MECHANISMS AND IMPLICATIONS OF SODIUM LOSS IN SWEAT DURING EXERCISE IN THE HEAT FOR PATIENTS WITH CYSTIC FIBROSIS AND HEALTHY INDIVIDUALS

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MECHANISMS AND IMPLICATIONS OF SODIUM LOSS IN SWEAT DURING EXERCISE IN THE HEAT FOR PATIENTS WITH CYSTIC FIBROSIS AND HEALTHY INDIVIDUALS

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This Thesis is dedicated to the individuals with cystic fibrosis and healthy volunteers who cheerfully gave their time, energy, body fluids, and, quite literally, ‘the skin off their back’ to improve our understanding of sweat glands and responses to exercise in the heat.
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<td>antibody</td>
<td></td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
<td></td>
</tr>
<tr>
<td>AQP5</td>
<td>aquaporin 5</td>
<td></td>
</tr>
<tr>
<td>AVP</td>
<td>arginine vasopressin</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
<td></td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium</td>
<td></td>
</tr>
<tr>
<td>CF</td>
<td>cystic fibrosis or cystic fibrosis subjects</td>
<td></td>
</tr>
<tr>
<td>CFTR</td>
<td>cystic fibrosis transmembrane conductance regulator</td>
<td></td>
</tr>
<tr>
<td>Cl⁻</td>
<td>chloride</td>
<td></td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
<td></td>
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<tr>
<td>CV</td>
<td>coefficient of variance</td>
<td></td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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</tr>
<tr>
<td>DEXA</td>
<td>dual energy x-ray absorptiometry</td>
<td></td>
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<tr>
<td>ECF</td>
<td>extracellular fluid</td>
<td></td>
</tr>
<tr>
<td>ENaC</td>
<td>epithelial sodium channel</td>
<td></td>
</tr>
<tr>
<td>FW</td>
<td>free water</td>
<td></td>
</tr>
<tr>
<td>Hb</td>
<td>hemoglobin</td>
<td></td>
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<tr>
<td>Hct</td>
<td>hematocrit</td>
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<tr>
<td>HR</td>
<td>heart rate</td>
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<tr>
<td>ICF</td>
<td>intracellular fluid</td>
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<tr>
<td>K⁺</td>
<td>potassium</td>
<td></td>
</tr>
<tr>
<td>Na⁺</td>
<td>sodium</td>
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</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
<td></td>
</tr>
<tr>
<td>NaK2Cl</td>
<td>sodium potassium 2 chloride transporter</td>
<td></td>
</tr>
<tr>
<td>NaKATPase</td>
<td>sodium potassium pump</td>
<td></td>
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<tr>
<td>NHE1</td>
<td>sodium hydrogen exchanger 1</td>
<td></td>
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<tr>
<td>osmo</td>
<td>osmolality</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PV</td>
<td>plasma volume</td>
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</tr>
<tr>
<td>RER</td>
<td>respiratory exchange ratio</td>
<td></td>
</tr>
<tr>
<td>RMANOVA</td>
<td>repeated measures analysis of variance</td>
<td></td>
</tr>
<tr>
<td>RPE</td>
<td>rating of perceived exertion</td>
<td></td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
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<tr>
<td>SR</td>
<td>sweat rate</td>
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<tr>
<td>SS</td>
<td>non CF salty sweater</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>TBW</td>
<td>total body water</td>
<td></td>
</tr>
<tr>
<td>USG</td>
<td>urine specific gravity</td>
<td></td>
</tr>
<tr>
<td>VAS</td>
<td>visual analog scale</td>
<td></td>
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<tr>
<td>VO(_2)</td>
<td>(is used in place of (\dot{\text{VO}}_2), rate of oxygen uptake)</td>
<td></td>
</tr>
<tr>
<td>VO(_{2\text{max}})</td>
<td>(is used in place of (\text{VO}_{2\text{max}}), maximal rate of oxygen uptake)</td>
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SUMMARY

Our aim was to understand mechanisms responsible for excessive electrolyte loss in the sweat gland and the potential impact on fluid balance during exercise in heat stress conditions. Human physiological testing under exercise/heat stress and immunofluorescence staining of sweat glands from skin biopsies were compared between healthy individuals (with normal and high sweat sodium concentration) and with cystic fibrosis patients (CF), who exhibit excessively salty sweat due to a defect of Cl− channel cystic fibrosis transmembrane conductance regulator (CFTR). Three novel findings are presented. First, excessively salty sweat may be associated with reduced expression of CFTR in the sweat gland reabsorptive duct of healthy individuals in addition to in those with cystic fibrosis (CF); however, although a link to a CF gene mutation in healthy individuals with high sweat sodium was not demonstrated, the possibility of an undetected CFTR mutation or polymorphism remains to be investigated as an underlying mechanism. Two, CF and healthy individuals with excessively salty sweat respond to moderate dehydration (3% body weight loss during exercise) with an attenuated rise in serum osmolality, greater relative loss in plasma volume, but similar perceived thirst compared to healthy individuals with “normal” sweat sodium. However, individuals with CF respond to rehydration (presentation of hypotonic beverage following dehydration) by drinking less ad libitum in response to reduced serum sodium chloride concentration, suggesting that thirst-guided fluid and electrolyte replacement may be more appropriate for CF patients rather than restoring 100% of sweat loss following dehydration as is often recommended in healthy individuals.
INTRODUCTION

1.1 Variability in the NaCl Content of Human Eccrine Sweat

There is considerable disagreement in values reported for the electrolyte composition of human sweat, particularly in the electrolyte sodium (Na+) for which the concentration can vary greatly within and between individuals. Across studies there is disparity in reported sweat electrolytes with values for mean sweat sodium concentration ([Na⁺]) from 30 mmol/L to as high as 90 mmol/L for healthy subjects. Discrepancies in sweat composition between studies can be related to differences in sweat stimulation method; rate of sweating elicited; acclimation status of subjects; anatomic location; and technique used (gauze pad, Brisson pouch, whole body washdown, arm bag, or ventilated capsule) for sweat collection. Variability in sweat composition among subjects within a single study, where such factors are controlled, is still commonly high. This is particularly true for [Na⁺] where reported mean values for typical subject pool are typically accompanied by large SDs (e.g. ± 10-20 mmol/L) with coefficient of variation ranging from 40 to 60%.

Electrolytes in the extracellular fluid (ECF), and specifically in the vascular space of the ECF, are maintained in a fairly tight physiological range in humans. It is puzzling, therefore, that humans are also known to exhibit large variability in Na⁺ loss during both thermal- and pharmacologically-induced eccrine sweating compared to other electrolytes such as potassium (K⁺). Sweat electrolyte composition at a given sweat
rate is not believed to be influenced by gender$^{28}$ or age$^{29}$. The impact of diet, specifically sodium ingestion, however, is debated$^{30-32}$. Sweat composition can be acutely altered by dehydration. Healthy subjects exercising in the heat exhibited higher mean sweat $[\text{Na}^+]$ and chloride concentration ($[\text{Cl}^-]$) during dehydration compared to when hydration was maintained via fluid ingestion (euhydration)$^6$. It is also well-documented that heat acclimation reduces sweat electrolyte loss by producing a more copious and dilute sweat$^{14-17,33}$; however, even when controlling for acclimation status, some individuals continue to excrete higher concentrations of sweat NaCl for reasons that have not yet been fully identified or understood. The underlying mechanisms responsible for this sweat [NaCl] variability might be attributed to individual differences in sweat duct absorption of electrolytes.

### 1.2 Eccrine Sweat Gland Physiology

Human skin adaptations are the result of physical modifications to climate and environment changes over the last 4 to 7 million years$^{34}$. In addition to loss of hair and skin pigmentation, the human eccrine sweat gland is thought to have been a key evolutionary adaptation for humans to become superior predators$^{35}$. Unlike humans and horses, other mammals are not capable of copious sweating in response to a rise in body temperature and, coincidentally, are also unable to perform sustained running$^{34,36}$. Sweating occurs through two types of glands in mammals: apocrine and eccrine sweat glands. Humans use primarily eccrine sweating while other mammals, including horses, exhibit apocrine sweating$^{34,37}$. Apocrine sweating is considered to be primitive, the quantity is small in most mammals (except in horses) making it less effective for dissipating heat$^{34}$. The human body contains typically 2 to 4 million eccrine sweat glands.
distributed over most body surfaces. The density of sweat glands varies by region, for instance, there are approximately ~ 64 per square cm on the back but two to three times as many over the forearm and forehead. The eccrine sweat gland is composed of two morphologically and functionally distinct components, a secretory coil and a reabsorptive duct. The secretory coil is responsible for the formation and secretion of an isotonic fluid when stimulated via cholinergic or adrenergic receptors. Originally thought to operate under the pump leak model, it is now understood that the Na-K-2Cl cotransport model better explains the mechanism of eccrine sweat secretion (Fig 1.1). Upon cholinergic or adrenergic stimulation, extracellular calcium (Ca\(^{2+}\)) enters the secretory cell and opens cAMP activated Cl\(^{-}\) channels (cystic fibrosis transmembrane conductance regulator, CFTR) in the luminal membrane, as well as Ca\(^{2+}\) sensitive K\(^{+}\) channels in the basolateral membrane. This results in the electrically-neutral KCl loss from the cytoplasm and the decrease of cell volume. Loss of KCl produces a favorable chemical potential gradient for the influx of K\(^{+}\) and Cl\(^{-}\) from the interstitial medium thru NaK2Cl co-transporters. The Na\(^{+}\) carried into the cell by the cotransporters is then pumped out across the basolateral cell membrane by Na`K`ATPase. Transport of Cl\(^{-}\) into the lumen produces lumen negative transepithelial potential and Na\(^{+}\) enters the lumen from the interstitial fluid through a paracellular route. Water enters the lumen as needed to balance the Na\(^{+}\) and Cl\(^{-}\).

The iso-osmotic initial fluid, or ‘primary’ sweat produced by the secretory portion of the gland is approximately isotonic with plasma, containing NaCl concentrations approximately equal to blood levels. The primary sweat travels through the coil and enters the lumen of the two-cell thick reabsorptive duct. In the duct, NaCl
in the primary sweat is partially reabsorbed by passive transport of Na\(^+\) and Cl\(^-\) into the ductal cell via apical membrane channels CFTR and epithelial sodium channel (ENaC)\(^{47-49}\) (Fig 1.2). Na\(^+\)K\(^+\)ATPase activity in the basolateral membrane maintains concentration

![Diagram of membrane transport proteins involved in the Na-K-2Cl model of sweat secretion in a secretory coil cell.](image)

**Fig 1.1:** Schematic of membrane transport proteins involved in the Na-K-2Cl model of sweat secretion in a secretory coil cell.

gradients and K\(^+\) is passively transported out basolateral K\(^+\) channels\(^{41}\). In addition, a proton pump present on the apical membrane of the luminal cell is suggested to be responsible for acidifying sweat as it passes through the lumen\(^{50,51}\). More recently, a sodium hydrogen exchanger (NHE1) was identified on the basolateral membranes and is likely responsible for intracellular pH and cell volume regulation as it is in other tissues\(^{51,52}\). Modification of primary sweat in the duct produces a hypotonic liquid for excretion at
the skin surface as ‘final’ sweat. The hypotonicity of excreted sweat can vary greatly across humans, however, and is a primary focus of this dissertation.

![Diagram of membrane transport proteins](image)

**Fig 1.2:** Schematic of membrane transport proteins involved in ductal cell modification of primary iso-osmotic secreted sweat into hypotonic final sweat by reabsorption of Na\(^+\) and Cl\(^-\). Only the inner ductal cell is represented in the schematic for simplicity.

### 1.3 Cystic Fibrosis Sweat

Individuals with the inherited autosomal recessive disease cystic fibrosis (CF) excrete sweat with a three to five times higher [NaCl] than typical. The primary sweat is not different in CF; it is failure of NaCl reabsorption in the CF sweat duct (calculated to be approximately 20% of normal based on the Thaysen-Schwartz model of isotonic secretion) that results in the excessively salty sweat of CF patients. The CF sweat duct is defective in NaCl absorption due to an absence or malfunction of the mutated CF gene product CFTR in the apical and basolateral membranes.
studies have attempted to demonstrate a relationship between sweat duct membrane
transport protein expression/activity and extent of sweat electrolyte loss during
physiological thermoregulatory sweating in CF compared to individuals without disease.
A whole-cell patch clamp study comparing normal and CF sweat gland cells found no
difference in channel types and proportions however their data is from secretory not
ductal cells. This is an important distinction because it has been known for some time that
“primary” sweat (entering the duct after exiting the secretory portion of the gland) is
isotonic with respect to ECF and any decrease in [electrolyte] in the excreted sweat
compared to ECF reflects the absorptive properties of the sweat duct. The study of
sweat from isolated microperfused sweat duct segments, and from pharmacologically-
induced local sweat tests, have helped characterize the CF defect. However,
physiologically-induced sweating only reflects one aspect of sweat gland function.
Physiological in situ sweating is controlled by multiple innervations to the sweat gland
under the influence of multiple endogenous agonists. For example, thermally-induced
sweat contains a significantly lower [K+] when compared to both pilocarpine- and
methacholine- induced sweat and could reflect endogenous agonist activity on K+
channel activity. This is in parallel with new evidence suggesting that CFTR activity in
the sweat duct may be regulated by relative kinase and phosphatase activity in response
to changes in relative intracellular concentrations of K+ and Na+. Furthermore, exercise
is known to induce greater increases in blood flow and periglandular region
temperature, as well as higher concentrations of circulating adrenergic hormones that
can serve as co-factors in the sudorific response, all of which may contribute to the
differences between pharmacologically-, passive heating-, and exercise-induced
sweating. Therefore, while elevated sweat electrolytes in pharmacologically-induced CF sweat is well-documented, electrolyte composition of in situ sweating (i.e. as encountered in the context of exercise) remains to be elucidated for this population.

Some healthy individuals without CF also exhibit sweat $[\text{Na}^+]$ approaching that of CF. The explanation for this is unknown and a genetic link to CF has been theorized, but never directly investigated in individuals with excessively salty sweat. In a study comparing CF heterozygote and homozygote newborns ($n > 700$) with the CFTR mutation $\Delta F508$ and non-CF newborns, heterozygotes had significantly higher pilocarpine-induced sweat $[\text{Na}^+]$ and $[\text{Cl}^-]$ than the non-carrier newborns. In a more recent study, healthy individuals that were CF heterozygotes ($n > 500$), were shown to have a lower rate of increase in systolic blood pressure with age; and the subjects with the lowest blood pressures were heterozygotes with the highest pilocarpine-induced sweat $[\text{Na}^+]$ and $[\text{Cl}^-]$.

It is not known if a CF mutation can explain differences in sweat $[\text{NaCl}]$ in healthy individuals (e.g. those with high and ‘typical’ $[\text{NaCl}]$). A recent review suggested that perhaps certain unidentified populations are at risk for the development of exercise-associated hyponatremia (blood $[\text{Na}^+] < 135$ mmol/L), such as those without disease but heterozygotes carrying variants of the CFTR gene. It was suggested that future research addressing this would be helpful in determining if this genetic basis could explain why some individuals develop low serum Na$^+$ levels during prolonged activity in the heat. To our knowledge, it has not been investigated whether apparently healthy individuals who exhibit excessively salty sweat (heretofore referred to as ‘salty sweaters’) are carriers of a CF gene mutation. Moreover, the quantity and function of
CFTR and ENaC in these non-CF ‘salty sweaters’ have not been examined and compared to those individuals with normal sweat [NaCl] or CF patients. The first Aim of this research was to investigate if a cellular mechanism similar to that in CF disease (i.e. decreased channel number) also explains heterogeneity in sweat [NaCl] between “healthy” individuals. Therefore, with an original investigation of ductal electrolyte transport channel expression and the incidence of CF mutations in apparently healthy ‘salty sweaters’ compared to those with ‘average’ sweat [NaCl], the first major aim of this dissertation research tested the hypothesis that a mechanism (similar to that in CF disease) explains the variance in sweat NaCl loss in human eccrine sweating. The findings related to this Aim are detailed primarily in chapter 2 of this thesis.

1.4 Impact of Sweat NaCl Loss on Physiological Responses to Exercise and Dehydration

As discussed above, some individuals without CF excrete sweat containing high [NaCl], with values approaching that of CF ⁶⁴. It is not known if other physiological responses such as blood osmolality and blood electrolytes during prolonged exercise are affected in ‘salty sweaters’ compared to their normal-to-low sweat NaCl counterparts. Furthermore, it is not known if the body water deficit commonly incurred during prolonged exercise in the heat differentially affects thirst drive and thermoregulation in salty sweaters. There is a strong positive cause-effect relationship between the sensation of thirst and plasma osmolality ⁷¹-⁷⁴. Approximately 0.8% body weight loss and 1-2% increase in plasma osmolality represent the hypovolemic, and hyperosmotic thresholds for the stimulation of thirst in humans, respectively ⁷³ ⁷⁵-⁷⁷. The hyperosmotic thirst mechanism (in response to plasma osmolality increases) is thought to have greater
sensitivity than the hypovolemic thirst mechanism (loss of blood volume)\textsuperscript{71 75 78}. As plasma osmolality increases, plasma vasopressin (AVP) is released, mediated by signaling from osmoreceptors of the preoptic anterior hypothalamus. In response to rise in plasma osmotic pressure (via either fluid loss or solute concentration increase), fluid shifts down a concentration gradient out of cells (cell dehydration) to maintain fluid volume in the vascular space\textsuperscript{79 80}. It is thought that this cell dehydration, in response to a rise in extracellular osmotic concentration, provides the strong stimulus for thirst because the activated volume-depleted osmoreceptor cell signals for increased release of AVP\textsuperscript{71 81 82}.

No study has examined if blood osmolality and blood $[\text{Na}^+]$ are lower and alter thirst drive in CF and healthy ‘salty sweaters’ compared to those with typical sweat NaCl loss at the same relative level of dehydration during exercise in the heat. One study\textsuperscript{83} that compared thirst-guided drinking behavior of CF to non-CF children during a three hour intermittent exercise session (20 min cycling at 45%$\text{VO}_{2\text{max}}$ with 25 min rest periods) found that CF children drank significantly less than controls and subsequently lost twice as much body weight. Unfortunately blood osmolality measures were taken only at the end of the three hour session and reflected not only the electrolyte loss but also the consumption of differing amounts of water. Therefore, while the authors postulated that the high NaCl loss in CF prevented the sweating-induced hyperosmolality of body fluids and thus deprived the CF children of a thirst trigger, they conceded that without serial blood samples during the protocol, they could not confirm the greater NaCl loss was accompanied by lower serum osmolality throughout exercise\textsuperscript{83}. Clearly, further research is required to understand the impact of high sweat NaCl on physiological
responses regulating fluid and electrolyte homeostasis during prolonged exercise. Therefore, in addition to investigating the mechanisms related to high sweat [NaCl], the present dissertation work included experiments of human physiological testing in order to explore questions related to implications. Findings and conclusions related to physiological testing are detailed primarily in chapter 3 of this thesis.

1.4.1 To Rely or Not to Rely on Thirst

Since first recognized in 1965, many researchers have confirmed that a lag in the thirst mechanism can lead to inadequate body fluid homeostasis and impair thermoregulation as well as exercise performance. Montain et al. reported impaired thermoregulatory capacity during exercise in the heat with dehydration to 3% and 5% body weight loss as compared to euhydration trials. With lag in the thirst mechanism potentially contributing to inadequate hydration during prolonged exercise in the heat, regimented or ‘programmed’ drinking according to a formula based on sweat losses has been recommended and published by expert organizations to ensure safety and improve performance in sport. It is argued by some that moderate dehydration during exercise is actually preferable such that reliance upon thirst to dictate fluid intake prevents the problem of water intoxication by regimented, overconsumption of hypotonic fluids. Most cases of exercise-induced hyponatremia (serum Na+ < 135 mmol/L) are attributed to overconsumption of hypotonic fluid (water, low sodium beverages), replacement in excess of fluid loss, and is indicated by post-event weight gain. Whether thirst-guided drinking may sufficiently maintain fluid balance during prolonged physical activity in the heat depends on the accuracy of the thirst drive and regulatory inputs to thirst such as blood osmolality. A second Aim of this research
investigated if thirst perception appropriately reflects body water loss in individuals with high sweat NaCl loss. These findings are presented in chapter 3 of this thesis.

### Contributing Factors to Exercise - Induced Hyponatremia

<table>
<thead>
<tr>
<th>FLUID OVERLOAD</th>
<th>EXCESSIVE SODIUM LOSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Overconsumption of fluids</td>
<td>• High sweat [Na⁺]</td>
</tr>
<tr>
<td>- drinking beyond thirst</td>
<td>- ductal Cl⁻ or Na⁺ channels</td>
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<tr>
<td>• fluid intake guidelines vs.</td>
<td>• CFTR variant gene</td>
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<tr>
<td>thirst-driven replacement</td>
<td>• ENaC variant gene</td>
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<tr>
<td>• impaired thirst sensitivity</td>
<td>• Prolonged sweating</td>
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<td>• Inappropriate AVP release</td>
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</tbody>
</table>

![Diagram](image)


**Fig. 1.3:** Model of Contributing Factors to Exercise-Induced Hyponatremia

### 1.4.2 Impact of Sweat NaCl Loss on Electrolyte Balance

While not as common, hyponatremia may also occur in conjunction with dehydration, particularly in longer, ultraendurance events. This ‘hypovolemic hyponatremia’ is possible presumably from excessive salt depletion via sweating and not from overdrinking, according to a recently-developed quantitative model (Fig. 1.3). It is believed that a large sweat NaCl loss during exercise presents a greater risk for the development of hyponatremia during prolonged activity in the heat. Mathematic predictions for change in serum [electrolytes] with progressive dehydration have not been
validated in a field or laboratory setting using individuals at extreme ends of the sweat [NaCl] continuum. Therefore, a secondary goal of the dissertation work was to evaluate implications related to mathematically-predicted and actual serum [electrolytes] in individuals with high vs. ‘typical’ sweat [NaCl] and will be discussed in chapter 4 of this thesis.

1.4.3 Cystic Fibrosis and Exercise in the Heat

This dissertation’s investigation into implications of high sweat [NaCl] loss was, for the most part, novel for the healthy and physically-fit individuals studied. However, research into differential responses related to fluid and electrolyte balance as a result of salty sweat was almost entirely unexplored territory for the CF subjects included in this work. Exercise physiology in the CF population has been an understandably low research priority given the average life expectancy for a CF patient, up until 1990, was not beyond the teenage years. Management of respiratory and digestive sequelae has greatly improved for CF and not only has the average life expectancy nearly doubled since 1990, CF are participating in recreational and competitive sports in increasing numbers. Some of the unique responses of CF are presented in chapter 3 of this thesis. It is hoped that these findings may contribute to the development of recommendations for CF, as well as non-CF exercisers with excessive sweat NaCl loss, to more safely and comfortably perform prolonged activity in the heat.
CHAPTER 2

REDUCED EXPRESSION OF CFTR IN SWEAT DUCTS OF
HEALTHY INDIVIDUALS AND CYSTIC FIBROSIS PATIENTS
WITH HIGH SWEAT SODIUM

2.1. Abstract

Humans exhibit large variability in sodium chloride loss through eccrine sweating. In an effort to understand the underlying mechanisms responsible for heterogeneity of human NaCl losses in sweat during exercise, we investigated the relationship between sweat duct membrane transport protein expression and sweat electrolyte concentration. We hypothesized that CFTR expression would be decreased in healthy individuals with high sweat sodium concentration ([Na⁺]) in a mechanism similar to CF. Skin biopsies and sweat samples were obtained from physically-active, healthy young adults and from six physically-active patients with CF. Half of the non-CF subjects were individuals identified to excrete sweat with high sweat [Na⁺], ‘salty sweaters’. CF and healthy subjects performed cycling ergometry in the heat to obtain sweat samples for electrolyte measurement. Immunofluorescent staining for the chloride channel CFTR, and for the sodium channel alpha-ENaC, was performed on cryosections of skin biopsies. Ductal and luminal CFTR and ENaC fluorescence was quantified by a blinded investigator. Immunofluorescent staining of sweat glands revealed significantly lower luminal membrane CFTR in ducts of non-CF ‘salty sweaters’ (p<0.05) and CF (p<0.005) when compared to Control. Epithelial Na⁺ Channel (ENaC) staining was similar among
the three groups. Genetic testing of healthy subjects to investigate a possible CF gene mutation underlying ductal Cl⁻ channel deficiency did not result in any CF heterozygotes (carriers) within non-CF groups. Availability of CFTR for facilitation of electrolyte transport across the ductal membrane may contribute to the high physiological variability known for eccrine sweat NaCl across apparently healthy humans. Yet, we found no direct evidence that lower CFTR in healthy individuals who excrete excess sodium chloride had a relationship to any of the known, common disease-causing CF gene mutations.

2.2. Background

2.2.1. NaCl Reabsorption in the Eccrine Sweat Gland

Fluid excreted by the sweat gland is the work of two morphologically and functionally distinct organ components, a secretory coil and a reabsorptive duct. In a two-step process common to all mammalian fluid secretory systems, a primary isotonic fluid secretion (i.e. similar in electrolyte concentration to blood) is modified as it travels through the lumen of the reabsorptive duct and, in humans, results in a final sweat that is hypotonic to the plasma. Despite controlling for factors known to influence measured sweat concentration such as acclimation status and sweat stimulation and collection technique, the diluteness of this final excreted sweat can vary greatly between individuals. Since primary sweat is always isotonic to the plasma, glandular mechanisms underlying this inter-individual ‘physiological’ variability are presumably due to differences in sweat duct electrolyte reabsorption. A principal source of Cl⁻ conductance in the reabsorption process, the cystic fibrosis transmembrane conductance regulator (CFTR) is highly expressed and constitutively active in the apical membrane of the normal sweat duct. CFTR activity regulates an
epithelial sodium channel (ENaC) \(^{100}\), the only channel for luminal \(\text{Na}^+\) entry into the ductal cell.

Cystic fibrosis (CF) is an inherited autosomal recessive disease of faulty electrolyte transport due to insufficient CFTR activity that results in a secondary loss of CFTR-dependent ENaC conductance. In the sweat gland, CFTR malfunction results in excretion of sweat with a three to five times higher sodium chloride concentration [NaCl] than typical \(^{53-55}\). The mechanism for and extent of insufficient CFTR activity is mutation-specific although the relationship between genotype and phenotype and sweat [NaCl] is debated \(^{101-104}\). The secreted primary sweat in CF is not different than in non-CF; it is failure of NaCl reabsorption in the sweat duct (calculated to be approximately 20% of normal based on the Thaysen/Schwartz model of isotonic secretion \(^{56}\)) that results in the excessively salty sweat of patients \(^{57}\).

Studies of the composition of pharmacologically- and thermally-induced sweat have identified that some non-CF subjects have sweat Na\(^+\) levels that approach that of CF \(^{64}\). It is possible that reduced expression of ductal transport proteins CFTR and ENaC mechanistically underlies these apparently-healthy ‘salty sweaters’, potentially linked to a genetic cause such as a heterozygous CF mutation \(^{70}\). A mutation of the CF gene occurs with high frequency in man, \(~1\) in 25 Caucasians of European descent \(^{105\, 106}\). Some reports have shown a greater incidence of heterozygocity for a CF mutation in non-CF persons prone to chronic CF-like conditions such as idiopathic pancreatitis \(^{107\, 108}\), bronchiectasis, nontuberculous mycobacterial infections \(^{109}\), and chronic rhinosinusitis \(^{110\, 112}\). Furthermore, sweat induction by beta-adrenergic stimulation (a secondary method of sweat gland stimulation \(^{11\, 38}\)) cannot occur in CF homozygotes \(^{113}\) and is not as
effective in CF heterozygotes as compared to non-carriers. Differences in sweat electrolyte composition of CF heterozygotes as compared to non-carriers are debated, mostly due to difficulty sorting out the major confounding factor of sweat rate. CF carriers have pilocarpine-induced sweat Cl\textsuperscript{−} and Na\textsuperscript{+} levels that are significantly higher than non-carriers (2-5 times normal has been reported) but lower than CF homozygotes. In pilocarpine-induced sweat tests performed on CF gene carriers and non-carriers with chronic pancreatitis (n=134), a step-wise increase in sweat [Na\textsuperscript{+}] and [Cl\textsuperscript{−}] was reported where non-carriers had the lowest concentrations and carriers had the highest concentrations. Thus, it is plausible that there exists a correlation between apparently-healthy individuals with greater NaCl loss in sweat and a non-disease causing CFTR mutation. The present study investigated eccrine sweat duct CFTR and ENaC expression, and electrolyte composition of control subjects with ‘average’ sweat [NaCl] compared to healthy ‘salty sweaters’ and to CF patients with phenotypically high sweat [NaCl] during exercise-induced sweating in a hot environment. We hypothesized an inverse association between ductal CFTR and/or ENaC expression and sweat [NaCl]; consequently, salty sweaters (including CF and healthy subjects) would exhibit decreased CFTR and/or ENaC expression at the ductal luminal membrane as compared to individuals with average sweat [NaCl]. Since a genetic link to CF has been theorized for individuals with high sweat [NaCl], but never directly investigated, incidence of CF mutations within the healthy subjects exhibiting large variability in sweat NaCl loss was also investigated.

2.3. Methods

2.3.1 Preliminary Screening and Subject Selection
Recreationally-active young adults (aged 18-40 yrs) were recruited from the campus community and endurance sports teams in the area. Normal, healthy volunteers (non-CF subjects) participated in a preliminary sweat collection session (i.e., 30-60 min of cycling or running at self-selected pace until ~1.5-2 ml of sweat was obtained). Twelve individuals were chosen to participate as subjects based on their measured sweat composition: six were ‘salty sweaters’ (SS) with sweat [Na\(^+\)] > 70 mmol/L (Mean ± SD = 87.6 ± 18.4 mmol/L, range 70-111 mmol/L), and six individuals (Control) with sweat [Na\(^+\)] < 60 mmol/L mmol/L (Mean ± SD = 41.2 ± 8.4 mmol/L, range 31-55 mmol/L). The cut-point of > 70 mmol/L was used for selecting SS because it represents ~ two SD higher than the mean recently reported for regional sweat [Na\(^+\)] collected under similar conditions using the same measurement site (i.e. upper back region) during exercise in the heat\(^{117}\).

In addition, six young adults with cystic fibrosis (CF) were recruited through the Emory University Cystic Fibrosis Center and the local community to participate as volunteers. All CF subjects had sweat [Cl\(^-\)] in previous diagnostic pilocarpine testing of > 75 mmol/L and had ΔF508 mutations on at least one allele. One CF subject was ΔF508/R1162X, one was ΔF508/1717-1G→A, and the remaining four were homozygous for ΔF508 mutations. All CF subjects were in stable clinical status with an FEV\(_1\) > 75% of predicted value, performed aerobic exercise for a minimum of four hours per week, and were cleared by their physician for participation. Informed written consent was obtained from both CF and non-CF subjects as approved by the Institutional Review Boards at the Georgia Institute of Technology and Emory University School of Medicine.

2.3.2. Study Design
Sweat duct membrane transport protein expression, genotype, and sweat electrolytes were compared among Control, SS, and CF using a cross-sectional design. Identification of SS subjects was performed first through sweat collections in preliminary screening. Subsequent matching of each SS subject with a Control subject was performed on the criteria that Control have lower sweat [Na⁺] by at least ~50% (mean ± SD % difference in sweat [Na⁺] for Control compared to matched SS = -50.7 ± 10.1%). Attempts were also made to match all non-CF pairs based on age, gender, anthropometry, and training status. In order, to control for natural heat acclimation (a well-documented modifier of sweat composition) 14-17, paired non-CF subjects were tested in the same month and not during summer months of June, July, and August. CF subjects were matched to non-CF subjects by gender but not necessarily tested within the same month as their non-CF counterparts. However, this was not a major study limitation since CF sweat composition does not appear modifiable, even with heat acclimation 118.

Sweat comparison among subject groups was performed at the same relative level of dehydration (from 0.5 to 3% body weight loss) and exercise intensity (50% of aerobic capacity) to minimize potential effects of these known modifiers on sweat electrolytes. Skin sampling via scapular biopsy for examination of sweat ducts was performed at approximately the same time of day (mid morning) to minimize potential differences in sweat duct membrane transport proteins under circadian influence 119 120. In order to examine the relationship between ductal membrane protein expression and measured sweat electrolytes, all biopsies were performed immediately prior to initiation of the prolonged exercise protocol circumventing potential differences in ductal channel expression invoked by exercise and sweating responses. To avoid potential influence of
estrogen and progesterone on ductal channel expression during the luteal phase \cite{121-123}, all female subjects were tested in the early follicular phase of the menstrual cycle.

2.3.3. Initial Testing Session: Aerobic Capacity Assessment and Familiarization

In the first test session, a graded, incremental cycling test was conducted in the heat (32-33°C and 35% relative humidity) to determine maximal oxygen uptake (VO$_{2\text{max}}$). Collection of expired gases to determine oxygen consumption (VO$_2$) and respiratory exchange ratio (RER), heart rate (HR) and rating of perceived exertion (RPE) \cite{124} were recorded each minute during the test. Subjects cycled until volitional exhaustion. VO$_{2\text{max}}$ was considered achieved at test termination based on attainment of at least two of the following criteria: a plateau in VO$_2$ during the last two stages (increase < 2.1 ml/kg/min), a HR within 10 beats/min of age-predicted HR$_{\text{max}}$, a respiratory exchange ratio (RER) $\geq$ 1.10, or a minute ventilation $>$ 115 L/min.

A 30 min familiarization ride in the heat (32-33°C and 35% relative humidity) followed the VO$_{2\text{max}}$ testing to validate the workloads corresponding to a work rate estimated to elicit 50%VO$_{2\text{max}}$ for the next test session. Nude dry body weight was obtained before and after the 30 min ride to determine individual whole-body sweat rates. During the familiarization ride, a regional sweat sample was collected from the right scapula in order to confirm group placement.

During the first test session, body composition was assessed for subject matching purposes using dual energy X-ray absorptiometry (DEXA) with a Lunar Prodigy whole body scanner (GE Medical Systems, Madison, WI). At the completion of this initial testing session, subjects were instructed in use of dietary logs to record ingested food and beverages for the three days prior to their second testing session.
2.3.4. Second Testing Session: Biopsy and Sweat Collection

2.3.4.1. Pre-testing Controls

   Subjects abstained from caffeine (12 hr minimum) and alcohol (32 hr minimum) prior to reporting to the research facility for skin biopsy and subsequent exercise testing. Twenty-four hour food logs indicated that subjects complied with instructions to consume a standardized breakfast meal consisting of bagel, toast, and/or English muffin with cream cheese, butter, and/or peanut butter, and juice (any desired, except tomato juice) on the morning of testing. There was no difference (p<0.05) among groups in mean macronutrient, sodium, or caloric intake relative to body weight (BW) for the three days prior to testing. There was also no difference among groups in macronutrient, sodium, or caloric intake relative to BW for the morning of testing except for CF who consumed significantly (p=0.02) more fat (0.61 ± 0.3 g/kg) than both Control (0.27 ± 0.1 g/kg) and SS (0.28 ± 0.1 g/kg). To minimize variation in pre-exercise hydration between subjects, a euhydration protocol was piloted and instituted. Subjects were given instructions to ingest a volume of water equivalent to 12 ml per kg of BW the evening before and morning of testing. Furthermore, subjects did not perform physical exercise for 24 hours prior to testing. Euhydration was confirmed prior to biopsy and again prior to beginning the exercise protocol with measurement of urine specific gravity (USG) < 1.021\textsuperscript{125}, and with subsequent measurement of serum osmolality <290 mOsm\textsuperscript{4}.

2.3.4.2. Skin Biopsies

   At the start of the second testing session, skin biopsies were removed from the right scapular region, identical to the site of sweat collection in the initial testing session,
and contralateral to the collection site of the subsequent prolonged exercise protocol. Two full-thickness 4 mm diameter punch biopsies were performed by a dermatologist under local anesthetic. Biopsied tissue was immediately rinsed in ice-cold lactate Ringers and maintained in a second ice-cold lactate Ringers with glucose for tissue transport until placed in OCT embedding medium (Miles Inc., Elkhart, IN) and frozen in isopentane cooled in liquid nitrogen. Frozen biopsies were stored at -80°C until sectioned for immunohistochemistry experiments.

2.3.4.3. Sweat Collection During Prolonged Exercise in the Heat

Sweat was collected following the skin biopsy during a prolonged exercise protocol in an environmental chamber (32-33°C and 35% relative humidity). Intermittent cycling was performed at the workload determined previously to correspond to an exercise intensity of 50% of VO₂max, in 20 minute bouts, separated by five min rest periods. No fluids were ingested by subjects during exercise. To estimate whole body fluid loss, nude dry body weight was obtained pre-exercise, and during the break periods between 20 min cycling bouts, until 3% body weight loss was achieved. Subjects undressed and towel dried in a screened area within the heat chamber prior to each weighing.

Sweat was collected with the modified Brisson method using a collection pouch constructed with impermeable Parafilm (7 cm x 8 cm) (American Can Co., Greenwich, CT) and Opsite wound dressing (10 cm x 14 cm) (Smith & Nephew Inc., Largo, FL). The sweat collection site used for all sweat sampling was the scapular region, an accessible area that correlates well to whole-body sweat constituent concentrations for [Na⁺] and [Cl⁻]. The skin of the scapula was cleaned with alcohol, deionized water, and sterile
gauze, and air dried before application of the collection pouch. Sweat was aspirated from the collection pouch every 20 min during cycling. Sweat $[\text{Na}^+]$, $[\text{Cl}^-]$, and $[\text{K}^+]$ were measured in triplicate using a chemistry analyzer (Nova 5, Nova Biomedical, Waltham, MA). Frequent removal of accumulated sweat in this protocol likely minimized electrolyte leaching from the epidermal layer into the sweat sample. Stable sweat $[\text{K}^+]$ values throughout the collection time points provided evidence that this potential source of error was minimal \(^{126}\).

2.3.5. **Cryosectioning and Immunostaining**

Skin biopsy sections at a thickness of 6 $\mu$M were cut using a cryostat (Leica CM3050-S, Bannockburn, IL) at a chamber temperature of -20°C, beginning at the epidermis and advancing into the dermis. To confirm that sections for immunostaining contained ductal portions of the gland, initial sections at each cut depth were treated with the nuclear stain hematoxlin and examined under light microscopy for ductal characteristics. Sweat ducts were distinguished from the secretory coil portion of the sweat gland by their two cell thickness and well-defined lumen (Fig 1). This was an important distinction because it has been known for some time that sweat entering the duct after exiting the secretory portion of the gland, ‘primary sweat’, is isotonic to extracellular fluid and any decrease in [electrolyte] in the final sweat compared to the extracellular fluid reflects the absorptive properties of the sweat duct \(^{98}\). In order to obtain representation from as much of the entire length of the sweat duct as possible for immunofluorescence assay, sections were inspected for presence of ducts at cut depths spaced every 90 to 150 $\mu$M. Sections cut in immediate succession from those found to contain adequate ducts were picked up onto silane-coated slides (Histobond, Marienfeld,
Lauda-Königshofen), air dried, and fixed in a -20°C solution of acetone-methanol (50:50) for 10 min. Following rinsing with phosphate-buffered saline (PBS), plated sections were permeabilized in 0.25% Triton in PBS for 10 min. After blocking in 1% bovine serum albumin (BSA) in PBS, sections were incubated with primary antibodies overnight at 4°C. M3A7 (sc-58615, Santa Cruz Biotechnology, CA), a mouse monoclonal Ab raised against recombinant CFTR NBF 2 domain (human origin), was used to detect CFTR at a dilution of 1:10. Of the eight main CFTR antibodies established in the literature, M3A7 has been used to distinguish healthy and ΔF508 sweat ducts by CFTR immunostaining \(^{60,127,128}\), and has an epitope outside of the ΔF508 deletion. ENaC was detected using at a dilution of 1:200 the rabbit polyclonal Ab H-95 (sc-21012, Santa Cruz Biotechnology, CA), which targets amino acids 131-225 near the N-terminus of human α-ENaC. After thorough washes to remove excess primary antibody, sections were incubated for 60 min at room temperature with anti-mouse AlexaFluor488 and anti-rabbit AlexaFluor 594 secondary antibodies at a 1:500 dilution (Invitrogen, Life Technologies, Carlsburg, CA). To confirm lack of nonspecific binding by secondary antibodies, omission of primary antibodies was performed on some sections from each subject. After thorough washes, the sections were stained with 4’,6-diamidino-2-phenylindole (DAPI) to demonstrate sweat gland morphology, and fixed with a mounting solution. Localization of CFTR and ENaC were examined using a Zeiss Axiovision microscope and photographed using Zeiss Axiocam-HR (Zeiss, Oberkochen, Germany). Captured images were analyzed by a blinded investigator using ImageJ analysis software (National Institutes of Health, Bethesda, MD) for quantification of ENaC and CFTR staining (mean pixel intensity per area) at ductal lumen. Background fluorescence was subtracted from
staining intensity. To assess reproducibility, cryosectioning, immunostaining and image analysis was performed on the biopsies of six subjects (3 non-CF pairs) a second time.

2.3.6. Statistical Analysis

Results are presented as means ± SE. For each subject, an overall mean pixel intensity per area for CFTR, and for ENaC, was computed as an average of all imaged ducts’ mean pixel intensity per area values. This consolidated value was used to determine between group differences in CFTR and ENaC expression. To minimize the effect of batch-to-batch variability in this cross-sectional analysis, staining intensity, cryosectioning and immunostaining procedures were always performed in the same run for matched subjects. Differences in subject characteristics, sweat electrolytes, and expression of membrane channels CFTR and ENaC among subject groups Control, SS, and CF were determined using a one way Analysis of Variance (ANOVA). Post-hoc testing (Tukey) was performed to determine between-group differences. For the six paired non-CF subjects for whom repeated immunostaining was performed, coefficient of variation for relative expression, calculated as mean pixel intensity per area divided by the total mean pixel intensity per area of both subjects within a matched pair, was determined with Pearson-Product-Moment Correlation. The relationship between sweat electrolytes and mean pixel intensity per area of CFTR staining, and ENaC staining, was analyzed using Pearson-Product Moment Correlation. All statistical testing was performed using SPSS (version 17.0, SPSS, Inc., Chicago, IL). An α level of 0.05 was used to indicate statistical significance.

2.3.7. Genetic Testing
A venous sample collected from non-CF subjects at the start of their second testing session was used for genetic testing. Genomic DNA (gDNA) was isolated from whole blood and tested by an outside laboratory (Emory Genetics) using an allele-specific primer extension assay (Tag-It Cystic Fibrosis Kit, Luminex, Toronto). This assay tested for 39 of the most common CF mutations in the US, including the recommended ACMG panel\textsuperscript{129} of 23 common mutations. Mutations tested in this panel were: ΔF508, R334W, S549N, 3659delC, ΔI507, I347P, A559T, S1255X, 1898+1G>A, R347H, N1303K, 1898+5G>T, 3876delA, A455E, 394delTT, 2183GG>A, 3905insT, 3120+1G>A, V520F, 2184delA, G85E, Y1092X, 711+1G>T, 2307insA, Y122X, S549R, M1101K, 1078delT, 2789+5G>A, G551D, G542X, 621+1G>T, R560T, W1282X, 1717-1 g>a, 3849 + 10KbC>T, R553X, R117H, and R1162X.

2.4. Results

2.4.1. Subjects

Mean (± SE) physical characteristics are presented in Table 2.1. The two groups of non-CF subjects (those with high and low sweat [Na\textsuperscript{+}]) were similar in their physical characteristics and exercise training volume. As expected given the nature of the disease, CF subjects were younger, and had lower aerobic capacity and weekly training volume compared to non-CF subjects.

2.4.2. Sweat Electrolytes and Sweat Rate

Mean (±SE) sweat electrolytes presented in Table 2.2 are calculated for each subject as their average across all collections (from 0.5 to 3% dehydration). As expected given the procedure for the subject selection, sweat [Na\textsuperscript{+}] and [Cl\textsuperscript{-}] were higher for SS
and CF compared to Control (p<0.001). Sweat [Na\(^+\)] and [Cl\(^-\)] values for CF were also higher than SS (p<0.001). Consistent with reports in the literature for CF sweat electrolyte composition\(^2\)\(^6\)\(^4\), CF sweat [K\(^+\)] tended to be higher than non-CF groups (p=0.09). Control and SS had similarly low sweat [K\(^+\)] (p=0.858).

**Table 2.1:** Mean (±SE) physical characteristics, exercise training volume, and aerobic fitness (VO\(_{2}\max\)) of Control, non-CF salty sweaters (SS), and cystic fibrosis (CF) subjects (n=18). * Significantly < Control, # CF significantly < SS (p<0.05).

<table>
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<th>Physical Characteristics</th>
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<td>Weight (kg)</td>
<td>68.4 ± 5.7</td>
<td>73.2 ± 6.4</td>
<td>64.0 ± 5.7</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>15.0 ± 3.3</td>
<td>18.0 ± 3.5</td>
<td>15.3 ± 2.3</td>
</tr>
<tr>
<td>Training Volume (hrs/wk)</td>
<td>12.2 ± 2.0</td>
<td>11.8 ± 1.8</td>
<td>5.1 ± 0.7 *#</td>
</tr>
<tr>
<td>VO(_{2}\max) (ml/kg/min)</td>
<td>53.9 ± 2.4</td>
<td>49.5 ± 2.8</td>
<td>39.9 ± 1.8 *#</td>
</tr>
</tbody>
</table>

**Table 2.2:** Mean (±SE) sweat electrolytes concentration (indicated by brackets), and sweat rate (SR) relative to body weight (BW), of Control, non-CF salty sweaters (SS), and cystic fibrosis (CF) subjects (n=18). * Significantly > Control (p<0.005), # CF significantly > SS (p<0.05).

<table>
<thead>
<tr>
<th>Sweat Characteristics</th>
<th>Control</th>
<th>SS</th>
<th>CF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sweat [Na(^+)] (mmol/L)</td>
<td>46.2 ± 4.2</td>
<td>94.9 ± 6.2 *</td>
<td>132.6 ± 2.6 *#</td>
</tr>
<tr>
<td>Sweat [Cl(^-)] (mmol/L)</td>
<td>47.8 ± 3.6</td>
<td>87.1 ± 7.1 *</td>
<td>127.0 ± 4.9 *#</td>
</tr>
<tr>
<td>Sweat [K(^+)] (mmol/L)</td>
<td>4.9 ± 0.2</td>
<td>4.3 ± 0.1</td>
<td>7.4 ± 0.6 #</td>
</tr>
<tr>
<td>SR (ml/hr/kgBW)</td>
<td>13.1 ± 0.9</td>
<td>12.4 ± 1.4</td>
<td>11.0 ± 0.7</td>
</tr>
</tbody>
</table>
[Na\(^+\)] and [Cl\(^-\)] increased with progressive dehydration in all three groups. Interestingly, there was a greater relative increase in sweat [Na\(^+\)] (p<0.05) with dehydration in Control (mean % change of 17.7 ± 3.2%) compared to CF (4.2 ± 2.1%), and tended to be greater (p=0.15) in Control compared to SS (8.7 ± 4.2%). There was also a trend (p=0.10) for greater relative increase in sweat [Cl\(^-\)] with dehydration in Control (16.5 ± 3.9%) compared to CF (6.2 ± 1.9%).

As designed, Control, SS, and CF achieved similar percent body weight loss (% dehydration) at the termination of exercise (3.0 ± 0.06% for Control, 2.9 ± 0.07% for SS, and 2.9 ± 0.07% for CF). There was also no difference among groups in sweat rate (SR) expressed relative to BW (Table 2.2).

2.4.3. CFTR and ENaC Expression at Ductal Lumen

Sections from the ductal portion of the sweat gland were distinguished morphologically from sections of the secretory coil portion of the gland by nuclear staining. The ductal segment (coiled and straight portion) has a two cell layered wall, as indicated by two layers of nuclei surrounding a clearly defined lumen (examples shown with arrow, Fig 2.1a and Fig 2.1b). In contrast, the secretory coil segment is only one cell wall thick, has a more poorly defined lumen, and is usually larger in diameter with larger nuclei (examples labeled with ‘S’, Fig 2.1a and Fig 2.1b). This distinction is important because CFTR and ENaC staining was quantified only in the lumen (example is circled in Fig 2.1b) of images identified clearly as ductal segments. Representative epifluorescence images of immunostaining for Control (row 1), SS (row 2), and CF (row 3) are presented in Fig 2.2. As expected, CFTR (stained green) was localized primarily to the ductal lumen. ENaC (stained red) was also located primarily at ductal lumen but additionally
Fig. 2.1: Eccrine sweat gland tubules in biopsied human skin. To demonstrate morphology of secretory coil (S) and reabsorptive duct (arrows), 6 µm cryosections were stained with (a and b) hematoxylin (brightfield image original magnifications 20X) and (c) 4',6-diamidino-2-phenylindole (DAPI, epifluorescence image original magnification 40X). An example of a ductal lumen (outlined) is shown in part (c).
distributed throughout the cytoplasm. Immunofluorescent staining for CFTR and ENaC was absent on negative control sections not incubated with primary antibodies (Fig 2.2, 4th column). In the three subject pairs for which repeat sectioning and immunostaining was performed, the coefficients of variation (CV) between initial and repeat relative expression of CFTR were 7.2%, 4.0%, and 4.3% (mean 5.5 ± 1.8%). The relative expression of ENaC in these repeated pairs had CVs of 1.7%, 19.1%, and 0.4% (mean 7.1 ± 10.4%).

CFTR expression was lower (p< 0.05) in SS than Control (Figs 2.2 and 2.3). CFTR expression in CF was lower than Control (p<0.005), but not SS (p=0.241) (Fig 2.2 and 2.3). ENaC expression was not different (p=0.957) among groups (Fig 2.2 and 2.3).

2.4.4. Relationship between Channel Expression and Sweat Electrolytes

Significant inverse relationships were observed between CFTR expression and sweat [Na⁺] (r = -0.639, p<0.005) (Fig 2.4), and for CFTR and sweat [Cl⁻] (r = -0.594, p<0.010); namely, greater sweat Na⁺ and Cl⁻ was associated with lower sweat duct CFTR. When only non-CF subjects (n=16) were examined, the same relationship with CFTR expression persisted but did not reach significance for sweat [Na⁺] (r = -0.461, p=0.132), or for sweat [Cl⁻] (r = -0.402, p=0.195). There was also no significant relationship between CFTR and sweat electrolytes in the six CF subjects (r = 0.143, p=0.788 for Na⁺; and r = 0.304, p=0.558 for Cl⁻).

Consistent with the finding of similar ENaC expression among groups, there was no significant relationship between ENaC expression and sweat [Na⁺] (r = 0.211, p=0.401)(Fig. 2.4), or sweat [Cl⁻] (r = -0.307, p=0.216). There was also no significant
Fig. 2.2: Immunofluorescence staining of the eccrine sweat gland reabsorptive duct in 6 µm cryosections of biopsied human skin. Anti-CFTR labeling with M3A7 (column 1, green), and anti-αENaC labeling with H-95 (column 2, red) revealed immunoreactivity particularly corresponding to the luminal plasma membranes. Overlay of images from column 1 and 2 demonstrates where immunoreactivity to CFTR and ENaC occurred at the same location (column 3, yellow). Sections without primary antibodies did not show immunoreactivity (column 4). CFTR staining was significantly more pronounced in Control subject with mean sweat [Na⁺] of 45 mmol/L and [Cl⁻] of 44 mmol/L (row 1) compared to paired non-CF salty sweater subject (SS) with sweat [Na⁺] of 88 mmol/L and [Cl⁻] of 85 mmol/L (row 2), and compared to paired cystic fibrosis (CF) subject (ΔF508/1717-1G→A) with sweat [Na⁺] of 136 mmol/L and [Cl⁻] of 130 mmol/L (row 3). Original magnification 40x.

relationship when analyzed for only non-CF subjects (r = 0.285, p=0.369 for Na⁺; and r = 0.398, p=0.200 for Cl⁻). However, unlike in non-CF subjects, there was a significant positive relationship in CF for ENaC and sweat [Cl⁻] (r = 0.846, p = 0.034), with a tendency for sweat [Na⁺] in CF subjects to be positively correlated with ENaC as well (r = 0.741, p = 0.092).
Fig. 2.3: Mean (±SE) sweat duct luminal membrane channel expression (mean pixel intensity per area) for cystic fibrosis transmembrane conductance regulator (CFTR, green), and epithelial sodium channel (ENaC, red) of Control subjects with mean±SE sweat [Na⁺] of 46.2 ±4.2 and [Cl⁻] of 47.8 ±3.6 mmol/L (solid bars), non-CF salty sweaters (SS) with mean±SE sweat [Na⁺] of 94.9 ±6.2 and [Cl⁻] of 87.1 ±7.1 mmol/L (striped bars), and cystic fibrosis (CF) subjects with mean±SE sweat [Na⁺] of 132.6 ±2.6 and [Cl⁻] of 127.0 ±4.9 mmol/L (checkered bars). * Significantly < Control (p<0.05). Number of (n) ducts and subjects for each mean are indicated below x axis.
**Fig. 2.4:** Relationship between sweat duct luminal membrane channel expression and [Na⁺] of thermally-induced sweat of CF (circles), non-CF salty sweaters (SS, diamonds), and Control (triangles) subjects. A significant inverse relationship (green line, $r=-0.639$, *p*<0.005) was demonstrated for mean pixel intensity per area of ductal luminal membrane CFTR (green symbols) and [Na⁺] of sweat collected during exercise in the heat to 3% dehydration. No relationship (p=0.401) was observed for mean pixel intensity per area of ductal luminal membrane ENaC (red symbols) and sweat [Na⁺].

2.4.5. Genetic Testing

Genetic testing determined that none of the healthy subjects were heterozygous for any of the 39 most common disease-causing CFTR mutations in the US. While the testing panel used in this study is considered to accurately predict 99% of all non-Hispanic Caucasian carriers of a disease-causing CFTR mutation in the US \(^{129}\), it does not detect CFTR mutations with milder or unknown phenotypes.
2.5. Discussion

This study investigated a potential mechanism that might account for the known inter-individual variability of NaCl loss in humans during thermoregulatory sweating. Given that primary sweat produced by the secretory coil of the sweat gland is isotonic to extracellular fluid \(^{38,97}\), and that extracellular [NaCl] is maintained in a tight physiological range across humans, we examined the reabsorptive duct of the sweat gland for differences in the abundance of membrane transport machinery that might explain variable electrolyte reabsorption. To accomplish this, measurement of sweat electrolytes (during exercise in the heat eliciting moderate dehydration) and luminal membrane CFTR and ENaC expression was performed in healthy subjects with salty sweat compared to those with ‘typical’ sweat [NaCl], and compared to subjects with CF. Immunoreactivity to anti-CFTR was localized primarily to the apical membrane of the inner luminal ductal cells, the principal site of NaCl reabsorption \(^{38}\). Immunoreactivity to anti-αENaC was greatest at the apical membrane of the inner luminal ductal cells, but was also observed between the inner and outer luminal ductal cells, and generally distributed more diffusely throughout the cell than CFTR. Intriguingly, a significant association was demonstrated for ductal luminal membrane CFTR expression and thermoregulatory sweat [NaCl]. Further, CFTR expression in sweat ducts of healthy salty sweaters was found to be less than in Control and not significantly different than in CF subjects.

In light of this finding of altered CFTR expression in SS, it is somewhat surprising then that none of these subjects were identified as heterozygotes for CF. This suggests that: 1) either the lower CFTR expression in the SS group was not related to CF carrier status, or, 2) the testing panel utilized was not complete and thus failed to detect
all other potential CFTR gene mutations. The test panel utilized in this study is the most commonly used for CF genetic testing to detect the most frequently occurring mutations of the CFTR gene that are associated with development of the classical form of the disease. Approximately 4% of individuals of European Caucasian descent are carriers of one of these CFTR mutations \(^{105,106}\). Over 1600 mutations and over 300 polymorphisms of the CFTR gene have been identified \(^{130}\), however, with no known clinical abnormalities associated with most \(^{70}\). The prevalence of carriers in the population for one of the many other CFTR variants is not known. Thus, it is still possible that a less common CFTR gene mutation associated with a non-disease causing phenotype, perhaps only manifesting in the sweat gland, was responsible for the lower CFTR expression and the higher sweat [NaCl] in SS. Given that SS were all well-trained endurance athletes who reported no chronic CF-like conditions (e.g. pancreatitis, recurrent sinus infections), this is more likely. Gene sequencing of non-CF subjects would be required to determine if such a discreet CF gene polymorphism is present with greater frequency in SS. Thus, we cannot yet exclude the possibility that a relationship exists between excess NaCl loss in sweat and CF carrier status.

While CFTR expression was reduced in SS and CF, ENaC expression was remarkably similar among groups. In addition to serving as a cAMP-activated Cl-channel, CFTR plays a critical role in transepithelial absorption through its influence on other ion transport proteins, including ENaC \(^{131-133}\). Functionally dependent on CFTR in several absorbing tissues including those of the sweat gland \(^{100,134}\), ENaC conductance for Na\(^+\) reabsorption should not be assumed intact when ENaC expression is normal. Therefore, it is reasonable that [Na\(^+\)] in sweat was markedly higher in SS despite no
difference in ENaC expression, and that no association was determined between sweat \([\text{Na}^+]\) and luminal expression of ENaC.

While our findings indicate that reduced CFTR expression at the luminal membrane of the sweat duct is related to the high sweat [\(\text{NaCl}\)] found in some apparently-healthy individuals, reduced CFTR expression accounts for only 21% of the variance in sweat [\(\text{Na}^+\)] within non-CF subjects which suggests that other factors may also play a mechanistic role. The possibility of other contributing factors is also suggested by the observation that sweat [\(\text{NaCl}\)] in SS was approximately twice that of Control while CFTR expression in SS compared to Control was only 25% less. Further, CF sweat [\(\text{NaCl}\)] was almost three times higher than Control yet mean CFTR expression in CF compared to Control was only approximately 50% less. It is not possible with these data to assess how differences in overall channel function for SS and CF compared to Control contributed to differences in their sweat [\(\text{NaCl}\)]. However, functional impairment of transepithelial transport channels may explain how sweat [\(\text{NaCl}\)] was almost twice that of matched Control despite CFTR expression that was similar for one of the six SS subjects, and unexpectedly greater for another of the six SS subjects, compared to Control counterparts.

Functional impairment of CFTR was almost certainly an additional factor in the high sweat [\(\text{NaCl}\)] for CF subjects, all of whom had \(\Delta F508\) mutations on at least one allele, and four of whom were homozygous for \(\Delta F508\). \(\Delta F508\) CFTR is translated, but misprocessing within the endoplasmic reticulum results in most of the protein being directed toward degradation instead of insertion into the plasma membrane\(^{135, 136}\). Therefore, it was expected that luminal CFTR immunostaining would be largely absent in
CF as other investigators have demonstrated for ΔF508 sweat ducts\textsuperscript{60,128}. Interestingly, there was not a complete absence of luminal CFTR staining in CF tissue. While many ductal sections showed scarce immunoreactivity to M3A7, some CF ducts were found to stain for CFTR similar to that in non-CF SS tissue, and overall mean CFTR expression in CF was still approximately half that of Control. This is unexpected as immunostaining with M3A7 at similar dilutions has previously been characterized by other investigators\textsuperscript{127,128}. One possible explanation for the slightly greater CFTR expression in these subjects compared to ΔF508 CF in previous studies is an increase secondary to patient medications. Aminoglycosides, especially tobramycin, are frequently prescribed for CF patients to combat respiratory infections from the opportunistic bacterial pathogen Pseudomonas aeruginosa\textsuperscript{137}. It is not known if aminoglycoside therapy results in pharmacologic effects that extend beyond control of infections. Four out of six CF subjects in this study were on tobramycin or other aminoglycosides at the time of participation. An important difference observed for CF compared to non-CF was the lack of association between sweat duct CFTR expression and sweat [Na\textsuperscript{+}] or [Cl\textsuperscript{-}]. Despite apparent presence of CFTR (albeit reduced) at the CF ductal lumen, NaCl sweat loss was still almost three fold higher for CF than Control. This is consistent with the belief that for the few ΔF508 CFTR channels that are somehow trafficked to the luminal membrane, channel function is compromised, possibly due to reduced channel activity\textsuperscript{138} and/or stability\textsuperscript{139}. The unexpected positive relationship between ENaC and sweat [Na\textsuperscript{+}] and [Cl\textsuperscript{-}] in CF subjects is congruent with the observed upregulation of ENaC expression in CF nasal epithelium\textsuperscript{140}. 
It is also possible that expression or function of ductal membrane transport proteins other than CFTR and ENaC is additionally reduced in SS or CF. One possibility is a Na\(^+\)/H\(^+\) exchanger (NHE1), thought to be responsible for intracellular pH regulation in ductal epithelium in concert with an apical concanamycin A-sensitive proton pump\(^{50,51}\). NHE1 is present at the basolateral and lateral membranes of human eccrine sweat duct cells\(^{51,52}\) and control of ductal intracellular pH may be mechanistically linked to luminal Na\(^+\) removal\(^{51}\). A deficiency in NHE1 expression or function would lead to a rise in intracellular Na\(^+\), thereby reducing the gradient for luminal Na\(^+\) entry through ENaC. This would decrease luminal Na\(^+\) reabsorption and acidification of sweat. While not examined in this study, it would be interesting to determine if sweat pH is elevated along with [NaCl] in apparently-healthy salty sweaters.

Looking for other potential mechanisms that can explain the remaining variability, it has been suggested that aquaporins might play a role in the saltiness of sweat particularly as a possible point of control for acute regulation of the sweat gland by vasopressin\(^{6,141}\). However, while secretory coil cells of mice and rat paw sweat glands appear to specifically express AQP5\(^{142}\), there is not good evidence supporting its presence in the cells of the human sweat duct. AQP5 is more likely to be found in the secretory not ductal portion of the human sweat gland, as has been shown to be the case in other glandular epithelium\(^{143-145}\). If AQP5 or other aquaporins are indeed localized to the secretory coil of the human sweat gland, they would not participate in the ductal modification of primary sweat prior to final sweat excretion. However, it is conceivable that decreased aquaporin expression or function in the coil could ultimately contribute to a more concentrated final sweat if transcellular water transport (into the lumen) during
the formation of primary secreted sweat is decreased. Volume of sweat produced would also be compromised, though, and salty sweat is not associated with a lower rate of sweating as observed in both CF and SS in this study. Nonetheless, further investigation is needed to clarify the role that aquaporins may play in the human sweat gland and if variable expression or function is a contributing factor to the excretion of saltier sweat in some individuals.

2.6. Conclusion

In the present study, we have demonstrated that reduced expression of the Cl⁻ channel CFTR at the apical membrane of the sweat gland’s reabsorptive duct may contribute to greater loss of NaCl in some apparently healthy individuals during thermoregulatory sweating. CF subjects had significantly lower ductal CFTR expression compared to healthy salty sweaters; moreover, these healthy salty sweaters had lower CFTR expression than their control counterparts. Differences in CFTR-mediated Cl⁻ and Na⁺ transport in ductal NaCl reabsorption could be a potential mechanism underlying variability in human sweat Na (i.e. promoting a CF-like phenotype). However, genetic testing failed to establish a link between healthy salty sweaters and one of the 39 most common disease-causing CFTR mutations; but, a relationship to other untested CFTR variants cannot be entirely ruled out. Further research is needed to investigate whether other epithelial transport proteins contribute to the variability in human eccrine sweat sodium chloride composition.
CHAPTER 3

HIGH SWEAT SODIUM LOSSES DO NOT DIFFERENTIALLY ALTER PERCEIVED THIRST DURING EXERCISE IN THE HEAT

3.1. Abstract

Purpose: Eccrine sweat sodium concentration ([Na\(^+\)]) varies greatly among individuals; but, whether this differentially affects physiological responses that dictate thirst during prolonged exercise in the heat has not been investigated. Methods: A total of twenty-one physically-active young adults performed cycling in the heat (32-33°C, 35% RH) at 50% of VO\(_{2\text{max}}\) until 3% body weight loss. Seven healthy subjects with high sweat [Na\(^+\)] (SS) were matched by physical characteristics, training, and acclimation status to healthy subjects with average sweat [Na\(^+\)] (Control). Six physically-active cystic fibrosis patients (CF), who phenotypically excrete extremely salty sweat, served as a third group. Results: Mean (± SD) regional sweat [Na\(^+\)] (91.0 ± 17.3) and [Cl\(^-\)] (84.0 ± 17.8 mmol/L) for SS were higher (p<0.001) than Control (43.7 ± 9.9, and 41.9 ± 6.9 mmol/L, respectively). CF sweat [Na\(^+\)] (132.6 ± 6.4) and [Cl\(^-\)] (127.0 ± 12.1) were also higher compared to Control (p<0.001) and SS (p<0.005). Whole body sweat rate relative to body mass (12.3 ± 2.5 mL/kg/hr) and exercise time required to reach 3% dehydration (125.7 ± 22.0 min) did not differ among groups. Rise in serum osmolality with dehydration was lower (p<0.05) in CF (6.1 ± 4.3 mOsm/kgH\(_2\)O), and SS (8.4 ± 3.0 mOsm/kgH\(_2\)O) compared to Control (14.8 ± 3.5 mOsm/kgH\(_2\)O). Relative change in plasma volume (%ΔPV) was significantly (p<0.05) greater in CF (-19.3 ± 4.5%), and SS
(-18.8 ± 3.1%) than Control (-14.3 ± 2.3%). Rating of thirst relative to dehydration and change in plasma aldosterone, vasopressin, and angiotensin II was not different among groups. During recovery, serum [Na+] (by 2.8 ± 2.1 mmol/L) and [Cl−] (by 4.8 ± 1.3 mmol/L) decreased below resting values in CF with ingestion of a sports beverage despite 30% lower (p<0.05) volume ingested ad libitum compared to SS and Control.

Conclusion: Individuals with CF and healthy individuals with high sweat [Na+] exhibit attenuated blood osmolality but greater relative plasma fluid loss at moderate levels of dehydration, resulting in similar ratings for thirst as Controls during exercise in the heat. However, drinking behavior in CF, unlike SS, was altered when presented with hypotonic fluids in recovery, presumably in response to the reduction in blood [NaCl].

3.2. Background

3.2.1. Variability in Human Eccrine Sweat Electrolytes

There is tremendous inter-individual variability in eccrine sweat sodium concentration ([Na⁺]) in healthy subjects tested under similar conditions that control methodological factors related to sweat collection and analysis. Representing the most extreme physiological example of extra salty sweat, [Na⁺] of sweat from individuals with the lethal autosomal recessive disease cystic fibrosis (CF) is typically three to five times higher than average due to defective chloride (Cl−) and sodium (Na⁺) channel transport in the CF sweat duct. ΔF508 is the most frequent mutation causing CF in the US, with more than 90% of all CF patients carrying at least one allele. As a result of the misprocessing and protein degradation associated with this mutation, CFTR fails to localize correctly at the sweat duct membrane and results in sweat that is nearly isotonic to plasma. Regardless of acclimation status (which is known to modulate sweat
electrolytes even within an individual\textsuperscript{14-17,33}, some apparently healthy (non-CF) individuals also excrete sweat containing high [Na\textsuperscript{+}], with values approaching that of CF\textsuperscript{64}. The implications of excess sweat sodium losses on physiological functions (e.g., thermoregulation, fluid balance, cardiovascular regulation) are not fully understood, especially for humans engaging in exercise where dehydration may occur.

3.2.2. Physiology of Thirst

Thermoregulatory sweating during exercise typically results in increased blood osmolality due to the relatively greater loss of water compared to electrolytes. There is a strong relationship between the sensation of thirst and blood osmolality\textsuperscript{71-73}. As osmolality increases, arginine vasopressin (AVP) release increases, mediated by signaling from osmoreceptors of the preoptic anterior hypothalamus\textsuperscript{77}. As little as a 1 or 2\% rise in osmolality induces release of this dipsogenic hormone\textsuperscript{73-77}, AVP, which results in insertion of aquaporin-2 channels into the apical membrane of the collecting duct and subsequently increases renal fluid retention\textsuperscript{77}. Vasopressin also stimulates a behavioral mechanism (drinking) for the correction of hyperosmolality by its influence on thirst drive\textsuperscript{73-76}. Individuals with high sweat [Na\textsuperscript{+}] have proportionately less free water (FW) loss during sweat-induced dehydration\textsuperscript{79}; however, the impact of lower FW loss on their hyperosmolality-dependent sensitivity of thirst relative to hydration deficit is not well known. This has important implications during exercise in the heat, because attenuated thirst and diminished drinking may promote a state of involuntary dehydration\textsuperscript{71}, with subsequent challenges for cardiovascular responses and thermoregulation.

3.2.3. Adequacy of Thirst Mechanism during Prolonged Exercise in the Heat
There is debate among exercise physiologists whether reliance on thirst is recommended during exercise in the heat. It has been common practice to recommend programmed drinking due to a potential lag in the thirst mechanism leading to inadequate fluid balance to maintain exercise performance. An alternative view is that thirst-guided drinking in humans is sufficient for fluid balance during exercise (as in other mammalian species) and that not relying upon the thirst drive could potentially lead to excess consumption of fluids and increased risk of hypervolemic hyponatremia. It is possible that both views are correct, but that the thirst drive is not reliable for all individuals. It has been observed that individuals with CF, who phenotypically exhibit elevated sweat [NaCl], may underestimate their fluid needs during exercise in the heat, resulting in a state of ‘involuntary dehydration.’ It has been suggested that this may be due to an attenuated hyperosmolality trigger for thirst. The impact of variability in sweat [Na\(^+\)] on thirst sensation has not been previously explored. The implications could be particularly important for individuals with high sweat [Na\(^+\)] such that, if thirst does not match actual fluid needs due to sweating, they may be at greater risk for dehydration if dependent upon thirst-guided drinking during prolonged activity in the heat. However, to recommend regimented drinking for these individuals is also possibly deleterious because the combination of excess sweat [Na\(^+\)] loss coupled with excessive water intake could result in lower serum sodium and risk for hyponatremia during prolonged activity in the heat.

For individuals with CF who excrete extremely salty sweat, the impact of a potentially greater cumulative sweat NaCl loss on blood electrolytes and thirst sensitivity has not been investigated systematically. With improved management of respiratory and
digestive sequelae, participation in recreational and competitive sports is increasing for patients with CF. Exercise is encouraged, as higher levels of aerobic fitness in CF patients have been shown to be associated with an increased life expectancy\textsuperscript{153,154}. However, with electrolyte and fluid losses during prolonged exercise not clearly defined for CF, fluid replacement guidelines have yet to be established for this population to maximize safety and performance during exercise in the heat. To our knowledge, CF responses to thermally-induced sweating during exercise have been investigated in only four studies to date in the literature\textsuperscript{83,118,151,155}. However, there are methodological limitations in some aspects of these studies, such as uncontrolled volume of fluid ingestion during exercise between groups\textsuperscript{83,118,155}, inadequate yield in blood sampling\textsuperscript{151}, use of CF subjects with low fitness\textsuperscript{83} or unequal fitness levels between CF and non-CF groups\textsuperscript{118}. In the present study, exercise-trained CF patients were compared to healthy exercise-trained individuals with high and “normal” sweat NaCl loss to determine the effect of dehydration on physiological responses and fluid/electrolyte balance during exercise. It was hypothesized that individuals with high sweat [NaCl] (e.g. both healthy and CF) would have attenuated increases in blood osmolality and thus decreased osmotic stimulation of thirst relative to change in hydration status compared to individuals with normal sweat [NaCl].

3.3. Methods

3.3.1. Subjects

3.3.1.1. Preliminary Screening and Subject Selection

Recreationally-active young adults (aged 18–40 yrs) were recruited from the campus community and endurance sports teams in the area. Normal, healthy volunteers
(non-CF subjects) participated in a preliminary sweat collection session (i.e., 30-60 min of cycling or running at self-selected pace until 1.5-2 ml of sweat was obtained). Sixteen individuals were chosen to participate as subjects based on their measured sweat composition: eight were ‘salty sweaters’ (SS) with sweat [Na⁺] > 70 mmol/L (Mean ± SD = 87.6 ± 18.4 mmol/L, range 70-111 mmol/L), and eight were normal (Control) with sweat [Na⁺] < 60 mmol/L mmol/L (Mean ± SD = 41.2 ± 8.4 mmol/L, range 31-55 mmol/L). The cut-point of > 70 mmol/L was used for selecting SS because it represents ~ 2 SD higher than the mean recently reported for regional sweat [Na⁺] collected under similar conditions and with a similar technique from the upper back region during exercise in the heat\textsuperscript{117}.

In addition, six young adults with cystic fibrosis (CF) were recruited through the Emory University Cystic Fibrosis Center and the local community to participate as volunteers. All CF had sweat [Cl⁻] in previous diagnostic pilocarpine testing of > 75 mmol/L. One CF subject was ΔF508/R1162X, one was ΔF508/1717-1G→A, and the remaining four were homozygous for ΔF508 mutations. All CF were in stable clinical status with an FEV\textsubscript{1} > 75% of predicted value, performed aerobic exercise for a minimum of four hours per week, and were cleared by their physician for participation. Informed written consent was obtained from both CF and non-CF as approved by the Institutional Review Boards at the Georgia Institute of Technology and Emory University.

3.3.1.2. Study Design and Subject Characteristics

Responses to progressive dehydration induced by prolonged exercise in the heat in Control, SS, and CF were compared using a cross-sectional design. Identification of SS subjects was performed first through sweat collections in preliminary screening.
Subsequent matching of each SS subject with a Control subject was performed on the criteria that Control have lower sweat [Na⁺] by at least ~50% (mean ± SD % difference in sweat [Na⁺] for Control compared to matched SS = -53.2 ± 11.6%). Non-CF pairs were also matched by age, gender, anthropometry, training history, and aerobic capacity.

Subject characteristics are presented in Table 3.1. Sample size among the groups was not equally balanced due to difficulty in recruiting CF subjects and a subject drop-out in SS. Compared to non-CF groups, CF were younger, had lower aerobic capacity, and lower weekly training volume as expected given the nature of the disease. Paired non-CF subjects were tested in the same month, and between the months of December through May, to control for natural heat acclimation (a well-documented modifier of sweat composition) 14-17. CF subjects were matched to non-CF subjects by gender but not necessarily tested within the same month as their non-CF counterparts. However, this was not a major study limitation since CF sweat composition does not appear to change with

### Table 3.1: Mean (±SD) physical characteristics, exercise training volume, and aerobic fitness (VO₂max) of Control, non-CF salty sweaters (SS), and cystic fibrosis (CF) subjects (n=21). * Significantly < Control and SS (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SS</th>
<th>CF</th>
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<tbody>
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<td>Gender</td>
<td>8 m, 2 f</td>
<td>7 m, 2 f</td>
<td>4 m, 2 f</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>30.5 ± 5.7</td>
<td>31.6 ± 6.5</td>
<td>22.2 ± 4.5 *</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>68.8 ± 11.8</td>
<td>74.4 ± 14.7</td>
<td>63.7 ± 13.9</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>14.5 ± 6.8</td>
<td>17.3 ± 8.1</td>
<td>15.3 ± 5.6</td>
</tr>
<tr>
<td>Training Volume (hrs/wk)</td>
<td>11.1 ± 4.9</td>
<td>12.0 ± 4.0</td>
<td>5.1 ± 1.6 *</td>
</tr>
<tr>
<td>VO₂max (ml/kg/min)</td>
<td>52.8 ± 5.7</td>
<td>50.1 ± 6.5</td>
<td>39.9 ± 4.5 *</td>
</tr>
<tr>
<td>HRpeak (bts/min)</td>
<td>184.9 ± 7.1</td>
<td>181.4 ± 6.2</td>
<td>190.0 ± 4.8</td>
</tr>
</tbody>
</table>
heat acclimation \textsuperscript{118}. All female subjects were tested in the early follicular phase of the menstrual cycle to avoid estrogen and progesterone influences on osmotic thirst and AVP responses during the luteal phase \textsuperscript{123}. Comparison of collected sweat among groups was performed at the same relative dehydration and exercise intensity to minimize potential effects of these known modifiers on sweat electrolytes.

\subsection*{3.3.2. Initial Testing Session: Aerobic Capacity Assessment and Familiarization}

In the first test session, a graded, incremental cycling test was conducted in the heat (32-33°C and 35\% relative humidity) to determine maximal oxygen uptake (\(\text{VO}_2\text{max}\)). Collection of expired gases to determine oxygen consumption (\(\text{VO}_2\)) and respiratory exchange ratio (RER), heart rate (HR) and rating of perceived exertion (RPE) \textsuperscript{124} were recorded during each stage the test. Subjects cycled until volitional exhaustion. \(\text{VO}_2\text{max}\) was considered achieved at test termination based on attainment of at least two of the following criteria: a plateau in \(\text{VO}_2\) during the last two stages (increase < 2.1 ml/kg/min), a HR within 10 beats/min of age-predicted HR\(_{\text{max}}\), a respiratory exchange ratio (RER) \(\geq 1.10\), or a minute ventilation > 115 L/min.

A 30 min familiarization ride in the heat (32-33°C and 35\% relative humidity) followed the \(\text{VO}_2\text{max}\) testing to validate the workloads corresponding to a work rate estimated to elicit 50\%\(\text{VO}_2\text{max}\) for the next test session. Nude dry body weight was obtained before and after the 30 min ride to determine individual whole-body sweat rates. During the familiarization ride, a regional sweat sample was collected from the right scapula in order to confirm group placement.

Body composition was assessed for subject matching purposes using dual energy X-ray absorptiometry (DEXA) with a Lunar Prodigy whole body scanner (GE Medical
Systems, Madison, WI). At the completion of their initial testing session, subjects were instructed in use of dietary logs to record ingested food and beverages for the three days prior to their second testing session.

3.3.3. Second Testing Session: Dehydration Induced by Prolonged Cycling in Heat

3.3.3.1. Pre-testing Controls

Subjects abstained from caffeine at least 12 hours prior and alcohol at least 32 hours prior to reporting to the lab for testing. Twenty-four hour food logs indicated that subjects complied with instructions to consume a standardized breakfast meal consisting of bagel, toast, and/or English muffin with cream cheese, butter, and/or peanut butter, and orange juice (if desired) on the morning of testing. There was no difference (p>0.05) between SS and Control in macronutrient, sodium, or caloric intake relative to body weight (BW) for the morning of testing, and for the average of three days prior to testing. CF consumed significantly more (p<0.05) total calories (14.7 ± 7.1 kcals/kg of BW) and protein (0.6 ± 0.3 g/kg BW) than SS (6.5 ± 2.7 kcals/kg, 0.3 ± 0.2 g/kg protein), and significantly more (p<0.05) fat (0.6 ± 0.3 g/kg) than both Control (0.3 ± 0.1 g/kg) and SS (0.2 ± 0.1 g/kg) the morning of testing. However, there was no difference (p>0.05) from Control or SS for CF 3 day average macronutrient, sodium, or caloric intake relative to BW.

To minimize variation in pre-exercise hydration between subjects, a euhydration protocol was piloted and instituted. Subjects were given instructions to ingest a volume of water equivalent to approximately 12 ml of per kg of BW the evening before and morning of testing. Furthermore, subjects did not perform physical exercise for 24 hours prior to testing. Euhydration was confirmed with measurement of urine specific gravity.
(USG) < 1.021 one hour prior to, and immediately prior to beginning the exercise protocol, and with subsequent measurement of serum osmolality < 290 mOsm. All subjects began the protocol well-hydrated with no difference (p<0.05) among groups in initial serum osmolality (Control 279.9 ± 2.8, SS 282.8 ± 2.5, and CF 284.0 ± 3.2 mOsm), or USG (Control 1.006 ± 0.002, SS 1.006 ± 0.001, and CF 1.009 ± 0.004).

3.3.3.2. Testing Protocols: Exercise and Recovery

The experimental test protocol consisted of prolonged cycling exercise in a heated environmental chamber (32-33°C and 35% relative humidity). Cycling was performed at 50% of VO₂max, in 20 minute bouts, separated by five min rest periods and continued until 3% body weight was lost. No fluids were ingested by subjects during exercise. To estimate whole body fluid loss, nude body weight was obtained pre-exercise and every 20 min during cycling; subjects undressed and towel dried in a screened area within the heat chamber prior to each weighing.

Following the exercise-induced dehydration protocol, subjects moved to a thermoneutral room (22°C) for a 60 min recovery period. Subjects were provided ad libitum a carbohydrate-electrolyte replacement beverage with 20 mmol Na⁺ (Gatorade, Pepsico, Purchase, NY). Due to CF subject complaints following the exercise-dehydration protocol presumably from substantial sweat NaCl losses, CF were also provided ad libitum salty foods (e.g. potato chips, salty crackers) beginning at 30 minutes of recovery and these subjects remained an additional 60 min for monitoring of blood [Na⁺] prior to departure.

3.3.3.3. Measurements
VO2 and RER were obtained five min prior to the end of every 20 min exercise stage by open-circuit spirometry using a PARVO Medics TrueOne 2400 Metabolic Measurement System (Parvo Medics, Inc., Salt Lake City, UT). HR was measured via telemetry (Polar Electro Inc., Woodbury, NY) and recorded every five min. Core temperature was monitored using an ingestible temperature sensor (CoreTemp, HTI Technologies Inc, Palmetto, FL) and recorded every 20 min. RPE with a 15 point Borg Scale was recorded at the end of each 20 min exercise stage. Rating of perceived thirst using a 10 mm Visual Analog Scale (VAS) was obtained prior to and every 20 min during cycling and every 10 min during the post-exercise recovery. Without giving specific rehydration instructions to subjects, post-exercise volume of beverage ingested ad libitum was also recorded.

3.3.3.4. Regional Sweat Analysis

Sweat was collected with the modified Brisson method using a collection pouch constructed with impermeable Parafilm (7 cm x 8 cm) (American Can Co., Greenwich, CT) and Opsite wound dressing (10 cm x 14 cm) (Smith & Nephew Inc., Largo, FL). The sweat collection site was the scapular region, an accessible area that correlates well to whole-body sweat concentrations for [Na\(^+\)] and [Cl\(^-\)]\(^{18}\). The skin of the scapula was cleaned with alcohol, deionized water, and sterile gauze, and air dried before application of the collection pouch. Sweat was aspirated from the collection pouch every 20 min during cycling. Sweat [Na\(^+\)], [Cl\(^-\)], and [K\(^+\)] were measured in triplicate using a chemistry analyzer (Nova 5, Nova Biomedical, Waltham, MA). Frequent removal of accumulated sweat minimized electrolyte leaching from the epidermal layer into the sweat sample.
Stable sweat [K+] values throughout the collection time points provided evidence that this potential source of error was minimal.

3.3.3.5. Blood and Urine Analysis

A forearm vein was cannulated with subjects in a supine position, and following 12 min in a sitting position, a resting blood sample was drawn. Blood samples were also drawn every 20 min during the cycling protocol, and following the 60 min post-exercise recovery period. For most CF subjects, additional recovery period samples were drawn at 30 min, 90 min, and 120 min. The catheter was kept patent with a sodium heparin lockflush solution between samplings. Following removal of a ~1.5 mL waste sample, venous blood was drawn into an EDTA-treated test tube and immediately analyzed for hemoglobin (Hb) (HemaCue AB, Angelholm) and hematocrit (Hct) (microhematocrit centrifugation). Blood was centrifuged at 3,000 rpms for 10 min and plasma was stored at -20°C. Plasma hormone assays were performed by an outside laboratory (Yerkes Biomarkers Core Lab, Emory University). Plasma samples corresponding to baseline (0%), 1.5%, and 3.0% dehydration were analyzed using commercially-available radioimmunoassay kits for aldosterone (ALDO) (Diagnostic Systems Laboratories, Beckman Coulter, Webster, TX), and angiotensin II (AngII) (American Laboratory Products Company, Windham, NH), and a commercially-available enzyme immunoassay kit for arginine vasopressin (AVP) (Assay Designs, Ann Arbor, MI). At all collection times, a venous sample was also drawn into a serum separator tube, allowed 30 to 60 min to clot, and centrifuged at 3,000 rpms for 10 min. Urine was collected pre- and post-exercise, and during the 60 min recovery period to assess volume, specific gravity (handheld refractometry), and [electrolytes]. There was no urine output during exercise.
except for one SS subject. In this case, the urine output during exercise was included in
the post-exercise urine volume. Urine and serum $[\text{Na}^+]$, $[\text{K}^+]$, and $[\text{Cl}^-]$ were measured
with a Nova5 chemistry analyzer (Nova Biomedical, Waltham MA). Serum was also
measured for osmolality via freeze point depression method (MicroOsmette Precision
Systems, Natick, MA).

3.3.3.6. Calculations

Total body water (TBW) loss was estimated from body weight loss. Total sweat
loss was calculated from net change in body weight assuming that water loss due to
respiration was negligible $^{156}$. Electrolyte losses in sweat and urine were calculated by
multiplying the volume lost by the [electrolyte] of each $^{157}$. Cation loss was calculated as
$\frac{([\text{Na}^+] + [\text{K}^+])_{\text{sweat}} \times \text{volumesweat} + ([\text{Na}^+] + [\text{K}^+]_{\text{urine}} \times \text{volumeurine}}$. Free water
(FW) loss was calculated as TBW loss – (cation loss x 2)/serum Osmopre. The ratio of
FW loss to TBW loss describes the nature of the dehydration challenge (isotonic vs.
hypotonic) and determines how the TBW loss is shared by the different body fluid
compartments $^{79,158}$. The relative change in plasma volume ($\%\Delta\text{PV}$) was calculated from
changes in Hb and Hct $^{159}$.

3.3.4. Statistical Analysis

The sample size required to obtain a measurable effect on serum $[\text{Na}^+]$ and
osmolality was estimated based on previously published research with eight cyclists
exercising in the heat that resulted in varied sweat electrolyte loss $^{6}$. Using G Power $^{160}$,
for a power of 0.9 and a minimum effect size of 0.75, comparisons between 16 subjects
was considered adequate. Analysis of variance with repeated measures (ANOVA-RM)
was used to assess differences among groups for physiological responses across
dehydration levels 0 to 3.0%, or across recovery time points. One-way Analysis of variance (ANOVA) was used to assess differences among groups for variables not expressed over progressive dehydration or time such as subject characteristics, pre-test measures, and change relative to baseline for variables measured post-exercise or post-recovery. When a significant main effect for group was observed, post-hoc analyses (Tukey) were performed to identify between-group differences. Association of sweat $[\text{Na}^+]$ with serum osmolality, actual and predicted serum $[\text{Na}^+]$, $\% \Delta \text{PV}$, and hormone responses; and association of physiological responses to dehydration with *ad libitum* drinking volume were analyzed with Pearson product-moment correlation. All statistical testing was conducted using SPSS (ver. 17.0, Chicago, IL). An $\alpha$ level of 0.05 was used to indicate statistical significance. All values are presented as mean (± SD).

3.4. Results

3.4.1. Sweating Characteristics and Responses

Sweating-related characteristics are presented in Table 3.2. Control, SS, and CF had similar exercise time, TBW loss, and percent body weight loss (3% dehydration) achieved at the termination of exercise. There was no difference among groups in sweat rates (SR) expressed relative to kg of body mass, or as absolute values (0.9 ± 0.2 L/hr for Control, 1.0 ± 0.3 L/hr for SS, and 0.7 ± 0.2 L/hr for CF). Sweat electrolytes presented in Table 3.2 reflect an average for all values obtained during the entire exercise protocol. As an expected outcome based on the subject recruitment procedure, sweat $[\text{Na}^+]$ and $[\text{Cl}^-]$ were significantly higher for CF and SS compared to Control ($p<0.001$) and also higher for CF compared to SS ($p<0.001$). Consistent with reports in the literature$^{2,64}$, CF sweat $[\text{K}^+]$ was also significantly higher compared to both non-CF groups ($p=0.04$ vs. Control and $p=0.02$ vs. SS); moreover, Control sweat $[\text{K}^+]$ did not differ from SS ($p=0.941$).
There was no significant relationship observed for SR and sweat [Na\(^+\)] (r = -0.278, p=0.223). Sweat [Na\(^+\)] and [Cl\(^-\)] increased with progressive dehydration as expected and consistent with the literature\(^6\). There was a trend (p=0.074) for sweat [Na\(^+\)] to increase more in Control compared to CF. Sweat [Na\(^+\)] was significantly and inversely associated with free water (FW) loss (Fig 3.1, r = -0.991, p<0.001). Calculated FW loss relative to kg of BW was significantly lower for SS (12.2 ± 3.0 ml/kg BW), p<0.001) and CF (1.1 ± 2.5 ml/kg BW, p<0.001) compared to Control (21.7 ± 1.8 ml/kg BW). FW loss in CF was also significantly less than SS (p<0.001). This was also observed when FW loss was expressed as a percentage of ΔTBW (Fig 3.2).

| Table 3.2: | Mean (±SD) characteristics for Control, non-CF salty sweaters (SS), and cystic fibrosis (CF) subjects (n=21) for exercise time to achieve 3% dehydration, total body water (TBW) loss relative to body weight (BW), free water loss relative to BW, sweat rate (SR) relative to BW, change in SR by 3% compared to 1% dehydration final exercise stage, sweat electrolytes concentration (indicated by brackets), and change in sweat electrolytes by final exercise stage. Percent change values are calculated relative to values at 1.0% dehydration. * Significantly > Control, # CF significantly > SS (p<0.05). |
|---|---|---|
| **Exercise time (min)** | Control | SS | CF |
| | 122.5 ± 16.7 | 125.7 ± 27.6 | 130.0 ± 24.5 |
| **TBW loss (ml/kg)** | 33.8 ± 3.3 | 32.1 ± 2.8 | 30.4 ± 2.8 |
| **Free water loss (ml/kg)** | 21.7 ± 1.8 | 12.2 ± 2.9 | 1.1 ± 2.5 |
| **Sweat Rate (ml/min/kgBW)** | 212.8 ± 32.5 | 215.8 ± 58.0 | 183.7 ± 29.0 |
| **% Change in SR** | -2.8 ± 12.5 | -1.6 ± 22.9 | -1.3 ± 20.2 |
| **Sweat [Na\(^+\)] (mmol/L)** | 43.7 ± 9.9 | 91.0 ± 17.3 | 132.6 ± 6.4 |
| **% Change in Sweat [Na\(^+\)]** | 14.2 ± 9.5 | 8.2 ± 7.4 | 4.2 ± 5.0 |
| **Sweat [Cl\(^-\)] (mmol/L)** | 41.9 ± 6.9 | 84.1 ± 18.0 | 127.0 ± 12.1 |
| **% Change in Sweat [Cl\(^-\)]** | 16.1 ± 9.7 | 9.4 ± 8.9 | 6.2 ± 4.7 |
| **Sweat [K\(^+\)] (mmol/L)** | 4.7 ± 0.5 | 4.4 ± 0.3 | 7.4 ± 3.3 |
| **% Change in Sweat [K\(^+\)]** | -4.2 ± 8.8 | 2.0 ± 10.3 | 1.8 ± 8.1 |
3.4.2. Blood Responses

Serum osmolality increased during exercise but was not different across groups at the same relative level of dehydration (p=0.589) (Fig 3.3a); however, the increase in serum osmolality was significantly less in CF (p=0.015) at 1.5 through 3.0% dehydration and in SS (p=0.034) at 3% dehydration compared to Control (Fig 3.3b). A significant relationship (r = -0.756, p<0.001) was determined between sweat [Na+] and change in osmolality (Fig 3.4a), such that individuals with the highest sweat [Na+] had smaller change in osmolality.

Fig. 3.1: Relationship between regional sweat sodium concentration (indicated by brackets), and change from baseline in free water relative to change in total body water (ΔFW/ΔTBW) incurred by exercise in the heat to 3% body weight loss (3% dehydration) for Control subjects (blue squares) with ‘typical’ sweat [Na+], non-CF salty sweaters (orange squares), and CF subjects (red squares) with phenotypically very high sweat [NaCl]. * p <0.001; n=21.
Fig. 3.2: Mean ± SD Free water loss relative to total body water loss (ΔFW/ΔTBW) incurred by exercise in the heat to 3% body weight loss (3% dehydration) for Control subjects (blue) with ‘typical’ sweat sodium concentration (indicated by brackets), non-CF salty sweaters (SS, orange), and CF subjects (CF, red) with phenotypically very high sweat [NaCl]. * Significantly less than Control, p <0.001; # CF significantly less than SS, p<0.001; n=21.
Fig. 3.3: Mean ± SD serum osmolality (A) and change (Δ) in serum osmolality (B) relative to percent body weight loss (percent dehydration) during exercise in the heat for Control subjects (blue) with ‘typical’ sweat sodium concentration (indicated by brackets), non-CF salty sweaters (SS, orange), and CF subjects (CF, red) with phenotypically very high sweat [NaCl]. * Significantly less than Control, p <0.05
Fig. 3.4: Relationship between regional sweat sodium concentration (indicated by brackets), and change from baseline in serum osmolality incurred from exercise in the heat to 3% body weight loss (3% dehydration) for Control subjects (blue squares) with ‘typical’ sweat [Na⁺], non-CF salty sweaters (orange squares), and CF subjects (red squares) with phenotypically very high sweat [NaCl].

* p <0.001, n=19.

Serum [electrolytes] during progressive dehydration and recovery are presented in Figures 3.5a-c. Control and SS had similar pre-exercise serum [Na⁺] (p=0.370) but CF had higher pre-exercise serum [Na⁺] than SS (p=0.023) (Fig 5a). However, CF developed lower serum [Na⁺] than Control (p=0.022) at 2.5% dehydration and CF values remained lower throughout exercise (p=0.003) and recovery (p=0.013) (Fig 3.5a). Net change in serum [Na⁺] was significantly less for CF (2.88 ± 1.7 mmol/L)
Fig. 3.5: Mean ± SD serum Na⁺ concentration (indicated by brackets) (a), serum [Cl⁻] (b), and serum [K⁺] (c), relative to percent body weight loss (percent dehydration) during exercise in the heat and at 60 min recovery for Control subjects (blue) with ‘typical’ sweat, non-CF salty sweaters (SS, orange), and CF subjects (CF, red) with phenotypically very high sweat [NaCl]. * CF significantly less than Control,  p < 0.05, # CF significantly greater than SS, p<0.05.

compared to SS (6.3 ± 1.0 mmol/L, p=0.001) and Control (7.5 ± 1.0 mmol/L, p<0.001), and less for SS compared to Control (p=0.05).

Unlike serum [Na⁺], serum [Cl⁻] was not different among groups at baseline (p=0.356) (Fig 3.5b). Serum [Cl⁻] in CF did not increase with progressive dehydration as it did for SS and Control, but decreased significantly below baseline at 2.5% dehydration (p=0.04) and was significantly lower (p=0.031) than Control after 1.5% dehydration (Fig 3.5b). SS serum [Cl⁻] was significantly lower than Control at 2% dehydration (p=0.05) and remained lower throughout exercise (p=0.03)(Fig 3.5b). Net change in serum [Cl⁻] was less for CF (-0.8 ± 0.7 mmol/L) compared to both SS (p<0.001), and Control (p<0.001), and less for SS (2.9 ± 1.5 mmol/L) compared to Control (4.9 ± 1.0 mmol/L, p=0.02).

There was no difference among groups in serum [K⁺] from 0-3% dehydration or following recovery (Figs 5c). There was also no difference in net change (pre- to post-exercise) in serum [K⁺] among groups.

There was no difference among groups in baseline Hb (13.4 ± 1.6, 14.1 ± 1.3, and 14.6 ± 1.4 g/dL) or Hct (40.3 ± 1.5%, 41.8 ± 1.9% for SS, 43.7 ± 3.7% for Control, SS, and CF, respectively). Percent reduction in plasma volume (PV) across progressive dehydration was significantly greater in CF (p=0.04) and SS (p=0.03) compared to
Control (Fig 3.6) and post-exercise percent loss in PV was significantly greater in CF (p=0.03) and SS (p=0.01) compared to Control. There was a significant relationship (r=0.53, p=0.02) between sweat [Na\(^+\)] and post-exercise relative change in plasma volume (Fig 3.7) such that those with higher sweat [Na\(^+\)] had greater relative loss of plasma volume.

![Graph showing percent dehydration and plasma volume change](image)

**Fig. 3.6**: Mean ± SD % change in plasma volume relative to body weight loss (percent dehydration) during exercise in the heat for Control subjects (blue) with ‘typical’ sweat Na\(^+\) concentration (indicated by brackets), non-CF salty sweaters (SS, orange), and CF subjects (CF, red) with phenotypically very high sweat [Na\(^+\)], * CF significantly < Control, p < 0.05, * SS significantly < Control, p < 0.05.
SS had significantly higher plasma [AVP] at baseline than Control (p=0.042) (Table 3.3) and remained higher during progressive dehydration and at 60 min of recovery (p=0.034) (Table 3.3). A significant positive relationship was determined for non-CF subjects between sweat [Na⁺] and plasma [AVP] at baseline (r = 0.707, p=0.007), post-exercise (r = 0.616, p<0.05), and recovery (r = 0.712, p=0.006) indicating in healthy subjects higher sweat [Na⁺] was associated with higher AVP. There were no differences among groups for change in plasma [AVP] relative to baseline across dehydration and at 60 min recovery (p=0.619), nor was there any relationship observed between change in

Fig. 3.7: Relationship between regional sweat sodium concentration (indicated by brackets), and relative change in plasma volume (%ΔPV) incurred from exercise in the heat to 3% body weight loss (3% dehydration) for Control subjects (blue squares) with ‘typical’ sweat [Na⁺], non-CF salty sweaters (orange squares), and CF subjects (red squares) with phenotypically very high sweat [NaCl]. * p <0.05, n=19
plasma [AVP] at any collection points and sweat [Na⁺]. Higher recovery plasma [AVP] was associated with greater fluid retention in recovery (calculated as % of ingested fluid that was not diuresed by 60 min post-exercise) \((r = 0.605, p = 0.049)\) and post-exercise urine volume \((r = -0.628, p=0.029)\) when considering only non-CF subjects. Pre- to post-exercise increase in plasma [AVP] was associated with greater dehydration-induced change (% increase) in sweat [Na⁺] \((r = 0.490, p=0.046)\) and smaller dehydration-induced reduction (% decrease) in SR \((r = -0.656, p=0.003)\).

There were no differences among groups in either plasma [AngII] or ALDO at baseline, 1.5% and 3.0% dehydration, or after 60 min post-exercise recovery (Table 3.3). There were also no differences among groups in change in plasma [AngII] or ALDO relative to baseline at any sample points.

**Table 3.3:** Mean (±SD) in Control, non-CF salty sweaters (SS), and cystic fibrosis (CF) subjects \((n=19)\) for plasma hormone concentration (indicated by brackets) of arginine vasopressin ([AVP]), angiotensin II ([angII]), and aldosterone ([ALDO]) at baseline (pre), in samples corresponding to 1.5% and 3% dehydration, and at 60 min post-exercise (post-recovery). * Significantly > Control \((p<0.05)\).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SS</th>
<th>CF</th>
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<tbody>
<tr>
<td>[AVP]</td>
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</tr>
<tr>
<td>Pre</td>
<td>48.1 ± 13.0</td>
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<tr>
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</table>
3.4.3. Physiological Responses

Relative exercise intensity (%VO₂max) was similar among groups as designed (51.9 ± 2.5% for Control, 51.2 ± 2.6% for SS, and 52.6 ± 2.8% for CF). Mean HR across the dehydration protocol were higher for CF compared to Control and SS (p=0.013), but mean %HRpeak, was not different (p= 0.223) among groups (72.5 ± 4.4% for Control, 72.8 ± 5.6% for SS, and 77.6 ± 7.1% for CF) and increased with progressive dehydration (by 8.1 ± 4.4 % for Control, 9.1 ± 4.7% for SS, and 6.1 ± 5.4 % for CF). There was no difference among groups in core temperature relative to dehydration level. Final core temperature was 38.4 ± 0.3 for Control, 38.1 ± 0.2 for SS, and 38.1 ± 0.4°C for CF and increased similarly among groups (net change of 1.5 ± 0.5 for Control, 1.4 ± 0.6 for SS, and 1.0 ± 0.2 °C for CF). There was no difference (p=0.696) in RPE between groups relative to dehydration across the exercise protocol. RPE increased with progressive dehydration similarly among groups (2.1 ± 1.7 for Control, 2.8 ± 2.2 for SS, and 2.9 ± 1.4 for CF).

3.4.4. Thirst Response

Thirst ratings via VAS are presented in Fig 8. Pre-exercise thirst rating was similar among groups (1.4 ± 1.2 for Control, 1.9 ± 0.9 for SS, and 1.2 ± 0.5 for CF). There was no difference among groups in thirst rating relative to dehydration across the exercise protocol (p=0.956) or recovery (p=0.724) (Fig 8). Net change in thirst ratings at 3% dehydration from pre-exercise values was also similar (p=0.298) among groups (5.95 ± 1.6 for Control, 5.33 ± 1.9 for SS, and 6.72 ± 0.9 for CF). There was also no difference among groups (p=0.798) in level of dehydration at the thirst rating of ‘4’, which is anchored by the descriptors, “a little thirsty” and “moderately thirsty” on the 10
cm VAS rating scale. Mean (± SD) % dehydration at the “4” rating was 1.20 ± 0.8% for Control, 1.11 ± 0.9 for SS, and 1.39 ± 0.2 for CF.

Post-exercise thirst rating (at 3% dehydration) tended to be higher in subjects with greater increase in plasma [AVP] (r = 0.413, p=0.08, n=16). Greater post-exercise thirst was also seen with greater dehydration-induced elevation in serum osmolality, but only for the control group (r = 0.833, p=0.01, n=8). For SS, there was a tendency for greater post-exercise thirst to occur in those with greater increase in plasma [AngII] (r = 0.670, p=0.14, n=6).

Individual responses for CF subjects are presented along with mean (± SD) volume of fluid ingested ad libitum (normalized by BW) for the three groups during recovery in Figure 9. There was no difference between Control and SS in beverage volume (p=0.70); however, CF drank significantly less compared to Control and SS when expressed in absolute volume in mL (p=0.018 vs. Control, 0.017 vs. SS) and relative to BW (Fig 9) (p=0.010 vs. Control, 0.029 vs. SS).
Fig. 3.8: Mean ± SD rating of thirst relative to percent body weight loss (percent dehydration) during exercise in the heat for Control subjects (blue) with ‘typical’ sweat, non-CF salty sweaters (SS, orange), and CF subjects (CF, red) with phenotypically very high sweat [NaCl].

Greater early ad libitum drinking (volume relative to BW, consumed in the first 30 min of recovery) was related to greater dehydration-induced elevation of serum [Cl\(^-\)] \((r = 0.645, p= 0.003)\), and [Na\(^+\)] \((r = 0.491, p=0.03)\), and lower sweat [Na\(^+\)] \((r = -0.490, p=0.024)\), and [Cl\(^-\)] \((r = -0.474, p=0.030)\). These relationships did not persist for healthy subjects \((n=14)\) when CF subjects were eliminated from the analysis \((p>0.05)\). Mean % dehydration (relative to pre-exercise?) at 60 min post-exercise despite ad libitum fluid ingestion in recovery was similar \((p=0.137)\) for Control \((0.3 ± 0.9\%)\), SS \((0.3 ± 0.4\%)\) and CF \((1.0 ± 0.5\%)\).
Annotated text for the image:

**Fig 3.9:** Mean ± SD cumulative volume of sports beverage consumed during *ad libitum* drinking of recovery period following exercise in the heat to 3% body weight loss (dehydration) for Control subjects (blue squares) with ‘typical’ sweat, non-CF salty sweaters (SS, orange squares), and CF subjects (CF, red squares) with phenotypically very high sweat [NaCl]. Individual data for CF is also shown (solid lines). * CF significantly < Control, p < 0.05; # CF significantly < SS, p<0.05.

### 3.4.5. Recovery Blood and Urine Responses

Individual and mean serum [electrolyte] responses during 60 min recovery (and an extended recovery period of 120 min for CF) are presented in Figure 3.9. Net *change* in serum [Na⁺] from baseline at 60 min recovery (Fig 10a) was significantly less for CF compared to Control (p=0.009) and compared to SS (p=0.005). Following the 60 min recovery, serum [Na⁺] was significantly lower (p=0.013) for CF (141.9 ± 0.6 mmol/L) compared to Control (145.1 ± 1.9 mmol/L), but not different from SS (144.0 ± 1.8 mmol/L).
mmol/L, p=0.129). There were no CF or SS subjects that exhibited hyponatremia (<135 mmol/L). Serum [Cl\(^-\)] at 60 min recovery was also significantly lower (p=0.032) for CF (103.9 ± 1.3 mmol/L) compared to Control (107.6 ± 2.5 mmol/L) but not compared to SS (105.6 ± 2.3 mmol/L). Serum osmolality at 60 min recovery was not different (p=0.886) (290.5 ± 11.1 mOsm/kgH\(_2\)O for Control, 287.5 ± 8.3 mOsm/kgH\(_2\)O for SS, and 288.3 ± 3.6 mOsm/kgH\(_2\)O for CF) and was different from baseline in Control (p<0.001), but not for CF (p=0.082) or SS (p=0.167).

Recovery values for %ΔPV at 60 min post-exercise following ad libitum fluid ingestion were still below baseline for all groups, and the differences across groups did not reach significance (p=0.685) (-3.7 ± 11.1% for Control, -3.6 ± 8.3% for SS, and -5.5 ± 3.6% for CF). Mean (± SD) plasma volume restoration (difference between %ΔPV post-exercise and %ΔPV following recovery at 60 min post-exercise) was greater (p<0.05) in SS (15.2 ± 4.1) than Control (10.6 ± 3.6), but not CF (13.8 ± 4.7, p=0.847); and the difference in plasma volume restoration between CF and Control did not reach significance (p= 0.178).

Effects of exercise-induced dehydration to 3% body weight loss and ad libitum drinking in the subsequent 60 min recovery period on urine [electrolytes], specific gravity, and volume are presented in Table 3.4. CF and SS appeared to concentrate their urine less during recovery compared to Control in terms of change in [electrolytes] and USG; and CF appeared to retain less of what was a significantly smaller fluid intake, however none of these observations for recovery urine reached statistical significance.
Table 3.4: Mean (±SD) 60 min post-exercise recovery values in Control, non-CF salty sweaters (SS), and cystic fibrosis (CF) subjects (n=21) for percent change in urine Na\(^+\) concentration (indicated by brackets) relative to baseline (pre), and relative to immediate post-exercise values (post-ex), urine volume, urine specific gravity (USG), fluids ingested ad libitum relative to body weight (BW), and percent of fluids ingested during ad libitum drinking not lost in urine (% of ingested fluid retained), n=21. * < Control and SS (p<0.05).

<table>
<thead>
<tr>
<th>60 min Recovery Variable</th>
<th>Control</th>
<th>SS</th>
<th>CF</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Δ urine [Na(^+)] from pre</td>
<td>210.4 ± 144.7</td>
<td>108.6 ± 97.1</td>
<td>49.6 ± 90.6</td>
</tr>
<tr>
<td>% Δ urine [Na(^+)] from post-ex</td>
<td>74.3 ± 50.6</td>
<td>36.1 ± 53.2</td>
<td>15.4 ± 62.1</td>
</tr>
<tr>
<td>Urine Vol (mL)</td>
<td>54.3 ± 27.8</td>
<td>58.7 ± 52.9</td>
<td>90.7 ± 79.1</td>
</tr>
<tr>
<td>USG</td>
<td>1.022 ± 0.004</td>
<td>1.021 ± 0.007</td>
<td>1.020 ± 0.010</td>
</tr>
<tr>
<td>Fluid ingested (mL/kgBW)</td>
<td>27.2 ± 8.3</td>
<td>26.7 ± 3.7</td>
<td>19.0 ± 4.2 *</td>
</tr>
<tr>
<td>% of ingested fluid retained</td>
<td>96.7 ± 2.1</td>
<td>96.7 ± 3.6</td>
<td>92.6 ± 5.4</td>
</tr>
</tbody>
</table>

3.5. Discussion

The present study provided a novel comparison of physiological responses to prolonged exercise in the heat among groups with varying NaCl loss in sweat. A key focus was the physiological impact of high sweat [NaCl] on factors known to drive thirst during progressive dehydration. The most salient finding was that perceived thirst was not differentially affected by sweat [NaCl] during exercise, despite our observation that, as hypothesized, the hyperosmolality that typically accompanies progressive dehydration
was attenuated in subjects with greater sweat NaCl loss. Since increased blood osmolality is a potent stimulator of thirst in humans \(^{75-78}\), similar perceived thirst with differential osmotic stimuli in our subjects with high sweat [NaCl] (Fig 3.3) was unexpected. We also did not anticipate that the loss of PV relative to dehydration would be greater in subjects with high sweat [NaCl] (Fig 3.6). Interestingly, despite similar post-exercise thirst ratings, we observed less \textit{ad libitum} fluid ingestion for individuals with CF compared to healthy subjects during the post-exercise recovery period.

That thirst sensitivity was largely unaffected by high sweat NaCl loss in CF and SS may be attributed to the well-documented redundancy in the control mechanisms for thirst drive (i.e. osmolality \textit{and} relative volume of fluid within the vascular space in the face of moderate dehydration) \(^{77,161}\). The fact that drinking behavior subsequently was different in CF subjects also suggests that thirst sensitivity (Fig 3.8) is not unidimensional (based solely on hyperosmolality or on hypovolemia). The greater loss of PV relative to dehydration (Fig 3.6) likely impacted thirst sensitivity in SS and CF as it is recognized that a reduction in extracellular fluid (ECF) volume, such as incurred with PV loss (although believed to be secondary \(^{162}\)) also serves as a signal for thirst. A 10\% decrease in plasma volume induces an animal to drink voluntarily \(^{77}\). In humans, the hypovolemic threshold for stimulation of thirst is a body water loss of \(\sim 0.8\%\) \(^{73,76}\). While all subjects experienced similar relative TBW loss in the dehydration protocol of this study (3\% of body weight), maintenance of fluid within the vascular space with dehydration was less in SS and CF (Fig 3.6). Therefore, while their hyperosmotic stimulus for thirst was less, SS and CF subjects may have had a greater hypovolemic stimulus relative to dehydration which maintained thirst sensitivity similar to Control.
Supporting the notion that thirst sensitivity in individuals with high sweat [NaCl] may rely additionally on hypovolemic stimuli, while individuals with more ‘typical’ hypotonicity of sweat rely primarily on hyperosmotic stimuli, post-exercise thirst was related to dehydration-induced elevation in serum osmolality only within Control, and there was a tendency for post-exercise thirst to relate to dehydration-induced elevation of the (primarily hypovolemic hormone) plasma [AngII] only within SS.

Congruent with the observation that relative loss of PV was greater in SS and CF compared to Control (Fig 3.6), a strong positive relationship was determined for sweat [Na+] and reduction in PV with dehydration (Fig 3.7). These observations are somewhat counterintuitive, for it might be expected that Controls, with greater fluid lost relative to solute in sweat and greater increases in blood osmolality, would also demonstrate greater PV loss. However, the opposite finding was observed: SS and CF did not maintain PV with dehydration as well as Control. This might be explained by differences in osmotic-driven shifts between fluid compartments. With exercise-induced dehydration (loss of hypotonic body water via sweating), fluid is drawn from the intracellular fluid (ICF) compartment into the vascular space of the ECF compartment, along an osmotic gradient determined primarily by the [NaCl] of the blood, in order to maintain circulating blood volume. Nose et al. a strong positive correlation between dehydration-induced change in blood osmolality and change in ICF volume (r = 0.738, p<0.02), and between sweat [Na+] and dehydration-induced change in ECF volume (r = 0.804, p<0.01). This led them to conclude that the [Na+] in sweat dictates the osmotic gradient that determines directional fluid shifts between compartments with dehydration whereby the greater the sweat [Na+], the greater the loss in ECF relative to total body water loss. We cannot
report changes in ICF and ECF that occurred in our subjects because the calculations require measurement of initial total plasma volume. However, given that the relationships observed for sweat [Na\(^+\)] with FW loss (fig. 3.1), and change in blood osmolality with FW loss (r = 0.719, p<0.01), are remarkably similar to those reported by Nose et al. 79 for subjects dehydrated to 2.3% body weight loss via exercise, it is likely, too, that a reduced shift from the ICF to ECF compartment occurred in SS and CF compared to Control due to the diminishing effect of high sweat [NaCl] on osmotic pressure to maintain fluid in vessels with dehydration. Since the major determinant of osmolality is NaCl levels in the blood, the finding of smaller dehydration- induced change in serum [Na\(^+\)] and [Cl\(^-\)] in SS and CF subjects with greater sweat [NaCl] loss is congruent with the finding of attenuated dehydration-induced increase in osmolality in these subjects as well.

The tendency for post-exercise thirst rating (at 3% dehydration) to be higher in subjects with greater dehydration-induced increase in plasma [AVP] is consistent with the well-documented dipsogenic effect of this hormone 72 73 123. A primary driver of hyperosmotic thirst, AVP is released from the posterior pituitary gland in response to cell shrinking (from loss of ICF), mediated through activated, volume-depleted osmoreceptor cells in the pre-optic anterior hypothalamus 77. It was anticipated that the plasma AVP response to dehydration might be blunted in SS and CF since their dehydration-induced relative hypernatremia and hyperosmolality (Figs 3.3, 3.5), and thus secondary dehydration of the ICF compartment, was less. Further, it was anticipated that the dipsogenic hormone most sensitive to loss of PV, angiotensin II 162, might demonstrate a more pronounced rise in SS and CF with greater relative PV loss over dehydration (Fig 3.6). However, change in plasma [AVP] and plasma [AngII] with dehydration was not
different among groups (Table 3.3). The absence of differential effect of sweat [NaCl] on
dipsogenic hormone response may be explained by the significant cross-talk that is
known to exist between hormones of the hyperosmotic and hypovolemic thirst pathways
For instance, peripherally-administered AngII can stimulate AVP release by acting on
circumventricular organs or directly on the pituitary, and intracranial AngII can also
cause vasopressin release.

Understanding human thirst is important since it has been recognized that the
decline to drink in humans may be blunted in healthy individuals under certain
physiological, psychological, and environmental stresses (i.e. athletic competition, cold)
as well as diseases such as CF, where involuntarily dehydration during exercise has
been speculated due to an impaired hyperosmotic trigger for thirst. Our results on
CF subjects are especially novel because serial blood sampling during the dehydration
and recovery protocols permitted drinking behavior differences in this population to be
related to measured changes in hydration and electrolyte balance. CF responses were
particularly unique because it appears that despite the predicted attenuated
hyperosmolality, perceived thirst per during moderate dehydration was not affected;
however, when presented with fluids during recovery, drinking behavior was different.
While sustaining similar relative TBW loss (3% dehydration), CF subjects ingested 30%
less fluid ad libitum during recovery than non-CF (even the saltiest of sweaters). Such
‘involuntary dehydration’ in our CF subjects is inconsistent with the similar perceived
thirst ratings throughout exercise that mirrored the other non-CF groups, but consistent
with the behavior observed by other investigators for CF children during ad libitum
fluid replacement following exercise. This suggests that volitional fluid intake for CF is
particularly influenced by factors other than just TBW loss, such as the unique challenge to [NaCl] balance that they incur with prolonged sweating. Volitional drinking may have been additionally repressed in CF by the hypotonicity of the recovery beverage provided ([Na⁺] = 20 mmol). As present evidence indicates, CF ingestion of the sports drink during recovery elicited marked decreases relative to baseline in serum [Na⁺] and, to a greater extent, [Cl⁻], within 30 min (Fig 3.10). It is possible that the relatively rapid decrease in serum [NaCl] for CF may have served to inhibit appetite for the hypotonic beverage provided. Consistent with this, it is known that drinking is also controlled by sodium appetite. Osmoreceptors in the organum vasculosum of the lamina terminals (OVLT) and subfornical organ (SFO) responsible for eliciting thirst also trigger one’s taste for salt. In addition to input from osmoreceptors, recent evidence in rats suggest that specialized salt sensors with Na⁺ sensitive channels, Naₓ, are also located on cells of the circumventricular organs and may also contribute to fluid intake behavior independently of plasma osmolality. While not well characterized in humans, Naₓ in laboratory rats is thought to function by responding to a rise in [Na⁺] of the cerebrospinal fluid and, via efferent projections to the pre-optic anterior hypothalamus, stimulate AVP release as well as signal other motor effector regions of the brain. Analysis of drinking patterns in rats with experimentally-induced hypertonic vs. hypotonic hypovolemia suggests that avoidance of salt imbalance is given preference over stimulation of fluid intake for blood volume restoration, with inhibitory signals originating from both the gastrointestinal tract and circumventricular organs involved in this response. Similar to that demonstrated repeatedly in animal models, the reduced ad libitum fluid ingestion in CF may reflect physiological cues directed at
preservation of salt balance over volume restoration. To illustrate this, individual serum [Na\(^+\)] and [Cl\(^-\)] responses during recovery are plotted for the CF subjects (Fig 10 a, b). Unique to CF, post-exercise serum [Cl\(^-\)] was reduced, and post-exercise serum [Na\(^+\)] was minimally increased, relative to baseline. By 30 minutes into the recovery period of ad libitum drinking, serum [Na\(^+\)] dropped below pre-exercise values for four CF subjects, and to pre-exercise value for another. Serum [Na\(^+\)] continued to decrease and by 60 minutes reached values 2-5 mmol/L below pre-exercise. At 90 min into recovery, serum [Na\(^+\)] was below pre-exercise values for all five CF subjects and by 2 hours post exercise remained lower for 3 of the subjects, equaled pre-exercise value for one subject, and in one was not measured.

At the start of the recovery, serum [Cl\(^-\)] was below pre-exercise values for all but one of the CF subjects. By 30 minutes into the recovery period, serum [Cl\(^-\)] had dropped further for all CF subjects, between 3 and 4.5 mmol/L below baseline. Serum [Cl\(^-\)] continued to fall for 4 of the subjects, and remained unchanged in one of the subjects, so that by 60 minutes reached between 3 and 7 mmol/L below baseline. By 90 min into recovery, serum [Cl\(^-\)] was still below baseline for all five CF subjects and by 2 hours post exercise remained lower relative to baseline for 4 of the subjects; and for one was not measured.

Illustrating the potential importance of a strategy aimed at preservation of salt balance over volume restoration, CF subjects CPE and SWH had the lowest ad libitum drinking volume relative to BW during the first 40 min of recovery (Fig 3.9) and by 60 min had the smallest remaining deficit relative to baseline in serum [Na\(^+\)] (Fig 3.10 a) and [Cl\(^-\)] (Fig 3.10 b). While no CF subject’s recovery serum [Na\(^+\)] decreased into the range
Fig. 3.10: Mean ± SD change relative to baseline in serum Na⁺ concentration (indicated by brackets) (a), and [Cl⁻] immediately following exercise in the heat to 3% body weight loss (dehydration), and during *ad libitum* drinking of subsequent recovery period, for Control subjects (blue squares) with ‘typical’ sweat, non-CF salty sweaters (SS, orange squares), and CF subjects (CF, red squares) with phenotypically very high sweat [NaCl]. Individual data for CF is also shown (solid lines). * Significantly < Control, p < 0.05 for SS, p < 0.01 for CF; # CF significantly < SS, p < 0.01.
of clinical hyponatremia (<135 mmol/L), it is tempting to speculate how much lower CF serum [Na\(^+\)] and [Cl\(^-\)] might have continued to fall without provision for *ad libitum* salty snacks along with sports drink replacement after 30 min in recovery (Fig. 3.11).

During progressive dehydration, there was no effect of sweat electrolytes on other physiological responses (e.g. SR, thermoregulation) important for safety during exercise in the heat. SR decreased and core temperature increased similarly for Control, SS and CF. While an increase in blood osmolality is believed to be a signal for the reduction in sweating sensitivity associated with dehydration during exercise in the heat\(^88\,168-170\), sweating sensitivity was not different in the saltiest sweat groups with attenuated rise in serum osmolality. However, analogous to the dual control mechanism for fluid intake, hyperosmolality and hypovolemia jointly contribute to sweating inhibition in order to preserve fluid and electrolyte balance during prolonged thermoregulatory demand\(^171\,172\).
Therefore, even though SS and CF may have experienced less hyperosmotic inhibition of sweating with dehydration, this may have been offset by their reduced maintenance of PV potentially contributing to a greater hypovolemic inhibition of sweating as dehydration progressed during exercise.

3.6. Summary and Practical Recommendations

In summary, unique findings from this study were that despite smaller FW loss and attenuated serum hyperosmolality with dehydration, thirst perception was not differentially affected in individuals with high sweat [NaCl] loss during exercise (both healthy and CF patients). The greater relative PV losses with dehydration observed in these individuals may serve as compensatory input to drive thirst in the absence of a strong hyperosmotic signal. Thirst drive appears to be appropriately maintained despite large variability in human sweat electrolyte loss, particularly when considering that drinking behavior in the CF patients following dehydration appeared to be more responsive to serum [NaCl] compared to body fluid loss. With a sweat concentration of salt nearly isotonic to blood, CF did not experience hyponatremia during exercise (2-2.5 hr duration) with moderate dehydration when not ingesting fluids, but ad libitum fluid replacement with a hypotonic sports beverage resulted in a fall in blood [NaCl] below pre-exercise values. These data suggest that CF should approach replacement with hypotonic fluids cautiously and ingest salt to minimize risk of electrolyte deficiency during recovery from prolonged exertion-related sweating. The ‘involuntary dehydration’ reported to occur in CF\(^{83,151}\), and observed in the recovery phase of this study, may reflect physiological cues directed at preservation of salt balance over volume restoration. Further research is needed to identify optimal fluid and electrolyte
replacement strategies for this population who, clearly, can gain the health-related benefits obtained from endurance exercise.
CHAPTER 4

PERSPECTIVES, REMAINING QUESTIONS, and FUTURE INVESTIGATIONS

4.1. Inter-individual Differences in Sweat Composition Related to Channel Function

A major conclusion from the present work is that differences in CFTR channel expression may be a mechanism underlying variability in human eccrine sweat [NaCl]. However, as discussed in chapter 2, the magnitude of difference from Control for sweat [NaCl] did not match the magnitude of difference from Control in CFTR expression for the excess salty sweaters studied. For instance, sweat [NaCl] was ~ 100% greater in non-CF salty sweaters (SS) compared to Control while CFTR expression was only ~ 25% less. Further, appearance of CFTR in the sweat duct lumen only explains ~ 21% of the variance in sweat [Na\(^+\)] in non-CF subjects. This discrepancy suggests that another mechanism beyond CFTR quantity as assessed by immunostaining may contribute to inter-individual differences in sweat [NaCl] in healthy individuals. However, it is also possible that the method used to quantify immunostaining intensity of channel expression (via selected cross-sectional sections along variable locations within the sweat duct) could not completely capture relative deficiencies in total sweat duct channel proteins. Use of immunofluorescence staining to evaluate channel expression is less ideal than more quantifiable methods of total channel protein expression, such as immunoblotting. However, it was not possible to obtain sufficient protein in the 4 to 6 sweat glands
homogenized from 4 mm skin biopsies to perform immunoblotting experiments, and increasing the size of human tissue biopsied was also not a reasonable option.

In addition to quantification limitations imposed by immunohistochemistry methods, it was not possible to assess with these methods how differences in overall channel function may also contribute to inter-individual differences in sweat [NaCl]. To evaluate cell physiology (i.e. channel function) and cell morphology unaffected by questions of tissue preservation, the study of living sweat gland cells would be preferred. Studies of living sweat gland cells have been largely limited to model systems such as cultured cells and isolated ductal segments. Unfortunately, morphological and functional observations that are secondary to cell culturing may be difficult to distinguish from physiological ones. For instance, if primary cultures of sweat duct cells obtained from isolated ducts would have been used to compare epithelial transport among subjects in the present study, known quantitative differences in active Na⁺ flux in cells of primary culture compared to intact sweat ducts may have led to flawed interpretations regarding the relationship between channel function and sweat [NaCl]. It was originally proposed that channel function would be compared among subjects using electrophysiology experiments on isolated sweat ducts. Measurement of size-relative currents of individual cells in isolated ducts could have allowed evaluation of differences in membrane transport among the three subject groups attributed to differences in maximal activation capability of electrolyte channels present. Unfortunately this electrophysiological data was not collected because the split tubule technique required for patch clamping experiments on apical membrane cells was determined not possible on isolated ducts. The split tubule model used extensively in the study of renal tubules
has never been performed with sweat gland tubules. In our hands, sweat ducts were successfully isolated from skin samples but the thick basement membrane, two-cell thickness, and frequently-closed lumen of the tubule segments (Fig 4.1) made it difficult to longitudinally split, open, and plate ducts for apical membrane patch clamping.

![Cut end of a microdissected sweat duct from a pilot subject skin biopsy viewed under light microscopy. Arrows indicate uniquely ductal characteristics. Original magnification 40x.](image)

An alternative method to determine differences in channel function among individuals with varying sweat [NaCl] is the tubule microperfusion technique. This technique is performed routinely in sweat glands but only by a relatively few number of laboratories because it is technically difficult and requires specialized instrumentation of an electrophysiology ‘rig’ in order to achieve and maintain tubule perfusion. Another limitation of the tubule microperfusion method that may make it less useful for comparisons between tissues is that it measures transepithelial currents representing the
summation of activity from thousands of cells lining the duct, rather than from a single cell, and therefore inter-individual differences in total measured current could be confounded by inter-individual differences in tubule morphology.

It is always a concern that experimental systems employing isolated cells and isolated tubules do not accurately represent the in vivo situation because they lack interactions with blood vessels, nerves, extracellular matrix, and other supporting tissues. An alternative to the microperfusion technique that may, in the future, facilitate the study of living sweat duct channel function in its native environment is the application of multicolor two-photon intravital microscopy to biopsied human skin samples. Two-photon fluorescence depends on the simultaneous absorption of two photons of light, and fluorescence excitation occurs only at a focal point where the density of illuminating photons is highest. Fluorescence generated in two-photon microscopy can be collected without a confocal aperture, which allows measurement of fluorescence generated deep in light-scattering tissue that is otherwise immeasurable. Two-photon microscopy has been recently adapted for use in explanted organs of living laboratory animals, for instance to obtain three dimensional information regarding the complex organization of the kidney. Two-photon intravital microscopy such as that providing in vivo and real time study of epithelial transport in nephrons has not been performed in living humans for obvious reasons. However, it may be possible to extend this technique to living human tissue. Intact sweat glands distributed throughout biopsies of human skin were noted to stay alive and capable of epithelial transport for several days following removal when maintained in cold lactate Ringers with glucose. Viability of sweat glands
was evidenced by secretion of Neutral Red into the ductal lumen within ten minutes following addition of the substance to the bath containing a whole biopsy (Fig 4.2a) or an a.

Fig. 4.2: Viability of the eccrine sweat gland of a 4 mm punch biopsy removed from a pilot subject 2 days earlier, shown (a) embedded in fat globule and dermis of intact skin plug, and (b) in an isolated sweat duct. Neutral Red added to bath is taken up into the gland and secreted into the ductal lumen (dark red stripe) within 10 min. Original magnification 10x (a) and 20x (b).
isolated gland (Fig 4.2b). Therefore, future work may be directed at novel adaptation of two-photon fluorescence microscopy to permit ‘ex vivo’ study of the living human sweat gland maintained in its physiological dermal environment. Two-photon microscopy employing intracellular probes like acytoxylmethyl esters could be used to measure inter-individual differences in ductal epithelial electrolyte transport, pH regulation, calcium flux, and the like. Skin samples containing sweat glands are readily accessible and require a minimally invasive punch biopsy to obtain. If two-photon ex vivo microscopy can be successfully adapted for use in fresh skin biopsies, the technique could be particularly useful in therapeutics research, such that directed at improving CFTR expression and function in CF epithelium. Strength of such an approach is that it would permit the simultaneous study of inter-individual differences in drug response mechanistically (e.g. ductal transport) and symptomatically (e.g. sweat composition) in order to improve target specificity.

4.2. Aquaporin Expression in Human Sweat Duct

It is possible that inter-individual differences in sweat [NaCl] could also be attributed to differences in expression of other membrane transport proteins in sweat gland epithelium. Membranous aquaporins of principal cells forming the collecting duct in renal tubules are largely responsible for dictating urine concentration by regulating the reabsorption of water through this tight epithelium of the nephron \(^{183}\). Differences in collecting duct aquaporins have been implicated in pathology related to fluid and sodium management such as hypertension \(^{184}\). It is possible that inter-individual differences in expression of aquaporins could contribute to inter-individual variability in sweat [NaCl]. However, as discussed in chapter two, evidence for the presence of aquaporins in the
human sweat duct is not convincing. In the one study of aquaporin expression to date that included human sweat glands, no quantitative assessment is provided and only a single fluorescence image of an immunostained gland is shown. It is not clear from the poorly visualized cell morphology in that image if the aquaporin staining was along the lumen of the ductal versus the secretory portion of the sweat gland. Therefore, a follow-up experiment should investigate the expression of aquaporins in the human sweat duct, and the relationship to [NaCl] in excreted sweat. This could be achieved with the same protocol employed in the present study except with the use of primary antibodies directed at aquaporins instead of at ENaC and CFTR when immunostaining cryosections. AQP5 appears to be expressed in the secretory coil of mice and rat paw sweat glands so AQP5 may be the most logical aquaporin to investigate first in human glands.

4.3. Differences in Hormonal Regulation of Sweat Duct Transport

Interestingly, basal plasma vasopressin concentrations ([AVP]), as well as levels measured in the plasma after 1.5% and 3% dehydration and recovery, were unexpectedly elevated for the non-CF subjects with high salt sweat examined in the present study. Elevated AVP has been suggested as a potential contributing mechanism to exercise-induced hyponatremia based primarily on retrospective investigation of post-exercise blood [AVP] in athletes receiving medical attention for symptomatic hyponatremia. In individuals susceptible to the condition dubbed the ‘syndrome of inappropriate secretion of AVP (SIADH)’, an inability to rapidly excrete relative fluid excess when excessive fluid intake is coupled to high [Na\(^+\)] losses is thought to cause and/or sustain low serum [Na\(^+\)]. It has been assumed that the elevated post-exercise [AVP] measured in these athletes is a consequence of failure to appropriately suppress AVP but
it is not clear in these retrospective studies if baseline AVP and thus net change in AVP was also different for these athletes. Higher baseline plasma [AVP] (that remained higher through dehydration and recovery) was observed in SS compared to Control of the present study, but change in plasma AVP across graded dehydration and following recovery was similar. If, in these SS, recovery plasma [AVP] were to have been examined without consideration of the higher starting values, it may have been assumed that they, like the hyponatremic athletes in the retrospective studies, had inappropriate secretion of AVP relative to their Control counterparts. Since greater sodium loss in sweat is associated with risk for hyponatremia, it is likely that many of the hyponatremic athletes in the retrospective studies were indeed ‘salty sweaters’ like SS in this study.

While the cause for the higher plasma [AVP] in SS is not apparent, it is possible that increased plasma [AVP] could have contributed to the greater [NaCl] of sweat in these individuals. AVP acts on aquaporin channels to increase water absorption across renal epithelium. As discussed above, the mere presence of aquaporins in the human sweat duct needs to be established prior to suggesting that differing vasopressin levels may be related to sweat [NaCl]. However, increased vasopressin could contribute to greater sweat [NaCl] in non-CF salty sweaters via another mechanism. In vitro vasopressin addition to rat collecting tubules transiently increases activity of the enzyme hydroxysteroid dehydrogenase type II (HSD2). The enzyme 11 beta-hydroxysteroid dehydrogenase type II (11 beta HSD2) confers specificity on mineralocorticoid receptors (MR) by inactivating glucocorticoids. In eccrine sweat ducts, MR is present on basolateral membranes and 11 beta HSD2 is present on luminal membranes. Recently
reported in Hypertension $^{190}$, HSD2 activity was found to be partially deficient in isolated sweat ducts from individuals with essential hypertension as compared to normotensives. This indicates that MR, 11 beta HSD2 and vasopressin could play an important role in the regulation of sweat duct Na$^+$ absorption and is a topic ripe for further investigation. Further, it would be interesting to investigate if the elevated plasma [AVP] found in the high salt sweaters of this study may have resulted in chronic, elevated in vivo exposure to vasopressin at the duct and subsequently increased luminal HSD2 activity, as it is known to do in other absorptive epithelium $^{188}$. Increased HSD2 would decrease Na$^+$ reabsorption across duct and could be a contributing mechanism for the greater salt losses in sweat for individuals with elevated vasopressin.

4.4. Colocalization of CFTR and ENaC in Human Eccrine Sweat Duct

A functional link between CFTR and ENaC has been suggested based on evidence from electrophysiological and biochemical methods in heterologous expression systems $^{191}$. Their interaction in native tissue is not clearly defined but is implicated in the pathophysiology of CF $^{192}$. One proposed mechanism is a direct protein-protein interaction between CFTR and ENaC, suggested based on evidence from electrophysiological $^{100,193}$, FRET $^{194}$, yeast-two-hybrid $^{195}$, and co-immunoprecipitation methods in heterologous expression systems. However, the overexpression required for these methods to work in mammalian cells can produce interactions between the channel proteins that are neither entirely specific nor physiologic. In this respect, investigation of a potential CFTR-ENaC relationship under conditions of endogenous channel expression is favored, but complicated by the large quantity of tissue required to obtain measurable membrane protein levels. While tangential to the primary aims of this
thesis work, in order to provide a novel characterization of the physical proximity of CFTR and ENaC in a native absorptive epithelium, immunostained cryosections of subjects’ biopsied tissues were additionally examined under laser confocal microscopy (Olympus Microscopes, Melville, NY). Photomicrographs of immunofluorescence were captured using FluoView software (Olympus) and colocalization of ductal immunoreactivity corresponding to CFTR and ENaC was quantified using ImageJ (National Institutes of Health, Bethesda, MD). Some sections were treated with a polyclonal antibody against an epitope near the N-terminus of the α1 subunit of Na⁺K⁺ATPase, which contains the binding sites for ATP and cations (N-15, sc-16041, Santa Cruz Biotechnology, CA). Na⁺K⁺ATPase is located primarily on the basolateral and apical membrane of the outer duct cell and the basolateral membrane of the inner duct cell. Therefore co-staining for Na⁺K⁺ATPase with CFTR served as a negative control for colocalization.

Similar to epifluorescence microscopy (chapter 2), immunostaining visualized under confocal microscopy marked abundant CFTR (green) and ENaC (red) as expected in the ductal portion of the sweat glands (Fig 3). Staining was primarily localized to the luminal membrane for both channels, but immunoreactivity for ENaC was additionally observed across the entire duct. Quantification of preliminary images indicated that lumen-specific staining was significantly greater for CFTR (by 195.3 ± 32%, p<0.001) and ENaC (by 98.2 ± 38%) compared to staining across the entire duct. CFTR and ENaC in preliminary images were also well-associated in the duct (R=0.48, p<0.05) but show even stronger correlation in the lumen (R= 0.69, p<0.01). Immunostaining for Na⁺K⁺ATPase was located primarily at the basolateral membrane of inner and outer
Fig. 4.3 a and b: Immunofluorescence staining of the eccrine sweat gland reabsorptive duct in 6 µm cryosections of biopsied human skin under laser confocal microscopy. Anti-αENaC labeling with H-95 (column 1, red) and anti-CFTR labeling with M3A7 (column 1, green) revealed immunoreactivity particularly corresponding to the luminal plasma membranes. Overlay of images from column 1 and 2 demonstrates where immunoreactivity to CFTR and ENaC was colocalized (column 3, yellow). Tubules without luminal staining in image ‘a’ are secretory coil. Original magnification 40x.
luminal ductal cells, the apical and outer membrane of outer ductal cells, and largely absent at the apical membrane of inner ductal cells (Fig 4). While image analysis and quantification of Na⁺K⁺ATPase localization with CFTR has not yet been performed, no significant colocalization is expected for these two proteins.

Fig. 4.4: Overlay image of immunofluorescence staining of eccrine sweat gland reabsorptive ducts in 6 µm cryosection of biopsied human skin under epifluorescence microscopy. Anti-α1-Na⁺K⁺ATPase labeling with N-15 (red) revealed immunoreactivity particularly corresponding to the basolateral plasma membranes while anti-CFTR labeling with M3A7 (green) stained lumen. Original magnification 40x.

While not a primary aim of the present investigations, immunofluorescent staining of subject biopsies additionally visualized under confocal microscopy has
provided a novel demonstration of localization of CFTR and ENaC in a native absorptive epithelium. These preliminary data suggest that colocalization of CFTR and ENaC is particularly rich along what operates as the reabsorptive epithelium of the duct. Their striking physical proximity at this principal site of transepithelial NaCl transport speaks to the functionality of the CFTR-ENaC physical association and lends support to a potential direct protein interaction between these two membrane transport channels. Continued experiments are planned in order to fully investigate this interesting preliminary finding. These follow-up experiments are 1) to perform co-immunostaining in sweat ducts for two subunits of ENaC, α and β, as a positive control for colocalization; 2) to additionally quantify colocalization in ~10 confocal images from each of ~ 6 different subjects’ immunostained sweat ducts (CF and non-CF) to build upon the current data set.

4.5. Indirect Evidence for Acute Regulation of Sweat Duct Reabsorption

Sweat [Na\(^+\)] and [Cl\(^-\)] increased as expected with progressive dehydration for all subjects participating in the present study (chapter 3). Differences were observed among subject groups in dehydration-induced change in electrolyte composition. Since rate of sweating and ductal electrolyte reabsorption are thought to be related\(^{12,13}\); it is important to note that sweat rate (SR) did not differ among groups and therefore differences in dehydration-induced change in sweat [NaCl] were not confounded by a varied SR response among groups. It is interesting that differences among Control, SS and CF in dehydration-induced elevation in sweat [Na\(^+\)] and [Cl\(^-\)] (Table 2, chapter 3) are similar to the between-group differences noted in dehydration-induced elevation in serum [Na\(^+\)] and [Cl\(^-\)] (Fig 5). In a stepwise fashion, Control had greatest increase in sweat [Na\(^+\)] and [Cl\(^-\)]
Fig. 4.5: Mean ± SD change in serum Na\(^+\) concentration (indicated by brackets) (a), and serum [Cl\(^-\)] (b), relative to baseline following exercise in the heat to 3% body weight loss (dehydration) for Control subjects (blue) with ‘typical’ sweat [NaCl], non-CF salty sweaters (SS, orange), and CF subjects (CF, red) with phenotypically very high sweat [NaCl]. * Significantly < Control (p < 0.05), # CF significantly < SS (p<0.05).

with dehydration, followed by SS, and then CF. Similarly, the greatest increase in blood
[Na\(^+\)] and [Cl\(^-\)] with dehydration occurred in Control, then SS, then CF. However, it is curious that the magnitude of increase in sweat [Na\(^+\)] and sweat [Cl\(^-\)] with dehydration did not match the magnitude of increase in serum [Na\(^+\)] and serum [Cl\(^-\)] with dehydration. For example, in Control, sweat [Na\(^+\)] increased by 13% while serum [Na\(^+\)] increased by ~3%. In SS, sweat [Na\(^+\)] increased by 8% while serum [Na\(^+\)] increased by only 2%. Morgan et al.\(^6\) noted a similar mismatch in the magnitude of change in serum [Na\(^+\)] and sweat [Na\(^+\)] with acute dehydration. This observation led them to be among the first to suggest that the sweat gland might be acutely regulated by hormonal or sympathetic input to adjust Na\(^+\) reabsorption in response to a stimulus such as elevated serum electrolytes. The concept of an acutely regulated sweat gland is not consistent with the traditional assumption that the increase in sweat electrolyte loss (e.g. with dehydration) is primarily a function of increased blood electrolytes delivered to the sweat gland due to relatively greater loss of fluid in sweat relative to electrolytes since sweat is typically quite hypotonic to sodium chloride compared to plasma concentration. If the increase in sweat [NaCl] with dehydration were to be simply due to blood levels increasing (e.g. more Na\(^+\) in = more Na\(^+\) out), then the percent change incurred with dehydration in sweat [Na\(^+\)] and serum [Na\(^+\)] should be nearly identical. The discrepancy in magnitude of change can be explained, however, if the sweat duct’s ion transport activity is considered potentially capable of acute regulation via alterations in transmembranous channel function. It is not known if hyperosmotic stimuli can signal for an acute compensatory down regulation of NaCl absorption in sweat duct epithelium as it does in renal and gut epithelium. However, the human eccrine sweat gland is well-evolved to provide copious sweating for cooling during even prolonged thermoregulatory
challenges. Furthermore, the sweat duct is equipped for acute regulation with epithelial transport proteins responsive to changes in intracellular and extracellular milieu. As evidence of regulation in the sweat duct that works on a simple negative feedback loop, Reddy and Quinton (2006) demonstrated using tubule microperfusion that intracellular \([K^+]\) helps to regulate NaCl influx to prevent the massive entry that could otherwise lead to cell swelling and destruction. They determined that relatively low basolateral \(K^+\) conductance in non-transporting cells helps to maintain higher intracellular \([K^+]\), which results in the tonic inhibition of endogenous phosphatase dephosphorylation of CFTR. Then, at the onset of the sweating when primary sweat first enters into the lumen of the duct, there is a massive rapid NaCl influx into the cell through ENaC and CFTR down their concentration gradients. This large NaCl influx increases \(Na^+K^+ATPase\) activity which serves to maintain concentration gradients. The activated CFTR results in an increased \(K^+\) conductance at the basolateral membrane for the exit of \(K^+\) from the ductal cells. The lowering of intracellular \(K^+\) serves as a feedback mechanism whereby the tonic inhibition of phosphatases is reduced, thus decreasing the PKA phosphorylation of CFTR which reduces the channel activation and the entry of \(Cl^-\) and \(Na^+\) from the lumen into the cell. Slowing the entry of \(Cl^-\) and \(Na^+\) from the lumen into the ductal cell lowers the \(K^+\) conductance at the basolateral membrane, and eventually a new steady state is reached where the influx balances the outflux to prevent the cell from swelling and bursting.

Another example of acute regulation of electrolyte transport in a different secreting and absorbing epithelium, increased cytosolic \([Na^+]\) or \([Cl^-]\) results in an acute down-regulation of luminal ENaC in salivary duct epithelium. It has not been
investigated if increases in serum [Na\(^+\)] or [Cl\(^-\)] may increase cytosolic [Na\(^+\)] or [Cl\(^-\)] of sweat duct cells and if this in turn is able to down-regulate luminal ENaC in ductal epithelium. However, supporting the concept of an acutely regulate-able sweat gland during prolonged thermoregulation, Buono et al.\(^ {199} \) determined that sweat Na\(^+\) reabsorption rate increases proportionately less than Na\(^+\) secretion rate with increases in sweat rate, and suggested that the inhibitory effect of decreasing cytosolic pH on ENaC could mechanistically explain this observation. If reabsorption across the sweat duct is acutely regulated in response to serum concentration changes, then in the present study where serum osmolality was observed to rise more in response to dehydration in Control due to their greater free water (FW) loss, the response to this stimulus would be greater signaling for reduction of NaCl reabsorption across the sweat duct epithelium. It follows, then, that the greatest magnitude of dehydration-induced increase in sweat [Na\(^+\)] and [Cl\(^-\)] is in Control. The fact that in CF subjects the percent change in dehydration-induced elevation in sweat [Na\(^+\)] and serum [Na\(^+\)] were curiously more similar to each other than they were in the non-CF subjects serves to reinforce the concept of an acutely regulate-able sweat duct, in a pathway that directly or indirectly involves the chloride channel CFTR. Without much functioning CFTR at the sweat duct, CF are quite possibly not able to perform the suggested regulation to the degree that non-CF are capable. While no group’s sweat or serum [K\(^+\)] changed significantly, it is interesting that the mean change in [K\(^+\)] is in the opposite direction in non-CF subjects. This makes physiological sense because K\(^+\) moves in the opposite direction of Na\(^+\) and Cl\(^-\) during epithelial transport and SS and Control had a relatively larger (and possibly intentional) increase in sweat [NaCl] with dehydration.
While indirect evidence provided by the present study suggests that the sweat duct may actually decrease NaCl reabsorption in order to attenuate hyperosmolality during progressive dehydration, more research is needed to determine if and how the sweat duct is capable of acutely regulating ductal reabsorption during prolonged thermoregulatory sweating. Taking the notion of an acutely regulate-able sweat gland even further, it was suggested in a recent paper that the sweat gland may actually serve as an integral regulator of fluid and [Na⁺] balance during exercise 141, possibly though AVP response, based on relationships observed for post-exercise plasma [AVP], and sweat and urine [Na⁺]. One limitation to this study, however, is that the authors do not report change values for plasma [AVP] so it is difficult to assess true hormone ‘response’. It is possible that baseline AVP was also related to sweat [Na⁺] in their subjects, as it was for the non-CF subjects in the present study, which would indicate that the relationship they were observing was not truly an acute regulatory response per se. Clearly, future experiments are needed to investigate potential mechanisms at the level of the gland for acute sweat gland regulation. Such experiments could employ sweat duct tubule microperfusion or ex-vital microscopy of sweat ducts in skin to assess acute changes in channel behavior in response to ligands (e.g. aldosterone and vasopressin), and in response to changes in intracellular and extracellular environments (e.g. pH, salinity).

4.6. Sweat Cl⁻ is Hypertonic to Serum Cl⁻ in CF

Consistent with compromised ductal NaCl reabsorption, sweat [Na⁺] in CF was higher than both non-CF groups and near-isotonic to serum. Sweat [Cl⁻] in CF was also higher than both non-CF groups and the difference between sweat [Cl⁻] and sweat [Na⁺] was smaller for CF subjects, as other researchers have reported with cholinergic-
stimulated sweat testing. Interestingly, sweat [Cl\(^-\)] was not only higher than non-CF but was hyper tonic to serum in all CF subjects. Using in vitro sweat induction in isolated glands, Sato and Sato\(^ {64}\) demonstrated that, relative to the extracellular bath, [Na\(^+\)] and [K\(^+\)] in the primary sweat produced by the secretory coil is nearly isotonic, but [Cl\(^-\)] is actually hypertonic, in both CF and non-CF tissue. Therefore, in the case of severely limited ductal Cl- reabsorption, such as that in CF, it is not unphysiological for the [Cl\(^-\)] of the final excreted sweat to be measured higher than serum levels. Other factors may also contribute to the hypertonic sweat [Cl\(^-\)] in CF, such as greater net Cl- transport out of the ductal cell into the lumen secondary to abnormal activity of a putative luminal HCO\(_3^-\) /Cl\(^-\) exchanger\(^ {200}\), particularly if HCO\(_3^-\) management is faulty in the CF sweat gland as it is in other CF epithelia. Potentially prompting greater HCO\(_3^-\)/Cl\(^-\) exchanger activity, sweat [HCO\(_3^-\)] may have been higher in CF subjects. Indeed, the anion gap (Na\(^+\) + K\(^+\) - Cl\(^-\)), thought to approximate HCO\(_3^-\) + lactate\(^ {201}\), was significantly greater in CF sweat than non-CF sweat, as others have shown in pilocarpine-stimulated sweat\(^ {56}\)\(^ {202}\). Future research should investigate how the sweat gland handles HCO\(_3^-\) and the role that CFTR may play in this process.

4.7. Predicting Changes in Serum Na\(^+\) and Susceptibility to Exercise-induced Hyponatremia

Exercise-induced hyponatremia (serum [Na\(^+\)] < 135 mmol/L) at endurance events, such as the marathon or ultradistance triathlon, is most commonly attributed to ingestion of hypotonic fluids in excess of fluid loss, ‘overdrinking,’ and is associated with post-event weight gain\(^ {93}\). While not as common, hyponatremia may also occur in conjunction with dehydration, particularly in longer, ultraendurance events\(^ {26}\)\(^ {70}\)\(^ {94}\)\(^ {203}\) or military
operations. This ‘hypovolemic hyponatremia’ is presumably from excessive salt depletion via sweating and not from overdrinking, according to a recently-developed quantitative model. This model, based on an equation developed by Kurtz and Nguyen, predicts post-exercise serum $[\text{Na}^+]$ on the basis of changes in the mass balance of cations $\text{Na}^+$, $\text{K}^+$, and water. The high sweat $[\text{Na}^+]$ in CF and also found in many healthy individuals may present a greater risk for the development of hyponatremia during prolonged activity in the heat. One example is the case reported by Smith et al. in 1995 of a British soldier who developed hyponatremia during desert marches on two occasions and was subsequently confirmed to have elevated sweat electrolytes and homozygous CF mutations. In the present study, examination of actual and calculated predicted change in serum [electrolytes] with progressive dehydration in individuals with salty sweat compared to those with normal sweat $[\text{NaCl}]$ was additionally performed to indicate whether or not hyponatremia may be possible and predictable in some individuals (i.e. those with excess salt loss in sweat) over the duration of ultraendurance events in the absence of overdrinking.

Predicted post-exercise serum $[\text{Na}^+]$ was calculated for each subject according to Nguyen and Kurtz: 

$$\text{Post-Exercise Serum } [\text{Na}^+] = \frac{([\text{Initial serum } [\text{Na}^+] + 23.8] \times \text{initial TBW} + 1.03 \Delta E)}{\text{(initial TBW – TBW loss)}} - 23.8.$$ 

Initial TBW assumed = 0.73 of fat-free mass. Individually-calculated predicted serum $[\text{Na}^+]$ values were compared to actual values with paired t-tests and association of sweat $[\text{Na}^+]$ with actual and predicted serum $[\text{Na}^+]$ was analyzed with Pearson product-moment correlation (SPSS, ver. 17.0, Chicago, IL).

### 4.7.1. Predicted vs. Actual Post-Exercise Serum $[\text{Na}^+]$
The difference between calculated (predicted) post-exercise serum $[\text{Na}^+]$ and measured (actual) values for all subjects was (mean ± SD) -1.5 ± 1.2 mmol/L. The discrepancy between actual and predicted values for post-exercise serum $[\text{Na}^+]$ was not different among the three groups (p=0.333). Mean ± SD for difference between actual and predicted values for post-exercise serum $[\text{Na}^+]$ was -1.12 ± 1.2 mmol/L for Control (p=0.03), -2.16 ± 1.2 mmol/L for SS (p=0.01), and -1.55 ± 1.4 mmol/L for CF (p=0.06).

\[ y = 0.96x + 6.9 \]

Pearson R = 0.926 *

* p<0.000

Fig. 4.6: Relationship between predicted (calculated) serum sodium concentration $[\text{Na}^+]$ and actual (measured) serum $[\text{Na}^+]$ following exercise in the heat to 3% body weight loss (3% dehydration) for Control subjects (blue squares) with ‘typical’ sweat $[\text{Na}^+]$, non-CF salty sweaters (orange squares), and CF subjects (red squares) with phenotypically very high sweat $[\text{NaCl}]$. Predicted value was calculated using equation by Kurtz and Nguyen which predicts post-exercise serum $[\text{Na}^+]$ on the basis of changes in the mass balance of cations $\text{Na}^+$, $\text{K}^+$, and water. * p <0.001, n=19.
A significant (p <0.001) relationship was determined between measured post-exercise serum [Na⁺] and predicted values (Fig 6). This relationship (y=0.96x + 6.9, Pearson r=0.92) was similar to that reported by Baker et al. (y = 0.84x + 23, Pearson r=0.90) for subjects following prolonged running in the heat with ending dehydration levels of 0, -2, and -4% body weight loss. The linear regression’s non-zero positive y-intercept, and the mean negative value for difference between predicted and measured post-exercise serum [Na⁺], indicates that the equation slightly over predicted Na⁺ losses for our subjects. There are a number of possible explanations for this. First, an overestimation of [Na⁺] lost in sweat could have resulted from collection artifact, such as that proposed by Weschler due to electrolyte leaching from the stratum corneum, or a discrepancy between regional and whole body sweat electrolyte loss. The low and extremely consistent values for sweat [K⁺] measured in our sweat sampling across the 80 to 160 min dehydration protocol indicate that electrolyte leaching as a source of sample artifact is not likely. It is possible that sweat collected from the scapular region was slightly higher in [Na⁺] than reflected in whole body sweat. While whole-body washdown is the criterion method to determine whole body sweat Na⁺ losses, it was not practical for use in this protocol. Stofan et al. recently put forth an equation, y=0.67x-2.56, for correction of regional samples obtained from the back to reflect whole body losses. When this equation is used to correct individual subjects’ sweat [Na⁺] values prior to use in the Nguyen-Kurtz equation, the gap between predicted and actual post-exercise serum [Na⁺] almost completely closes (mean ± SD difference = -0.20 ± 1.4 mmol/L for Control, -0.42 ± 1.2 mmol/L for SS, 0.72 ± 1.2 mmol/L for CF, and 0.03 ± 1.3 mmol/L for the whole group). A final possible explanation for the small discrepancy between predicted and
actual post-exercise serum $[\text{Na}^+]$ is the mobilization of osmotically-activated $\text{Na}^+$ from exchangeable internal stores\(^{93}\), a notion put forth by Noakes et al. as a potential mechanism determining susceptibility to hyponatremia under conditions of overdrinking. The existence and identity of such an internal store, and its potential function in regulating extracellular fluid space electrolyte balance during prolonged sweating have not yet been elucidated.

### 4.7.2. Risk for Exercise-Induced Hyponatremia- Evaluating Actual and Predicted Post-Exercise Serum $[\text{Na}^+]$

The relationship between sweat $[\text{Na}^+]$ and actual (open circles) and predicted (closed circles) post-exercise serum $[\text{Na}^+]$ is shown in Fig 7. Actual and predicted post-exercise serum $[\text{Na}^+]$ values with 3% dehydration did not fall into hyponatremia range ($< 135 \text{ mmol/L}$, shaded region Fig 7) for any of the subjects following their $\sim 2$ hr dehydration cycling protocol. Using the Nguyen-Kurtz equation\(^{204}\) in the model proposed by Montain et al.\(^{26}\) to predict post-exercise values for serum $[\text{Na}^+]$ if all body water loss were to be exactly replaced by water (0% dehydration) indicated that one CF and one SS subject would have become hyponatremic (closed squares, Fig 4.7) at the same exercise duration. Calculating post-exercise serum $[\text{Na}^+]$ values at 3% dehydration but doubling each subject’s total exercise time (to closer reflect marathon and other ultraendurance events finishing times) indicated that one CF subject would have become hyponatremic (closed diamonds, Fig 4.7). Finally, post-exercise serum $[\text{Na}^+]$ values calculated for both double the exercise duration and with all body water loss exactly replaced by water (0% dehydration) indicated that all subjects ($n=11$) with sweat $[\text{Na}^+] > 60 \text{ mmol/L}$ would have become hyponatremic (closed triangles, Fig 4.7). As expected
with an exercise protocol of dehydration and relatively short duration (~ 2 hrs), no subjects developed exercise-induced hyponatremia. However, examination of the relationship between sweat $[\text{Na}^+]$ and predicted post-exercise serum electrolytes when each subject’s own exercise duration is extended two-fold (4-5 hr typical of ultra-endurance events) predicts that hypovolemic hyponatremia may be possible in extremely high salty sweaters (i.e. CF). To assess if hyponatremia may be possible in the absence

![Graph showing sweat $[\text{Na}^+]$ vs. post-exercise serum $[\text{Na}^+]$](image)

**Fig. 4.7:** Actual and predicted post-exercise serum $[\text{Na}^+]$ with 0% dehydration and with 3% dehydration, for two different total exercise durations. Open circles and black trend line: actual values at 3% dehydration, $r=0.761$, $y = -0.042x + 151.6$. Closed circles and green trend line: predicted values at 3% dehydration, $r = 0.798$, $y = -0.045x + 150.2$. Actual and predicted lines were not different. Closed diamonds and brown trend line: predicted values at 3% dehydration and 2x the subject’s exercise duration, $r=0.925$, $y = -0.093x + 149.7$. Closed squares and purple trend line: predicted values at 0% dehydration (all sweat loss replaced 100% with water), $r=0.693$, $y = -0.03x + 140.3$. Closed triangles and light blue trend line: predicted values if 0% dehydration (all fluids exactly replaced with water) and 2x the subject’s exercise duration, $r=0.871$, $y = -0.080x + 139.7$. Hyponatremia is indicated by yellowed area.
of dehydration and overdrinking, predicted serum $[\text{Na}^+]$ calculated for a condition of euhydration (0% body weight loss/gain, water exactly replaces fluid volume loss) predicts that one CF and one SS subject would have become hyponatremic at the end of their exercise testing session. Furthermore, when calculated again for a condition of euhydration but at an exercise duration arguably more representative of a typical recreational marathon finish time (~4 to 5 hrs), all CF and SS subjects were predicted to be in the range of clinical hyponatremia when water intake exactly replaces fluid volume loss. It is important to note that none of these predictions factor in electrolyte replacement of any kind and would not likely represent what most competitors would practice during endurance exercise. These data suggest that exercise-induced hyponatremia appears to be possible in the absence of overdrinking within the typical exercise duration required to finish a marathon (4-5 hours) for CF and healthy individuals with sweat $[\text{Na}^+]$ above ~65 mmol/L. Future experiments should investigate the impact of sweat $[\text{NaCl}]$ loss on fluid and electrolyte balance during exercise in the heat with ad libitum vs. regimented fluid and/or electrolyte replacement. In conjunction with ad libitum vs. regimented fluid and electrolyte replacement, actual and predicted during- and post-exercise serum $[\text{Na}^+]$ could be assessed for incidence and potential risk of exercise-induced hyponatremia.

4.8. Self-assessment of Sweat Composition

Findings from the present study, as well as from other investigations, suggest that individuals with high sweat $[\text{Na}^+]$ may be at greater risk for exercise-induced hyponatremia during prolonged exercise in the heat, such as military operations or performance of ultra-distance marathons and triathlons. Since measurement of
thermoregulatory sweat [electrolytes] requires specialized collection and a chemistry analyzer, it is often not practical in field settings. Therefore, it important to understand how well individuals are able to assess their own sweat electrolyte losses to formulate personal fluid and electrolyte replacement strategies during prolonged endurance events. It has been suggested that it may be possible to subjectively identify individuals with high sweat salt [NaCl] from complaints of a salty taste of sweat in their mouth or eye irritation when sweat drips into their eyes; or from visible salt stains on clothing worn during prolonged exertion in the heat. To date, there have been no studies to investigate the validity of such assessment. To address this need, subjects in the present study completed a brief survey for self-assessment of sweat rate and salt content prior to being informed of actual sweat rate and [NaCl]. The survey was comprised of five questions with answers indicated by subjects placing a vertical line along a horizontal visual analog scale line (Appendix item E). The relationship between predicted (self-assessed) and actual (measured) sweat [NaCl] and sweat rate was analyzed with Pearson product-moment correlation (SPSS, ver. 17.0, Chicago, IL).

No significant correlation was determined between self-assessed rate of sweating (question #1) and actual rate of sweating (r = 0.393, p = 0.119). There was also no significant correlation for answers to the first three questions related to saltiness of sweat (questions 2-4) with actual (measured) sweat [Na\(^+\)] or [Cl\(^-\)] (r = 0.149, and p = 0.567 for question # 2, r = 0.355, and p = 0.162 for question # 3, and r = 0.032, and p = 0.902 for question # 4. There was a significant correlation for answers to the last question on the survey (question #5, ‘how much salt do you think is in your sweat compared to others?’) and sweat [Na\(^+\)] (r = 0.761, and p< 0.001). This indicates that factors other than ‘stinging
of sweat in the eyes’ and white salt residue on skin may have led subjects to correctly self-assess their sweat NaCl losses. However, before conclusions can be drawn from these correlations, additional population sampling will be needed to expand this dataset from its current limited size of n= 22 (16 males and 6 females) to include the additional 47 healthy subjects for whom preliminary screening of sweat content was performed. An additional ~20 subjects should also be tested to balance gender and age groups. Future research should examine the predictability of other survey questions that may be useful to subjectively identify subjects with high versus low to normal sweat NaCl loss during exercise. If eventually determined to be effective, a survey such as this could be further developed into a testing tool for coaches, athletes, military personnel, and others that work with exercisers and laborers in the heat in order to identify those that may need to pay particular attention to electrolyte replacement during prolonged activity.

4.9. Summary of Future Directions

Several potentially fruitful lines of investigation remain based on the primary dissertation work: 1) novel adaptation of two-photon microscopy to study inter-individual differences in ductal epithelial transport in living sweat glands of fresh skin biopsies; 2) quantifying expression of aquaporins in the human sweat duct and the relationship to [NaCl] of excreted sweat; 3) quantifying luminal HSD2 activity in the sweat duct of individuals with high sweat [Na+] and elevated basal plasma [AVP]; 4) quantifying colocalization of CFTR and ENaC in the sweat duct using laser confocal microscopy; 5) evaluating fluid and electrolyte balance during exercise in the heat with ad libitum vs. regimented fluid and/or electrolyte replacement and assessment of actual and predicted during- and post-exercise serum [Na+] for incidence and potential risk for exercise-
induced hyponatremia; and 6) examining the predictability of survey questions that may be useful to subjectively identify individuals with high vs. normal sweat NaCl loss during prolonged exercise in the heat.
APPENDIX A

PROTOCOL FOR CRYOSECTIONING AND IMMUNOSTAINING

BIOPSIED HUMAN SKIN

- Remove tissue to be cut from -80°C freezer and place in -20°C cryochamber to thaw for at least 30 min but optimally > 60 min prior to cutting.
- Pour acetone methanol solution into coplin jar (1:1) and place in -20°C cryochamber for at least 30 min prior to use.
- Begin gross sectioning at the surface of the epidermis, remove ~ 300 µm of tissue then cut a 6 µm sections and transfer to (any) slide. Let air dry at room temperature for at least 30 sec.
- Apply a large droplet of hematoxylin to section.
- After 2 min, rinse hematoxylin off slide carefully with light drizzle of distilled water or submerge very briefly in water.
- View hematoxylin-stained section under light microscopy, 10-40x, to look for sweat ducts.
- If an insufficient number of ductal cross sections are observed, then return to cutting. Remove only 90 to 150 µm of tissue each time during cutting before cutting another 6 µm section to check for ducts with hematoxylin staining.
- When a desired section is found under light microscopy (presence of sweat ducts), cut another 6 µm section and transfer to silane-coated slide.
- When three sections are on a silane-coated slide, place in acetone and methanol solution in coplin jar in cryochamber (-20°C) for 10 min.
- Air dry slides at RT and put in slide holder, keep slide holder in cryochamber and keep lid on so that tissue debris during cutting does not land on slides.
- When desired slides are prepared, perform immunostaining as soon as possible, keeping slides cold (cooler with ice packs) if transport is necessary.
- Prepare PBS solution: use a 10x PBS prepared to 1x (so 100 ml of the 10x PBS + 900 mL distilled water). Make two 1L bottles of this.
- Prepare permeabilization solution: 0.25% Triton in PBS for 10 min. For coplin jar size use 162.5 μL of Triton in 65 ml of PBS. For larger slide holder container, double that. Be sure to clip end off of pipet tip to make it easier.

- Prepare blocking solution, the 1%BSA solution: 10ul of BSA serum+90 ul of PBS which is also 0.05g of dry BSA into 5 ml of PBS.

- Submerge slides to rinse in PBS solution in coplin jar for 2x3 min at room temp.

- Permeabilization step: 0.25% Triton in PBS for 10 min

- Submerge slides to rinse in PBS solution in coplin jar again, for 3x3 min at room temp.

- Blocking in 1%BSA solution: Apply 100 ul per slide. Incubate 10-30 min at RT in humidifier box.

- Prepare primary antibodies while blocking solution is on. But first decide which going to use together and make those up in the same vial. Dilutions are done with the 1%BSA solution. Vortex vial(s) containing primary antibodies and 1%BSA for 3 sec, then keep on ice until used.
  - 1:10 CFTR M3A7
  - 1:200 αENaC
  - 1:500 Z01
  - 1:50 NaKATPase

- After blocking step, just tip and firmly tap slide to clean surface to get excess off the serum. May also dab with a chemwipe to carefully dry around sections, this will keep antibody solutions from running all over the slide.

- Apply primary antibody. Apply 80 μL per slide. Let sit in humidifier box sealed tightly at 4°C overnight.

- Next day, tip and tap slides to get Ab excess off the slide, and do three times three minute soaks in PBS in coplin jar.

- Make up secondary antibodies: Alexa Fluor made up in 1:500 strength in PBS with 1%BSA. Vortex mixture in vial for 3 sec. Keep away from light and on ice until use.

- Apply secondary antibody, 100 μL per section, let sit in humidifier box sealed tightly for 1 hour at room temp in dark, then tip slides to get Ab excess off the slide.

- Do three times three minute soaks in PBS in coplin jar
- Make up DAPI. The DAPI solution is made up for the coplin jar as 6 uL of the DAPI (already made up in vial in foil in fridge as 1 mg per ml) into 60 mL of PBS. Mix in flask and pour into coplin jar. Keep in dark. Soak in DAPI solution for 10 min in dark followed by 3x3 min rinses in PBS. Double this if making for the larger slide tray holder. OR, apply DAPI in Vecta Shield, apply per section about 1-2 drops, then carefully coverslip so get no air bubbles. Before coverslapping whether with or without DAPI in the coverslapping material, be sure to remove excess solution from front and back of slide with tissue carefully, particularly around the section itself.

- Let dry in dark at least 90 min. Then can nailpolish sides, be carefully not to move coverslip while doing this because will smear DAPI staining. Store in dark at room temperature.
APPENDIX B

DIAGRAM OF OVERALL THESIS METHODS

Research Protocol

SKIN BIOPSY

PHYSIOLOGICAL TESTING

BLOOD MEASURES

[Electrolytes]
%Δ plasma volume
Osmolality
Genetic testing (CF panel)
Hormone assays

SKIN CRYOSECTIONING

IF
APPENDIX C

SCHEMATIC OF PROLONGED EXERCISE TESTING PROTOCOL

- Cycling at 50% aerobic capacity (VO₂max) to 3% body mass loss
- Post-exercise recovery period

Measures: SWEAT  WEIGHT  BLOOD  THIRST  URINE  VO₂

(HR, RPE, core temp recorded every 5 min)
APPENDIX D

PROLONGED EXERCISE TESTING DATA COLLECTION FORM

PAGE 1:

PROLONGED CYCLE DATA

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<th>Sensor #</th>
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<th>50% of VO2max is:</th>
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<td>Sensor swallow time:</td>
<td>Pre-ride drink amount (2ml/kg) to swallow sensor (ml)</td>
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<td>Dry bulb</td>
<td>Wet bulb</td>
<td>%RH</td>
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<td>USG (=1.021) Time of urine sample</td>
<td>Vol of urine sample (ml) (refrig. 2 vials)</td>
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<td>Nude Wt (lbs/kg)</td>
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<td>Wt after 3% loss will =</td>
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<td>Fit headgear</td>
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<td>Clean scapula</td>
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<td>Draw 1 purple top (genetics)</td>
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<td>Draw baseline blood</td>
<td>(after 10 min sit rest)</td>
<td>Resting core temp</td>
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<td>Subject gets on bike. Record time of day: START CLOCK when pedaling begins.</td>
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ID: ___________ Goal VO₂ ___________ 3% loss weight will be ________ lbs or ________ kg

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Name: ______________________________________

RECOVERY PERIOD

0-60 min recovery clock time: (to be performed in this order)

1. Be sure a recovery clock was started at end of exercise
2. Remove sweat collection patch, give dry clothes to put on and urine jug
3. Urine volume __________ (be sure to save 2 vials, put in fridge), USG ________
4. Thirst rating ________
5. Sit subject down, give post-ride drink (2 L), and start ad lib clock
   (goal is within 10 min into recovery time)
   Starting volume (ml): ______ time of day: ______ recovery clock time: ______
   Record cumulative drink level CONSUMED every 10 min from time ad lib began
   Ad lib clock time 10:00 ______ Thirst rating: ______
   Ad lib clock time 20:00 ______ Thirst rating: ______
   Ad lib clock time 30:00 ______ Thirst rating: ______
   Ad lib clock time 40:00 ______ Thirst rating: ______
   Ad lib clock time 50:00 ______ Final thirst rating: ______
6. After 50 min of ad lib drinking, take drink away from subject and perform
   final steps below

At 1 hr recovery clock time:

1. Blood draw, then remove catheter
2. Nudge weight ________
3. Urine collection (be sure to save 2 vials and put in fridge on ice)
   Volume ________ USG ________
4. Core temperature ________
5. Check biopsy wound and re-bandage if necessary

Check that all paperwork is completed before subject is released ________

Lab crew present: _________________________________________
APPENDIX E

SURVEY FOR SELF-ASSESSMENT OF SWEATING

Name:_________________________         ID:_______       Date:________________

This questionnaire asks about your current opinion regarding your own sweating. There are no right or wrong answers. Please be as honest and accurate as possible in responses. Make a vertical line through each horizontal line below to indicate the intensity of your current opinion. If you have no answer, write “N/A” next to the question.

1. How much do you sweat compared to others?
   Much, much more                                                        Much, much less
   ________________________________________________________________

2. Do you have stinging if sweat drips in your eyes?
   Very, very much                                                                      Not at all
   ________________________________________________________________

3. Do you have salt dried on your skin after sweating?
   Very, very much                                                   Not at all
   ________________________________________________________________

4. Do you have white salt stains on your clothing after sweating?
   Very, very much                                                                Not at all
   ________________________________________________________________

5. How much salt do you think is in your sweat compared to others?
   Much, much more                                                        Much, much less
   ________________________________________________________________
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VITA

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BROWN was born in Tampa, Florida. She attended public schools in St. Petersburg, Florida, received a B.A. in Exercise Physiology and Athletic Training Certification from Lenoir-Rhyne College, Hickory, North Carolina in 1993 and a M.S. in Physical Therapy from University of Miami School of Medicine, Coral Gables, Florida in 1995. She practiced physical therapy in St. Petersburg, Florida for nearly a decade before coming to Georgia Tech to pursue a doctorate in Applied Physiology. When she is not working on her research, Ms. Brown enjoys triathlon, traveling with her husband, and running with her dog, Meishka.