution (Sigma-Aldrich) was performed on two different skin sites, for 10 min, on a 1.4 cm^2 area on the ventral forearm. Neutral electrodes for current dispersion were placed >10

cm away from the wrist. This protocol was based on previous studies (24, 25, 35, 51). ACh was administered following the L-NAME iontophoresis. Basal flow was measured for a minimum of 5 min at a site treated solely with ACh (Baseline 1, Figure 1) and then at a site treated with both ACh and L-NAME (Baseline 2, Figure 1). Following these measurements, 6 'doses' of ACh 2% were applied at 100 μA (anodal current) for 10, 20, 30, 40, 60, and 80 s, with total charges of 1, 2, 3, 4, 6, and 8 millicoulombs (mC), respectively (i.e., 6 'doses' – each 'dose' is a product of the ACh solution concentration and the applied current) (24). Each of the first 4 'doses' were followed by 120s rest interval, which was increased to 135s following each of the final two 'doses', in order to allow the response to plateau (24).

Submaximal exercise response: Following measurement of SkBF at the ACh and ACh + L-NAME sites, thermocouples (PVC-T-24-190, Omega Environmental Inc., Laval, QC, Canada) were taped to the participant's skin on 4 sites (over the bicep, quadriceps, calf and chest) to calculate weighted mean skin temperature ($\overline{T}_{sk}$) (39). Participants were also fitted with a HR monitor.

Prior to exercise, following 5 min of rest, baseline measurements (Baseline 3, Figure 1), which included manual BP, HR, $\overline{T}_{sk}$, and RPE were recorded. Participants pedaled on the semi-recumbent cycle ergometer for 30 min at the power corresponding to 60% of their predetermined $\dot{V}O_2$max (in visit 1). A fixed relative workload (i.e., %$\dot{V}O_2$max) was chosen as it has previously been shown to elicit similar thermoregulatory responses (core temperature and heart rate) among individuals of differing $\dot{V}O_2$max (20). Additionally, exercise intensity and duration were based on Rowland et al. (42), who demonstrated a similar increase in body temperature (<1°C) in boys and men following 30 min of cycling at 65% $\dot{V}O_2$max. A semi-recumbent cycle ergometer was chosen in order to minimize forearm movement artifacts. SkBF, local skin temperature (T_{loc}),

and $\overline{T}_{sk}$ values were recorded continuously (5 min intervals were used for analysis), while BP, HR, RPE and thermal discomfort and sensation were recorded every 5-min throughout exercise.

During exercise, participants were asked to refrain from drinking, although a specific pre-weighed water bottle, at room temperature was available if participants asked to drink. It was weighed pre- and post-exercise to determine volume of consumed beverage. Body mass was measured pre- and post-exercise, at 10g resolution for the estimation of sweat loss.

Local heating response: Following the 30-min cycling, local skin heating was applied to determine peak SkBF response. The laser-Doppler system (PeriFlux 5010 laser-Doppler perfusion monitor; Perimed; Järfälla, Sweden) was used to heat the skin, using an integrated laser-Doppler local heating probe (Probe 413; Perimed). A calibration device (PF 1000, Perimed) standard was used to adjust the laser-Doppler fluxmeter readings to coincide with the readings obtained with Perimed's Motility Standard. Manipulation of local skin temperature was achieved with local heating unit and heating probe holders (PF5020 local heating units and PeriFlux 5020 Temperature Unit; Perimed) that controlled and monitored skin surface temperature. The peak SkBF response was measured only at the control site. The temperature was increased at a rate of 1°C·20 s^{-1} to 42°C and then 1°C·min^{-1} to 44°C (18, 19). The local heating procedure lasted 5 min, following which data were collected for 30 min or until a stable plateau was observed. Throughout local heating, thermal comfort and sensation were assessed and HR and $\overline{T}_{sk}$ were measured and recorded.

Measurements

All measurements were performed by the same investigator.

Body mass was recorded to an accuracy of ± 10g (GFK 330aH, AE Adam, USA), without shoes, while wearing shorts and a T-shirt. Standing and sitting height were measured to

the nearest 0.5 cm on a stadiometer (Ellard Instrumentation Ltd.). Body surface area (BSA) was calculated according to DuBois & DuBois (12). Skinfold thickness of the triceps and subscapularis, were measured using Harpenden skinfold calipers (Baty International, England) and were used to calculate percentage of body fat (46).

Sexual maturity was self-assessed using secondary sex characteristics (pubic hair) (48). Somatic maturity was assessed using the predicted years from age of peak height velocity (maturity offset), as described by Mirwald et al. (36).

During the submaximal protocol (in session 1) expired gas was collected and analyzed using the Moxus metabolic cart (AEI technologies, PA, USA), calibrated prior to each test and HR was recorded using a HR monitor (Suunto M1, Finland). $\dot{V}O_2$ and HR were recorded during the last 60 and10s, respectively, of each 4-min stage. During the incremental $\dot{V}O_2$max test, $\dot{V}O_2$ and HR were recorded in the last 30 and10s, respectively, of every 1-min interval. During the supramaximal test, $\dot{V}O_2$ was recorded for the last 30s and HR was 10s of every 30-s. HR was recorded every 5 min during the 30-min exercise protocol. Manual systolic and diastolic BP at the brachial artery were recorded (WelchAllyn sphygmomanometer, Welch Allyn Inc. Skaneateles Fall, NY) at baseline prior to exercise, every 5 min during the 30-min cycle exercise, and during local heating at 10 and 30 min.

Mean skin temperature ($\overline{T}_{sk}$) was measured with standard T-type thermocouples (PVC-T-24-190, Omega Environmental Inc., Laval, QC, Canada) and an estimate was produced based on a weighted average of four sites that include skin over the biceps (20%), quadriceps (30%), calf (20%), and chest (30%) (39). The thermocouples for the calf, quadriceps and the chest were placed on the left side of the body, while the thermocouple for the biceps was placed on the right side of the body.

Sweating rate was estimated as the change in body mass from pre- to post-exercise, divided by the duration (30 min). None of the participants consumed any beverage during this time.

Data Analysis

The values during local heating, ACh dose-response measurements and exercise were separately normalized to baseline values and are presented as percentage change from baseline, in perfusion units (PU). Baseline values were not different between boys and girls (Table 2). Note that peak SkBF response could not be determined for the ACh and the L-NAME sites. Therefore, the SkBF response is expressed as percentage change from baseline of ACh administration (for the ACh response) and from pre-exercise values (for the exercise response and local heating response). For the ACh dose-response, individual trend lines were constructed, from which the concentration eliciting 50% of the SkBF response (EC_{50}) was calculated. Finally, since peak thermal SkBF was measured at the control site, we *also* analyzed the SkBF response during exercise as a percent of peak SkBF at the control site only.

The data are also presented as CVC, which is calculated as perfusion units divided by mean arterial pressure ($CVC=PU \cdot MAP^{-1}$). CVC is used to normalize the data to allow for individual or group differences in BP.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism v7 (GraphPad Software, Inc. USA) and SPSS V.24 for windows (SPSS Inc. USA). All data were normally distributed, as determined using skewness and kurtosis measures. Data were also inspected visually. Data were considered to be normally distributed if the skewness was less than ±3 and kurtosis was less than ±9, similar to previous studies using LDF (31). Group differences in physical characteristics,

body mass changes, sweating rate, SkBF response during local heating, ambient temperature and humidity were assessed using independent t-tests. Separate three-way ANOVAs for repeated measures were used to determine main effects of Group (boys and girls), Treatment (L-NAME vs. Control) and Time (throughout exercise) or 'Dose' (throughout ACh protocol). Interactions were also assessed (Group-Time/Dose, Time/Dose-Treatment, Treatment-Group and Treatment-Group-Time/Dose). Additionally, two-way ANOVAs for repeated measures determined the main effects of Group (boys vs. girls) and Time (throughout exercise) on HR, BP, $\overline{T}$sk, thermal comfort and thermal sensation. Two-way ANOVA for repeated measures was also used to determine the main effect of Group and treatment (Control vs. L-NAME) on EC_{50}. The % contribution of NO to each of the responses was calculated using the formula ((Control – treatment) / Control) * 100. The acceptable level of significance for all tests was set to $p<0.05$. Data are presented as Mean and SD, unless otherwise indicated.

Results

The 24 participants completed all study visits. No adverse events were observed or reported as a result of study participation.

$\dot{V}O_2$max, was not different between boys and girls (p=0.29) (Table 1). However, $\dot{V}O_2$max was significantly higher in boys compared with girls when adjusted for body mass (p=0.001), or for lean body mass (p=0.006). During the 30-min submaximal exercise (power output corresponding to 60% $\dot{V}O_2$max), workload was not different between girls and boys (p=.57). However, when expressed relative to lean body mass, boys exercised at a slightly higher relative workload (p=.06).

Local heating to 44°C

The increase in SkBF PU in boys (1445±901%) was similar to that in girls (1432±583%) (group effect; $F_{1,22}$ = .34, p =0.57) (Figure 2). This was also the case for CVC (1551±932% vs. 1420±696%, respectively, $F_{1,22}$ = .24, p =0.62).

Insert Figure 2

Acetylcholine response

The 'dose'–response curves for both skin sites are presented in Figure 3. There were main effects for ACh delivery ($F_{6,22}$=37.74, p<0.0001), treatment (L-NAME) ($F_{1,22}$=19.60, p<0.0001), and treatment-by-'dose' ($F_{5,5}$=22.09, p<0.0001). The latter interaction reflects that with increasing ACh delivery, L-NAME had a greater (blunting) effect. There was no significant group ($F_{1,22}$=1.95, p=0.18), group-by-'dose' interaction ($F_{1,22}$=1.58, p =0.16), group-by-treatment interaction ($F_{1,22}$=0.003, p=0.95), or group-by-treatment-by-'dose' interaction ($F_{6,22}$=0.10, p=0.996). The calculated EC_{50} was significantly lower with L-NAME treatment (Control: 4.6±0.6 mC; L-NAME: 4.1±1.0 mC; $F_{1,22}$=4.30, p=0.05), with no significant group effect ($F_{1,22}$=4.17, p=0.053), nor group-by-treatment interaction ($F_{1,22}$=1.24, p=0.28).

Insert Figure 3

Exercise Response

During exercise, there was a statistically significant increase in SkBF in both boys and girls (Figure 4). There were group effects for the SkBF response expressed in PU (group effect; $F_{1,22}$ = 5.48, p=0.03) and CVC (group effect; $F_{1,22}$ = 4.76, p=0.04), reflecting greater increase in SkBF in boys. There was no treatment effect, reflecting that overall, the increase in the control site for both boys and girls (528±290 and 374±192%, respectively) was not different than the

283 increase in the L-NAME site (444±273 and 259±195%, respectively) in terms of PUs (treatment
284 effect; $F_{1,22}$ = 1.63, p=0.22) or CVC (treatment effect; $F_{1, 22}$ = 1.47, p=0.24). As expected, there
285 was a significant time effect for the increase in SkBF in terms of both, PUs ($F_{5, 22}$ = 54.29,
286 p<0.0001) and CVC ($F_{5,22}$ = 48.8, p<0.0001), and a significant time-by-treatment interaction in
287 terms of PUs ($F_{5,22}$ =5.08, p=<0.0001) and for CVC ($F_{5,22}$ =5.01, p=<0.0001), reflecting that the
288 blunting effect of L-NAME increased with increase in exercise time. The group-by-time
289 interaction effect for the increase in SkBF in terms of PUs was ($F_{1, 22}$ = 2.27, p=0.052) and in
290 terms of CVC was ($F_{1, 22}$ = 1.74, p=0.13), reflecting that, with time, the increase in SkBF was
291 somewhat greater in boys, although it did not reach the 0.05 significance level. There was no
292 significant group-by-treatment interaction in terms of PUs ($F_{1,22}$ =0.54, p=0.82) or CVC ($F_{1,22}$=
293 0.09, p=0.77). Finally, there was no significant group-by-treatment-by-time interaction in terms
294 of PUs ($F_{5,22}$ =0.22, p=0.95) or CVC ($F_{5,22}$ =0.26, p=0.93).

295 Insert Figure 4

296 The SkBF response was also analyzed as percent of peak values, in the control site only.
297 The results demonstrated a similar pattern to the above in that there was a significant time effect
298 ($F_{6,132}$ = 61.2, p<0.0001), and no group-by-time interaction ($F_{5,132}$ = 67.6, p=0.23). While SkBF
299 response was consistently higher in the boys, the difference did not reach statistical significance
300 (($F_{1, 22}$ = 3.38, p=0.079).

301 Boys had a greater loss in mass from the beginning of exercise to the end of exercise,
302 compared with girls (133±82 vs. 77±34g, respectively, p=0.030). Consequently, the calculated
303 sweating rate was higher in boys compared with girls (4.44±2.75 vs. 2.57±1.14 g·min^{-1},
304 respectively, p=0.030). Relative to BSA, sweating rate was significantly greater in boys
305 compared with girls (216.6±125.4 vs. 112.8±46.8 ml·m^{-2}·h^{-1}, p=0.01).

306 Due to technical problems with the thermocouples, $\overline{T}$sk was recorded in 12 boys but only
307 9 girls. At baseline, there was no difference (p=0.20) in $\overline{T}$sk between boys (31.1±1.9°C) and girls
308 (30.1±1.4°C) (Figure 5). Throughout exercise, the increase in $\overline{T}$sk was significant ($\Delta\overline{T}$sk of
309 1.1±1.0°C and 0.41±1.1°C, in boys and girls, respectively) (time effect; $F_{6, 19}$ = 15.77,
310 p<0.0001). At the end of exercise, boys' $\overline{T}$sk was 32.1±2.1°C, while girls $\overline{T}$sk was 30.6±1.8°C,
311 but group differences were not statistically significant (group effect; $F_{1, 19}$= 2.79, p=0.11; group-
312 by-time interaction: $F_{6, 19}$ =1.79, p=0.11).

313 Insert Figure 5

314 Forearm skin temperature (Tloc) was not different between boys (30.9±1.0°C) and girls
315 (30.4±1.2°C) (p=0.41). From the beginning to the end of exercise, $\overline{T}$loc increased in both boys
316 (30.9±1.0 to 32.4±1.0°C) and girls (30.4±1.2 to 32.1±2.6°C), with a significant time effect ($F_{6, 14}$
317 = 16.27, p=<0.0001). There were no differences in $\overline{T}$loc between boys and girls (group effect; $F_{1,}$
318 $_{14}$ = 0.30, p=0.87), and there was no significant sex-by-time interaction ($F_{6, 14}$ =0.35, p=0.91).
319 Heart rate increased in both boys and girls from baseline (77±9 and 81±7 beats·min^{-1},
320 respectively) until the end of exercise (160±15 and 160±11 beats·min^{-1}, respectively) (time
321 effect; $F_{6, 22}$ = 605.8, p<0.0001), with no difference between groups (group effect: $F_{1, 22}$ =0.05,
322 p=0.82) and no group-by-time interaction ($F_{6, 22}$ =1.26, p=0.28). There was no significant
323 difference between groups in RPE at the end of exercise (17.0±1.9 and 16.6±2.2 in boys and
324 girls, respectively).
325 There were no sex-related differences for systolic BP, diastolic BP and MAP (group
326 effect; $F_{1, 22}$ = 1.85, p=0.19, $F_{1, 22}$ =0.08, p=0.78, $F_{1, 22}$ = 0.66, p=0.42, respectively) (Figure 6).
327 Throughout exercise, there were increases in systolic BP, diastolic BP and MAP (time effect; $F_{6,}$
328 $_{22}$ =49.49, p<0.0001, $F_{6, 22}$ = 1171, p=0.0001, $F_{6, 22}$ = 23.39, p<0.0001, respectively). There was

no group-by-time interaction for diastolic BP or MAP ($F_{6, 22}$ =0.37, p=0.90, $F_{6, 22}$ =1.34, p=0.24, respectively), but there was a significant interaction for systolic BP ($F_{6, 22}$ = 3.60, p=0.002). The latter reflects a higher systolic BP in boys at the beginning of exercise, with no apparent difference between groups after 10 min. Note that during exercise and in all conditions, MAP was similar in boys and girls, varying by 3 mmHg, at most. Therefore, the patterns of the SkBF response were similar, whether expressed as PU or as CVC.

Insert Figure 6

Thermal sensation increased similarly from the start to the end of exercise in boys (3.6±1.4 to 6.8±0.6) and girls (3.7±1.4 to 6.5±0.8) (time effect; $F_{6, 22}$ =66.85, p<0.001). There was no difference between boys and girls (group effect; $F_{1, 22}$ = 0.54, p=0.47) and there was no group-by-time interaction ($F_{6, 22}$ =0.16, p=0.987). Thermal discomfort also significantly increased from the start to end of exercise in boys (1.8±0.5 to 2.9±0.9) and girls (1.4±0.5 to 2.9±0.8) (time effect; $F_{6, 22}$ =24.93, p<0.001), with no difference between groups (group effect; $F_{1, 22}$ =0.37, p=0.56) and no group-by-time interaction ($F_{6, 22}$=1.26, p=0.28).

Discussion

This study examined possible sex-related differences among children in the SkBF response to local heating, ACh-mediated vasodilation, and exercise, as well as the contribution of NO to these responses. As expected, SkBF response to local heating and ACh-induced vasodilation was not different between boys and girls. However, the SkBF response to exercise (30 min at a power output corresponding to 60%$\dot{V}O_2$max) was greater in the boys. This difference may be explained by greater relative heat production in the boys. The contribution of

NO to the SkBF response during ACh-mediated vasodilation or during exercise was not different between the sexes, suggesting that the mechanism of cutaneous vasodilation is similar in boys and girls.

Local Heating

Local heating can demonstrate the role of local skin temperature in the cutaneous vasodilatory response, and when *T*loc is sufficiently high, local heating can induce maximal thermal SkBF. The SkBF response at *T*loc of 44˚C, presumed to elicit maximal thermal SkBF, was similar in boys and girls. This observation is in agreement with some previously reported findings in men and women (19, 47). On the other hand, other studies reported higher forearm SkBF in men compared with women at rest and in response to 42˚C local heating (23), and higher hand and finger SkBF in women (8). These apparent contradictions could be due to methodological differences in the heating protocol (42° vs. 44°C), or to the measurement site. Contrary to the forearm, the hand and fingers are glabrous skin sites, abundant in arteriovenous anastomoses and prone to larger SkBF variability (26). While the forearm may not be fully representative of the whole body in terms of maximal thermal SkBF, we suggest that based on our data and those in adults (19, 47), the maximal thermal SkBF response is similar in boys and girls.

ACh-mediated vasodilation

The SkBF response to ACh was consistently lower in girls (Figure 3), although the difference did not reach statistical significance (p=0.18). In older adolescents (11–14 yrs), Khan et al. (30) reported *greater* ACh-induced SkBF response in girls than in boys. Inconsistent findings are also seen in studies examining adults, where some studies report no sex-related differences in response to ACh iontophoresis (15, 17), while others report greater vasodilatory

response in women than in men (1, 5). Some of those inconsistencies may be due to different ACh administration protocols of diverse doses, concentrations, and currents. As ACh-mediated vasodilation is dependent on the dose and duration of the infusion (7), it is possible that different doses and administration regimens elicited different responses. It is evident that sex-related differences in ACh-mediated vasodilation require further research.

ACh-mediated cutaneous vasodilation is highly NO-dependent, but in adults, the contribution of NO to ACh-mediated vasodilation is diminished at high ACh concentrations (34). In the present study, the L-NAME effect on the SkBF response to increasing ACh dosage was not statistically different between boys and girls (16 and 30%, respectively), which is in line with previous findings in adults (29). However, contrary to findings by Medow et al. (34), we observed increased NO contribution with increasing ACh delivery (Figure 3). This apparent contradiction may be a reflection of the different ACh delivery methods. That is, our highest ACh delivery (via iontophoresis) may have resulted in lower ACh than the highest dose delivered by Medow et al. (via microdialysis). Nevertheless, these findings indicate that factors other than NO also contribute to ACh-induced vasodilation in both children and adults. Future research is needed to examine the role of other vasodilators, such as prostaglandins and endothelial-derived hyperpolarizing factors in ACh-induced vasodilation in children, as well as in adults.

Exercise

This is the first study to examine sex-related differences in the SkBF response to exercise in children. We observed a greater increase in SkBF in boys compared with girls. In adults, several studies report no sex-related differences in the SkBF response to exercise (18, 37). The apparent discrepancy between those adult studies and the present findings is partly explained by

the different manners in which exercise intensity was equated between males and females. That is, in the adult studies, exercise intensity was assigned relative to BSA, while in the present study exercise was assigned relative to the children's $\dot{V}O_2$max. Previous studies in adults demonstrated that the thermoregulatory response, namely core temperature and HR, to exercise at a fixed relative intensity (65%$\dot{V}O_2$max) was similar in individuals of differing $\dot{V}O_2$max (20). Indeed, in the present study, exercising at the same relative $\dot{V}O_2$max (60%), the boys' and girls' physiological (HR) and perceptual (RPE, thermal sensation and discomfort) responses were similar. However, exercising at the same relative $\dot{V}O_2$max may lead to different sweating response, as recently demonstrated by Cramer and Jay (9), and potentially, different SkBF response. In the present study, the boys had 26% higher maximal aerobic power relative to body mass (p=0.001, Table 1), and 7% lower BSA (p=0.11, Table 1). Consequently, relative to their BSA, the boys' workload was ~14% higher (54.1 vs. 47.6 $W \cdot m^{-2}$, respectively, p=0.11), which likely required them to dissipate more heat. This somewhat greater heat-dissipation requirement is congruent with their higher sweating rate and higher SkBF response. Generally, children rely more on dry heat dissipation, specifically via higher SkBF, compared with adults (14, 41), which may also require a larger fraction of their cardiac output. It should be noted that in the present study, while dry heat dissipation (via SkBF) may have been the predominant thermoregulatory mechanism, as previously shown in children (10), sweating also played a role, and SkBF reached only ~40% of its potential peak (see Fig. 4 vs. Fig. 2).

In view of the above, we suggest that the boys' greater SkBF increase during exercise compared with the girls' stemmed mainly from their likely greater heat-dissipation needs. This is in line with the boys' greater (not significant) increase in $\overline{T}$sk (~0.5°C, p=0.1). Thus, as has been demonstrated in adults (18, 37), we suggest that in children, apparent sex-related differences in

420 the SkBF response to exercise are a result of differences in body size, composition, and
421 metabolic capacity, which affect heat production and dissipation and therefore, the SkBF
422 response. This is in line with our recent study, in which we reasoned that the SkBF response to
423 exercise (60% $\dot{V}O_2$max) in boys and men was related to differences in body dimensions,
424 specifically, relative BSA (50, 51). To address this issue, future studies are needed in which
425 exercise intensities are related to (or normalized to) BSA, rather than to $\dot{V}O_2$max.

426 In the present study, we found that NO played a similar role in the vasodilatory response
427 to exercise in boys and girls. This is the first study to examine potential sex-related differences in
428 the mechanisms involved in SkBF response during exercise in either adults or children. In adults,
429 previous studies have estimated that approximately 30–45% of the vasodilatory response during
430 exercise or passive heating is NO-dependent, although sex-related differences were not reported
431 (27, 33, 49). In the present study, the girls' NO-dependent response during exercise (~30%) is
432 similar to previous studies in adults, while the boys' NO-dependent portion of the response
433 (~16%) is somewhat lower than that reported in adult studies. No statistical difference was
434 observed in the present study between boys and girls. However, it should be recalled that the two
435 groups exercised at different absolute workloads, making it difficult to directly compare NO-
436 mediated vasodilation between groups during exercise. Thus, the inference that NO contribution
437 is not different between sexes should be treated with caution.

438 **Strengths and limitations**

439 The two groups in this study were similar in age and physical activity level. While there
440 was no significant group differences in somatic and pubertal maturity, the girls were physically
441 larger/heavier and with greater adiposity, as would be expected at their age (~11 years). These

differences were reflected in the girls' significantly lower mass-specific $\dot{V}O_2$max and lower SkBF response during exercise.

One of the limitations of the study was that core body temperature was not measured. As mentioned above, in view of the boys' higher $\dot{V}O_2$max and smaller body size (specifically, BSA-to-mass ratio), relative heat production, and therefore, heat dissipation needs were different, possibly resulting in higher core temperature in the boys. Body core temperature values would have complemented the findings of higher sweat loss and SkBF during exercise in the boys. Another limitation is that an iontophoretic current was not applied at the control site (i.e., "sham control"). However, it should be noted that LDF measurements at the control site (during exercise) were performed 60 min following the completion of iontophoresis. Pilot work and a prior study (24) suggested that in response to exercise, local heating and ACh application, the skin sites are not affected by the electrical current application. Nevertheless, application of a similar iontophoresis protocol (charge and duration) to the control skin site should be considered in future studies, so as to account for the possible effect of skin perturbations. Finally, it should be acknowledged that LDF measures (arbitrary PUs) are variable within and between individuals. Between-subject variability is often addressed by expressing changes in PU relative to maximal values (i.e., relative to local heating at 44°C). However, in the present study, maximal values could only be determined for the control site (in the other sites, values were influenced by ACh and/or L-NAME and therefore, did not represent maximal SkBF). Therefore, between-subject variability was addressed by examining changes in PU relative to baseline, always at a given skin site. Baseline values did not differ between groups. However, given the between-subject variability, it is possible that some potential between-group differences were not detected.

Conclusions

Under the conditions of this study, the peak SkBF response (to local heating) and the ACh-mediated vasodilation were similar in girls and boys, while during exercise, boys had a greater increase in SkBF. The contribution of nitric oxide to cutaneous vasodilation was not different in the boys compared with the girls. We suggest that the SkBF response, in general, is similar in boys and girls and that during exercise, as is the case in adults, observed sex-related differences in SkBF response are likely due to dimensional differences. Thus, it is suggested that during systemic heat stress (e.g., during exercise), thermoregulation is similar in boys and girls. Further study is needed to test this dimensionality-related suggestion by ascribing heat production (i.e., exercise intensity) in relation to the skin surface area available for heat dissipation.

Conflicts of interest

The authors have no conflict of interest to declare.

Author Contributions

All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Figure Legends

Figure 1. Timeline of visit 2. Where BM = body mass, HR= heart rate, TC=thermal comfort, TS=thermal sensation, T_{sk}= mean skin temperature, ACh = acetylcholine, L-NAME = Nω-nitro-L-arginine methyl ester., $\dot{V}O_2$max = maximum oxygen consumption, T_{loc} = local temperature, LDF = laser Doppler fluxmetry, and SkBF = skin blood flow

Figure 2. LDF skin blood flow response (percent change in perfusion units from baseline) to local heating (LH) to 44˚C in boys and girls. There was no difference between groups.

Figure 3. LDF skin blood flow response (percent change in perfusion units from baseline) to increasing administration of acetylcholine (ACh) (millicoulombs, mC) in boys and girls at skin sites treated with ACh and ACh + L-NAME. Skin blood flow increased with increasing ACh delivery (p<0.0001) and was lower in the L-NAME site (p<0.0001). There was a treatment-by-delivery interaction (p<0.0001), reflecting L-NAME's greater blunting effect with increasing ACh delivery. The curve was fitted with a non-linear regression and a variable slope.

Figure 4. Percent change of skin blood flow, from baseline through the end of exercise, in boys and girls, represented by changes in LDF perfusion units. Skin blood flow increased with time (p<0.001) and was higher in boys compared with girls (p<0.05). The blunting effect of L-NAME increased over time in both groups (treatment-by-time interaction, p<0.05).

Figure 5. Mean skin temperature ($\overline{T}$sk) of boys (n=12) and girls (n=9) from beginning to end of exercise. There was a significant time effect (p<0.01), but $\overline{T}$sk group differences did not reach statistical significance (group effect p=0.11) (Mean±SD).

Figure 6. Mean arterial blood pressure (MAP) during semi-recumbent cycling at 60% $\dot{V}O_2$max in boys and girls. (Mean±SD). There were no differences between groups.

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Tables

Table 1 – Participants' physical characteristics and exercise capacity

	Boys	Girls
Age (yrs)	10.9 ± 1.1	11.1 ± 1.2
Stature (cm)	145.8 ± 8.1	149.4 ± 8.6
Body Mass (Kg)	36.6 ± 6.9	41.9 ± 5.3*
Body surface area (m^2)	1.23 ± 0.14	1.31 ± 0.12
Surface area-to-mass ratio ($cm^2 \cdot kg^{-1}$)	339 ± 25	314 ± 19*
Time to Peak Height Velocity (yrs)	2.53 ± 0.85	2.40 ± 0.81
Body fat (%)	15.8 ± 2.6	20.2 ± 3.2*
Leisure Physical Activity score	74 ± 28	68 ± 34
$\dot{V}O_2$max ($ml \cdot min^{-1}$)	1665 ± 282	1537± 296
$\dot{V}O_2$max ($ml \cdot kg^{-1} \cdot min^{-1}$)	46.2 ± 7.3	36.6 ± 5.1*
$\dot{V}O_2$max ($ml \cdot LBMkg^{-1} \cdot min^{-1}$)	54.8 ± 7.6	45.9 ± 6.5*
Workload at 60% $\dot{V}O_2$max (W)	66.1 ± 14.8	62.8 ± 13.3
Workload relative to LBM ($W \cdot LBMkg^{-1}$)	2.17 ± 0.43	1.87 ± 0.30

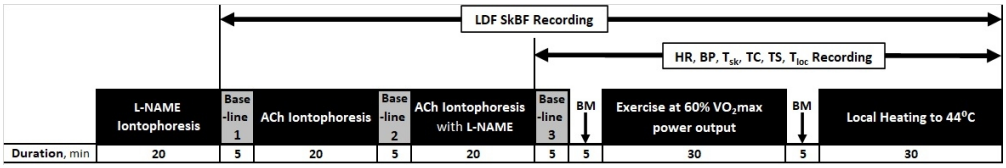
* = significant difference ($p<0.05$).
LBM = lean body mass, $\dot{V}O_2$max = maximum oxygen consumption.

673 Table 2: Laser-Doppler fluxmetry perfusion units (PU) at baseline in boys and girls (mean±SD)
674
675

	Pre-ACh		Pre-exercise	
	ACh	**ACh + L-NAME**	**Control**	**L-NAME**
Boys	10.5 ± 4.3	12.1 ± 7.8	13.5 ± 5.7	13.6 ± 6.0
Girls	9.0 ± 2.7	10.1 ± 5.3	11.1 ± 2.7	16.2 ± 8.9

676
677 Where ACh = acetylcholine, L-NAME = N^{ω}-nitro-L-arginine methyl ester

678



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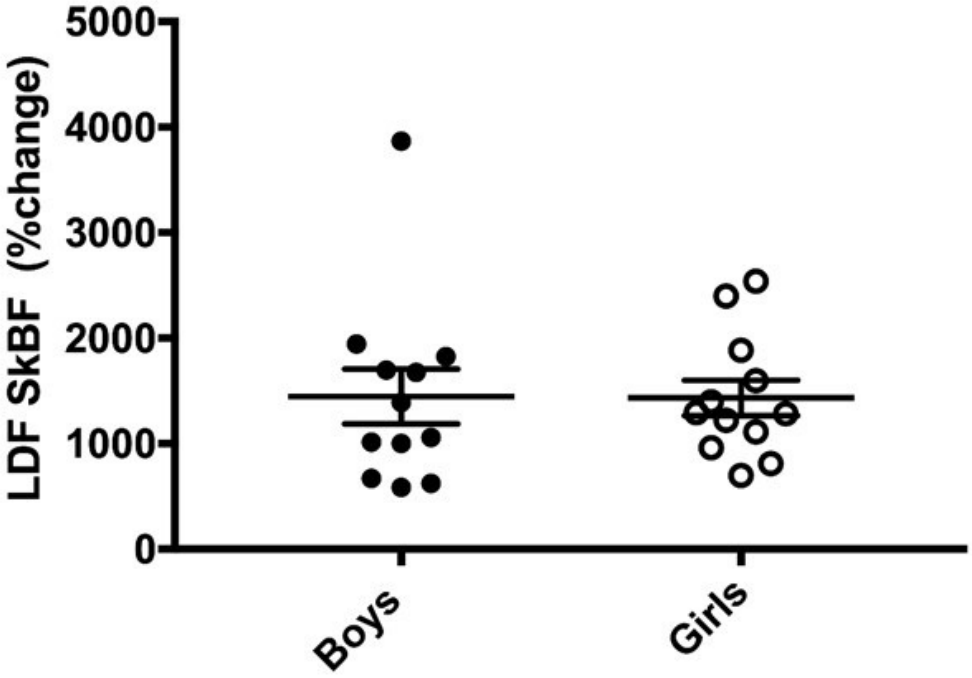

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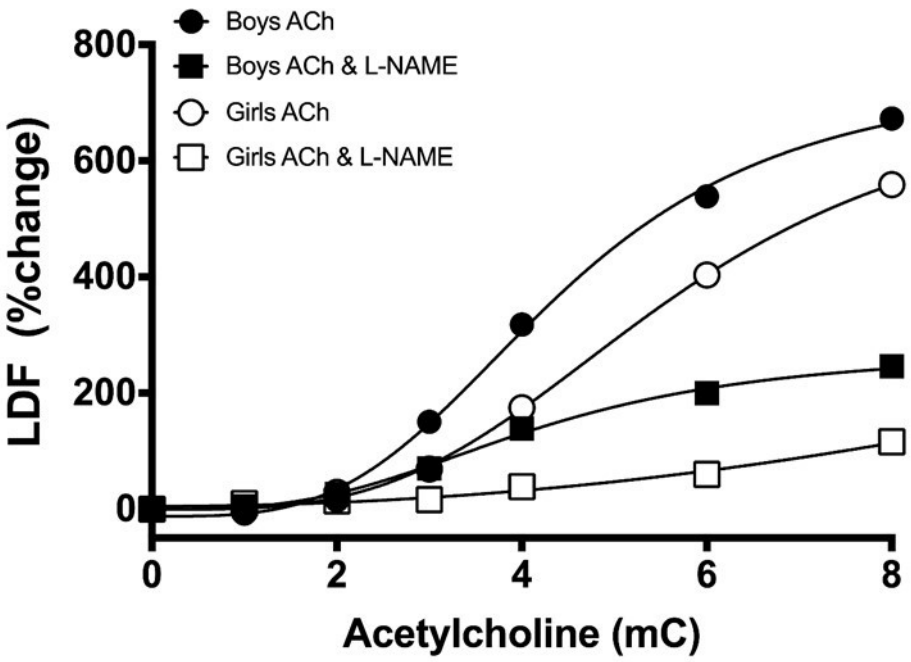

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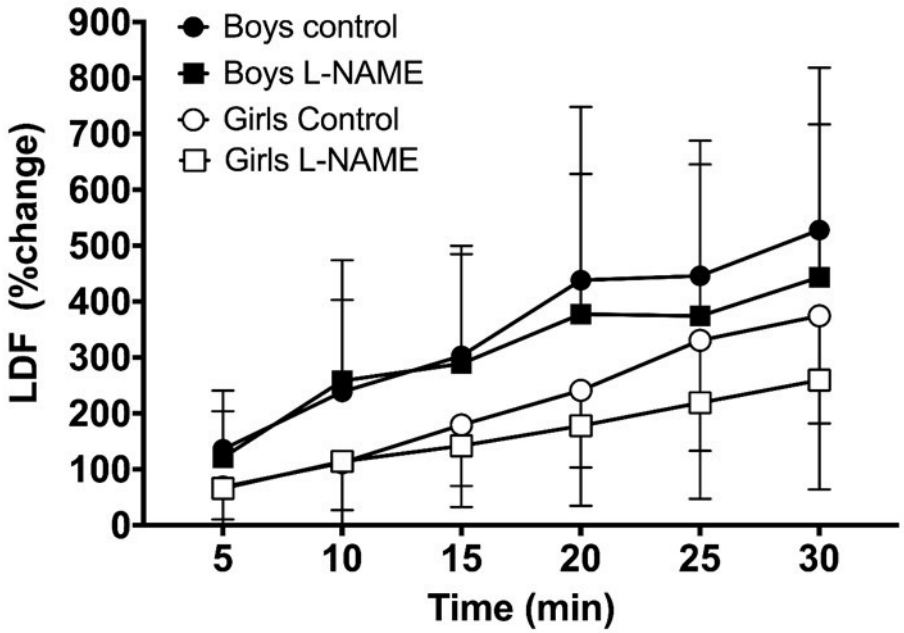

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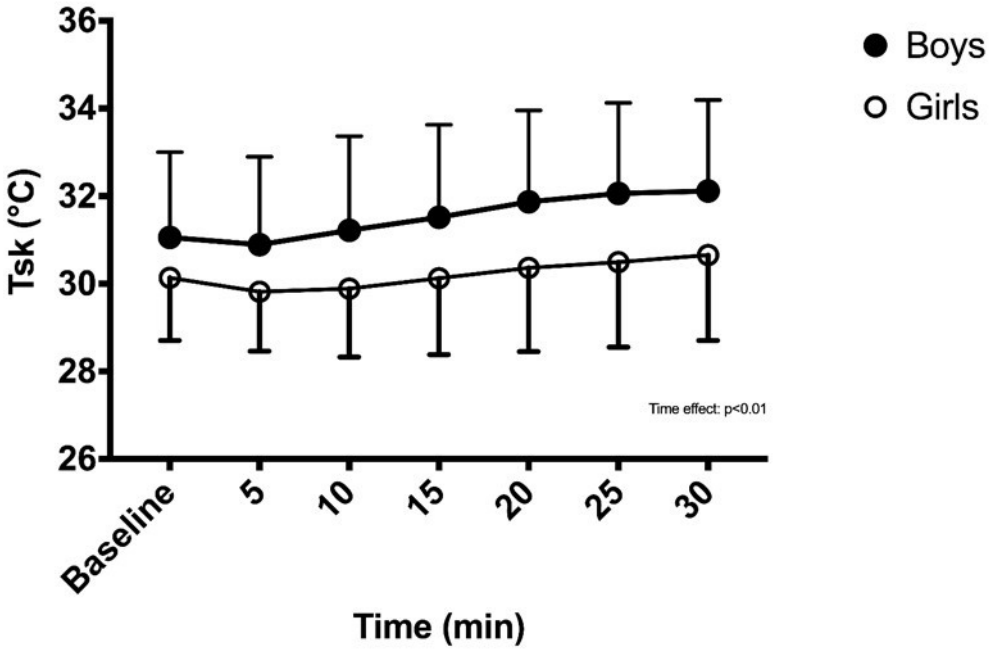

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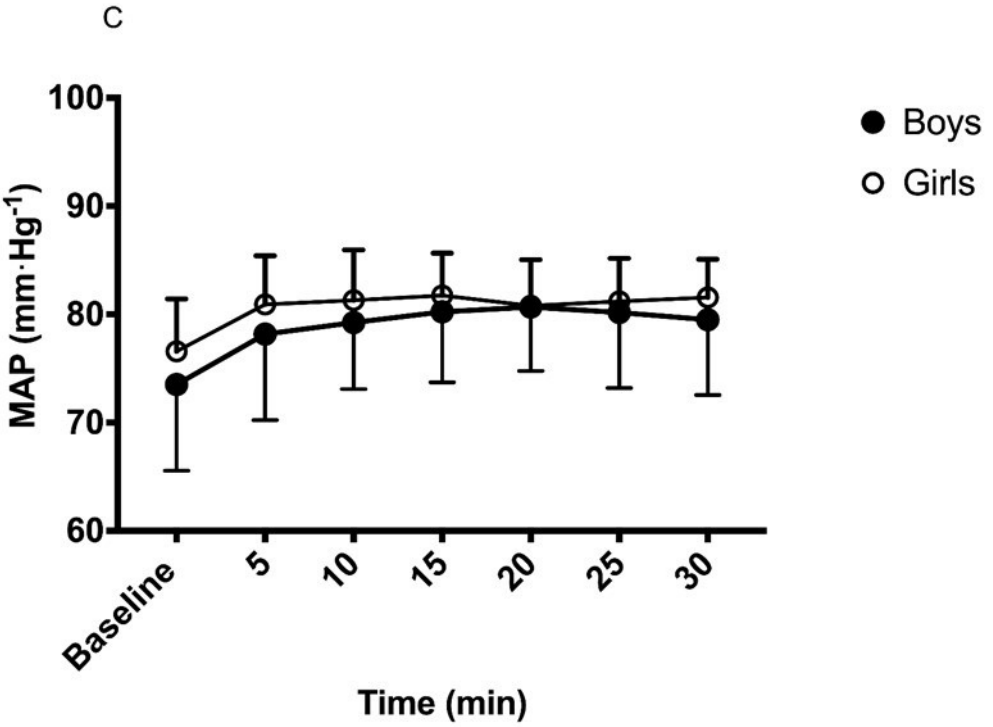

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