Sex-related differences in the microvascular function of pre-pubertal children

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Abstract

There is little research examining the effect of sex on skin blood flow (SkBF) in adults, and less in children. Sex-related differences in SkBF may help explain known thermoregulatory differences between males and females. The purpose of this study is to determine whether there are sex-related differences in the SkBF response to exercise, local heating, and acetylcholine (ACh). Additionally, the role of nitric oxide (NO) was examined. Laser-Doppler fluxmetry was used to assess forearm SkBF. Responses to exercise (30 min cycling, 60%$\dot{V}O_2$max), local heating (44˚C), and ACh iontophoresis were assessed in 12 pre-pubertal boys (age=10.9 ±1.1y, $\dot{V}O_2$max =1665 ± 282 ml·min^{-1}) and 12 girls (age=11.1±1.2y, $\dot{V}O_2$max =1537± 296 ml·min^{-1}), with and without NO synthase inhibition, using N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME) iontophoresis. Exercise-induced increase in SkBF was greater in boys compared with girls (528±290 and 374±192% of baseline, respectively, p=0.03). L-NAME blunted the SkBF response to exercise in boys and in girls (group-by-treatment interaction, p=.82). Local heating-induced SkBF was not significantly different between boys and girls (1445 ±900% and 1432±582%, respectively, p=.57). The ACh-induced SkBF was not different between the boys and girls, with no difference in the increase in SkBF (673± 434% and 558±405%, respectively, p=.18). L-NAME blunted the SkBF response to ACh in boys and girls (group-by-treatment interaction, p=.19). These findings demonstrate that there are no differences between boys and girls in the responses to ACh and to local heating (44˚C). Additionally, the role of NO in the SkBF response appears similar in boys and girls both during exercise and ACh-mediated vasodilation. The greater SkBF response in the boys during exercise may be workload-related.

Absolute and relative (%$\dot{V}O_2max$) exercise intensity were not different between the two groups. Therefore, it is possible that the greater SkBF response in boys may be related to their greater workload relative to body mass (p=0.01). Additionally, sex-related factors (e.g., hormones) may interact with the exercise response or other vasodilators may be involved, resulting in the observed sex-related difference in the SkBF response to exercise.

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Abbreviations/Key Terms

ACh	Acetylcholine
AVA	Arteriovenous anastomoses
BKca	Large calcium-activated hyperpolarizing factors
BIA	Bioelectrical impedance analysis
bpm	Beats per minute
BSA	Body surface area
BSA/M	Body Surface-Area-to-Mass Ratio
%BF	Body Fat Percentage
COX	Cyclooxygenase
COX-1	Cyclooxygenase type 1
COX-2	Cyclooxygenase type 2
CVC	Cutaneous vascular conductance
EDHF	Endothelial derived hyperpolarizing factor
eNOS	Endothelial nitric oxide synthase
FBF	Forearm blood flow
FMD	Flow-mediated dilation
IKca	Intermediate calcium-activated potassium channels
KETO	Ketorolac
LDF	Laser Doppler fluxmetry
L-NAME	Nω-nitro-L-arginine methyl ester
L-NNA	N^G-nitro-L-arginine
L-NMMA	L-NG-monomethyl arginine citrate
NA	Noradrenaline
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NPY	Neuropeptide Y
PORH	Post-occlusive reactive hyperemia
PU	Perfusion Units
PWV	Pulse Wave Velocity
RH	Relative Humidity
RPE	Rate of Perceived Exertion
SKca	Small calcium-activated potassium channels
SkBF	Skin blood flow
SRCE	Semi-recumbent cycle ergometer
TEA	Tetraethylammonia
$\overline{T}_{sk}$	Skin temperature
$\dot{V}O_2max$	Maximum volume of oxygen consumption
VOP	Venous Occlusion Plethysmography

1.0 Introduction

Microvascular function is a general term used to describe the complex interactions that occur in the microcirculation. Endothelial function is one of the microvascular mechanisms which affect microcirculation. Endothelial cells line the internal surface of blood vessels and play an important role in controlling blood flow. The endothelial cells control the behaviour of blood vessels by releasing vasoconstrictive and vasodilating factors (Cracowski et al., 2006). Endothelial dysfunction observed in large and small blood arteries can serve as a marker of the overall atherosclerotic risk (Deanfield et al., 2007). Endothelial dysfunction appears to occur in the microcirculation prior to any changes at the macrovascular level, before structural atherosclerotic changes develop in the conduit arteries (Duprez et al., 2005). The early detection of endothelial dysfunction is a major clinical goal to assess individuals that are at risk for cardiovascular morbidity and to ultimately initiate strategies to reduce the risk.

The human skin microcirculation plays an important role in thermoregulation. Juvenile growth has been linked with the growth of microvascular networks and an increase in microvascular wall mass (Boegehold, 2010). These changes occur jointly with alterations in an individual's microvascular perfusion pressure and microvascular blood flow (Wang & Prewitt, 1991) affecting skin blood flow and therefore, thermoregulation. Heat is exchanged between the body and the environment via evaporation or through dry heat exchange (radiation, convection, conduction). When exercising or exposed to a hot environment, there are various physiological means for heat dissipation. Sweating, enhances the evaporative heat loss and cutaneous venous dilation and increased skin blood flow enhances heat transfer from the body's core to

the periphery and then ultimately to the surrounding environment (Falk & Dotan, 2008). Thus, with cutaneous vasodilation more blood is transferred from the core to the skin surface allowing dissipation of heat to occur.

Cutaneous blood flow is an accessible vascular bed that is commonly used to assess microvascular function and dysfunction (Holowatz et al., 2008). There are various factors that affect microvascular function and skin blood flow (SkBF) response such as age, fitness level and sex. Currently, there is limited research that examines the impact of sex on skin blood flow in adults, and even less in children. The sex-related differences that are seen in the SkBF in adults may help explain the thermoregulatory differences between men and women. However, thermoregulatory response to heat stress, and specifically the SkBF response, is different between children and adults (Woloschuk et al., 2019). Investigating sex-related differences in the SkBF response in children would contribute to the understanding of whether or not the sex-related differences in adults are inherent or develop during puberty. Potential sex-related differences in microvascular function may also result in sex-related differences in heat dissipation mechanisms among children.

2.0 Literature Review

2.1 Sex-related differences in thermoregulation

This section highlights sex-related differences that are seen in adults' ability to thermoregulate and the physical and physiological factors which may affect them. The observed differences in thermoregulation between men and women may be due to several factors: physical factors (body size or proportions, body composition), or physiological factors (e.g., sweating pattern, microvascular function) (Gagnon & Kenny, 2012b).

2.1.1 Physical Differences related to thermoregulation

The main physical characteristics that influence temperature regulation are body mass and surface area. Body mass represents the capacity of an individual to store heat, where individuals with a greater body mass typically have smaller increases in core temperature during heat stress (Havenith et al., 1995). On the other hand, the heat transfer that occurs between the body and the environment relies on the exposed body surface area (BSA), which is generally associated with a lower core temperature during heat stress (Havenith et al., 1995). In general, females are smaller and tend to have greater BSA-to-mass ratio in comparison to males, which may indicate that females rely more on cutaneous vasodilation instead of sudomotor activity in order to thermoregulate (Notley et al., 2017). Similarly, children's greater BSA-to-mass ratio allows them to rely more on dry heat loss through means of conduction, convection and radiation, instead of on evaporative cooling (Falk & Dotan, 2008).

Metabolic energy expenditure is proportional to body mass during weight-bearing exercises such as walking. When walking at a fixed external workload, females elicit a lower rate of metabolic heat production due to their lower body mass, which can help to explain their lower observed sweating rates (Notley et al., 2017). Not only are females smaller, they also have less muscle mass when compared to males, resulting in less heat produced relative to their body size. Adipose tissue is known to act as the body's natural insulator: it increases heat storage putting individuals with higher fat mass at a disadvantage for heat dissipation (Alexander et al., 2015). As females mature, they attain a greater fat mass in comparison to girls and males (Alexander et al., 2015). However, since body composition is similar in pre-pubertal boys and girls, adiposity likely does not play a role in any potential sex-related differences in thermoregulation in children.

2.1.2 Physiological Differences related to thermoregulation

The majority of the studies that have looked at the physiological aspects in temperature regulation in females have focused on differences due to menstrual cycle (Gagnon & Kenny, 2012b). Core temperature, an indicator of thermoregulatory function, fluctuates in females due to changes in sex hormones concentrations. Sex hormones, such as estrogen and progesterone have an influence on core temperature and thermoregulation in females (Charkoudian & Stachenfield, 2014) and may impact cutaneous vascular responses (Charkoudian & Johnson, 2000). The rise in plasma concentrations of progesterone and estrogen during the luteal phase of the menstrual cycle increases resting body temperature by ~0.3-0.5˚C in relation to the follicular

phase, which in turn causes an increase (rightward shift), in the core temperature at the onset for sweating and cutaneous vasodilation (Stephenson & Kolka, 1985). Houghton, Holowatz, & Minson (2005) have shown that estrogen is necessary for endothelial-dependent nitric oxide (NO)-mediated cutaneous vasodilation, suggesting estrogen may upregulate NO production.

Another physiological factor that needs to be considered when comparing the heat loss responses between sexes is aerobic fitness, reflected in maximum oxygen consumption. Fitness may affect one's ability to thermoregulate (Gagnon & Kenny, 2012b). On average women's VO_2max is lower than men's, which may explain some of the sex-related differences in thermoregulation previously observed (Gagnon & Kenny, 2012b). This is particularly the case when comparing the thermoregulatory response of men and women at given external load (Davies, 1979). When working at a given external load, physically active females have similar cardiovascular responses and heat tolerance despite exhibiting lower sweating rates when compared to physically active males (Gagnon & Kenny, 2012b). One way to address this issue is to compare their response at a given relative load (e.g., % of $\dot{V}O_2$max). When working at a relative load, females tend to have a lower end-of exercise core temperature despite having lower sweating rates (Ichinose-Kuwahara et al., 2010). The sex-related differences in fitness are greater among adults than among children. Therefore, fitness may play a smaller role in potential sex-related thermoregulation differences among children.

Sex-related differences in sweating rates have been reported in adults (Gagnon & Kenny, 2011; Shapiro et al., 1980), where women tend to have a lower sweating rate than men, but the results are not as clear in children. Most notably, Rees & Shuster,

(1981) examined the difference in sweating rate via a pharmacological stimulus, during rest between males and females prior to and following puberty. They reported that when corrected to relative surface area, the sweating rate was similar in girls, boys and women, while in men, it was almost double that of the women. Thus, it appears that in adulthood, sweat evaporation may play a greater role in heat dissipation in men compared with women. This sex-related difference may not be apparent in children.

While sweat evaporation is an efficient avenue for heat dissipation, dry heat dissipation also contributes to body temperature regulation. In view of their greater BSA-to-mass ratio, children rely on dry heat exchange for heat dissipation to a greater extent than adults. Dry heat exchange is influenced by the SkBF response. Therefore, the subsequent section discusses physiological factors which may specifically affect SkBF, highlighting sex-related differences.

2.2 Physiological factors that may affect the skin blood flow response

2.2.1 Sex

The studies that describe the effects of sex on skin blood flow are limited, where the majority of studies focus on sex comparisons in adults and the results are inconsistent. This may be due to the use of various vasoactive provocations such as exercise, local heating, reactive hyperemia following arterial occlusion and various vasoactive substance administrations. During exercise, some studies in adults report no sex-related differences in the SkBF response to various workloads (Gagnon & Kenny, 2011, 2012a), while others (Notley et al., 2017), indicate that there are in fact sex-related differences in the forearm blood flow (FBF) response to exercise, where women exhibit higher SkBF responses. When determining whether or not there are sex-related

differences to local heating, again there are incongruent results. Some studies find that there are sex-related differences (Cooke et al., 1990; Hodges, Sharp, Clements, et al., 2010) in response to local heating, where women tend to have lower baseline and peak blood flow values, while others do not (Gagnon & Kenny, 2011; Stanhewicz et al., 2014). Lastly, in response to vasoactive substances such as ACh, yet again there are conflicting results. Ferrell et al. (2004) and Gagnon et al. (2013) indicate that there are no sex-related differences (Ferrell et al., 2004; Gagnon et al., 2013), while Algotsson and colleagues (1995) showed that women have a greater increase in perfusion following ACh iontophoresis (Algotsson et al., 1995). The inconsistent results in these studies may arise from the various techniques used to assess SkBF responses (see section 2.5.), the different protocols that were implemented and the anatomical areas where SkBF was assessed. These adult sex-related responses are described in more detail, later in this chapter (Section 2.3 & 2.4).

The studies mentioned above focused on adults. To my knowledge, there are only a few studies that have examined the differences in SkBF between boys and girls. Baboshina (2018) recently demonstrated that during rest in a thermoneutral environment SkBF was higher and more variable in adolescents (12-16yrs old) compared to children (8-11yrs old) and pre-adults (17-20yrs old) and this pattern was similar in males and females (Baboshina, 2018). Schlager et al., (2014) assessed the impact that age and sex have on microcirculation before, during, and after post-occlusive reactive hyperemia (PORH). SkBF was measured by laser Doppler Fluxmetry (LDF). It was determined that in children and adolescents, microvascular reactivity is related to age and sex has an impact on microvascular reactivity only during

adolescence. They found that there was a lower PORH with an increase in age, from 8 to 18 years. Additionally, sex-related differences in the SkBF response were apparent only during adolescence (12-18 years), with males exhibiting both higher baseline and peak perfusion than females. There were no sex-related differences exhibited between the ages of 4 and 11 years. Although this was a very extensive study in that it included a very large sample (n=896), the authors examined the SkBF response only to post-occlusive reactive hyperemia (PORH), which is not a physiological condition. Another study conducted by Radtke and colleagues (2012) assessed the reactive hyperemic index in children aged 10-16 years using peripheral arterial tonometry. Peripheral arterial tonometry noninvasively measures changes in distal pulse volumes at rest and after induction of reactive hyperemia. They found significant correlations between reactive hyperemic index and Tanner stage (r = 0.569; P <.001), age (r = 0.567; P <.001), but there was no difference between sexes (Radtke et al., 2012). Lastly, Khan et al., (2003) showed that as a result of ACh iontophoresis, females between the ages of 11-14 years exhibited a greater microvascular response when compared to males. These differences may have occurred due to the females being more sexually mature (advanced in their pubertal status) in comparison to the males.

Further research is warranted in order to determine the impact that sex has on the SkBF response to heat stressors, such as exercise, local heating and pharmacological stimuli (i.e. ACh). Currently, there are also no studies in children that examine sex-related differences in the mechanisms involved in the SkBF response to various stimuli. With the limited research assessing SkBF response in boys and girls,

further research is required to determine whether or not sex has an impact in younger children.

2.2.2 Age

Brien, Bar-Or, Iwata, Timmons, & Wilk (2011) examined the influence of pubertal development on post-exercise FBF of girls in a hot environment. Pre-, early- and late-pubertal girls cycled for two 20-minute bouts at 50% $\dot{V}O_2$max in a climate chamber that was set to 35˚C±1, with a relative humidity (RH) of 50±5%. FBF was assessed using venous occlusion plethysmography (VOP) prior, during and following the exercise bouts. They found that early- and pre-pubertal girls demonstrated higher FBF in comparison with late-pubertal girls. In another study, Drinkwater, Kupprat, Denton, Crist, & Horvath (1977) compared women to pre-pubertal girls during treadmill walking at (30% $\dot{V}O_2$max) in hot environments. They also reported that there was a tendency for pre-pubertal girls to have a higher FBF in comparison with women. Although not statistically significant, the authors suggested a greater shift from the central to the peripheral circulation in pre-pubertal girls compared to women. This reflects the larger BSA-to-mass ratio in smaller individuals. The girls in this study had lower blood volume per skin surface area, when compared with the women (2,269 ml.m-^{2} vs. 3,095 ml.m-^{2}). In contrast, Rivera-Brown, Rowland, Ramírez-Marrero, Santacana, & Vann (2006) found no difference in FBF between pre-pubertal girls and women while cycling (60%$\dot{V}O_2$max) in the heat (33.4˚C, 20%RH). The discrepancy in the results can be attributed to two factors; participants in Rivera-Brown et al., (2006) were heat-acclimatized and competitive athletes, which may suggest that the effect of puberty on FBF might be more apparent in females who are either less fit or not heat-acclimatized.

The higher SkBF reported by Brien et al. (2011) in pre- and early-pubertal girls is consistent with previous work in non-athletic and non-acclimatized males (Falk, Bar-Or, Calvert, & MacDougall, 1992), supporting the idea that growth and maturation may have an impact on the regulation of SkBF following exercise in the heat. Similarly, Martin, Loomis, & Kenney (1995) compared the SkBF response of children and adults while heating the forearm to 42˚C and found a lower forearm SkBF with an increase in age. In a more recent study, Hodges, Mueller, Cheung, & Falk (2018) compared the cutaneous vasomotor responses between boys and men during local heating, post occlusive reactive hyperemia and an isometric handgrip exercise. During rest and when the skin was locally heated to 39˚C, boys exhibited a higher SkBF and endothelial activity compared with men. However, there were no age-related differences during the maximal response to local heating at 44˚C during PORH or isometric handgrip. In this study children were more sensitive to an increase in local skin temperature when compared with men. This higher sensitivity may be a result of the responsiveness of the mechanisms governing vasodilation, specifically of the endothelial tissue. These results are consistent with the greater endothelial-mediated cutaneous vasodilator response to local skin heating in young versus older men that has been shown by Hodges, Stewart, Davison, & Cheung (2017) and in several large cross-sectional studies (Hodges, Sharp, Clements, et al., 2010; Martin et al., 1995). Additionally, maturity-related lower SkBF has been demonstrated in late- compared with pre-pubertal boys (Falk, Bar-Or, Calvert, & MacDougall.,1992). The above studies have shown the age-related decline in blood flow response, which may imply that the aging process of the endothelium begins at a young age.

2.2.3 Training Status

Training has been shown to have benefits on the vascular reactivity of human skin microcirculation in adults (Hodges, Sharp, Stephenson, et al., 2010). Generally, findings indicate that training enhances endothelium-dependent vasodilation. Additionally, training has been shown to improve the decline of endothelium-dependent vasodilation of cutaneous microcirculation in both disease and aging (Black et al., 2008; Colberg et al., 2005). There have been multiple studies to determine the effect that training has on the microcirculation of individuals. One study, conducted by Lenasi & Sturcl (2010), compared highly trained endurance athletes with healthy age-matched controls. They tested both glabrous (finger pulps) and non-glabrous (forearm) sites and it was concluded that there were apparent differences in the finger SkBF of the trained athletes compared to controls, but not in the forearm. There was an increased responsiveness in the glabrous sites to both ACh and PORH in the trained athletes. This was to be expected as they are abundant in arteriovenous anastomoses (AVAs). These findings suggest that AVAs are more involved in thermoregulatory exercise and that the exercise-induced shear stress can lead to adaptations.

Among youth, Roche, Rowland, Garrard, Marwood, & Unnithan (2010) found that trained adolescents have higher baseline and higher peak forearm SkBF response to local heating to 44˚C, higher cutaneous vascular conductance (CVC) and peak hyperemia following arterial occlusion than untrained adolescents. This indicates that in youth, chronic exercise training is associated with enhanced endothelial-dependent vasodilation in non-glabrous skin.

2.3 Interventions that cause vasodilation

2.3.1 Exercise

During dynamic exercise, there is a redistribution of blood flow to the coronary circulation and active muscles (Rowell, 1974). There has been some disagreement as to whether or not the cutaneous circulation is a target of that redistribution. There is a dilemma that occurs due to conflicting signals as an increase in body temperature with exercise signals for an increase in SkBF, however with exercise there are also signals to reduce skin and visceral blood flow (Christensen et al., 1942). At the onset of exercise there is an immediate skin vasoconstrictor response (Christensen et al., 1942) and this decrease in SkBF will be greater when baseline levels are highest (Taylor et al., 1984). As exercise continues, competing reflexes of temperature regulation from rising body temperature and from heat production play a role (Benzinger, 1963). SkBF will increase linearly with increasing exercise duration and core temperature, until core temperature reaches approximately 38-39˚C, and subsequently plateaus (Benzinger, 1963). The above has been demonstrated in adults but has not been examined in children.

Mortensen, González-Alonso, Damsgaard, Saltin, & Hellsten (2007) sought to determine the role of NO, prostaglandins and endothelial derived hyperpolarizing factors (EDHFs) in endothelium-mediated vasodilation during an acute bout of exercise. When the cyclooxygenase (COX) inhibitor was infused to inhibit the formation of prostaglandins, the increase in SkBF was similar with and without the COX inhibitor. Similarly, this was true when the EDHFs inhibitor, tetraethylammonia (TEA) was administered, showing no reduction in exercise hyperemia. However, when both NO and prostaglandins were inhibited simultaneously, there was a clear reduction in the

blood flow during exercise. Another study conducted by McNamara, Keen, Simmons, Alexander, & Wong (2014) investigated possible mechanisms underlying reflex cutaneous vasodilation in response to hyperthermia induced by dynamic exercise. Participants cycled at 60% $\dot{V}O_2$max in a thermoneutral environment until there was an increase of about 0.8˚C in core temperature (~35-45mins). They found that in response to dynamic exercise endothelial nitric oxide synthases (eNOS) is required for the NO-mediated cutaneous vasodilation. Additionally, they showed that NO directly contributes ~30% of the increase in the skin blood flow response during dynamic exercise, which is similar to what is seen during passive heating (~30-45%) (Kellogg et al., 1998). These mechanisms have not been examined in children.

Sex-related differences in the SkBF response to exercise have only been examined in adults. Gagnon & Kenny (2011) reported that there were no sex-related differences in CVC during 90 minutes of upright seated cycling at 50% $\dot{V}O_2$max or at a fixed rate of metabolic heat production (500W). Interestingly, during both exercise tests (50% $\dot{V}O_2$max and 500W) men exhibited a greater whole body sweat production than females. Another study conducted by Gagnon & Kenny (2012) found similar results to their previous research. Participants exercised at three different metabolic workloads (200, 250,300 W/m^2), and only at the higher intensities, sex-differences in the sweating patterns were apparent. While, at each of those workloads there were no sex-related differences in the SkBF response.

The above findings are inconsistent with Notley et al., (2017) who reported sex-related differences in the forearm skin blood flow response to semi-recumbent cycling at both 150 and 200 W/m^2. Women were shown to have higher forearm blood flow and

CVC than men during both exercise bouts, as well as during rest. Interestingly, when the participants were matched for mass-specific BSA, there were no longer differences in either the forearm blood flow or vascular conductance. This suggests that the SkBF response is related to body size or proportions, and not necessarily to sex-related factors. Thus, small individuals (women, children) who are characterized by higher surface area-to-mass ratio, appear to rely more on vasomotor activity to facilitate heat loss, while larger individuals (men) appear to rely more on sweating. That is, it is suggested that observed sex-related differences in the thermoeffector function among adults (Gagnon & Kenny, 2011, 2012a) are explained by morphological differences. Further research is warranted to determine whether these sex-related differences are a result of physical differences or physiological differences.

2.3.2 Local Heating

Direct localized warming of the skin, without changes in core or body temperature, causes a local vasodilation via multiple mechanisms (Johnson & Kellogg, 2010). The classic local thermal hyperemic response is known as the 'bisphasic response'. The biphasic response is characterized by an early, transient peak, followed by a brief nadir and a prolonged secondary rise to a sustained dilation, entirely mediated by locally produced chemical factors (Minson et al., 2001). The initial vasodilation is mediated predominately by local sensory nerves (Minson et al., 2001; Tew et al., 2011) and the sympathetic neurotransmitters noradrenaline (NA) and neuropeptide Y (NPY) (Del Pozzi & Hodges, 2015; Hodges, Kosiba, et al., 2009; Tew et al., 2011). The sustained vasodilation to a plateau, is mediated by eNOS (Dean L. Kellogg et al., 2008) and EDHFs (Brunt & Minson, 2012). Prolonged local heating leads to a plateau in SkBF that is maintained for roughly 90 minutes, at which point, blood

flow has been observed to decrease. This has been termed the “die away” phenomenon, (Barcroft & Edholm, 1943). This ‘die-away’ phenomenon was shown to be of sympathetic origin (Hodges, Kosiba, et al., 2009). One thing to note when heating the skin is that the rate at which it is being heated can affect the vascular mechanisms responsible for the vasodilation (Del Pozzi et al., 2016; Del Pozzi & Hodges, 2015; Hodges, Kosiba, et al., 2009).

The evidence supporting NO as a major contributor to vasodilation in local skin heating is compelling. The first study to examine the role for NO in local heating was conducted by Kellogg, Liu, Kosiba, & O′Donnell (1999), where they infused the nonselective nitric oxide synthase (NOS) inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) to an area of the skin via microdialysis while heating the skin to 41˚C. L-NAME blockade of NOS activity produced a significant ~60% reduction in SkBF (Kellogg et al., 1999; Minson et al., 2001). EDHFs contribute substantially to the local hyperemic response. Brunt & Minson, (2012) found that EDHFs are responsible for a large portion of the initial peak and for 40-50% of the plateau phase that is not abolished by NOS inhibition. During sustained dilation, nearly all of the hyperemic response was abolished when L-NAME was used in conjunction with TEA. As for prostaglandins, they have little or no effect on the response to local heating. McCord et al., (2006) assessed the local heating response with the combination of L-NAME and KETO (ketorolac) (a prostaglandin inhibitor) and found no significant differences when compared to L-NAME only. Also, there was no difference between the control site and the site with only KETO. Similar results were reported by Holowatz & Kenney (2009). The majority of these studies that assess the mechanisms involved in the local heating response were

done in adult males, currently there are no studies in children that examine sex-related response differences to local heating. Very limited research exists in understanding the mechanisms involved in the local heating of children.

There are few studies that have assessed the sex-related differences in response to local heating in adults and to my knowledge, none in children. The SkBF response to local heating is commonly used to examine microvascular dysfunction in clinical populations, due to the highly reproducible vasodilatory response and the large NO-dependent contribution. Cooke, Creager, Osmundson, & Shepherd, (1990) assessed a local heating protocol to 42˚C in the hand and the finger in both, men and women. At rest, women exhibited lower finger and hand SkBF than men, but during both, local and whole-body heating the women's hand blood flow increased to a greater degree than the men's. On the other hand, Stanhewicz, Greaney, Kenney, & Alexander (2014) found that following local heating to 42˚C there were no differences in SkBF between men and women at rest, nor in the magnitude of the SkBF plateau in either, the forearm or the calf.

In contrast, a cross-sectional study by Hodges et al., (2010) found sex-related differences where males were shown to have higher resting and peak forearm blood flow responses, as assessed using venous occlusion plethysmography, following a 5 min vascular occlusion. Similarly, during local heating (42°C), they reported higher SkBF responses, as measured with LDF, in males compared to females. All of the studies mentioned above were performed in adults and three of them had assessed the response to submaximal local heating (42˚C), while only Gagnon & Kenny, (2011) measured the SkBF response to maximal heating (44˚C) and found no sex-related

differences. There are no studies that have examined the sex-related differences in the SkBF response to maximal heating (44˚C) or submaximal heating in children.

2.3.3 Post-Occlusive Reactive Hyperemia

Post-occlusive reactive hyperemia (PORH) refers to an increase in SkBF after the release of a transient occlusion of a proximal artery and has been used as an index of microvascular reactivity. PORH has been characterized by an initial peak flux that is reached within in a few seconds following the release of occlusion, which depends on the duration of the occlusion (Yvonne-Tee et al., 2008) and then a return to baseline values. PORH is characterized by a release of metabolic and endothelial vasodilators, as well as a sensory component and myogenic response, making it a complex phenomenon (Lorenzo & Minson, 2007). The endothelial vasodilators (NO, prostaglandins and EDHFs) are released from the endothelium as a result of the augmented shear stress due to an increase in blood flow following occlusion. In the upper extremity, usually the brachial artery is compressed under a suprasystolic blood pressure (BP), usually for 3 or 5 minutes (Lenasi, 2011). The duration of the occlusion impacts the response of the microvasculature, with a positive relationship between post-occlusive response and the duration of arterial occlusion (Yvonne-Tee et al., 2008).

The endothelial component of the reactive hyperemia response is thought to result from various vasodilators (NO, prostaglandins and EDHFs). As for the roles of NO and prostaglandins, the results are controversial (Binggeli et al., 2003; Medow et al., 2007; Wong et al., 2003; Zhao et al., 2004). The role of NO seems to have less of an influence in the response to PORH than other vasodilators. Wong et al., (2003) have shown that there was no effect of NOS inhibition on the PORH response in the forearm. On the other hand, studies conducted by Medow et al., (2007) and Binggeli et al.,

(2003) confirmed the involvement of NO in the PORH response, but demonstrated that it only has a minor importance in comparison to other mechanisms. It seems that NO may play a minor role in the vasodilatory PORH response, if at all. Therefore, other modulators (e.g. EDHFs) (Lorenzo and Minson 2007) must play a greater role in the hyperemic response, although these modulators are not clear.

In adults, Schank, Acree, Longfors, & Gardner (2007) assessed the sex-related differences in the vascular reactivity of the calf and found that resting blood flow levels were similar between males and females, while following occlusion, women had a greater percentage change in comparison to men. Similarly, Stupin et al., (2019) found that women exhibit greater skin microvascular reactivity in their forearm to PORH of 1,2, and 3 minutes than age-matched males.

Results of studies that assessed the sex-related differences to reactive hyperemia in children are inconsistent. Schlager et al., (2014) found sex-related differences to PORH in children aged 12-18 years, but none in children aged 4-11 years. Male adolescents were shown to have both higher baseline and peak perfusion when compared to female adolescents. On the other hand, Radtke et al., (2012) found no sex-related differences in children between the ages 10-16 years in the blood flow response following reactive hyperemia.

2.4 Pharmacological interventions that affect vasodilation

2.4.1 Acetylcholine

Acetylcholine-mediated vasodilation in the skin has been studied extensively and it is commonly used to examine cutaneous endothelial function. ACh initiates vasodilation through muscarinic endothelium receptors, but it is unclear as to whether this is due to prostaglandins, NO or other mechanisms. Kellogg, Zhao, Coey, & Green

(2005) found that exogenous ACh impacts vasodilation in human skin by binding to muscarinic receptors, which in turn increases NO production by NOS and increases prostaglandin production by COX.

EDHFs have been shown to be involved in ACh-mediated dilation in other vascular beds and in cutaneous vasodilatory responses to other stimuli. Most EDHFs elicit vasodilation through calcium-activated potassium channels (KCa) which are found on the vascular smooth muscle and the endothelium. Brunt, Fujii, & Minson (2015) used TEA (a KCa channel inhibitor) to show that EDHFs account for the NOS- and COX-independent portion of ACh-mediated vasodilation. Peak dilation was inhibited when TEA was administered alone and in combination with N-nitro-$_{L}$-arginine (L-NNA) and KETO. Their data suggest that EDHFs may have a more vital role during the earlier phases of the vasodilatory response or they influence the speed at which relaxation of blood vessels occur. The use of ACh allows the investigation of possible alterations to these pathways that may occur under various disease states and to potentially identify therapeutic interventions.

Algotsson, Nordberg, & Winblad (1995) found no correlation between age and the ACh-mediated vasodilatory response in forearm SkBF in men, but there was a weak, yet significant correlation in women. Additionally, women were shown to have lower baseline levels, but a greater increase in perfusion following iontophoresis of ACh. Similarly, Brar et al., (2015) showed that women had a greater vasodilator response to ACh in relation to men. On the other hand, Ferrell, Wong, Lockhart, & Ramsay (2004) found no sex-related differences in the hand SkBF response to ACh iontophoresis between men and women. Gagnon, Crandall, & Kenny (2013) used LDF and

microdialysis in order to measure the response to ACh and, similar to Ferrell, Wong, Lockhart, & Ramsay (2004), females exhibited lower baseline values in comparison to males, but there were no differences in the response to ACh. Currently, there are no studies in children that use the combination of LDF and iontophoresis to assess the SkBF response to ACh and no studies that have considered the mechanisms involved in the Ach-induced SkBF response in children.

The studies mentioned above were performed in adults. Khan et al., (2003) evaluated the response to ACh in 11-14 yr old children. The authors reported that girls had significantly greater SkBF response to ACh than males, although this may be related to the fact that girls were more advanced in their pubertal status. Attenuation or abolishment of ACh-mediated vasodilation has been associated with various health conditions, such as atherosclerosis, diminished coronary microcirculation, hypocholesteremia and obesity (Hedvall Kallerman et al., 2014; Zeiher et al., 1993). Therefore, it is important to study in various populations.

2.4.2 Nitric Oxide and L-NAME

Endothelial nitric oxide synthase (eNOS) is expressed in all endothelial cells, causing the secretion of NO. Nitric oxide is a powerful endogenous vasodilator found in humans and many animals (Clough, 1999). The enzyme NOS produces NO in many cells from L-arginine and O_2 (Clough, 1999). eNOS can be further activated by a rise in either different agonists or calcium ions to induce changes in the phosphorylation of certain amino acid residues of the enzyme (Fleming, 2010). Its actions depend on the bioavailability of cofactors or substrates (Lenasi, 2011).

Primarily, studies have used L-NAME to inhibit NOS (Kopincová et al., 2012). L-NAME is an L-arginine analogue that inhibits NOS and ultimately, NO production. L-NAME has been shown to be effective at blocking the effects of NO both during local heating (Casey et al., 2013) and exercise (Welch et al., 2009).

Currently, only one study has looked at the sex-related differences in the NO-dependent portion of vasodilation. Stanhewicz, Greaney, Larry Kenney, & Alexander (2014) assessed the NO-dependent response to a local heating protocol to 42˚C. They determined that during local heating, women may have a lower reliance on NO-dependent mechanisms to increase SkBF in the forearm in comparison with men, but there was no sex-related difference in the absolute magnitude of the local heating plateau. The women in this study were tested during the follicular phase of their menstrual cycle (female hormones are at their lowest concentration) and they were not on oral contraceptives. Previously, it has been shown that female hormones play a small role in the SkBF plateau in response to local heating, which is NO-dependent (Nisha Charkoudian et al., 1999). To our knowledge, L-NAME has not been used to investigate the mechanisms involved in the microvasculature function of boys and girls.

2.5. Methods to introduce pharmacological agents

2.5.1 Iontophoresis

Iontophoresis is an externally applied direct electrical current that enables soluble charged substances to enter the skin (Kalia et al., 2004). The discrete application of predetermined pharmacological substances by iontophoresis, into small skin regions, allows for a localized understanding of how SkBF is controlled. The quantity of the drug is proportional to the magnitude of the electrical current that is used and the concentration of the drug (Kalia et al., 2004). Depending on the substance that is being

used, different polarities are applied. Iontophoresis is commonly used in combination with LDF to explore cutaneous vascular response, especially in non-glabrous skin.

One caveat of using iontophoresis is that prolonged administration of a current (with or without a pharmacological agent) can induce a transientn increase in blood flow. Therefore, a delay (about 45-60 min) is generally required between the iontophoresis procedure and the measurement of SkBF at the site. In order to combat this, there is usually a delay between iontophoresis and the experimental portion of the study to allow SkBF to return to baseline levels (around 45-60 mins) (Johnson et al., 2014). Another disadvantage with iontophoresis is skin resistance, but this can easily be combatted by cleaning the skin with alcohol (Ramsay, Ferrell, Greer, & Sattar, 2002). Iontophoresis also has some advantages over other techniques. Iontophoresis does not induce trauma that may affect SkBF, such as microinjection or microdialysis. As well, the quantity of the drug is minimal, which will not cause systematic effects (Cracowski et al., 2006). Hence this technique has been widely used both in the child and adult populations.

2.5.2 Microdialysis

Microinjection or microdialysis are common invasive techniques used for the application of various active agents into the skin. It involves a microdialysis fibre being inserted intradermally through a needle, that is withdrawn, leaving a fibre in place (Anderson et al., 1994). One advantage that microdialysis has is the ability to administer higher molecular weight and non-polar compounds. There is constant delivery of the agent throughout the experiment, eliminating chances of washout and the duration of drug efficacy (Anderson et al., 1994). When the microdialysis probe is administered a vasodilator response is elicited due to tissue injury (Anderson et al., 1994). Then, a

waiting period of roughly an hour or more is required prior to beginning the experiment (Anderson et al., 1994). Hodges, Chiu, Kosiba, Zhao, & Johnson (2009) found another disadvantage of microdialysis is that; that the presence of the probe slightly increases the measured internal threshold temperature and reduces the peak response of blood flow during whole body heating ((Hodges, Chiu, Kosiba, Zhao, & Johnson (2009). A major disadvantage of microdialysis is its invasiveness.

2.6 Methods to examine skin blood flow or vasodilation

2.6.1 Plethysmography

Venous occlusion plethysmography (VOP) is a technique that is used extensively to assess SkBF in both children and adults. VOP is measured by placing a cuff on an extremity (usually the arm), between the more distal part of the limb and the heart. The forearm is positioned above the level of the heart to allow adequate venous emptying during the period of cuff deflation. Initially, the cuff is inflated to a pressure that is greater than venous pressure, but less than arterial diastolic pressure, which prevents venous return. Then the cuff is deflated to allow venous emptying. Because the hand contains a high proportion of arterio-venous shunts and SkBF is highly dependent on temperature, the physiology of SkBF to the hand is different from muscle blood flow. Therefore, the hand is excluded from the measurement of forearm blood flow (Wilkinson & Webb, 2002) by inflating a cuff that is placed around the wrist, to well above systolic pressure allowing forearm blood flow to be measured between the two cuffs. Blood flow to the extremity is then calculated as the change in limb volume (Greenfield, 1960) or the change in circumference (Whitney, 1953). Those values are then normalized to limb volume and expressed as ml blood per minute per 100 mL tissue.

The caveat to using VOP is that the measurement of limb blood flow includes all tissues of the limb, notably skin and muscle. Therefore, VOP measurements require a limb to remain inactive, and hence is usually not performed during exercise (Wilkinson & Webb, 2002).

2.6.2 Laser Doppler Fluxmetry

Laser-Doppler fluxmetry (LDF) is a non-invasive approach to the measurement of skin circulation. It allows a continuous, real-time assessment of blood flow, not influenced by underlying skeletal muscle blood flow (Iredahl et al., 2015). The methodology is based on the Doppler shift of the emitted laser light as it travels through tissue and is reflected off moving objects (Oberg, 1990). LDF measures red cell flux (closely related to blood flow), which is the number of moving red blood cells multiplied by the velocity (Oberg, 1990). It does this by calculating the number of Doppler-shifted and reflected photons and their mean shift in wavelength. It can be used in conjunction with other techniques (iontophoresis, microdialysis and local heating) to determine the role the endothelium plays in the local control of SkBF.

One of the caveats of using LDF is that it measures only ~1 mm^3 of skin and there is significant local variation in the number of microvessels within the forearm (Cracowski et al., 2006). There are single and multiple fibre probes, which can integrate the information from a larger area. The pattern of blood flow change is known to be consistent among different sites, though the absolute levels may vary. The advantages of using LDF when exploring cutaneous circulation is that the signal is continuous and has high temporal resolution (Kvandal et al., 2006). Additionally, it is not influenced by the underlying skeletal muscle blood flow (Saumet et al., 1988). When interpreting results there are two recommendations. First, is to apply a perturbation to induce

maximal vasodilation (i.e. heating skin to 44˚C) in order to achieve peak flow to express the quantity of the measured LDF relative to peak flow (Cracowski et al., 2006). This allows the comparison between other experimental conditions or values from other devices since flow is usually expressed as a percent of maximal flow. The other recommendation is to express SkBF in terms of cutaneous vascular conductance (CVC) instead of perfusion units, thus accounting for differences in blood pressure between individuals. CVC is obtained by dividing LDF flux by mean arterial pressure (Cracowski et al., 2006).

3.0 Statement of purpose

There is a dearth of work examining sex-related differences in SkBF in children, which have produced inconsistent results (Baboshina, 2018; Radtke et al., 2012; Schlager et al., 2014)(Khan et al., 2003). Importantly, sex-related differences in SkBF response to exercise have not been examined. The SkBF response to exercise will provide insight into whether there is a difference in the way boys and girls thermoregulate during exercise.

Additionally, while there are several recent studies in adults that have used iontophoresis to examine sex-related differences in the mechanisms of the microvascular response to various stimuli (Algotsson et al., 1995; Ferrell et al., 2004), there is limited information in healthy children (Khan et al. 2003). Such studies can potentially provide insight into the possible mechanistic differences between boys and girls.

This study aims to examine sex-related differences in SkBF response to exercise, local heating, and ACh iontophoresis among boys and girls. Additionally, it aims to elucidate sex-related differences in the role of NO in the vasodilation response, using L-NAME. It was hypothesized that the SkBF response, as well as the role of NO will be similar in boys and girls.

4.0 Materials/Methods

4.1 Participants

Twelve boys and 12 girls, aged 7-13y were included in this study. Inclusion criteria required participants to be healthy, currently not on any medications, non-smokers, as well as have no current or recent lower leg injuries (within the past 6 months). Other exclusion criteria included any negative indicators reported on the medical screening questionnaire (Applied Physiology Research Group, Brock University) and metabolic disorders (Appendix A).

4.2 Protocol

Participants were asked to come to the laboratory for two testing sessions. The first session lasted roughly 1-hour and the second session lasted 3 hours. The study was cleared by the Brock University Research Ethics Board (REB #17-045).

4.2.1 Visit 1 - procedures

Parents or guardians of the participant provided written informed consent. Child participants provided written informed assent. The participants completed a medical screening questionnaire, an activity questionnaire (Godin & Shephard, 1985) (Appendix B) and a pubertal stage questionnaire (Tanner, 1962) (Appendix C). Upon completion of the questionnaires, participants were asked to change into shorts, a t-shirt and running shoes. Body mass, standing and sitting height were assessed (without shoes) to estimate the participants' age at peak height velocity (Mirwald et al., 2002). Peak height velocity is an indicator of somatic maturity. Additionally, body composition was estimated using triceps and subscapularis skinfolds using calipers (Baty International, England), as described by Slaughter et al., (1988).

Participants were familiarized with the equipment and the exercise protocol during the initial visit. Prior to beginning the exercise protocol, both resting heart rate

(HR) and blood pressure were recorded. Participants then performed submaximal, maximal and 'supramaximal' exercise on a semi-recumbent cycle ergometer (SCRE). During exercise, oxygen consumption, heart rate, power, and rate of perceived exertion (RPE) (Borg, 1973) were recorded (Appendix D).

Submaximal exercise consisted of 4 stages, each 4 min in duration. The workload progressively increased after each stage, while participants maintained a constant cadence between 60-80 rpm. The submaximal exercise bouts were used to create a regression equation (power vs. $\dot{V}O_2$) to allow for the determination of the exercise intensity at 60% of the participant's $\dot{V}O_2$max.

Next, participants completed a progressive test to exhaustion (in which the $\dot{V}O_2$max was determined). The workload increased each minute and cadence was maintained between 60-80 rpm. Lastly, in order to ensure that maximal $\dot{V}O_2$ was achieved, following a 10 min rest the participants performed a 'supramaximal' exercise bout at 105% of the highest workload achieved during the progressive test (Barker et al., 2011; Sansum et al., 2019).

4.2.2 Visit 2 – procedures

Upon arrival, participants were asked to change into a t-shirt and shorts and drink water. This session included a period of ACh and L-NAME iontophoresis, followed by 30 min of submaximal exercise (60% $\dot{V}O_2$max), and then local heating of the forearm.

During the iontophoresis procedure and subsequent SkBF measurements, participants sat in a comfortable position. The iontophoresis probes were placed on the left dorsal forearm (avoiding superficial veins, cuts, bruises etc.). If necessary, hair was

trimmed and the skin was cleaned with alcohol swabs. There were 4 sites: one site with ACh, one site with ACh and L-NAME, one site with L-NAME only, and one site served as a control site. The first two sites were used to examine the ACh dose response relationships and the last two sites were used during the exercise and the local heating procedures.

Local iontophoresis of L-NAME (MilliporeSigma Canada, Oakville, Canada) to the dermis began once the participant was seated in a comfortable upright position. A 100 μA dose of 2% L-NAME solution was iontophoresed to an area of 1.4 cm^2 for 10 min, twice. Neutral electrodes for current dispersion were placed >10 cm away (wrist). This protocol is based on previous studies (Hodges et al., 2017; Iredahl et al., 2013; Medow et al., 2005).

Following iontophoresis of L-NAME, ACh was administered, first at an untreated skin site and then a skin sited pre-treated with L-NAME. Basal flow was measured for a minimum of 5 min at a site treated solely with ACh (baseline 1) and then at a site treated with both ACh and L-NAME (baseline 2). Following baseline measurements, 6 doses of ACh (anodal current) were applied at 100 μA 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) (Hodges et al., 2017). Following each of the first 4 doses, there were 120 s intervals and following each of the final two doses, there were 135 s intervals of rest in order to allow the response to plateau (Hodges et al., 2017). Iontophoresis of ACh at each site took approximately 20 minutes.

Steady and normal breathing was encouraged to limit any movement artifacts. Two LDF probes were used to measure SkBF – initially, one probe was placed on the

site where ACh was administered and the other probe was positioned at the site where ACh and L-NAME were administered. The probes were secured to the skin with Transpore 3M tape. The SkBF response during the last 30 seconds of the rest period was analyzed.

Following measurement of SkBF at the ACh and ACh + L-NAME sites, thermocouples (PVC-T-24-190, Omega Environmental Inc., Laval, Canada) were taped to the participant's skin on 4 sites: the left calf, quadriceps and chest and on the right bicep to assess mean skin temperature ($\bar{T}_{sk}$) (Ramanathan, 1964). Participants were also fitted with a heart rate monitor.

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. Two Laser Doppler fluxmetry (LDF) probes were used to measure SkBF – one probe was placed on the site where L-NAME was administered and the other probe was positioned at the control site. The probes were secured to the skin with Transpore 3M tape. The participant's SkBF was measured using the LDF probes for the remainder of the session.

Baseline measurements (Baseline 3) were recorded for 5 minutes, which included manual blood pressure (BP), HR, RPE, thermal comfort and thermal sensation. Thermal comfort was assessed using an adapted four-point scale from the Bedford seven-point rating scale and thermal sensation was assessed using the ASHRAE seven-point scale (Gagge et al., 1969) (Appendix E). Participants pedaled on the SRCE (Corvical recumbent cpet, Lode, Netherlands) for 30-min at 60% of their predetermined $\dot{V}O_2$max (from the first visit). The 30-min exercise served as the systemic heat stress

stimulus. The exercise intensity and duration were adapted from Rowland, Hagenbuch, Pober, & Garrison (2008) who demonstrated a similar increase in body temperature (<1°C) in boys and men cycling at 65% $\dot{V}O_2$max. Throughout exercise, at 5-min intervals, manual systolic and diastolic pressure, HR, thermal comfort, thermal sensation, RPE, T_{loc} and $\bar{T}_{sk}$ were monitored and recorded.

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

Subsequent to the 30-minute cycle, local heating was performed using the LDF probes to determine maximal SkBF. Local heating commenced at a typical thermoneutral temperature of the skin surface, which served as the baseline phase. The temperature was increased at a rate of 1°C·20 s^{-1} until 42°C and 1°C·min^{-1} until 44°C for the determination of maximal SkBF. The local heating procedure lasted 5 minutes, following which data were collected for 30 minutes. Throughout local heating, thermal comfort, HR, thermal sensation and $\bar{T}_{sk}$ were assessed.

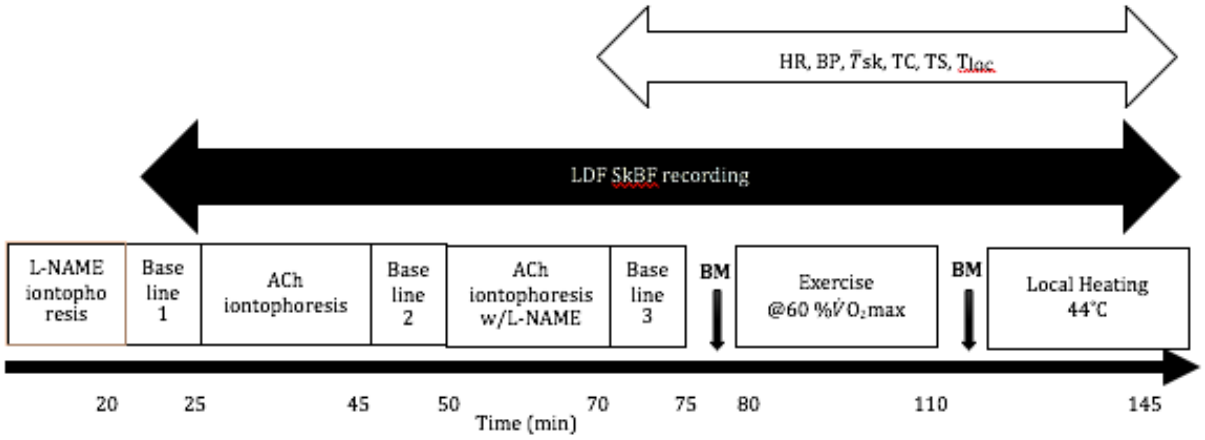


Figure 1. Timeline of visit 2. Where BM = body mass, HR= heart rate, TC=thermal comfort, TS=thermal sensation, $\bar{T}_{sk}$ = mean skin temperature, ACh = acetylcholine, L-NAME = Nω-nitro-L-arginine methyl ester., $\dot{V}O_2$max = maximum oxygen consumption, Tloc = local temperature, LDF = laser Doppler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

4.3 Measurements

All measurements were performed by the same investigator.

4.3.1 Anthropometry

Body mass was recorded to an accuracy of ±10g (GFK 330aH, AE Adam, USA). Children removed any excess clothing and their shoes (only in the first visit) prior to body mass measurement. Standing and sitting height was measured to the nearest 0.5 cm on a stadiometer (Ellard Instrumentation Ltd.). Body surface area (BSA) was calculated according to Du Bois (1916), where BSA (m^2) = 0.007184 × weight(kg)$^{0.425}$ × height (cm)$^{0.725}$

4.3.2 Maturity

Sexual maturity was assessed using secondary sex characteristics (pubic hair & breast development) (Tanner, 1962). Both male and female participants were provided with images of pubic hair at different stages. Girls were also provided with images of breast development and boys were also provided with images of penis development.

The participants were then asked to circle the image that most resembles them currently.

Somatic maturity was assessed using the predicted years from age of peak height velocity (maturity offset). The years from age of peak height velocity was calculated based on age, body mass, standing height, and sitting height, as described by Mirwald et al., (2002).

4.3.3 Body composition

Skinfold thickness of the triceps and subscapularis, were measured using a Harpenden skinfold caliper (Baty International, England) and were used to calculate percent body fat, using the equations and guidelines by Slaughter et al., (1988).

4.3.4 Laser Doppler fluxmetry

Laser-Doppler fluxmetry was used for the measurement of SkBF, expressed as LDF arbitrary units (AU) (Oberg, 1990). Two integrated laser-Doppler local heating probes (Probe 413; Perimed) were used. A calibration apparatus (PF 1000, Perimed) was used to adjust the laser-Doppler fluxmeter readings to correspond with the readings that were obtained with Perimed's Motility Standard. Local heating units and heat probe holders were used to manipulate local skin temperatures (T_{loc}).

The values during exercise, local heating and ACh dose-responses measurements were separately normalized to baseline values and presented as percentage change from baseline, in perfusion units (PU). Typically, when locally heating with LDF, data is normalized to the maximal response. However, in the present study, a 'maximal' response to local heating in the L-NAME-treated site was not achieved since L-NAME is known to blunt the SkBF response. Therefore, results were not normalized to maximal vasodilation values, but rather to baseline values, and

expressed as percentage change. Baseline values were not different between boys and girls. Therefore, comparison of relative (%) changes from baseline between groups are not biased.

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 blood pressure.

4.3.5 Iontophoresis Agents

A pharmacological micro-iontophoresis system was used to transdermally administer pharmacological agents. L-NAME was used to locally (area of a dime) inhibit the NOS-based vasodilatory response in the skin, preventing the synthesis of NO. (Kellogg et al., 1999). The drug-delivery electrode (Perimed, PF383) was filled with 200 µL of 2% L-NAME solution (Sigma-Aldrich).

Iontophoresis of ACh was performed using the same system. The drug-delivery electrode was filled with 200 µL of 2% ACh solution (Sigma-Aldrich).

4.3.6 Heart rate

A HR monitor (Suunto, Finland) was strapped around the participant's chest, just below their pectoral muscles. During the submaximal protocol (session 1) HR was recorded during the last 10-s of each 4-min stage. During the incremental $\dot{V}O_2$max test, HR was recorded in the last 10-s of every 1-min interval. During the supramaximal test, HR was recorded in the last 10-s of every 30-s interval. During session 2, HR was recorded every 5-min during the 30-min exercise protocol and during the local heating protocol.

4.3.7 Blood pressure

Manual systolic and diastolic BP of the brachial artery was measured by the same investigator at rest and throughout session 2. A pressure cuff was placed on the

right arm of the participant and it was inflated until blood flow has stopped. Then the cuff was slowly deflated and both the systolic and diastolic readings were recorded. BP was recorded at baseline prior to exercise, every 5 minutes during the 30-minute cycle exercise, and during local heating at 10 minutes and 30 minutes by auscultation.

4.3.8 Rate of perceived exertion

The Borg RPE Scale (Borg, 1973) is a 15-point scale that ranges from 6, which corresponds to 'no exertion', to 20, which corresponds to 'maximal exertion'. During the $\dot{V}O_2$max protocol, RPE was recorded during the last 10-s of each stage (after HR recording), and during the 30-min submaximal cycle RPE was recorded every 5-min (Appendix D).

4.3.9 Thermal sensation

The American Society of Heating, Refrigerating and Air-Conditioning Engineers (ASHRAE) seven-point scale ranging from cold (1) to hot (7) was administered to assess the participant's thermal sensation, every 5-min during experimental session 2.

4.3.10 Thermal comfort

A four-point scale ranging from comfortable (1) to very uncomfortable (4) was used to assess the participant's thermal comfort, every 5-min during experimental session 2.

4.3.11 Mean skin temperature

Mean skin temperature ($\overline{T}_{sk}$) was measured with standard T-type thermocouples (PVC-T-24-190, Omega Environmental Inc., Laval, 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%) (Ramanathan, 1964). 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. There was no interference when taking blood pressure.

4.3.12 Sweating rate

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.

4.3.13 Godin Leisure Physical Activity Questionnaire

This questionnaire asks participants to report on their physical activity during a typical 7-day period. In total, there are 3 types of physical activity, strenuous, moderate and minimal/light exercise. Participants indicated the number of times they engage in strenuous, moderate or light activity for 15 minutes or more. An activity score is calculated using the equation = (9* strenuous activity) + (5* moderate activity) + (3* minimal/light activity) (Godin & Shephard, 1985).

4.4 Exercise Protocols

4.4.1 Visit 1

On the day of testing, the metabolic cart was calibrated using known gas mixtures. Prior to exercise, participant data such as height, body mass, sex, age as well as atmospheric pressure, temperature and humidity were entered into the MOXUS metabolic cart (AEI Technologies, PA, USA). The cycle ergometer was adjusted to the preference of the participant.

Throughout the exercise protocols, the participant was asked to maintain a cadence between 60-80rpm. Heart rate and RPE were recorded for the last 10 seconds of each stage. The first protocol consists of 4 submaximal bouts. Children pedaled at 20-30 watts, depending on the size of the child, for 4 minutes and then the wattage

increased by 10-15 watts for a maximum of 16 minutes. An average of the last minute of every stage, measured every 15s (four readings), was used to determine the $\dot{V}O_2$ at that stage. Following the submaximal test, participants rested for 5-10 minutes or until they were ready for the next test where they completed a progressive $\dot{V}O_2$ test to exhaustion. Participants began the test at a pre-determined wattage based on their results from the submaximal test. The wattage increased by 10-15 watts every minute until the child reached volitional exhaustion. Cadence was maintained around 60-80 rpm. The average of the highest two consecutive readings were used to determine $\dot{V}O_2$max. Approximately 10 minutes after completion of the progressive phase, a 'supramaximal' test (105% of the maximum power reached during the progressive $\dot{V}O_2$max protocol) was administered to ensure that a true maximal $\dot{V}O_2$ was reached.

4.4.2 Visit 2

The SCRE was adjusted according to each individual's height, as measured in session 1. The wattage was set at 60% of the predetermined $\dot{V}O_2$max. Participants were required to pedal for a total of 30 minutes at a cadence between 60-80rpm.

5.0 Statistical Analysis

All statistical analyses were performed using SPSS, GraphPad Prism (GraphPad Software, Inc.) or Excel 2017. All data were normally distributed, as determined visually. Additionally, 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 (Mallette et al., 2016). Physical characteristics, body mass changes, sweating rate, ambient temperature and humidity were assessed using Independent t-tests to determine differences between the two groups. 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 response (throughout ACh protocol). Interactions were also assessed (Group-Time/Dose, Time/Dose-Treatment, Treatment-Group and Treatment-Group-Time/Dose). A separate two-way ANOVA for repeated measures was used to assess main effects of Group (boys and girls) and Treatment (L-NAME vs. control) for the local heating protocol. Additionally, two-way ANOVAs for repeated measures determined the main effects of Group (boys vs. girls) and Time (throughout exercise) on heart rate, blood pressure, $\bar{T}$sk, thermal comfort and thermal sensation. The % contribution of NO in 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

6.0 Results:

In total, 24 boys and 22 girls were tested, but only the data of 12 boys and 12 girls are presented. Participants were excluded from analysis due to various reasons: being over-weight or obese (3 boys and 4 girls), not completing all visits (7 boys and 6 girls), and technical measurement issues (higher than normal baseline SkBF) (2 boys). The remaining 24 participants completed all study visits. No adverse events were observed or reported as a result of study participation.

6.1 Baseline Characteristics

There were no differences between groups in physical activity level, somatic maturity or physical characteristics, except in body mass (p=.0388) (Table 1). Participants were classified as pubertal stage 1 (8 boys, 4 girls), 2 (3 boys, 7 girls) or 3 (1 boy, 1 girl).

$\dot{V}O_2$max, was not different between boys and girls (p=0.2882) (Table 1). However, when adjusted for body mass (Kg), or for lean body mass, $\dot{V}O_2$max was significantly higher in boys compared with girls (p<0.05). During the 30-min submaximal (60% $\dot{V}O_2$max) exercise, workload was not different between girls and boys (p=.5702). When expressed relative to lean body mass boys exercised at a slightly higher relative workload (p=.0565) (Table 1). No significant differences were shown for the mean years from age of peak height velocity for the girls and for the boys (p=.9529). There was no difference in engagement in leisure physical activities between boys and girls (*p*=.6425).

Table 1 – Personal Characteristics of Boys and Girls

	Boys	**Girls**
Age (yr)	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*
Years from Age of Peak Height Velocity	2.53 ± 0.85	2.4 ± 0.81
Body fat (%)	15.7 ± 2.6	20.2 ± 3.2*
Leisure Physical Activity score	74 ± 28	68 ± 34
$\dot{V}O_2$max (ml·min^{-1})	1665 ± 282	1537± 296
$\dot{V}O_2$max (ml·kg^{-1}·min^{-1})	46.2 ± 7.3	36.6 ± 5.1*
$\dot{V}O_2$max (ml·LBMkg^{-1}·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·LBMkg^{-1})	2.17 ± 0.43	1.87 ± 0.30

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

6.2 Exercise Response

6.2.1 SkBF during exercise

During exercise, there was a statistically significant ($p<.0001$) increase in SkBF in both boys and girls (Figure 2). The increase in the control site for both boys and girls (528±290 and 374±192%, respectively) was not significantly greater than at the L-NAME site (443±273 and 259±195%, respectively) for PU (treatment effect; $F_{1,22}$ = 1.628, p=.215) or for CVC (treatment effect; $F_{1, 22}$ = 1.473, p=*.238*). There were group effects for PU (group effect; $F_{1,22}$ = 5.479, p=.029) and CVC (group effect; $F_{1, 22}$ = 4.762, p=*.040*), reflecting greater increases in PU and CVC in boys. As expected, there was a significant time effect for both PU ($F_{5, 22}$ = 54.29, p<0.0001) and CVC ($F_{5,22}$ = 48.8, p<.0001) and a significant time-by-treatment interaction for PU ($F_{5,22}$ =5.082, p=<.0001) and for CVC ($F_{5,22}$ =5.006, p=<.0001), reflecting that the blunting effect of L-NAME was greater with an increase in exercise time. The group-by-time interaction effect for PU was ($F_{1, 22}$ = 2.27, p=0.052) and for CVC was ($F_{1, 22}$ = 1.738, p=.132), reflecting that, with time, the increase in SkBF was somewhat greater in boys, although it did not reach

the 0.05 significance level. There was no significant group-by-treatment interaction for PU ($F_{1,22}$ =.54, p=.818) or CVC ($F_{1,22}$= .086, p=.772). Finally, there was no significant group-by-treatment-by-time interaction for PU ($F_{5,22}$ =.223, p=.952) or CVC ($F_{5,22}$ =.260, p=.934).

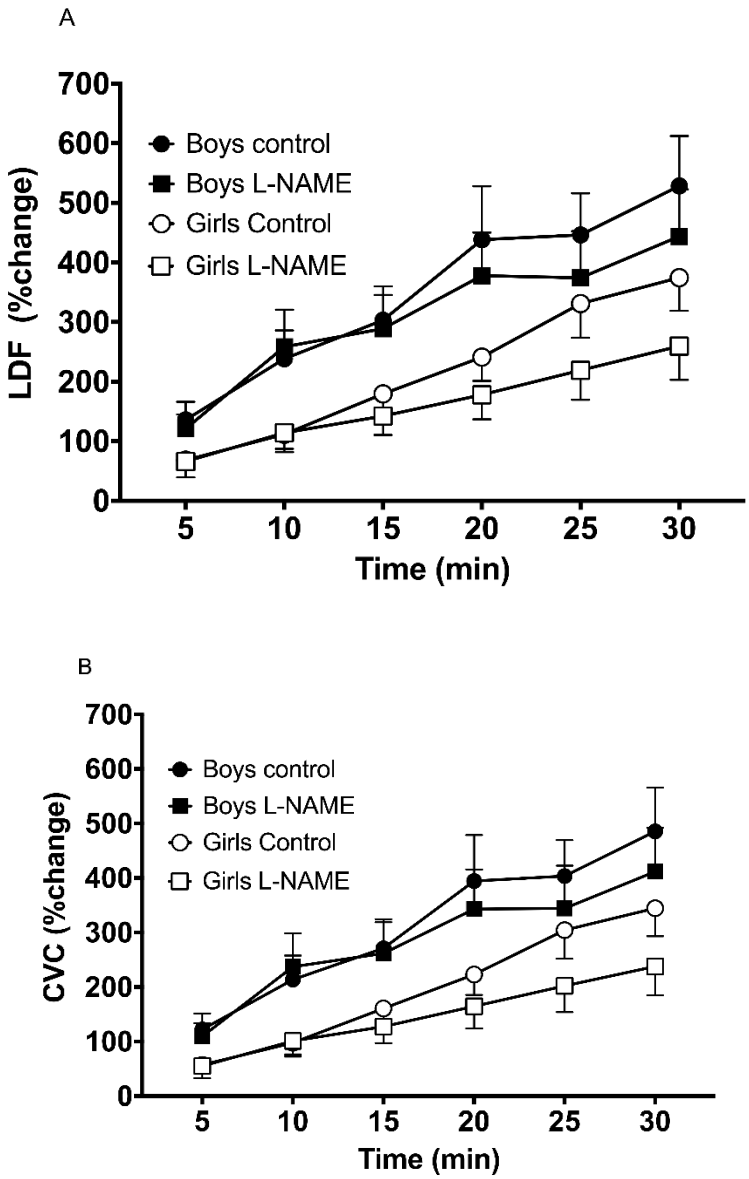


Figure 2. The percent change in SkBF from baseline to the end of exercise in boys and girls in perfusion units (PU) (A) and expressed in cutaneous vascular conductance (CVC) (B) (Mean±SD) LDF = laser Doppler fluxmetry, L-NAME = N^{ω}-nitro-L-arginine methyl ester.

6.2.2 Ambient Air Temperature and Humidity

The ambient air temperatures in the study room were similar when both the boys (23.4± 1.9°C) and girls were tested (23.6±1.2°C) (p>0.05). Likewise, there was no difference (p>0.05) in relative humidity in the room when the boys (31.7±6.6%) or girls were tested (35.1±16.7%).

6.2.3 Change in Body Mass, Sweating Rate

Boys had a greater loss in mass from the beginning of exercise to the end of exercise when compared to girls (133±82 vs. 77±34g, respectively, p=.0301). Similarly, sweating rate was higher in boys compared with girls (4.44±2.75 vs. 2.57±1.14 $g \cdot min^{-1}$, respectively, p=.0301). The estimated sweating rate relative to BSA was significantly greater in boys compared to girls (3.61±2.09 vs. 1.96±.84 $ml \cdot m^{-2} \cdot min^{-1}$, p=0.0095).

6.2.4 Mean Skin Temperature

Due to technical issues with the thermocouples, $\bar{T}$sk was recorded in 12 boys but only 9 girls. At baseline, boys (31.1±1.9°C) tended to have higher $\bar{T}$sk compared with girls (30.14±1.5°C), although the difference did not reach statistical significance (p=.23) (Figure 3). Throughout exercise, the increase in $\bar{T}$sk was significant ($\Delta\bar{T}$sk 1.05°C and $\Delta\bar{T}$sk 0.47°C, in both boys and girls, respectively) (time effect; $F_{6, 19}$ = 15.77, p=<.0001). At the end of exercise, boys (32.1±2.1°C) had higher $\bar{T}$sk compared with girls (30.7±1.9°C), although this difference was not statistically significant (group effect; $F_{1, 19}$= 2.785, p=.112; group-by-time interaction: $F_{6, 19}$ =1.787, p=.108).

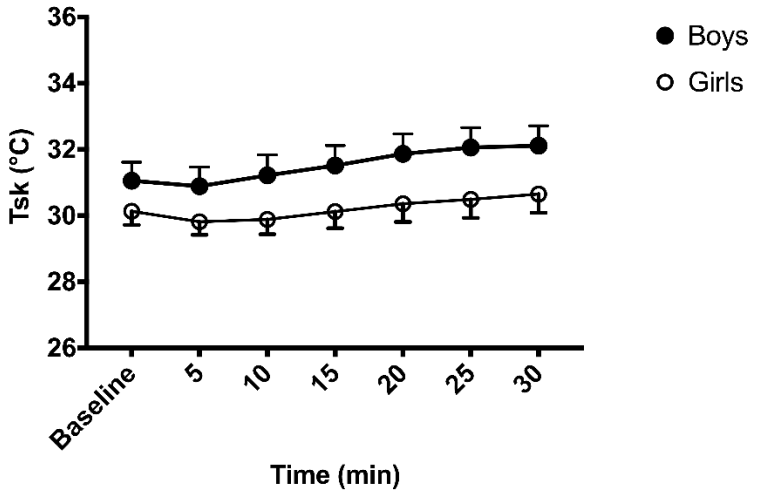


Figure 3. Mean skin temperature ($\bar{T}$sk) of boys (n=12) and girls (n=9) from beginning to end of exercise. There was a significant group difference (p<0.01) and no significant time difference (p>0.01) (Mean±SD).

6.2.5 Local (Forearm) Skin Temperature

At baseline, boys and (30.9±1.0°C) girls (30.4±1.2°C) had similar forearm skin temperatures (Tloc) (p=.41) (Figure 4). From the beginning to the end of exercise, $\bar{T}$loc increased in both boys (30.9±1.0 to 32.4±1.0°C) and girls (30.4±1.2 to 32.1±2.6°C). There was a significant increase in $\bar{T}$loc during exercise (time effect; $F_{6, 14}$ = 16.27, p=<.0001). There were no differences in local $\bar{T}$loc between boys and girls (group effect; $F_{1, 14}$ = .30, p=.865) and there was no significant sex-by-time interaction ($F_{6, 14}$ =.348, p=.909).

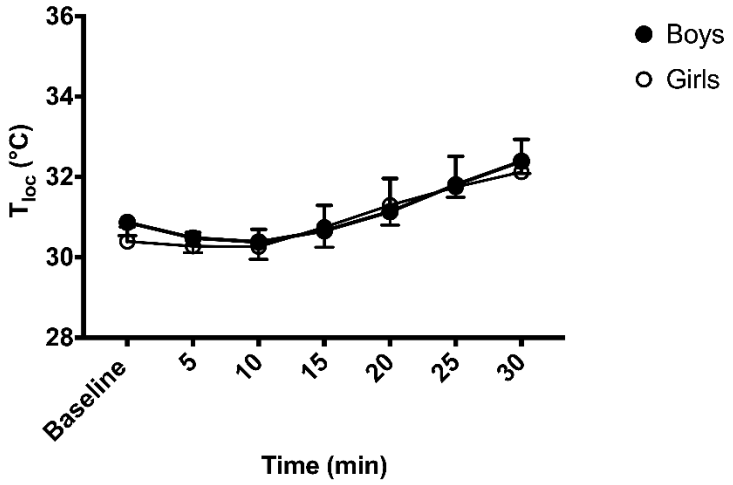


Figure 4. Local skin temperature (T_{loc}) from beginning to end of exercise. There was a significant time effect (p<0.01), but no significant group effect (p=0.63) (Mean±SD).

6.2.6 Heart Rate during exercise

Heart rate increased in both boys and girls from baseline (76±9 and 81±7 beats·min^{-1}, respectively) until the end of exercise (160±15 and 160±11 beats·min^{-1}, respectively) (Figure 5). There was no sex effect for HR response to exercise (group effect: $F_{1, 22}$ =.051, p=.823). There was a significant increase from the start to the end of exercise (time effect; $F_{6, 22}$ = 605.8, p<.0001) and no significant group-by-time interaction ($F_{6, 22}$ =1.258, p=.281).

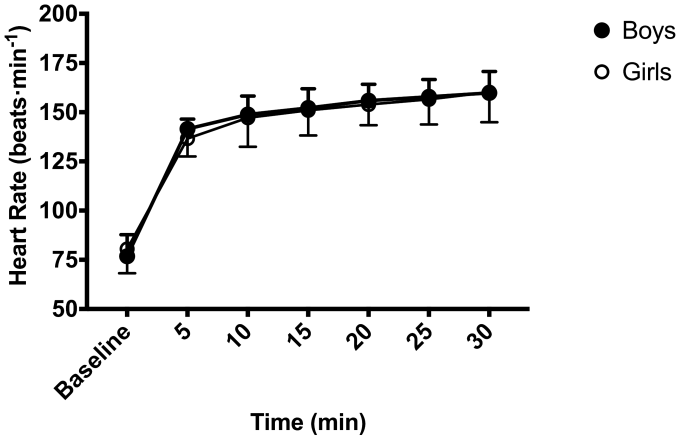
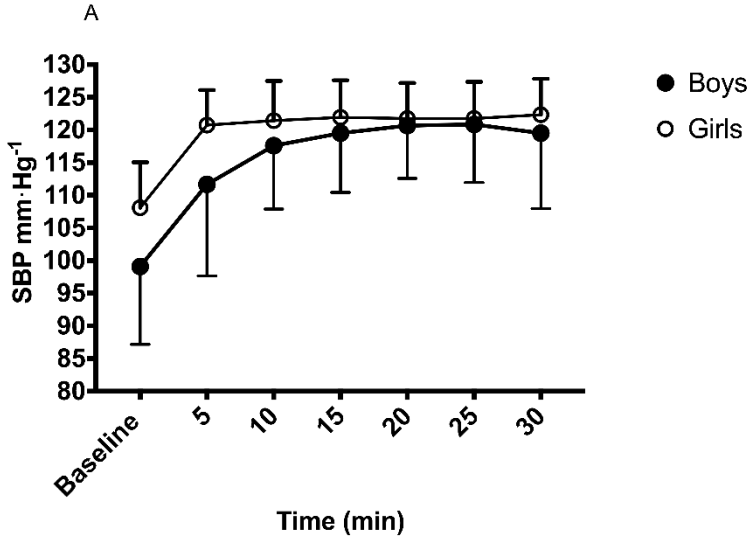


Figure 5. Heart rate (HR) (beats·min^{-1}) from beginning to end of exercise. Heart rate increased with time (p<0.001) but there was not a significant group effect (p=0.59) (Mean±SD).

6.2.7 Blood Pressure

There were no sex-related differences for systolic BP, diastolic BP and mean BP (group effect; $F_{1, 22}$ = 1.851, p=.187, $F_{1, 22}$ =.082, p=.777, $F_{1, 22}$ = .663, p=.424, respectively) (Figure 6). Throughout exercise, there were increases in systolic BP, diastolic BP and mean BP (time effect; $F_{6, 22}$ =49.49, p<.0001, $F_{6, 22}$ = 1171, p=0001, $F_{6, 22}$ = 23.39, p=<.0001, respectively). There were no group-by-time interaction effects for diastolic BP or mean BP ($F_{6, 22}$ =.370, p=.897, $F_{6, 22}$ =.1.343, p=.243, respectively), but there was for systolic BP ($F_{6, 22}$ = 3.601, p=.002). The latter reflects a higher systolic BP in boys at the beginning of exercise, with no apparent difference between groups after 10 min.



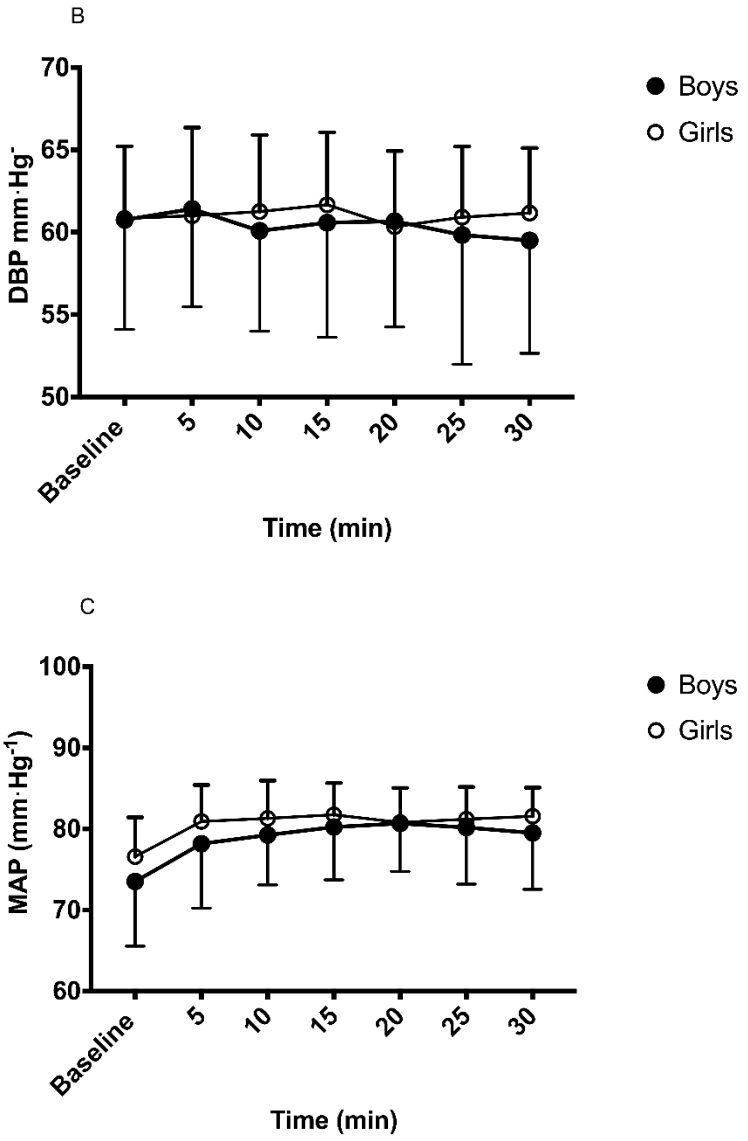


Figure 6. Systolic blood pressure (SBP) (Panel A), diastolic blood pressure (DBP) (Panel B), and mean arterial blood pressure (MAP) (Panel C) during semi-recumbent cycling at 60% $\dot{V}O_2$max in boys and girls. (Mean±SD).

6.2.8 Thermal Comfort and Thermal Sensation

Thermal sensation increased similarly from the start to the end of exercise in boys (4±1 to 6±1) and girls (4±1 to 6±1) (time effect; $F_{6, 22}$ =66.853, p<0.001) (Figure 7). There was no difference between boys and girls (group effect; $F_{1, 22}$ = .541, p=.470) and there was no group-by-time interaction ($F_{6, 22}$ =.158, p=.987). Thermal comfort also significantly decreased from the start to end of exercise in boys (2±1 to 3±1) and girls

(2±1 to 3±1) (time effect; $F_{6, 22}$ =24.93, p<0.001). Both boys and girls reported similar thermal comfort (group effect; $F_{1, 22}$ =.356, p=.557) and there was no group-by-time interaction ($F_{6, 22}$ =1.264, p=.278).

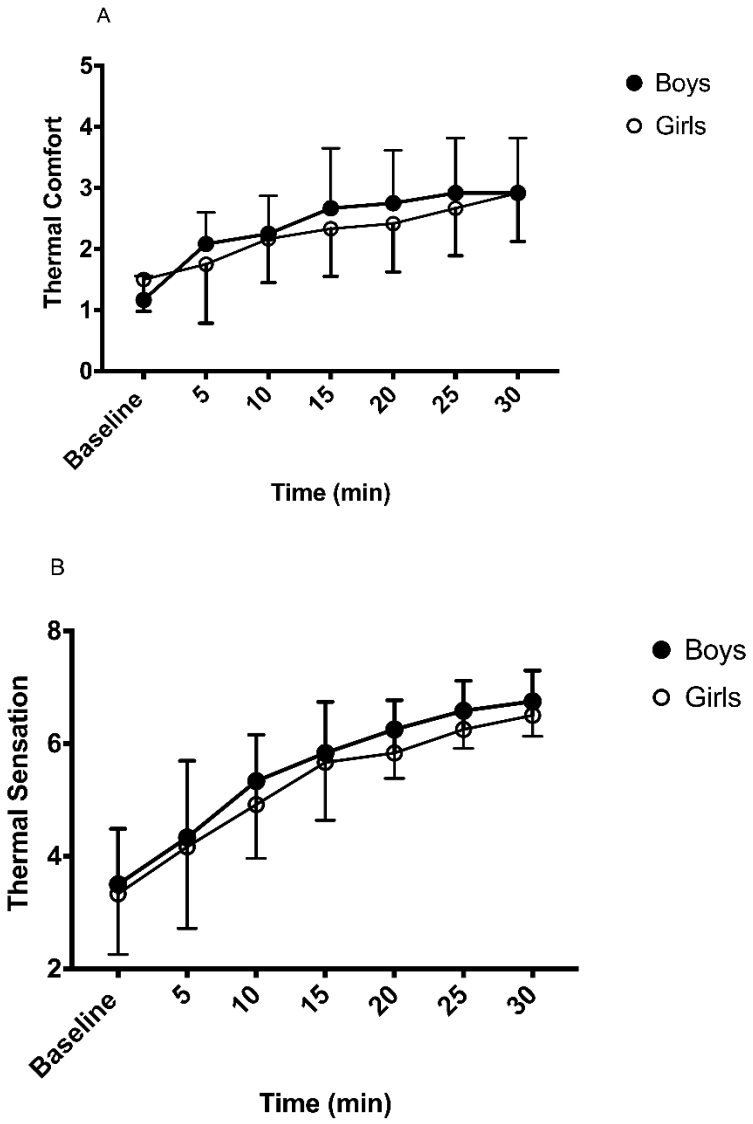


Figure 7. Thermal Comfort (Panel A), and thermal sensation (Panel B) during semi-recumbent cycling at 60% $\dot{V}O_2$max in boys and girls. (Mean±SD).

6.3 Local heating to 44°C

The increase in SkBF PU in boys (1445±900%) was similar to the increase in girls (1432±582%) (group effect; $F_{1,22}$ = .342, p =.565) (Figure 8). This was also the case for CVC (1551±932% vs. 1420±696%, respectively, $F_{1,22}$ = .243, p =.627). The increase in PU at the control site was not significantly different than at the L-NAME site for both PU ($F_{1,22}$ =1.4707, p =.248). and CVC ($F_{1,22}$ = 1.5202, p =.233). There was no group-by-treatment interaction either for PU ($F_{1,22}$ = .660, p= .425) or in CVC ($F_{1,22}$ = .727, p =.403).

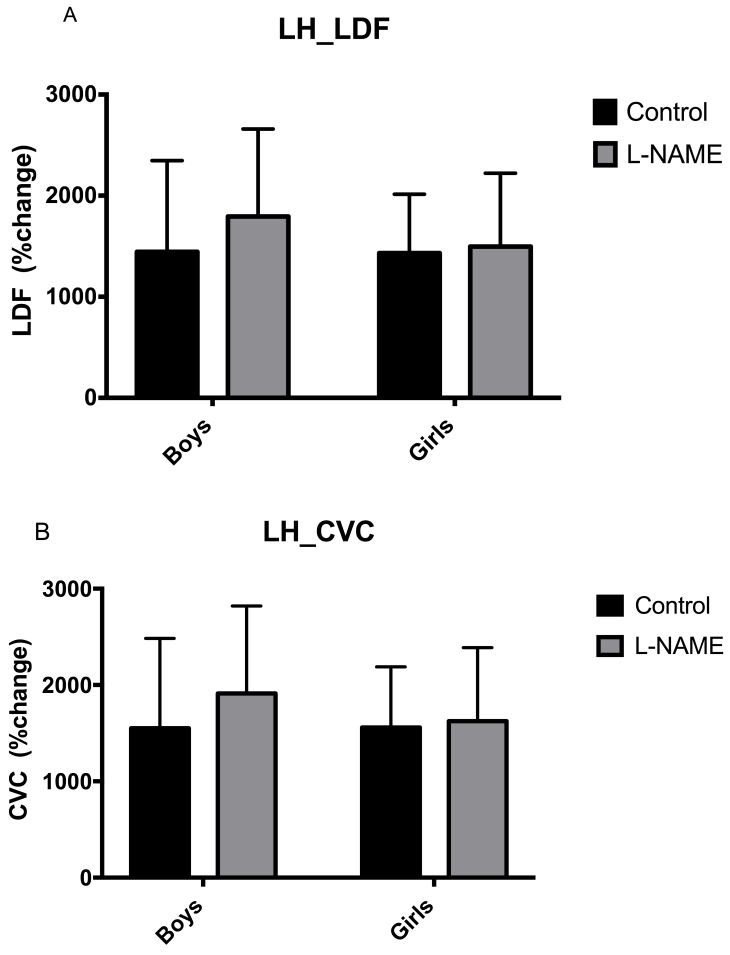


Figure 8. The percent change in PU (A) and in cutaneous vascular conductance (CVC) (B) in response to local heating (LH) to 44˚C in boys and girls at untreated (control) and treated with Nω-nitro-L-arginine methyl ester (L-NAME) skin sites. LDF = laser Doppler fluxmetry

6.4 Acetylcholine dose response curve

The stimulus-response curves for both skin sites are presented in Figure 9. There were main effects for ACh concentration ($F_{6,22}$ = 37.92, p < .0001), treatment (L-NAME) ($F_{1,22}$ = 19.63, p < .0001), and treatment-by-concentration ($F_{5,5}$ = 22.47, p < .0001). The latter interaction reflects that with increasing ACh concentration, L-NAME had a greater (blunting) effect. There was no significant group ($F_{1,22}$ = 1.949, *p=.177*), group-by-concentration interaction ($F_{1,22}$=1.51, p =.192), group-by-treatment interaction ($F_{1,22}$ =.003, p =.954), or group-by-treatment-by-concentration interaction ($F_{6,22}$=.113, p =.989).

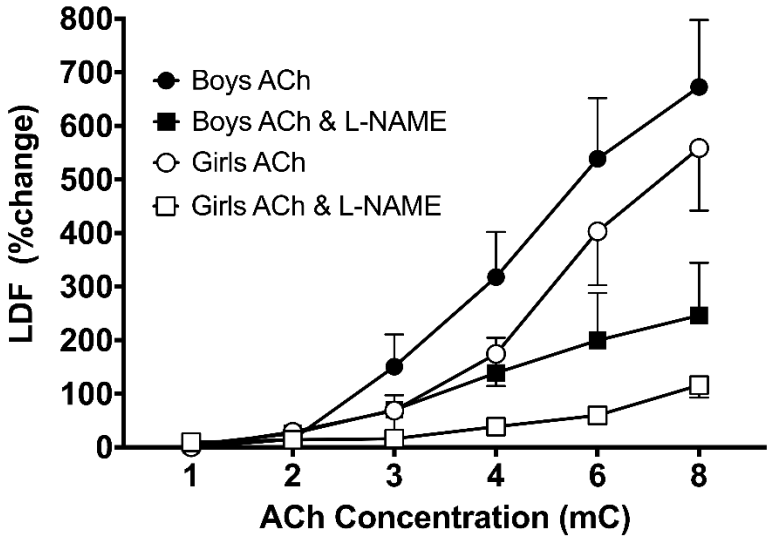


Figure 9. The percent change in PU in response to increasing concentrations in millicoulombs (mC) of acetylcholine (ACh) administration in boys and girls at untreated (control) and Nω-nitro-L-arginine methyl ester (L-NAME) treated sites. LDF = laser Doppler fluxmetry

7.0 Discussion

7.1 Summary

Laser-Doppler fluxmetry was used to examine the sex-related differences in the SkBF response to exercise, local heating, and ACh-mediated vasodilation. Additionally, the contribution of NO in these three assessments was assessed by using L-NAME iontophoresis. The SkBF (%change from baseline in PU or CVC) response to 30 minutes of exercise at 60%$\dot{V}O_2$max was greater in boys than in girls. However, there was no sex-related differences in the blunting effect of L-NAME, reflecting a similar role of NO in boys and girls. The ACh-mediated vasodilation was similar in boys compared with girls, with no differences in the role of NO. There were no sex-related differences in maximal SkBF, as examined during local heating. Note that in all conditions, MAP was similar in boys and girls, varying by 3 mmHg, at most. Therefore, the pattern of results in the SkBF and CVC were similar.

Currently, the majority of the studies that examine the sex-related differences in the response to either exercise (Gagnon & Kenny, 2011, 2012a; Notley et al., 2017), ACh-mediated vasodilation (Algotsson et al., 1995; Brar et al., 2015; Ferrell et al., 2004; Gagnon et al., 2013) or local heating (Cooke et al., 1990; Gagnon & Kenny, 2011; Hodges, Sharp, Clements, et al., 2010; Stanhewicz et al., 2014) are in adults. At present, there is only one study that examined the sex-related differences in response to ACh-mediated vasodilation in children (Khan et al., 2003), reporting a higher response to ACh in girls. There are no studies which have assessed the sex-related differences in the SkBF response to exercise or to local heating in children. Additionally, this is the first study to examine sex-related differences in the NO-dependent SkBF responses to exercise, local heating, and ACh-mediated vasodilation in children.

7.2 Exercise

When examining the sex-related differences in the response to exercise, boys had a greater increase in SkBF than girls. No previous study has examined the sex-related differences in SkBF response to exercise in children, but there are a few studies in adults (Gagnon & Kenny, 2011, 2012a; Notley et al., 2017) . Gagnon & Kenny (2011) observed that during upright cycling at either a fixed percentage (50%) of $\dot{V}O_2$max or a fixed rate of absolute metabolic heat production (500 W), there were no sex-related differences in the SkBF response. Another study conducted in the same lab (Gagnon & Kenny, 2012a), in which participants exercised at a given heat production relative to BSA (200 $W \cdot m^{-2}$, 250 $W \cdot m^{-2}$, 300 $W \cdot m^{-2}$) found similar results. Likewise, Notley, Park, Tagami, Ohnishi, & Taylor (2017) had men and women cycle at a fixed surface area-specific metabolic heat production rate (135 $W \cdot m^{-2}$ and 200 $W \cdot m^{-2}$) and found that, when matched for BSA, there were no sex-related differences in SkBF. Taken together, these studies suggest that among adults, there were no sex-related differences in the SkBF response to increasing requirements for heat dissipation.

Unlike the studies in adults, boys demonstrated a greater SkBF response during exercise compared with girls. One possible explanation may be the environmental and exercise heat stress. In the studies above, participants exercised for a longer duration (45-90 min) and at higher environmental temperatures compared with the present study (Gagnon & Kenny, 2011, 2012a; Notley et al., 2017). Thus, it is possible that these conditions required a greater SkBF response which masked any potential differences between males and females that may be observed at lower heat stress.

Additionally, the workloads were assigned differently than in the present study, where participants exercised at 60%$\dot{V}O_2$max. In one of the above studies, participants worked at 50% $\dot{V}O_2$max (Gagnon & Kenny, 2011). In the other studies participants exercised at a given metabolic heat production scaled to BSA. As such, when expressed relative to their $\dot{V}O_2$max, men exercised at lower relative intensity compared with women. For example, in the study by Notley et al. (2017), men exercised at 25.5±4.2 and 48.6±6.9% of $\dot{V}O_2$max, while women exercised at 32.2±6 and 63.8±13.4% $\dot{V}O_2$max. It is possible that at the same relative exercise intensity (%$\dot{V}O_2$max), men would have exhibited a greater SkBF response compared with women. Notley et al., (2017) demonstrated that in both men and women, those with a greater mass-specific surface area preferentially recruited cutaneous vasodilation to facilitate heat loss, while sweat-gland activation was relied upon more heavily in individuals with a lower mass-specific surface area. However, when accounting for differences in mass-specific surface area, neither the cutaneous vascular response nor the sweating response differed between men and women. In the present study, accounting for mass-specific surface area (as a covariate) did not affect the pattern of the results. That is, surface-area-to-mass ratio was not a significant covariate, and the increase in SkBF during exercise remained greater in boys than in girls.

The greater SkBF response during exercise in the boys is difficult to explain, since both groups exercised at the same relative intensity (60%$\dot{V}O_2$max) and at similar absolute intensity (~62-66 W). However, relative to LBM, boys exercised at a slightly higher (p=.056, ns) workload (2.17 vs. 1.87 $W \cdot kg^{-1}$, see Table 1). This may have resulted in greater heat production, and an associated greater need for heat dissipation.

The latter may have been achieved with a higher SkBF response. Although core temperature was not recorded, potentially greater heat production and heat dissipation is supported by the higher sweating rate in the boys.

In the present study, we found that NO played a similar role in the vasodilatory response to exercise in boys and girls. This is the first study to examine potential sex-related differences in the mechanisms involved in SkBF response during exercise in either adults or children. Welch, Foote, Hansen, & Mack (2009) reported that in adults cycling at 60%$\dot{V}O_2$max for 30 minutes, approximately ~50% of the vasodilatory response is NO-dependent. While participants included both men and women, no analysis of sex differences were conducted. McNamara, Keen, Simmons, Alexander, & Wong (2014) reported that in men exercising at 60%VO_2max, ~30% of the increase in the SkBF response is NO-dependent. This is similar to what has been shown with passive heating, where NO contributes ~30-45% to the cutaneous vasodilation response (Kellogg et al., 1998). In the present study, the NO-dependent response in the girls (~30%) appears to be similar to previous studies in adults, while the NO-dependent portion of the response in boys is somewhat lower (~16%).

7.4 Local Heating

Boys and girls had a similar increase in SkBF during local heating to 44˚C. Only one study (Gagnon & Kenny, 2011) previously assessed the sex-related differences in response to a local heating protocol of 44˚C in adults (15-min compared to 30-min in this present study), reporting no sex-related differences in the maximum CVC. Similarly, Stanhewicz, Greaney, Larry Kenney, & Alexander (2014) found no sex-related

differences in the local heating-induced vasodilatory plateau, albeit it was to 42˚C not 44˚C. On the other hand, Hodges et al., (2010) found sex-related differences in SkBF at rest and in response to local heating to 42˚C. This is the first study to assess the sex-related differences in the response to local heating in children, demonstrating that, as is the case in adults, there is no apparent difference in maximal SkBF between boys and girls.

Contrary to our findings, Cooke et al., (1990) found that both hand and finger blood flow in women were lower at rest compared with men, but following a local heating protocol to 42˚C, women exhibited greater increases. However, it should be noted that rather than measuring SkBF response in the forearm, such as in the present study, Cooke et al. assessed the response in the hand and fingers. The hand and the finger are glabrous skin sites, while the forearm is a non-glabrous skin site. Glabrous skin sites are abundant in arteriovenous anastomoses, leading to larger variability in the SkBF response in comparison to non-glabrous sites (Johnson et al., 2014). Contrary to Cooke et al., (1999) as well as to the present study, Hodges et al., (2010) showed that males had both higher resting and peak forearm blood flow when compared to women, following local heating to 42˚C. In the two previous studies, VOP was used to assess the blood flow responses. As mentioned before, the caveat of using VOP is that all tissues of the limb are assessed, not solely skin. Thus, the contradictory results may be explained by methodological differences, namely in the sites being measured (glabrous vs. non-glabrous) and in the measurement techniques (VOP vs. LDF).

This is the first study to examine the mechanisms/vasodilators involved in the SkBF response to local heating in children. The results suggest that NO did not have a

significant effect on the SkBF response in either boys or girls, which is contrary to our expectations. In adults, ~60% of the local heating response is dependent on the endothelial vasodilator NO (Kellogg et al., 1999; Minson et al., 2001), while EDHFs account for the remaining ~40% (Brunt & Minson, 2012). EDHFs were not examined in this present study. The apparent contradiction between the present study and previous studies in adults may be explained by the extended protocol in the present study. The role of NO on the local heating response was examined following exercise. This was done so that the local skin temperature (44°C) would not affect the measurement of the exercise response. Thus, the local heating response was examined about 2h after the iontophoresis of L-NAME. Experiments in our laboratory, with adults, have shown that under resting conditions L-NAME is effective for over 4h (not published). In the present study, the elevated SkBF during the preceding exercise conditions may have "washed out" much of the local L-NAME, thereby diminishing or eliminating its effect. Indeed, in 42% of the boys and 42% of the girls, examination of the raw LDF signal revealed irregularities. An example can be observed in Appendix F. Thus, the contribution of NO to the local heating SkBF response in children cannot be concluded from the present study.

7.5 ACh-mediated vasodilation

We observed a similar vasodilatory response to ACh (increase in SkBF with an increase in ACh dose) in boys and girls. In line with our results, Ferrell, Wong, Lockhart, & Ramsay (2004) assessed the influence of ACh iontophoresis in adults by examining the area under the curve and found no sex-related differences. Likewise, Gagnon,

Crandall, & Kenny (2013) reported that men and women had similar increases in SkBF in response to ACh microdialysis.

In contrast to these findings, when comparing sex-related differences in the response to ACh iontophoresis, Algotsson, Nordberg, & Winblad (1995) found that women exhibited greater increases in SkBF following ACh iontophoresis than men. This pattern was also apparent in 11-14 yr old youth as Khan et al., (2003) found that girls had a greater increase in SkBF to ACh iontophoresis than boys. Similarly, Brar et al., (2015) found that in response to ACh, both men and women had significant vasodilation, but the response was greater in women. These findings suggest that pharmacological stimuli cause a greater cutaneous vasodilation in females, (Inoue et al., 2005) which is not in-line with the present study. Thus, there is considerable variability in the literature regarding ACh-mediated vasodilation, with some studies reporting a greater response in females and some reporting no sex-related differences.

Some of the above inconsistencies may be related to the use of different methodologies. Both, Algotsson, Nordberg, & Winblad (1995) and Khan et al., (2003) used laser Doppler imaging rather than LDF in order to assess the SkBF responses and Brar et al., (2015) used VOP. Importantly, the ACh administration protocols differed among all studies. The protocol in Ferrell et al., (2004) involved incremental current delivery with four scans at 5 μA, 10 μA, 15 μA and 2 at 20 μA, resulting in a total charge of 8 mC. In Khan et al., (2003) only 3 doses were applied for 20, 40 and 80s at charges of 2, 4, 8 mC, respectively and the skin perfusion was measured for 100s between doses. Brar et al., (2015) used microdialysis to infuse 7.5, 15 and 30 $\mu mg \cdot min^{-1}$of ACh. In the present study a 2% solution of ACh was used, while in Algotsson, Nordberg, &

Winblad (1995) at 1% concentration was used and both Brar et al., (2015) and Khan et al., (2003) did not report the concentrations used. Previous work in men has shown that the mechanisms involved in the response to ACh-mediated vasodilation is dependent on the dose and the duration of the infusion (Brunt et al., 2015). Thus, it is possible that the different doses and techniques of administrations elicited different responses.

Currently, no literature exists that examine the sex-related differences in the mechanisms involved in the response to ACh vasodilation in either adults or children. In the present study, it was shown that boys and girls had similar NO-dependent responses. That is, L-NAME blunted the SkBF response to increasing ACh doses to a similar extent in the boys and girls. Brunt, Fujii, & Minson (2015) concluded that EDHFs are involved during the ACh-mediated dilation, but play a larger role only during the earlier phases of the response. Kellogg, Zhao, Coey, & Green (2005) showed that the response is only partially NO-dependent (~40%) and the majority of the remaining response was due to prostaglandins (~50%). When treated with L-NAME, both girls (~79%) and boys (~63%) displayed a significantly lower ACh-induced vasodilation, but there were no differences between groups. Other vasodilators, such as prostaglandins and EDHFs play a role in the ACh-induced vasodilation, but these were not examined in this study.

8.0 Conclusion

The current study examined the sex-related differences in the SkBF response to exercise, local heating and ACh-mediated vasodilation. As expected, maximal SkBF response (local heating to 44°C) and ACh-induced vasodilation was similar in boys and girls. This was not the case during exercise, where the SkBF response was higher in boys. The boys' higher response to exercise may be related to a greater heat production. That is, while absolute (W) and relative (% of $\dot{V}O_2$max) intensity was not different between the boys and girls, the workload per LBM was somewhat higher in the boys, although not statistically significant.

Nitric oxide appears to play a similar role in boys and girls. L-NAME iontophoresis, an inhibitor of NOS, resulted in a blunted SkBF response in both boys and girls during ACh-mediated vasodilation, as well as during exercise. The effect of L-NAME was similar in boys and girls, suggesting that the mechanism of cutaneous vasodilation is similar in boys and girls.

9.0 Strength, Limitations and Future Directions

9.1 Strengths

Careful consideration was given to the type of instrumentation and the design of the study. A SCRE was chosen to stabilize the participants in order to limit movement artifacts, but still provide a systemic heat stress. The two groups in this study were similar and homogenous in nature, with similar age, maturity level, body size, and physical activity level, although they were different in body composition. Importantly, they were of similar aerobic fitness level (absolute $\dot{V}O_2$max). Therefore, by design, when exercising at the same relative (%) $\dot{V}O_2$max, they also worked at the same absolute workload. Additionally, SkBF responses were assessed in CVC, which allows to control for the individual differences in blood pressure. Lastly, beyond the magnitude of the SkBF response, the NO contribution to the SkBF response to various stimuli (exercise, local heating and ACh-mediated vasodilation) was also examined.

9.2 Limitations

One of the main limitations was that core body temperature was not measured. Absolute (and relative) workload was similar in the two groups. Therefore, it was assumed that heat production was also similar. Nevertheless, it is possible that the change in body temperature was not the same in the two groups. In women, core temperature is known to differ between stages of the menstrual cycle, due to changes in estrogen and progesterone (Charkoudian et al., 1999). Although all girls in the present study self-identified as pre- or early-pubertal, it is possible that estrogen and progesterone were already beginning to fluctuate. Hormonal levels were not measured or controlled for in the present study.

NO activity was examined using a NOS inhibitor known as L-NAME. Beyond the magnitude of the SkBF response to various stimuli, we also attempted to get insight at

the responsible mechanisms. In this study, NO was the only mechanism that was assessed. However, it is possible that other vasodilators, such as prostaglandins and EDHFs, contribute to the SkBF response and may explain potential sex-related differences. Additionally, since L-NAME was administered via iontophoresis, NO dilatory effects in the smooth muscles of the vasculature were not specifically measured. Additionally, an iontophoretic current was not applied at the control site (i.e., “sham-control”). However, it should be noted that LDF measurements were performed 90 min following iontophoresis. Pilot work and prior study (Hodges et al., 2017) has suggested that in response to exercise, local heating and acetylcholine application, the skin sites are not affected by the current application. Further studies, should consider applying a similar iontophoresis protocol (charge and duration) to the control site in order to account for the possibility of any skin perturbations. Finally, it is possible that due to the extended protocol and the preceding exercise-related increase in SkBF, L-NAME was no longer effective during local heating.

9.3 Future Directions

In addition to considering the limitations in this present study, future studies should consider additional steps which will further our understanding of sex-related differences in cutaneous reactivity. First, future studies should consider other vasodilators, in addition to NO, such as prostaglandins and EDHFs that may impact the SkBF response to the stimuli explored in this study. This may elucidate some of the sex-related differences in the SkBF response observed in this present study. Second, the responses to these stimuli should be explored in various populations. There is very limited work examining the SkBF responses and mechanisms in children and even less work focusing on the sex-related differences in children. By exploring these responses

in children of varying ages/maturity levels it may help to explain whether the sex-related differences shown in adults is inherent or develops during maturation.

Another possible future direction is to examine populations of various chronic conditions. For example, obesity is known to have a negative impact on the vasculature. Changes in the microvascular circulation are known to occur prior to changes in the macrovascular circulation. Therefore, determining the SkBF response in children with obesity could provide a basis for early intervention to combat the negative effects of adiposity.

References:

Alexander, C. M., Kasza, I., Yen, C.-L. E., Reeder, S. B., Hernando, D., Gallo, R. L., Jahoda, C. A. B., Horsley, V., & MacDougald, O. A. (2015). Dermal white adipose tissue: A new component of the thermogenic response. *Journal of Lipid Research*, *56*(11), 2061–2069. https://doi.org/10.1194/jlr.R062893

Algotsson, A., Nordberg, A., & Winblad, B. (1995). Influence of Age and Gender on Skin Vessel Reactivity to Endothelium-Dependent and Endothelium- Independent Vasodilators Tested With Iontophoresis and a Laser Doppler Perfusion Imager. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *50A*(2), M121–M127. https://doi.org/10.1093/gerona/50A.2.M121

Anderson, C., Andersson, T., & Wårdell, K. (1994). Changes in Skin Circulation After Insertion of a Microdialysis Probe Visualized by Laser Doppler Perfusion Imaging. *Journal of Investigative Dermatology*, *102*(5), 807–811. https://doi.org/10.1111/1523-1747.ep12378630

Baboshina, N. V. (2018). Parameters of Microcirculation in Both Sexes at Different Ages. *Human Physiology*, *44*(4), 466–473. https://doi.org/10.1134/S0362119718010048

Barcroft, H., & Edholm, O. G. (1943). The effect of temperature on blood flow and deep temperature in the human forearm. *The Journal of Physiology*, *102*(1), 5–20. https://doi.org/10.1113/jphysiol.1943.sp004009

Barker, A. R., Williams, C. A., Jones, A. M., & Armstrong, N. (2011). Establishing maximal oxygen uptake in young people during a ramp cycle test to exhaustion. *British Journal of Sports Medicine*, *45*(6), 498–503. https://doi.org/10.1136/bjsm.2009.063180

Benzinger, T. H. (1963). Peripheral cold- and central warm-reception, main origins of human thermal discomfort. *Proceedings of the National Academy of Sciences of the United States of America*, *49*, 832–839.

Binggeli, C., Spieker, L. E., Corti, R., Sudano, I., Stojanovic, V., Hayoz, D., Lüscher, T. F., & Noll, G. (2003). Statins enhance postischemic hyperemia in the skin circulation of hypercholesterolemic patients: A monitoring test of endothelial dysfunction for clinical practice? *Journal of the American College of Cardiology*, *42*(1), 71–77.

Black, M. A., Green, D. J., & Cable, N. T. (2008). Exercise prevents age-related decline in nitric-oxide-mediated vasodilator function in cutaneous microvessels: Nitric oxide, ageing and exercise. *The Journal of Physiology*, *586*(14), 3511–3524. https://doi.org/10.1113/jphysiol.2008.153742

Boegehold, M. A. (2010). Endothelium-Dependent Control of Vascular Tone during Early Postnatal and Juvenile Growth. *Microcirculation*. https://doi.org/10.1111/j.1549-8719.2010.00035.x

Borg, G. A. (1973). Perceived exertion: A note on "history" and methods. *Medicine and Science in Sports*, *5*(2), 90–93.

Brar, V., Gill, S., Cardillo, C., Tesauro, M., Panza, J. A., & Campia, U. (2015). Sex-Specific Effects of Cardiovascular Risk Factors on Endothelium-Dependent Dilation and Endothelin Activity in Middle-Aged Women and Men. *PLOS ONE*, *10*(3), e0121810. https://doi.org/10.1371/journal.pone.0121810

Brien, E. K., Bar-Or, O., Iwata, M., Timmons, B. W., & Wilk, B. (2011). Influence of Pubertal Development on Post-Exercise Forearm Blood Flow of Girls Exercising

in the Heat. *Medicina Sportiva*, *15*(1), 1–5. https://doi.org/10.2478/v10036-011-0003-3

Brunt, V. E., Fujii, N., & Minson, C. T. (2015). Endothelial-derived hyperpolarization contributes to acetylcholine-mediated vasodilation in human skin in a dose-dependent manner. *Journal of Applied Physiology*, *119*(9), 1015–1022. https://doi.org/10.1152/japplphysiol.00201.2015

Brunt, V. E., & Minson, C. T. (2012). KCa channels and epoxyeicosatrienoic acids: Major contributors to thermal hyperaemia in human skin: KCa channels and EETs in cutaneous local heating. *The Journal of Physiology*, *590*(15), 3523–3534. https://doi.org/10.1113/jphysiol.2012.236398

Casey, D. P., Walker, B. G., Ranadive, S. M., Taylor, J. L., & Joyner, M. J. (2013). Contribution of nitric oxide in the contraction-induced rapid vasodilation in young and older adults. *Journal of Applied Physiology*, *115*(4), 446–455. https://doi.org/10.1152/japplphysiol.00446.2013

Charkoudian, N., & Johnson, J. M. (2000). Female reproductive hormones and thermoregulatory control of skin blood flow. *Exercise and Sport Sciences Reviews*, *28*(3), 108–112.

Charkoudian, Nisha, Stephens, D. P., Pirkle, K. C., Kosiba, W. A., & Johnson, J. M. (1999). Influence of female reproductive hormones on local thermal control of skin blood flow. *Journal of Applied Physiology*, *87*(5), 1719–1723. https://doi.org/10.1152/jappl.1999.87.5.1719

Christensen, E. H., Nielsen, M., & Hannisdahl., B. (1942). Investigations of the Circulation in the Skin at the Beginning of Muscular Work. *Acta Physiologica Scandinavica*, *4*(2), 162–170. https://doi.org/10.1111/j.1748-1716.1942.tb01451.x

Clough, G. F. (1999). Role of nitric oxide in the regulation of microvascular perfusion in human skin in vivo. *The Journal of Physiology*, *516 (Pt 2)*, 549–557.

Colberg, S. R., Parson, H. K., Nunnold, T., Holton, D. R., Swain, D. P., & Vinik, A. I. (2005). Change in cutaneous perfusion following 10 weeks of aerobic training in Type 2 diabetes. *Journal of Diabetes and Its Complications*, *19*(5), 276–283. https://doi.org/10.1016/j.jdiacomp.2005.02.006

Cooke, J. P., Creager, M. A., Osmundson, P. J., & Shepherd, J. T. (1990). Sex differences in control of cutaneous blood flow. *Circulation*, *82*(5), 1607–1615. https://doi.org/10.1161/01.CIR.82.5.1607

Cracowski, J.-L., Minson, C. T., Salvat-Melis, M., & Halliwill, J. R. (2006). Methodological issues in the assessment of skin microvascular endothelial function in humans. *Trends in Pharmacological Sciences*, *27*(9), 503–508. https://doi.org/10.1016/j.tips.2006.07.008

Davies, C. T. M. (1979). Thermoregulation during exercise in relation to sex and age. *European Journal of Applied Physiology and Occupational Physiology*, *42*(2), 71–79. https://doi.org/10.1007/BF00421907

Deanfield, J. E., Halcox, J. P., & Rabelink, T. J. (2007). Endothelial Function and Dysfunction: Testing and Clinical Relevance. *Circulation*, *115*(10), 1285–1295. https://doi.org/10.1161/CIRCULATIONAHA.106.652859

Del Pozzi, A. T., & Hodges, G. J. (2015). Comparison of the noradrenergic sympathetic nerve contribution during local skin heating at forearm and leg sites in humans. *European Journal of Applied Physiology*, *115*(5), 1155–1164. https://doi.org/10.1007/s00421-014-3097-1

Del Pozzi, A. T., Miller, J. T., & Hodges, G. J. (2016). The effect of heating rate on the cutaneous vasomotion responses of forearm and leg skin in humans. *Microvascular Research*, *105*, 77–84. https://doi.org/10.1016/j.mvr.2016.01.004

Drinkwater, B. L., Kupprat, I. C., Denton, J. E., Crist, J. L., & Horvath, S. M. (1977). Response of prepubertal girls and college women to work in the heat. *Journal of Applied Physiology*, *43*(6), 1046–1053. https://doi.org/10.1152/jappl.1977.43.6.1046

Du Bois, D. (1916). Clinical Calorimetry: Tenth paper for a formula to estimate the approximate surface area if height and weight be known. *Archives of Internal Medicine*, *XVII*(6_2), 863. https://doi.org/10.1001/archinte.1916.00080130010002

Duprez, D. A., Somasundaram, P. E., Sigurdsson, G., Hoke, L., Florea, N., & Cohn, J. N. (2005). Relationship between C-reactive protein and arterial stiffness in an asymptomatic population. *Journal of Human Hypertension*, *19*(7), 515–519. https://doi.org/10.1038/sj.jhh.1001860

Falk, B., Bar-Or, O., Calvert, R., & MacDougall, J. D. (1992). Sweat gland response to exercise in the heat among pre-, mid-, and late-pubertal boys. *Medicine and Science in Sports and Exercise*, *24*(3), 313–319.

Falk, B, & Dotan, R. (2008). Children's thermoregulation during exercise in the heat—A revisit. *Applied Physiology, Nutrition, and Metabolism*, *33*(2), 420–427. https://doi.org/10.1139/H07-185

Ferrell, W. R., Wong, B. B., Lockhart, J. C., & Ramsay, J. E. (2004). Gender differences in regional cutaneous microcirculatory responses to capsaicin. *Fundamental and Clinical Pharmacology*, *18*(2), 195–200. https://doi.org/10.1111/j.1472-8206.2004.00218.x

Fleming, I. (2010). Molecular mechanisms underlying the activation of eNOS. *Pflügers Archiv - European Journal of Physiology*, *459*(6), 793–806. https://doi.org/10.1007/s00424-009-0767-7

Gagge, A. P., Stolwijk, J. A. J., & Saltin, B. (1969). Comfort and thermal sensations and associated physiological responses during exercise at various ambient temperatures. *Environmental Research*, *2*(3), 209–229. https://doi.org/10.1016/0013-9351(69)90037-1

Gagnon, D., Crandall, C. G., & Kenny, G. P. (2013). Sex differences in postsynaptic sweating and cutaneous vasodilation. *Journal of Applied Physiology*, *114*(3), 394–401. https://doi.org/10.1152/japplphysiol.00877.2012

Gagnon, D., & Kenny, G. P. (2011). Sex modulates whole-body sudomotor thermosensitivity during exercise: Sex differences in temperature regulation. *The Journal of Physiology*, *589*(24), 6205–6217. https://doi.org/10.1113/jphysiol.2011.219220

Gagnon, D., & Kenny, G. P. (2012a). Sex differences in thermoeffector responses during exercise at fixed requirements for heat loss. *Journal of Applied Physiology*, *113*(5), 746–757. https://doi.org/10.1152/japplphysiol.00637.2012

Gagnon, D., & Kenny, G. P. (2012b). Does sex have an independent effect on thermoeffector responses during exercise in the heat?: Sex differences in human temperature regulation. *The Journal of Physiology*, *590*(23), 5963–5973. https://doi.org/10.1113/jphysiol.2012.240739

Godin, G., & Shephard, R. J. (1985). A simple method to assess exercise behavior in the community. *Canadian Journal of Applied Sport Sciences. Journal Canadien Des Sciences Appliquees Au Sport*, *10*(3), 141–146.

Greenfield, A. D. (1960). Venous occlusion plethysmography. *Methods in Medical Research*, *8*, 293–301.

Havenith, G., Luttikholt, V. G. M., & Vrijkotte, T. G. M. (1995). The relative influence of body characteristics on humid heat stress response. *European Journal of Applied Physiology and Occupational Physiology*, *70*(3), 270–279. https://doi.org/10.1007/BF00238575

Hedvall Kallerman, P., Hagman, E., Edstedt Bonamy, A.-K., Zemack, H., Marcus, C., Norman, M., & Westerståhl, M. (2014). Obese children without comorbidities have impaired microvascular endothelial function. *Acta Paediatrica*, *103*(4), 411–417. https://doi.org/10.1111/apa.12549

Hodges, G. J., Chiu, C., Kosiba, W. A., Zhao, K., & Johnson, J. M. (2009). The effect of microdialysis needle trauma on cutaneous vascular responses in humans.

Journal of Applied Physiology, *106*(4), 1112–1118. https://doi.org/10.1152/japplphysiol.91508.2008

Hodges, G. J., Kosiba, W. A., Zhao, K., & Johnson, J. M. (2009). The involvement of heating rate and vasoconstrictor nerves in the cutaneous vasodilator response to skin warming. *American Journal of Physiology-Heart and Circulatory Physiology*, *296*(1), H51–H56. https://doi.org/10.1152/ajpheart.00919.2008

Hodges, G. J., Mueller, M. C., Cheung, S. S., & Falk, B. (2018). Cutaneous vasomotor responses in boys and men. *Applied Physiology, Nutrition, and Metabolism*, *43*(10), 1019–1026. https://doi.org/10.1139/apnm-2018-0083

Hodges, G. J., Sharp, L., Clements, R. E., Goldspink, D. F., George, K. P., & Cable, N. T. (2010). Influence of age, sex, and aerobic capacity on forearm and skin blood flow and vascular conductance. *European Journal of Applied Physiology*, *109*(6), 1009–1015. https://doi.org/10.1007/s00421-010-1441-7

Hodges, G. J., Sharp, L., Stephenson, C., Patwala, A. Y., George, K. P., Goldspink, D. F., & Tim Cable, N. (2010). The effect of 48 weeks of aerobic exercise training on cutaneous vasodilator function in post-menopausal females. *European Journal of Applied Physiology*, *108*(6), 1259–1267. https://doi.org/10.1007/s00421-009-1330-0

Hodges, G. J., Stewart, D. G., Davison, P. J., & Cheung, S. S. (2017). The role of shear stress on cutaneous microvascular endothelial function in humans. *European Journal of Applied Physiology*, *117*(12), 2457–2468. https://doi.org/10.1007/s00421-017-3732-8

Holowatz, L. A., & Kenney, W. L. (2009). Chronic low-dose aspirin therapy attenuates reflex cutaneous vasodilation in middle-aged humans. *Journal of Applied Physiology*, *106*(2), 500–505. https://doi.org/10.1152/japplphysiol.91215.2008

Holowatz, L. A., Thompson-Torgerson, C. S., & Kenney, W. L. (2008). The human cutaneous circulation as a model of generalized microvascular function. *Journal of Applied Physiology*, *105*(1), 370–372. https://doi.org/10.1152/japplphysiol.00858.2007

Houghton, B. L., Holowatz, L. A., & Minson, C. T. (2005). Influence of Progestin Bioactivity on Cutaneous Vascular Responses to Passive Heating: *Medicine & Science in Sports & Exercise*, *37*(1), 45–51. https://doi.org/10.1249/01.MSS.0000150075.81511.FE

Ichinose-Kuwahara, T., Inoue, Y., Iseki, Y., Hara, S., Ogura, Y., & Kondo, N. (2010). Experimental Physiology - *Research Paper*: Sex differences in the effects of physical training on sweat gland responses during a graded exercise: Sex differences in sweating. *Experimental Physiology*, *95*(10), 1026–1032. https://doi.org/10.1113/expphysiol.2010.053710

Inoue, Y., Tanaka, Y., Omori, K., Kuwahara, T., Ogura, Y., & Ueda, H. (2005). Sex- and menstrual cycle-related differences in sweating and cutaneous blood flow in response to passive heat exposure. *European Journal of Applied Physiology*, *94*(3), 323–332. https://doi.org/10.1007/s00421-004-1303-2

Iredahl, F., Löfberg, A., Sjöberg, F., Farnebo, S., & Tesselaar, E. (2015). Non-Invasive Measurement of Skin Microvascular Response during Pharmacological and

Physiological Provocations. *PLOS ONE*, *10*(8), e0133760. https://doi.org/10.1371/journal.pone.0133760

Iredahl, F., Tesselaar, E., Sarker, S., Farnebo, S., & Sjöberg, F. (2013). The microvascular response to transdermal iontophoresis of insulin is mediated by nitric oxide. *Microcirculation*, n/a-n/a. https://doi.org/10.1111/micc.12071

Johnson, J. M., & Kellogg, D. L. (2010). Local thermal control of the human cutaneous circulation. *Journal of Applied Physiology*, *109*(4), 1229–1238. https://doi.org/10.1152/japplphysiol.00407.2010

Johnson, J. M., Minson, C. T., & Kellogg, D. L. (2014). Cutaneous Vasodilator and Vasoconstrictor Mechanisms in Temperature Regulation. In R. Terjung (Ed.), *Comprehensive Physiology* (pp. 33–89). John Wiley & Sons, Inc. https://doi.org/10.1002/cphy.c130015

Kalia, Y. N., Naik, A., Garrison, J., & Guy, R. H. (2004). Iontophoretic drug delivery. *Advanced Drug Delivery Reviews*, *56*(5), 619–658. https://doi.org/10.1016/j.addr.2003.10.026

Kellogg, D. L., Crandall, C. G., Liu, Y., Charkoudian, N., & Johnson, J. M. (1998). Nitric oxide and cutaneous active vasodilation during heat stress in humans. *Journal of Applied Physiology*, *85*(3), 824–829. https://doi.org/10.1152/jappl.1998.85.3.824

Kellogg, D. L., Liu, Y., Kosiba, I. F., & O'Donnell, D. (1999). Role of nitric oxide in the vascular effects of local warming of the skin in humans. *Journal of Applied Physiology*, *86*(4), 1185–1190. https://doi.org/10.1152/jappl.1999.86.4.1185

Kellogg, D. L., Zhao, J. L., Coey, U., & Green, J. V. (2005). Acetylcholine-induced vasodilation is mediated by nitric oxide and prostaglandins in human skin.

Journal of Applied Physiology, *98*(2), 629–632. https://doi.org/10.1152/japplphysiol.00728.2004

Kellogg, Dean L., Zhao, J. L., & Wu, Y. (2008). Endothelial nitric oxide synthase control mechanisms in the cutaneous vasculature of humans in vivo. *American Journal of Physiology-Heart and Circulatory Physiology*, *295*(1), H123–H129. https://doi.org/10.1152/ajpheart.00082.2008

Khan, F., Green, F. C., Forsyth, J. S., Greene, S. A., Morris, A. D., & Belch, J. J. F. (2003). Impaired Microvascular Function in Normal Children: Effects of Adiposity and Poor Glucose Handling. *The Journal of Physiology*, *551*(2), 705–711. https://doi.org/10.1113/jphysiol.2003.045351

Kopincová, J., Púzserová, A., & Bernátová, I. (2012). L-NAME in the cardiovascular system – nitric oxide synthase activator? *Pharmacological Reports*, *64*(3), 511–520. https://doi.org/10.1016/S1734-1140(12)70846-0

Kvandal, P., Landsverk, S. A., Bernjak, A., Stefanovska, A., Kvernmo, H. D., & Kirkebøen, K. A. (2006). Low-frequency oscillations of the laser Doppler perfusion signal in human skin. *Microvascular Research*, *72*(3), 120–127. https://doi.org/10.1016/j.mvr.2006.05.006

Lenasi, H. (2010). Assessment of Human Skin Microcirculation and Its Endothelial Function Using Laser Doppler Flowmetry. *Medical Imaging*, 28.

Lenasi, H. (2011. *Regular physical activity alters the postocclusive reactive hyperemia of the cutaneous microcirculation*. 11.

Lorenzo, S., & Minson, C. T. (2007). Human cutaneous reactive hyperaemia: Role of BK_{Ca} channels and sensory nerves: Mechanisms of reactive hyperaemia in skin.

The Journal of Physiology, *585*(1), 295–303. https://doi.org/10.1113/jphysiol.2007.143867

Mallette, M. M., Hodges, G. J., McGarr, G. W., Gabriel, D. A., & Cheung, S. S. (2016). Investigating the roles of core and local temperature on forearm skin blood flow. *Microvascular Research*, *106*, 88–95. https://doi.org/10.1016/j.mvr.2016.03.010

Martin, H. L., Loomis, J. L., & Kenney, W. L. (1995). Maximal skin vascular conductance in subjects aged 5-85 yr. *Journal of Applied Physiology*, *79*(1), 297–301. https://doi.org/10.1152/jappl.1995.79.1.297

McCord, G. R., Cracowski, J.-L., & Minson, C. T. (2006). Prostanoids contribute to cutaneous active vasodilation in humans. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *291*(3), R596–R602. https://doi.org/10.1152/ajpregu.00710.2005

McNamara, T. C., Keen, J. T., Simmons, G. H., Alexander, L. M., & Wong, B. J. (2014). Endothelial nitric oxide synthase mediates the nitric oxide component of reflex cutaneous vasodilatation during dynamic exercise in humans: Skin blood flow and dynamic exercise. *The Journal of Physiology*, *592*(23), 5317–5326. https://doi.org/10.1113/jphysiol.2014.272898

Medow, M. S., Minson, C. T., & Stewart, J. M. (2005). Decreased microvascular nitric oxide-dependent vasodilation in postural tachycardia syndrome. *Circulation*, *112*(17), 2611–2618. https://doi.org/10.1161/CIRCULATIONAHA.104.526764

Medow, M. S., Taneja, I., & Stewart, J. M. (2007). Cyclooxygenase and nitric oxide synthase dependence of cutaneous reactive hyperemia in humans. *American*

Journal of Physiology. Heart and Circulatory Physiology, *293*(1), H425-432. https://doi.org/10.1152/ajpheart.01217.2006

Minson, C. T., Berry, L. T., & Joyner, M. J. (2001). Nitric oxide and neurally mediated regulation of skin blood flow during local heating. *Journal of Applied Physiology*, *91*(4), 1619–1626. https://doi.org/10.1152/jappl.2001.91.4.1619

Mirwald, R. L., Baxter-Jones, A. D. G., Bailey, D. A., & Beunen, G. P. (2002). An assessment of maturity from anthropometric measurements. *Medicine and Science in Sports and Exercise*, *34*(4), 689–694.

Mortensen, S. P., González-Alonso, J., Damsgaard, R., Saltin, B., & Hellsten, Y. (2007). Inhibition of nitric oxide and prostaglandins, but not endothelial-derived hyperpolarizing factors, reduces blood flow and aerobic energy turnover in the exercising human leg: Effect of triple blockade on exercise hyperaemia. *The Journal of Physiology*, *581*(2), 853–861. https://doi.org/10.1113/jphysiol.2006.127423

Notley, S. R., Park, J., Tagami, K., Ohnishi, N., & Taylor, N. A. S. (2017). Variations in body morphology explain sex differences in thermoeffector function during compensable heat stress. *Experimental Physiology*, *102*(5), 545–562. https://doi.org/10.1113/EP086112

Oberg, P. A. (1990). Laser-Doppler flowmetry. *Critical Reviews in Biomedical Engineering*, *18*(2), 125–163.

Radtke, T., Khattab, K., Eser, P., Kriemler, S., Saner, H., & Wilhelm, M. (2012). Puberty and Microvascular Function in Healthy Children and Adolescents. *The Journal of Pediatrics*, *161*(5), 887-891.e1. https://doi.org/10.1016/j.jpeds.2012.04.033

Ramanathan, N. L. (1964). A new weighting system for mean surface temperature of the human body. *Journal of Applied Physiology*, *19*, 531–533. https://doi.org/10.1152/jappl.1964.19.3.531

Ramsay, J. E., Ferrell, W. R., Greer, I. A., & Sattar, N. (2002). Factors Critical to Iontophoretic Assessment of Vascular Reactivity: Implications for Clinical Studies of Endothelial Dysfunction: *Journal of Cardiovascular Pharmacology*, *39*(1), 9–17. https://doi.org/10.1097/00005344-200201000-00002

Rees, J., & Shuster, S. (1981). Pubertal Induction of Sweat Gland Activity. *Clinical Science*, *60*(6), 689–692. https://doi.org/10.1042/cs0600689

Rivera-Brown, A., Rowland, T., Ramírez-Marrero, F., Santacana, G., & Vann, A. (2006). Exercise Tolerance in a Hot and Humid Climate in Heat-Acclimatized Girls and Women. *International Journal of Sports Medicine*, *27*(12), 943–950. https://doi.org/10.1055/s-2006-923863

Roche, D. M., Rowland, T. W., Garrard, M., Marwood, S., & Unnithan, V. B. (2010). Skin microvascular reactivity in trained adolescents. *European Journal of Applied Physiology*, *108*(6), 1201–1208. https://doi.org/10.1007/s00421-009-1328-7

Rowell, L. B. (1974). Human cardiovascular adjustments to exercise and thermal stress. *Physiological Reviews*, *54*(1), 75–159. https://doi.org/10.1152/physrev.1974.54.1.75

Rowland, T. (2008). Thermoregulation during exercise in the heat in children: Old concepts revisited. *Journal of Applied Physiology*, *105*(2), 718–724. https://doi.org/10.1152/japplphysiol.01196.2007

Rowland, T., Hagenbuch, S., Pober, D., & Garrison, A. (2008). Exercise Tolerance and Thermoregulatory Responses during Cycling in Boys and Men: *Medicine & Science in Sports & Exercise*, *40*(2), 282–287. https://doi.org/10.1249/mss.0b013e31815a95a7

Sansum, K. M., Weston, M. E., Bond, B., Cockcroft, E. J., O'Connor, A., Tomlinson, O. W., Williams, C. A., & Barker, A. R. (2019). Validity of the Supramaximal Test to Verify Maximal Oxygen Uptake in Children and Adolescents. *Pediatric Exercise Science*, *31*(2), 213–222. https://doi.org/10.1123/pes.2018-0129

Saumet, J. L., Kellogg, D. L., Taylor, W. F., & Johnson, J. M. (1988). Cutaneous laser-Doppler flowmetry: Influence of underlying muscle blood flow. *Journal of Applied Physiology*, *65*(1), 478–481. https://doi.org/10.1152/jappl.1988.65.1.478

Schank, B. J., Acree, L. S., Longfors, J., & Gardner, A. W. (2007). Differences in Vascular Reactivity Between Men and Women. *Angiology*, *57*(6), 702–708. https://doi.org/10.1177/0003319706295474

Schlager, O., Giurgea, A., Hammer, A., Charwat-Resl, S., Margeta, C., Mueller, M., Ehringer, T., Zehetmayer, S., Willfort-Ehringer, A., Koppensteiner, R., & Gschwandtner, M. E. (2014). Impact of age and gender on microvascular function. *European Journal of Clinical Investigation*, *44*(8), 766–774. https://doi.org/10.1111/eci.12293

Shapiro, Y., Pandolf, K. B., Avellini, B. A., Pimental, N. A., & Goldman, R. F. (1980). Physiological responses of men and women to humid and dry heat. *Journal of Applied Physiology*, *49*(1), 1–8. https://doi.org/10.1152/jappl.1980.49.1.1

Slaughter, M. H., Lohman, T. G., Boileau, R. A., Horswill, C. A., Stillman, R. J., Van Loan, M. D., & Bemben, D. A. (1988). Skinfold equations for estimation of body fatness in children and youth. *Human Biology*, *60*(5), 709–723.

Stanhewicz, A. E., Greaney, J. L., Larry Kenney, W., & Alexander, L. M. (2014). Sex- and limb-specific differences in the nitric oxide-dependent cutaneous vasodilation in response to local heating. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *307*(7), R914–R919. https://doi.org/10.1152/ajpregu.00269.2014

Stephenson, L. A., & Kolka, M. A. (1985). Menstrual cycle phase and time of day alter reference signal controlling arm blood flow and sweating. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *249*(2), R186–R191. https://doi.org/10.1152/ajpregu.1985.249.2.R186

Tanner, J. M. (1962). *Growth at adolescence: With a general consideration of the effects of hereditary and environmental factors upon growth and maturation from birth to maturity* (2nd ed.). Oxford, Blackwell Scientific Publications.

Taylor, W. F., Johnson, J. M., O'Leary, D. S., & Park, M. K. (1984). Modification of the cutaneous vascular response to exercise by local skin temperature. *Journal of Applied Physiology*, *57*(6), 1878–1884. https://doi.org/10.1152/jappl.1984.57.6.1878

Tew, G. A., Saxton, J. M., Klonizakis, M., Moss, J., Ruddock, A. D., & Hodges, G. J. (2011). Aging and aerobic fitness affect the contribution of noradrenergic sympathetic nerves to the rapid cutaneous vasodilator response to local heating.

Journal of Applied Physiology, *110*(5), 1264–1270. https://doi.org/10.1152/japplphysiol.01423.2010

Wang, D. H., & Prewitt, R. L. (1991). Microvascular development during normal growth and reduced blood flow: Introduction of a new model. *American Journal of Physiology-Heart and Circulatory Physiology*, *260*(6), H1966–H1972. https://doi.org/10.1152/ajpheart.1991.260.6.H1966

Welch, G., Foote, K. M., Hansen, C., & Mack, G. W. (2009). Nonselective NOS inhibition blunts the sweat response to exercise in a warm environment. *Journal of Applied Physiology*, *106*(3), 796–803. https://doi.org/10.1152/japplphysiol.90809.2008

Whitney, R. J. (1953). The measurement of volume changes in human limbs. *The Journal of Physiology*, *121*(1), 1–27.

Wilkinson, I. B., & Webb, D. J. (2002). Venous occlusion plethysmography in cardiovascular research: Methodology and clinical applications: Venous occlusion plethysmography. *British Journal of Clinical Pharmacology*, *52*(6), 631–646. https://doi.org/10.1046/j.0306-5251.2001.01495.x

Woloschuk, A., Hodges, G. J., Massarotto, R. J., Klentrou, P., & Falk, B. (2019). The skin blood flow response to exercise in boys and men and the role of nitric oxide. *European Journal of Applied Physiology*. https://doi.org/10.1007/s00421-019-04286-4

Wong, B. J., Wilkins, B. W., Holowatz, L. A., & Minson, C. T. (2003). Nitric oxide synthase inhibition does not alter the reactive hyperemic response in the

cutaneous circulation. *Journal of Applied Physiology (Bethesda, Md.: 1985)*, *95*(2), 504–510. https://doi.org/10.1152/japplphysiol.00254.2003

Yvonne-Tee, G. B., Rasool, A. H. G., Halim, A. S., Wong, A. R., & Rahman, A. R. A. (2008). Method optimization on the use of postocclusive hyperemia model to assess microvascular function. *Clinical Hemorheology and Microcirculation*, *38*(2), 119–133.

Zeiher, A. M., Drexler, H., Saurbier, B., & Just, H. (1993). Endothelium-mediated coronary blood flow modulation in humans. Effects of age, atherosclerosis, hypercholesterolemia, and hypertension. *Journal of Clinical Investigation*, *92*(2), 652–662. https://doi.org/10.1172/JCI116634

Zhao, J. L., Pergola, P. E., Roman, L. J., & Kellogg, D. L. (2004). Bioactive nitric oxide concentration does not increase during reactive hyperemia in human skin. *Journal of Applied Physiology (Bethesda, Md.: 1985)*, *96*(2), 628–632. https://doi.org/10.1152/japplphysiol.00639.2003

Appendices

Appendix A: Participant Screening and Medical Health Questionnaire

Your responses to this questionnaire are confidential and you are asked to complete it for your own health and safety. If you answer “YES” to any of the following questions, please give additional details in the space provided and discuss the matter with one of the investigators.

1. Have you ever been told that you have a heart problem?

YES NO

2. Have you ever been told that you sometimes experience seizures?

YES NO

3. Have you ever had any major joint instability or ongoing chronic pain such as in the knee, back or elbow?

YES NO

4. Have you had any allergies to medication?

YES NO

5. Have you had any allergies to food or environmental factors?

YES NO

6. Have you had any stomach problems such as ulcers?

YES NO

7. When you experience a cut do you take a long time to stop bleeding?

YES NO

8. When you receive a blow to a muscle do you develop bruises easily?

YES NO

9. Are you currently taking any medication (including aspirin) or have you taken any medication in the last two days?

YES NO

10. Have been diagnosed by a physician with cardiovascular disease (e.g. atheroscolsis, high blood pressure)?

YES NO

11. Have been diagnosed by a physician with respiratory issues (e.g. asthma, bronchitis)?

YES NO

12. Have been diagnosed by a physician with neuromuscular disease (e.g. multiple sclerosis, mysasthenia gravis)?

YES NO

13. Have been diagnosed by a physician with metabolic disease (e.g. diabetes, kidney or liver problems)?

YES NO

Appendix B: Godin-Shephard Leisure Time Exercise Questionnaire

GODIN-SHEPHARD Leisure-Time Exercise Questionnaire

1. During a typical **7-Day period** (a week), how many times on the average do you do the following kinds of exercise for **more than 15 minutes** during your free time?

a) STRENUOUS EXERCISE
(HEART BEATS RAPIDLY)
(e.g., running, jogging, hockey, football, soccer, squash, basketball, cross country skiing, judo, roller skating, vigorous swimming, vigorous long distance bicycling)

Times per Week _____

b) MODERATE EXERCISE
(NOT EXHAUSTING)
(e.g., fast walking, baseball, tennis, easy bicycling, volleyball, badminton, easy swimming, alpine skiing, popular and folk dancing)

Times per Week _____

c) MILD EXERCISE
(MINIMAL EFFORT)
(e.g., yoga, archery, fishing from river bank, bowling, horseshoes, golf, snowmobiling, easy walking)

Times per Week _____

2. During a typical **7-Day period** (a week), in your leisure time, how often do you engage in any regular activity long enough to work up a sweat (heart beats rapidly)?

- OFTEN
- SOMETIMES
- NEVER/RARELY

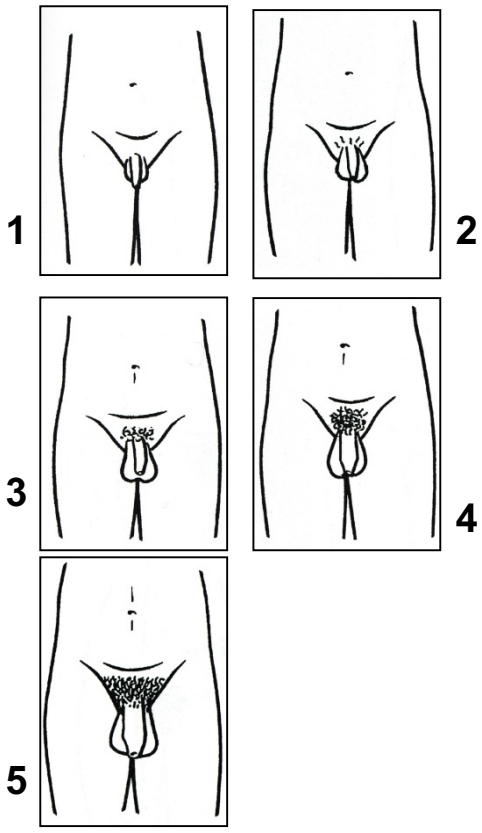
Appendix C: Pubertal Stage Questionnaire

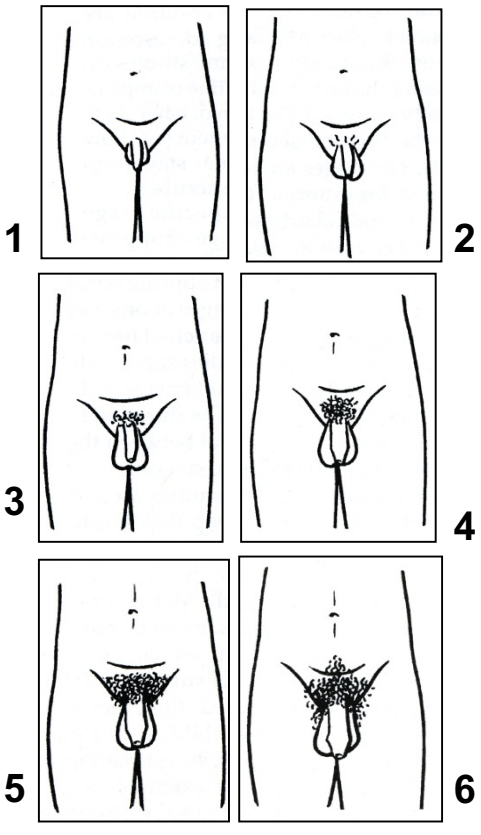
Male Pubertal Stage

This survey will be used to assess the maturational levels of the participant.

ID: _____ Date: _____

Please circle the box below that looks most like you	• Please look at the **pubic hair only** • Please circle the box that looks most like you





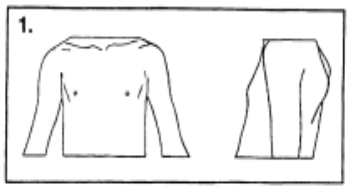
Female Pubertal Stage

Directions: You should choose only one of the stages shown below. One stage for Breast development and one stage for Pubic Hair development.

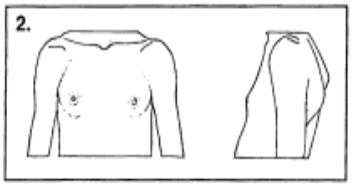
ID: _____Date: _____

Study Subject No:

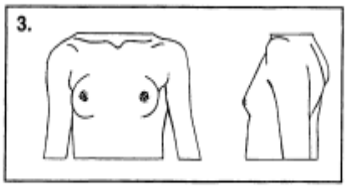
- Please put a tick in the box that looks most like you now....



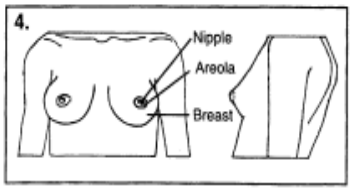
The breasts are flat.



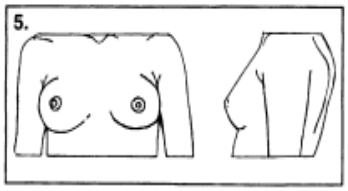
The breasts form small mounds.



The breasts form larger mounds than in 2.



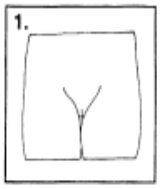
The nipple and the surrounding part *(the Areola)* make up a mound that sticks up above the breast.

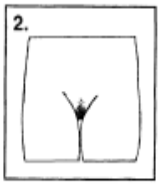


Only the nipple sticks out beyond the breast.

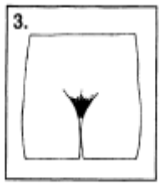
- Please put a tick in the box that looks most like you now....

No hairs

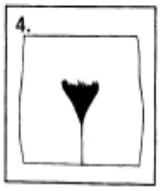




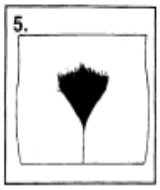
Very little hair



Quite a lot of hair



The hair has not spread over the thighs



The hair has spread over the thighs

Appendix D: RPE Scale

Rating	Perceived Exertion
6	No exertion
7	Extremely light
8	
9	Very light
10	
11	Light
12	
13	Somewhat hard
14	
15	Hard
16	
17	Very hard
18	
19	Extremely hard
20	Maximal exertion

Appendix E: Bedford Thermal Comfort Scale, SHRAE Thermal Sensation Scale

THERMAL COMFORT

1	Comfortable
2	Slightly uncomfortable
3	Uncomfortable
4	Very uncomfortable

THERMAL SENSATION

1	COLD
2	COOL
3	SLIGHTLY COOL
4	NEUTRAL
5	SLIGHTLY WARM
6	WARM
7	HOT

Appendix F: SkBF Traces

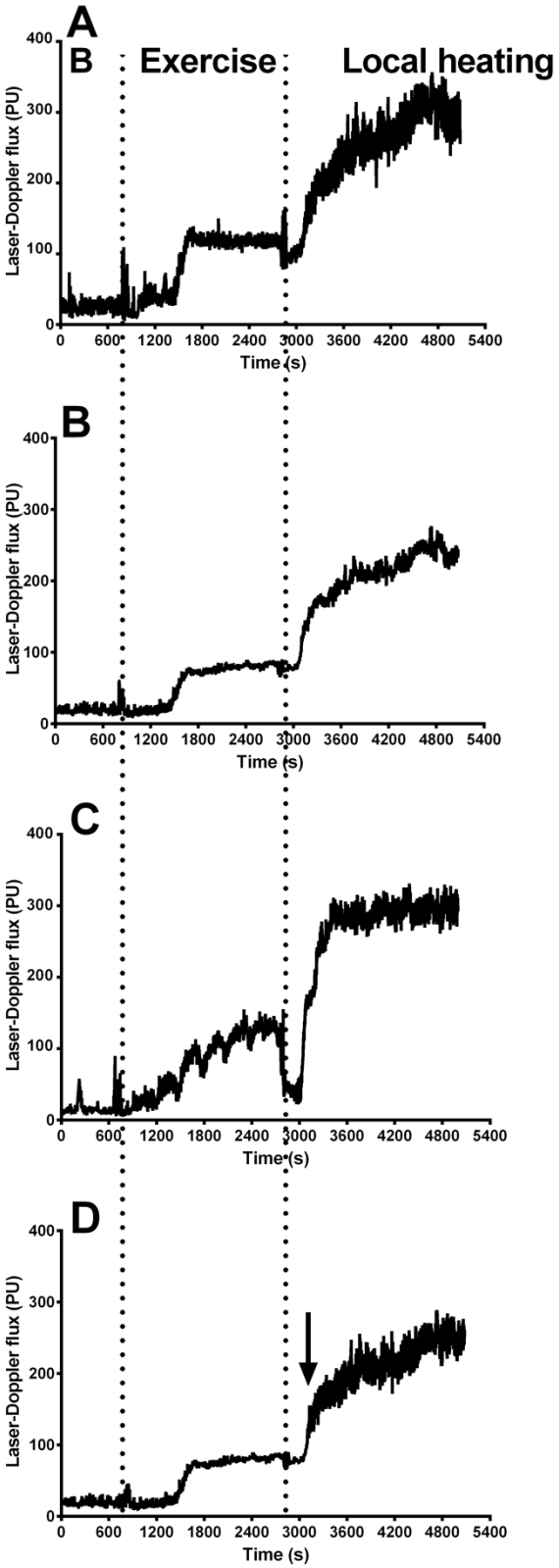


Figure 10 presents raw Laser-Doppler data from this study, providing examples of two sites for two participants. Participant 1's skin sites are shown in panel A and panel B,

respectively, while participant 2's skin sites are illustrated in panel C and panel D, respectively. Panel A and C present untreated sites, while panels B and D present the tracings for L-NAME-treated site. The larger amplitude and the higher absolute levels of the LDF flux illustrated in panel A when compared to panel B, indicate that L-NAME was effective at inhibiting NO throughout the entire protocol. On the other hand, when comparing the tracings in panels C and D, the amplitude and the absolute levels of the LDF flux are very similar. This similarity suggests that L-NAME lost its efficacy during the local heating portion of the protocol (as indicated by the arrow).

Appendix G: Assent Form for Child Participants

Assent Form for Child Participants

INVITATION

You are invited to participate in a study that involves examining the way the body regulates (controls) its temperature during exercise.

Project Title: Thermoregulatory mechanisms during exercise in Children and Adults

INVESTIGATORS: DEPARTMENT: CONTACT:

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Dr. Deborah O'Leary FAHS, Brock university (905) 688-5550 ex. 4339

PURPOSE

The purpose of this study is to see if there are differences in skin blood flow between children and adults.

WHAT'S INVOLVED

If you choose to volunteer for this study, you will visit the lab three times. There will be three sessions. One will be 1 hour and the other two will be 2.5 hours, respectively. For all of the visits, you will need to change into a t-shirt, shorts, and running shoes. Visits will be 3-14 days apart.

The next part describes what will happen when you come visit the lab:

A. Session 1

1. You will have a chance to see the equipment used for this experiment. You will fill out some questionnaires to find out about your medical history, regular physical activity and maturity. You may ask for help at any time.

2. We will take some measurements of your body such as, height, weight, sitting height, and body composition. We will measure your body composition by squeezing the skin on your triceps (back of the arm) and subscapularis (near the shoulder blade). This is safe and will not cause you any harm. In order to check your arterial stiffness, a probe that looks like a pencil will be placed on the left side of your neck and a small cuff will be attached to the left middle toe. This procedure does not hurt.

3. You will do a fitness test (maximum oxygen consumption, VO_2max) on a stationary bicycle (a bicycle that does not move). For the first part of the test you will pedal at an easy-to-moderate intensity for up to 20 minutes. Then, you will pedal until the maximal intensity you can reach (~8-10 mins). After a short rest, you will pedal again at a high intensity (~2-3 minutes). You will wear a head and mouth piece during the tests. You will also wear a heart rate monitor around your chest. Please do not exercise on the day of the test and drink plenty of water beforehand so that you're not thirsty.

B. Session 2

1. A painless procedure (iontophoresis) in which a weak electrical current is used to make small particles pass through intact skin will be performed on your forearm. Your skin will need to be wiped with alcohol swabs to clean it. The procedure involves 2 gel stickers being placed on your skin to allow a weak current to move a solution into the skin. The solution will make your blood vessels widen. We will need to wait ~40 minutes before setting up for the next phase. This procedure does not hurt.

2. Laser Doppler flowmetry probes (small disks, the size of a dime) will be taped on your forearm area at 2 places and kept there for the rest of the session. Laser Speckle contrast imaging (a camera) will also be placed over your arm, but will not touch your arm. Both, Laser Doppler flowmetry and laser speckle contrast imaging look at the blood flow in the blood vessels in the skin without breaking the skin. This procedure does not hurt.

3. You will exercise for 30 minutes on a stationary bicycle (a bicycle that does not move) at a moderate intensity. We will ask that you try not to drink during the exercise, but if you need water, there will be some available. Please try and drink lots of water to stay hydrated before you come to the session. Blood pressure will be measured every 10 minutes.

4. We will also warm up your skin on a small area of your forearm. The temperature will begin at 33°C and raised to 44°C in a 5-minute time period. Then, you will need to sit for ~30 minutes. Local heating is not painful and will not cause you any harm.

C. Session 3

This session is identical to Session 2, except without the exercise.

1. As in Session 2, a painless procedure in which a weak electrical current is used to make small particles pass through your skin will be performed on your forearm. This requires cleaning the skin with alcohol swabs. The procedure involves 2 gel stickers being placed on the skin to allow a weak current to move a solution into the skin. The solution allows blood vessels to widen. We will need to wait ~40 minutes before setting up for the next phase of the session.

2. Laser Doppler flowmetry probes (small disks, the size of a dime) will be taped to the forearm area at 2 spots and kept there for the remainder of the session. Laser Doppler

flowmetry looks at the blood flow in the blood vessels in the skin without breaking the skin.

3. On the forearm skin, at the site of the Laser Doppler flowmetry probe, we will perform local heating on an area the size of a dime. The temperature will begin at 33°C and raised to 44°C in a 5-minute time period. Then, you will need to sit for ~30 minutes. Local heating does not hurt and leaves no lasting effects on the skin or underlying tissue.

Note: None of the measurement and exercise procedures listed above should harm you in any way.

Experimental sessions will be terminated if:

You decide, for any reason, to end the experiment.

The investigators determine that you appear uncomfortable or stressed with the procedures.

POTENTIAL RISKS and BENEFITS

Use of skinfold calipers may cause a slight pinching sensation.

The tape used to attach the machines may bother your skin; If that happens, another tape is available.

The alcohol used for cleaning the skin may bother your skin. Cream will help, if that happens.

Your muscles may also be sore after the exercise tests. If you become sore, it goes away within 1-2 days.

If you experience any strange symptoms after the testing session, you should tell your parents or guardians.

Direct Benefits

You will gain information about the human body, including what your body is made of and your fitness level.

As a thank you for being in the study, $10.00 cash will be given at the end of each exercise sessions ($20 total). If you come in for one visit, you will get $10 in total.

Possible Benefits to the scientific community

This research will help science by providing a better understanding of the thermoregulatory mechanism(s) responsible for differences in heat loss strategies between children and adults.

CONFIDENTIALITY

The people working on the study will ask for your name, and contact information (phone, email) which will be kept in a master sheet but not on the sheets used to record the measurements. The master list matching participants to data will be kept by Dr. Falk on a password-protected computer, and will be destroyed following data publication.

All information you provide is considered private. You will not be identified individually in any way in written reports of this research.

Electronic data will be stored on password-protected computers. Paper data will be kept safe in Dr. Falk's lab. or office at Brock University. Only those people who work on the study, Dr. Falk, and student helpers will be able to look at the data. If you do not allow us to keep the data (see below), it will be deleted/shredded five years after publication.

VOLUNTARY PARTICIPATION

You can choose if you want to be in this study or not and you may take your data out of the study if you wish by telling one of the investigators. Note that you can take your data out only until the point at which the master list (which includes participant information) is destroyed. That is, after the results are published. After that, it will not be possible to find your data. You may also decide not to answer any question during the study (except the screening questionnaire) and still continue as a participant in the study. The investigators are able to take you out from the study if they believe that it is needed.

PUBLICATION OF RESULTS

Results of this study may be reported in scientific journals and presented, your personal information in this study will remain private. After testing is done and the data is reviewed, we will provide you with your results, and the overall group results. Feedback about this study will be available from Alexandra Woloschuk (aw16vq@brocku.ca 905-688-5550 x 5623) or Raffaele Massarotto (rm17ui@brocku.ca 905-688-5550 x 5623).

FUTURE RESEARCH

At the end of this study, we may wish to use the data for another study that might or might not be directly related to the current study. (Given that the master list will be destroyed, future studies will involve data where your name and other personal information will not be used). An appropriate research ethics board would clear any more research with the current data.

Check this box if you agree for us to keep your data for use in future work ☐

Check this box if, in the case that you want to stop your participation in the study, you allow us to use the partial data collected. ☐

If you would like to be contacted in the future about other studies, we will need to keep your personal information.

Check this box if you agree for us to keep your contact information □

Signature: _____ Date:

_____

CONTACT INFORMATION AND ETHICS CLEARANCE

If you have any questions about this study or want more information, please ask the Principal Investigator using the information above. This study has been given ethics clearance through the Research Ethics Board at Brock University. If you have any questions about your rights as a research participant, please contact the Research Ethics Office at (905) 688-5550 Ext. 3035, reb@brocku.ca.

ASSENT FORM

Project Title: Thermoregulatory mechanisms during exercise in Children and Adults (REB file #17-045)

I agree to participate in the study described above. I have made this decision based on the information I have read in this Informed Consent Letter. I have had the opportunity to get any added details I wanted about the study and understand that I may ask questions in the future. I understand that I may take back this approval at any time by telling or writing Dr. Bareket Falk (bfalk@brocku.ca 905-688-5550 x4979) Alexandra Woloschuk (aw16vq@brocku.ca 905-688-5550 x 5623) or Raffaele Massarotto (rm17ui@brocku.ca 905-688-5550 x 5623). My participation, non-participation, or removal from the study will not affect my standing at Brock.

Name: _____

Signature: _____ Date:

_____

Principal Investigator: Bareket Falk, Ph.D

Co-Investigator: Gary J. Hodges, Ph.D.

Student Investigator: Alexandra Woloschuk, MSc. Candidate & Raffaele Massarotto, MSc. Candidate

Signature: _____ Date:

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Thank you for your assistance in this project. Please keep a copy of this form for your records.

Appendix H: Consent Forms for Parents of Child Participants
Parent Informed Consent:

INVITATION

Your child is invited to participate in a study that involves examining thermoregulatory mechanisms during exercise. The overall aim of this study is to examine the way the body regulates its temperature during exercise in children compared to adults.

Project Title: Thermoregulatory mechanisms during exercise in Children and Adults

INVESTIGATORS: DEPARTMENT: CONTACT:

Dr. Bareket Falk FAHS, Brock University (905) 688-5550 ex. 4979

Alexandra Woloschuk FAHS, Brock University (905) 688-5550 ex. 5623

Dr. Gary Hodges FAHS, Brock University (905) 688-5550 ex. 4364

Dr. Nota Klentrou FAHS, Brock University (905) 688-5550 ex. 4538

Raffaele Massarotto FAHS, Brock University (905) 688-5550 ex. 5623

Dr. Deborah O'Leary FAHS, Brock university (905) 688-5550 ex. 4339

PURPOSE

The specific purpose of this study is to investigate skin blood flow responses during exercise between children and adults.

WHAT'S INVOLVED

If you allow your child to volunteer for this study, you and your child will visit the Applied Physiology Laboratory, at Brock University, for three experimental sessions. There will be three sessions. One will be 1 hour and the other two will be 2.5 hours, respectively, 3-14 days apart. At the start of the experimental sessions, your child will need to change into a T-shirt, shorts, and running shoes. Appropriate change rooms are provided.

Listed below are the measurements and procedures that your child will complete for the study. Your child may choose to complete the questionnaire portion of the study alone, or with your assistance.

A. Session 1

1. Your child will have a chance to be familiarized with the equipment used for this experiment. We will ask your child to fill out a series of questionnaires and screening tools to assess regular physical activity and medical history.

2. Measurements of your child's body such as, height, weight, and body composition will be taken. Skinfold thickness of the triceps (back of the arm) and subscapularis (near the shoulder blade) will be used to determine body composition. In order to assess your child's arterial stiffness, a pencil-like probe will be placed on the left carotid artery and a small cuff will be attached to the left middle toe. There is no pain associated with this procedure.

3. A maximum oxygen consumption (VO2max) test on a cycle ergometer (an exercise machine) will be administered. This test consists of cycling at submaximal intensities for up to 20 minutes, cycling until maximum intensity is reached (~8-10 mins), i.e., cycling until your child feel that he/she cannot cycle any longer. Following a short rest, we will ask your child to perform a supra-maximal test (~2-3 minutes). A head and mouthpiece must be worn during the cycling. Since it is difficult to communicate with the mouthpiece in place, we will establish hand signals which will allow us to communicate (e.g., thumb up, thumb down). A heart rate monitor worn around the chest will also be required. We ask that your child abstain from exercise or drinking caffeine on the day of the test.

B. Session 2

1. Iontophoresis (a painless procedure in which a weak electrical current is used to stimulate ions to pass through intact skin) will be performed on your child's forearm. This requires disinfecting the skin with alcohol swabs. Iontophoresis involves 2 gel stickers placed on the skin to allow a weak current to move a solution into the skin. The solution is a vasodilator, meaning it allows blood vessels to widen. We will need to wait ~40 minutes before setting up for the next phase of the session.
2. Laser Doppler flowmetry probes will be taped to the forearm area at 2 locations and kept there for the remainder of the session. Laser speckle contrast imaging will also be placed over the arm but will not make any contact with the arm. Both, Laser Doppler flowmetry and Laser speckle contrast imaging are non-invasive techniques which are used to assess blood flow in blood vessels in the skin.
3. Your child will exercise for 30 minutes on a semi-recumbent cycle ergometer at a moderate intensity. We will ask that your child abstains from drinking during the exercise, however, water will be available if needed. Blood pressure will be assessed every 10 minutes.
4. On the forearm skin, at the site of the Laser Doppler flowmetry probe, we will perform local heating on an area the size of a dime. The temperature will begin at 33°C and raised to 44°C in a 5-minute time period. We ask that your child remains seated for ~30 minutes after to record data. Local heating is non-painful and leaves no lasting effects on the skin or underlying tissue.

C. Session 3

1. Iontophoresis (a painless procedure in which a weak electrical current is used to stimulate ions to pass through intact skin) will be performed on your child's forearm. This requires disinfecting the skin with alcohol swabs. Iontophoresis involves 2 gel

stickers placed on the skin to allow a weak current to move a solution into the skin. The solution is a vasodilator, meaning it allows blood vessels to widen. We will need to wait ~40 minutes before setting up for the next phase of the session.

2. Laser Doppler flowmetry probes will be taped to the forearm area at 2 locations and kept there for the remainder of the session. Laser Doppler flowmetry is a non-invasive technique that is used to assess blood flow in blood vessels in the skin.

3. On the forearm skin, at the site of the Laser Doppler flowmetry probe, we will perform local heating on an area the size of a dime. The temperature will begin at 33°C and raised to 44°C in a 5-minute time period. We ask that your child remains seated for ~30 minutes after to record data. Local heating is non-painful and leaves no lasting effects on the skin or underlying tissue.

Note: None of the measurement and exercise procedures listed above should harm you in any way.

Experimental sessions will be terminated if:

5. You or your child decide, for any reason, to end the experiment.

6. The investigators determine that your child appears uncomfortable or agitated with the procedures.

POTENTIAL RISKS and BENEFITS

Instrumentation and maximal oxygen consumption test

Use of skinfold calipers may cause a slight pinching sensation.

The adhesive tape used to secure instrumentation may cause slight skin irritation for some people; alternative tape is available.

The alcohol used for prepping the skin for iontophoresis and laser Doppler flowmetry may leave the skin red and irritated. Moisturizer can be provided in such cases.

Your child may also feel some muscle soreness following the exercise. If soreness occurs, it generally disappears within 1-2 days.

If your child experiences any unusual symptoms after completing a testing session, your child should immediately seek medical attention and inform Dr. Falk, Alexandra Woloschuk or Raffaele Massarotto (see contact information above).

Direct Benefits

Your child will gain personal and general knowledge about the human body, including body composition and aerobic fitness (maximum oxygen consumption).

As compensation for participating in the study a $10 honorarium (cash or gift card) will be provided at the end of each exercise sessions ($20 total). i.e., compensation is pro-rated.

Potential Benefits to the scientific community/society

This research will benefit both the scientific and human performance communities by providing a better understanding of the thermoregulatory mechanism(s) responsible for differences in heat loss strategies between children and adults.

CONFIDENTIALITY

Investigators will require disclosure of your child's name, and contact information (phone, email), and therefore your child's participation is not anonymous during the conduct of the research. All participants will have their names removed from any data. The master list matching participants to data will be kept by Dr. Falk on a password-protected computer, and will be destroyed following data publication.

All information you and your child provides is considered confidential and because our interest is in the average responses of the entire group of participants, your child will not be identified individually in any way in written reports of this research.

Electronic data will be stored on password-protected computers. Paper data will be kept secured in Dr. Falk's lab or office at Brock University. Only those people involved with the study, Dr. Falk, and student investigators involved with the study, will have access to the data. Data will be deleted/shredded five years after publication if participants do not provide permission for the researchers to retain their data indefinitely (see exception below).

VOLUNTARY PARTICIPATION

Your child can choose whether to participate in this study or not and may remove his/her data from the study if they wish by telling one of the investigators. Note that removing of data can be done only until the master list (which includes participant identification) is destroyed at the end of data publication. After that, it will not be possible to identify your child's data. Your child may also refuse to answer any questions posed during the study (except the screening questionnaire) and still remain as a participant in the study. The investigators reserve the right to withdraw your child from the study if they believe that it is necessary.

PUBLICATION OF RESULTS

Results of this study may be published in professional journals and presented at conferences, your personal information and participation will remain confidential. After we finish testing all participants and analyzing the data, we will provide you with a summary of your child's results, and the overall group results. Feedback about this

study will be available from Alexandra Woloshcuk aw16vq@brocku.ca 905-688-5550 x 5623) or Raffaele Massarotto (rm17ui@brocku.ca 905-688-5550 x 5623).

FUTURE RESEARCH

At the completion of this study, we may wish to use the data for a future study that might or might not be directly related to the current research. Given that the master list will be destroyed, future studies will involve anonymized data (i.e., names and other personally identifying information will not be included). An appropriate research ethics board would clear any future research with the current data.

Check this box if you consent for us to retain your child's data for use in future work □

Check this box if, in the case of withdrawal from the study, you consent for us to use the partial data collected. □

If you would like to be contacted in the future about potential studies, we will need to keep your child's personal information.

Check this box if you consent for us to retain your contact information □

Signature: _____ Date:

_____

CONTACT INFORMATION AND ETHICS CLEARANCE

If you have any questions about this study or require further information, please contact the Principal Investigator using the contact information provided above. This study has been reviewed and received ethics clearance through the Research Ethics Board at Brock University. If you have any comments or concerns about your child's rights as a research participant, please contact the Research Ethics Office at (905) 688-5550 Ext. 3035, reb@brocku.ca.

CONSENT FORM

Project Title: Thermoregulatory mechanisms during exercise in Children and Adults (REB file #17-045)

I agree for my child to participate in the present study described above. I have made this decision based on the information I have read in this Informed Consent Letter. I have had the opportunity to receive any additional details I wanted about the study and understand that I may ask questions in the future. I understand that I may withdraw this consent at any time by telling or writing Dr. Bareket Falk (bfalk@brocku.ca 905-688-5550 x4979), Alexandra Woloschuk (aw16vq@brocku.ca 905-688-5550 x 5623) or Raffaele Massarotto (rm17ui@brocku.ca 905-688-5550 x 5623). My participation, non-participation, or withdrawal from the study will not affect my standing at Brock.

Name: _____

Signature: _____ Date:

_____

Principal Investigators: Bareket Falk, Ph.D

Co-Investigator: Gary J. Hodges, Ph.D.

Student Investigator: Alexandra Woloschuk, MSc. Candidate & Raffaele Massarotto, MSc. Candidate

Signature: _____ Date:

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Thank you for your assistance in this project. Please keep a copy of this form for your records.