Investigating the efficacy of the NASA fluid loading protocol for astronauts: The role of hormonal blood volume regulation in orthostasis after bed rest

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Despite years of research, the role that hypovolemia plays in orthostatic intolerance after head down bed rest (BR) and spaceflight remains unclear. Additionally, the efficacy of oral saline countermeasures, employed in an attempt to restore plasma volume (PV) after BR is questionable. Several previous studies have suggested that a new homeostatic set point is achieved in space or during BR, making attempts to restore PV temporary at best. We tested the hypotheses that one day of BR would induce a transient increase in PV followed by hypovolemia and new hormonal balance; that a salt tablet and water fluid loading (FL) countermeasure would be ineffective in restoring PV; and also that the FL would not attenuate the exaggerated hormonal responses to orthostatic stress that are expected after 28hr of BR. Plasma volume, serum sodium and osmolarity, and plasma ANP, AVP, renin, angiotensin II, aldosterone, and catecholamines were measured in nine male subjects undergoing 5 different protocols (28hr Bed Rest without Fluid Loading = 28NFL, 28hr Bed Rest with Fluid Loading = 28FL, 4hr Seated Control = 4NFLS, 4hr Seated Control with Fluid Loading = 4FLS, and 4hr Bed Rest = 4BR) in a randomized repeated measures design. The FL countermeasure was 15 ml/kg of body weight of water with 1g of NaCl per 125ml of water. Orthostatic testing by lower body negative pressure (LBNP) was performed before and after all protocols. In agreement with our first hypothesis, we observed transient reductions in renin, angiotensin II, and aldosterone, which after 25.5hr were restored to baseline, slightly augmented, and suppressed, respectively. Also after 25.5hr, PV was reduced in the 28hr BR protocols and was not restored in 28FL; however, the FL protocol increased PV during 4FLS. We additionally observed augmented renin and aldosterone responses, as well as generally elevated angiotensin II after 28NFL, but not after 28FL or any of the 4hr protocols. Furthermore, no changes in plasma norepinephrine responses to LBNP were

documented from Pre-Post test in any protocol. Our results indicate that: 1) PV is reduced after short term BR and is not restored by an oral FL; 2) renin-angiotensin-aldosterone system (RAAS) responses to orthostatic stress are augmented after 28hr of BR and the amplified response can be abrogated by FL; and 3) plasma norepinephrine responses during orthostatic stress are not affected by BR or FL, suggesting that RAAS activity may be modulated by FL independently of sympathetic activity and PV during orthostasis after bed rest.

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4BR – 4hr bed rest protocol
4FLS – 4hr seated fluid load protocol
4NFLS – 4hr seated protocol
28FL – 28hr bed rest fluid loading protocol
28NFL – 28hr bed rest protocol

ANP – Atrial Natriuretic Peptide
ANOVA – Analysis of variance
AVP – Arginine Vasopressin
BR – Head-down tilt bed rest
CO – Carbon Monoxide
ECG – Electrocardiogram
FL – Fluid loading
Hct - Hematocrit
HR – Heart rate
LBNP – Lower body negative pressure
NASA – National Aeronautics and Space Administration
OI – Orthostatic intolerance
PV – Plasma volume
Q – Cardiac Output
RAAS – Renin-Angiotensin-Aldosterone System
RSNA – Renal sympathetic nerve activity
SV – Stroke volume

1.0 Review of Literature

1.1 Introduction

Manned spaceflight constitutes a large part of many current space programs and will continue do so for the foreseeable future. Therefore, maintaining the health of astronauts in space is an important task and investigating and understanding the physiological consequences of microgravity on the human body is of the utmost importance. In general, spaceflight causes a substantial deconditioning of physiological systems due to the lack of gravitational stress. Bone demineralization (Collet *et al.*, 1997;Smith *et al.*, 2005) and muscle atrophy (Fitts *et al.*, 2001;Alkner & Tesch, 2004;Tesch *et al.*, 2005) are particularly common after long term missions. However, cardiovascular deconditioning leading to reduced maximal exercise capability (Convertino *et al.*, 1982;Greenleaf & Kozlowski, 1982;Convertino, 1997), and orthostatic intolerance (OI) (Buckey, Jr. *et al.*, 1996b) is very prevalent, even after short duration flights. To reduce or prevent the deconditioning response, many countermeasures are often employed. One of the most common is the NASA fluid loading protocol, which attempts to restore blood volume immediately prior to re-entry. This thesis will explore the effects of simulated microgravity, as well as the putative effects of oral fluid loading (FL), on the hormonal responses to orthostatic stress and their impact on cardiovascular function.

Orthostasis refers to the control of blood pressure within an appropriate range during variations in gravitational stress. The cardiovascular system achieves orthostasis by employing both neural and hormonal negative feedback mechanisms, which are generally capable of correcting perturbations in pressure. The pathophysiological condition of orthostatic intolerance is one in which the cardiovascular system's feedback mechanisms are unable to counter a decline in arterial pressure in the face of a stress which tends to pull blood away from the central

circulation. The compromise of pressure eventually reduces brain blood flow to the extent that a person may experience presyncope or syncope. Presyncope (the prodromal symptoms of syncope) is characterized by feelings of dizziness, nausea, narrowing or graying of vision, flushing and cold sweats and generally precedes syncope, the outright loss of consciousness (Van Lieshout *et al.*, 2003).

Despite extensive research of orthostatic intolerance after spaceflight (Buckey, Jr. *et al.*, 1996b) the underlying mechanisms remain poorly understood. There are likely many factors contributing to reduced orthostatic control after exposure to 0G. Alterations of baroreflex mechanisms (Convertino *et al.*, 1990;Fritsch *et al.*, 1992;Hughson *et al.*, 1994), changes in regional vascular structure and responsiveness (Hargens & Watenpaugh, 1996;Arbeille *et al.*, 2005;Bleeker *et al.*, 2005;Edgell *et al.*, 2007), reduced plasma volume (PV) (Frey *et al.*, 1994;Iwasaki *et al.*, 2000;Waters *et al.*, 2005) and altered heart structure and function (Levine *et al.*, 1997;Perhonen *et al.*, 2001;Dorfman *et al.*, 2007) can play a role in the degraded ability of astronauts to maintain appropriate blood pressure in the face of an orthostatic challenge. Importantly for the study discussed here, hormonal responses to orthostatic challenges are often altered and these changes can occur after very short periods of deconditioning (Butler *et al.*, 1990;Fischer *et al.*, 2007).

1.2 Head Down Bed Rest Simulation

For three decades head down tilt bed rest (BR) has been used as a model to simulate the micro-gravitational and hypokinesic effects of spaceflight on physiological systems. It was observed by the Russians in the late 1970's that tilted bed rest better simulated microgravity than horizontal bed rest (Krupina *et al.*, 1976;Kakurin *et al.*, 1976a;Kakurin *et al.*, 1976b;Mikhailov *et al.*, 1979) and it is currently accepted by many different space agencies that 6° of head down

tilt is the best approximation of spaceflight. While this model appears to be a somewhat accurate simulation of the blood redistribution experienced by astronauts, its usefulness has been questioned due to some differences in how the body reacts to the fluid shift and how it maintains intravascular volume afterwards (Drummer *et al.*, 1993;Gerzer *et al.*, 1994;Norsk *et al.*, 2001). Despite this, BR is a valuable model due to its capacity to induce the same general cardiovascular effects and orthostatic intolerance that are experienced upon return from 0G. Furthermore, its practicality and cost effectiveness make it very attractive for research into microgravity physiology.

While BR is a helpful research tool, several issues need to be considered to employ it effectively. BR of as little as 4hr has been shown to compromise orthostatic tolerance (Butler *et al.*, 1990). With such a short length of BR, circadian rhythms could be partly responsible for changes in hormone levels and orthostatic tolerance, since pre and post testing must necessarily occur at a different time of day. Thus, controls need to be employed to account for a time of day effect, while a deconditioning stimulus is still present through hypokinesia. However, there are few studies in the relevant literature that employed a control for this effect and these controls were often performed in different postures. Still, these studies (Gharib *et al.*, 1988;Butler *et al.*, 1990;Hughson *et al.*, 1995) highlight the importance of time control particularly regarding the effect of meals and drinking upon relevant regulatory hormones (Hughson *et al.*, 1995). Thus, employing a seated condition is critical to understanding the true effects of short term BR. Furthermore, the time length required to observe a reduction in blood volume should also be considered. Within the first 24hr of either spaceflight or BR, a reduction in blood volume of 10-15% is normally observed and maintained for the duration of simulation or flight (Watenpaugh, 2001). Thus, extending BR through to just 28hr would provide a useful model that should reduce

blood volume significantly, while also providing a time overlap with a 4hr model to control for any circadian effects. So, previous research suggests that using BR in 4hr and 28hr models with seated controls can be an effective method of investigating the effects of acute microgravity on the human body.

1.3 Head-Down Bed Rest and Plasma Volume

The hypovolemia experienced by astronauts can be the result of two separate mechanisms that reduce intravascular volumes. If the duration of deconditioning is long enough, a loss of red cell volume contributes significantly to reductions in blood volume (Fortney *et al.*, 1991). However, a loss of plasma, the fluid component of the blood, occurs much faster upon exposure to microgravity (Johansen *et al.*, 1997). Upon introduction to the simulated 0G in BR, there is a rapid shift of blood from the lower body into the central circulation. This is exacerbated by a slower movement of interstitial fluid into the intravascular compartment due to the relief of hydrostatic forces in the legs. Therefore, there is a transient increase of PV followed by a decline to below resting levels (Nixon *et al.*, 1979;Gharib *et al.*, 1988;Johansen *et al.*, 1997). The adaptation of the body to this position is appropriate in that the central circulating volume is maintained, despite the reduction of total blood volume. However, upon an orthostatic challenge, this hypovolemia may contribute to orthostatic intolerance (Frey *et al.*, 1994;Waters *et al.*, 2005).

Interestingly, it appears that although spaceflight and BR result in very similar changes in plasma volume, the mechanisms behind this loss may be slightly different (Norsk *et al.*, 1995;Drummer *et al.*, 2000a;Norsk, 2000;Norsk *et al.*, 2001;Christensen *et al.*, 2001). The diuresis and natriuresis observed in the initial hours of BR are not present when entering microgravity, but instead fluid consumption tends to decrease leading to reduced PV (Leach *et al.*, 1996). Furthermore, observations of fluid handling during intravenous FL trials in space have

conflicted with the expected observations based on ground simulations. One previous study examining blood volume expansion during BR found that urine flow and sodium excretion were similar compared to the acute supine position (Drummer *et al.*, 1992). However, it has also been observed that after exposure to BR, renal responses to isotonic fluid loads are augmented compared to the acute supine posture (Bestle *et al.*, 2001). In contrast, the limited data gathered in space appears to indicate that diuretic and natriuretic responses to intravenous saline loads are attenuated compared to supine controls on Earth (Norsk *et al.*, 1995). While in-flight observations are not as strictly controlled as during BR studies, it has been suggested that this effect may be due to the complete removal of gravitational stress on the tissues (Norsk *et al.*, 2001;Norsk, 2005). Yet, in studies examining fluid expansion after just 3 days of BR, another group showed attenuated responses to isotonic loads similar to the results of Norsk *et al.* (Mauran *et al.*, 1999;Mauran *et al.*, 2003). Therefore, it remains somewhat uncertain precisely how the body will respond to a fluid load after short duration BR, though it appears some of the confusion may be due to the particular conditions applied in the different studies. Additionally, it is unknown how the body will react to an oral fluid load as opposed to the intravenous load applied by the studies mentioned.

1.4 Fluid Loading as a Countermeasure

Studies in the past have indicated that while PV loss may play a role in orthostatic intolerance, it is not the sole determinant and it correlates poorly with the incidence of (pre)syncope (Traon *et al.*, 1998;Pavy-Le Traon *et al.*, 1999;Meck *et al.*, 2001). Yet because blood volume is a determinant of the pressure within the intravascular compartment, restoring this volume might provide some benefit in combating orthostatic intolerance after BR or spaceflight. Several methods of increasing PV after microgravity have been investigated: direct

intravenous injection of saline or pharmaceutical volume boosters and the ingestion of water with salt tablets. Recent studies indicate that PV restoration through acute intravenous saline injection is an effective countermeasure to resolve hypovolemia-induced alterations in baroreflex mechanisms (Iwasaki *et al.*, 2000;Iwasaki *et al.*, 2004) and improve orthostatic tolerance (Takenaka *et al.*, 2002). However, these studies involved a rapid volume expansion and did not investigate the hormonal responses to the infusion. Increasing PV by using fludrocortisone, a synthetic corticosteroid with sodium and water retention properties similar to aldosterone, Vernikos and Convertino (1994) observed a definite advantage compared to oral ingestion of water and salt after BR. In this study, the pharmacological countermeasure was able to restore PV and protect against OI and baroreflex changes. However, a similar study in astronauts concluded that while PV was increased with fludrocortisone, this method of plasma volume restoration was not appropriate for astronauts, because it did not improve orthostatic tolerance (Shi *et al.*, 2004).

The results of some spaceflight research also suggest that attempting to reduce OI in astronauts with water and salt prior to an orthostatic challenge is ineffective, since those who performed oral saline FL just prior to re-entry were no more likely to finish a stand test after landing (Buckey, Jr. *et al.*, 1996b). Similarly, Vernikos and Convertino (1994) found that plasma volume was not increased by this method. In direct contrast to this, Waters *et al.* (2005) concluded that this method was viable, as it was observed to restore plasma volume and no incidences of orthostatic intolerance were observed after BR. Thus, it appears controversial whether plasma volume can in fact be increased by the ingestion of water and salt, and also, whether the increase in plasma volume can alter tolerance to orthostatic stress. Additionally, none of the aforementioned PV restoration studies addressed fluid volume regulating hormones

during the ingestion period, while only some measured these variables during tilt protocols. Therefore, it is uncertain whether plasma volume restoration is truly capable of ameliorating orthostatic intolerance after BR and how the hormonal milieu may contribute to any putative effect.

1.5 Hormonal Responses to Bed Rest and Spaceflight

During BR or spaceflight deconditioning, the cardiovascular system and its hormonal control mechanisms undergo adaption to the altered physiological environment. Additionally, the cardiovascular system's response to orthostatic stress after BR or spaceflight is often altered, with larger increases in heart rate (HR), reduced cardiac output (Q), and greater reductions in arterial pressure, which are associated with exaggerated hormonal responses. An increase in PV may combat these problems by better maintaining stroke volume (SV), and therefore HR and Q regulation. This would tend to reduce the need for augmented sympathetic stimulation and compensatory changes in vasoactive hormones during orthostasis.

Generally, declines in blood pressure during orthostasis activate feedback mechanisms responsible for increasing blood pressure, as well as water and salt retention. Conversely, increases in blood pressure, such those during the initial stages of BR, inhibit these control mechanisms. Briefly, declines in arterial pressure reduce afferent activity of both cardiopulmonary and arterial baroreceptors and consequently enhance activity in the tractus solitarii within the medulla of the brain. This results in inhibition of vagal activity, as well as increased sympathetic nervous activity, which act to increase heart rate and peripheral vasoconstriction in order to restore pressure. If the drop in pressure is sustained, however, various hormonal compensatory mechanisms are also engaged. Importantly, when arterial pressure is reduced for a long enough period, renin is released from the juxtaglomerular cells of

the kidney due to both increased sympathetic activity as well as reduced pressure at the afferent arterioles of the glomerli. The release of renin increases concentrations of angiotensin II, which in turn stimulates aldosterone release from the pituitary. Angiotensin II is a potent vasoconstrictor and a stimulator of thirst, while aldosterone causes sodium and water retention and together they act to preserve blood volume and pressure. However, while the renin-angiotensin-aldosterone system (RAAS) is integral in the control of blood pressure, it also simultaneously regulates the body's sodium content by directly responding to sodium increases and decreases with inhibition and activation, respectively.

An observation common to both bed rest and spaceflight studies is the activation of the circulating RAAS (Norsk *et al.*, 1995;Sigaudo *et al.*, 1998;Millet *et al.*, 2001). Interestingly, several BR studies have also observed an increased renin:aldosterone ratio, suggesting some sort of disconnection in the hormonal cascade (Vernikos *et al.*, 1993;Waters *et al.*, 2005). Additionally, there is sometimes an elevation of circulating norepinephrine (Johansen *et al.*, 1997;Ertl *et al.*, 2002), which may be the result of elevated sympathetic activity in space (Ertl *et al.*, 2002;Fu *et al.*, 2002).

Several studies have noted that the inability to recruit the sympathetic nervous system in the face of declining blood pressure is characteristic of orthostatically intolerant subjects as well as women vs. men after spaceflight (Fritsch-Yelle *et al.*, 1996;Waters *et al.*, 2002;Meck *et al.*, 2004), while low alpha-adrenergic responsiveness may also play a role (Meck *et al.*, 2004). Additionally, plasma epinephrine levels may be elevated when subjects become acutely stressed after BR (Waters *et al.*, 2005). Also, after BR, the RAAS response may be augmented during orthostatic stress (Millet *et al.*, 2001) and it has been demonstrated that OI is associated with an inability to recruit this system during an orthostatic challenge (Greenleaf *et al.*, 2000).

Specifically, those with lower tolerance tend to be less able to release active renin and subsequently are less able to produce angiotensin II, which would increase peripheral resistance and help maintain blood pressure. However, in dehydrated subjects, higher plasma renin activity was associated with *lower* tolerance (Harrison *et al.*, 1985) and after BR, activation of this system does not appear to separate tolerant and intolerant subjects (Fritsch-Yelle *et al.*, 1996). While FL reduces the activity of the RAAS, and subsequently its component hormone concentrations (Norsk *et al.*, 1995;Bestle *et al.*, 2001;Mauran *et al.*, 2003), it is unclear if this suppression has any effect on the ability to withstand an orthostatic stress.

The role of atrial natriuretic peptide (ANP) in fluid regulation during BR is initially one of diuresis, extravasation and to a small extent natriuresis. When assuming the head down tilt position, the shift of blood into the central circulation increases cardiac filling pressure and elicits the release of ANP from atrial myocytes (de Bold *et al.*, 1996;Dietz, 2005;McGrath *et al.*, 2005). This mechanism is different from that experienced in space, as central venous pressure actually declines (Buckey, Jr. *et al.*, 1996a). However, the ANP response remains intact because of an increase in atrial transmural pressure due to the relief of gravitational forces on the heart, which results in similar atrial distension (Videbaek & Norsk, 1997). However, the natriuresis observed during the initial BR period appears to be mediated more by urodilatin, a natriuretic peptide of the kidney, than by ANP (Drummer *et al.*, 1991), though ANP may play a role in systemic extravasation and diuresis causing hemoconcentration when an increase in volume is sensed in the right atrium (Drummer *et al.*, 1992;Watenpaugh *et al.*, 1992). ANP responses to FL have been observed to be augmented after 12 days of BR (Bestle *et al.*, 2001) and during long periods of orthostatic stress, ANP levels may decline due to significant decreases in cardiac filling pressure.

Arginine vasopressin (AVP) tends to increase during spaceflight (Drummer *et al.*, 2000a;Drummer *et al.*, 2000b), even beyond the initial launch and microgravity period where large accelerations, motion sickness, and other launch stresses increase AVP. A series of studies performed in the 1980's and 90's investigated the role of the baroreflex in the release of AVP (Leimbach, Jr. *et al.*, 1984;Goldsmith *et al.*, 1985;Norsk *et al.*, 1986;Goldsmith, 1988;Norsk *et al.*, 1993;Goldsmith, 1994). The conclusion of these studies was that while AVP responds primarily to changes in plasma osmolarity (Goldsmith *et al.*, 1985), circulating AVP can also be modulated through baroreflex mechanisms. Specifically, reductions in pulse pressure appear to play a key role in increasing AVP concentrations in humans, which would counteract the reduction in pressure (Norsk *et al.*, 1993). However, AVP also feeds back to the sympathetic nervous system inhibiting outflow in a way that buffers the pressor effects of this compound on smooth muscle (Hasser *et al.*, 1997). Plasma AVP has been shown to remain unchanged or be reduced after short term BR (Annat *et al.*, 1986;Bestle *et al.*, 2001); however, due to the increased incidence of intolerance after BR (and importantly reduced pulse pressures), AVP sometimes increases to a larger extent at higher levels of orthostatic stress (Vernikos *et al.*, 1993;Meck *et al.*, 2004). Generally, however, changes in plasma osmolarity are not observed during BR, and this tends to keep plasma AVP levels stable.

1.6 The NASA Fluid Loading

The National Aeronautics and Space Administration (NASA) currently employs a fluid loading protocol for its astronauts involving the ingestion of 15ml/kg of water and 1g of NaCl per 125ml of fluid several hours before re-entry in an attempt to combat the effects of lowered plasma volume, combined with large G-forces. The salt to water ratio in this protocol provides a slightly hypotonic fluid load (0.8% compared to 0.9% isotonic saline). However, the efficacy of

this countermeasure is questionable based upon the fact that astronauts are often orthostatically intolerant after flight despite FL (Buckey, Jr. *et al.*, 1996b). Notwithstanding this, Waters *et al.* (2005) showed restoration of plasma volume that was accompanied by the maintenance of orthostatic tolerance after BR. The usefulness of this study is limited, however, as it employed no controls for non fluid loading after BR. Furthermore, no study has examined the endocrine responses during the FL phase before performing the orthostatic tests. The intent of this study was to examine the role of an oral fluid load on hormonal responses during short term simulated microgravity and during an orthostatic stress after simulation.

2.0 Hypotheses

The preceding discussion outlined several questions for this research project. What is the hormonal and plasma volume status of the body after a 28hr period of bed rest? How does the body respond to fluid loads when in this newly adapted state? Does fluid loading increase plasma volume and if so, how do the fluid loading induced alterations in the hormonal milieu affect the response to orthostatic stress? Subsequently, several hypotheses were formed:

1) Bed rest will result in a transient change in plasma volume that is compensated for by neural and hormonal mechanisms, specifically reductions in RAAS hormones, and catecholamines, as well as increases in ANP. Due to the head down position there will be an over-compensation and hormonal resetting, such that after 25.5hr a reduced plasma volume and new hormonal balance will be observed.

2) Fluid loading will result in diuresis, natriuresis and extravasation and will not increase blood volume or improve blood pressure regulation compared to null fluid loading after bed rest. The hormonal response to the fluid load will be reductions in the circulating components of the RAAS, reductions in norepinephrine, and potentially, increased ANP. The changes during BR will be similar to those of seated control fluid loading; however, differences may be observed due to the altered hormonal set points.

3) Exaggerated hormonal responses to orthostatic stress will be observed after BR and the longer BR period will amplify this effect. This will be manifested by larger increases in circulating concentrations of renin, angiotensin II, and aldosterone and possibly catecholamines and AVP. Fluid loading will not affect hormonal orthostatic responses and no changes will be observed after seated control protocols.

3.0 Study Design and Methods

This study involved five sessions in the laboratory for each participant, ranging between 8 and 30 hours per session. The order of sessions was randomized to avoid any crossover effects that may have resulted from a repeated measures design. Particularly, any large changes in blood volume that might be the result of a large number of blood samples were to be avoided. A washout period of at least one week was given after 28hr sessions, or if two 4hr sessions are performed in one week. However, if hemoglobin and hematocrit changed too much after several testing sessions, a longer washout period was allowed for the subjects in order to replenish their red blood cell volume.

The five experimental sessions involved five distinct treatments designed to separate circadian, bed rest, and blood volume effects. Two 28hr models examined the combined effect of BR and an expected reduction in PV. One of these 28hr sessions required the subjects to perform the NASA fluid loading protocol and one was a null fluid loading control session. Comparison of the orthostatic responses to lower body negative pressure (LBNP) after 28hr BR indicated whether the FL was in fact an effective countermeasure after BR to attenuate the anticipated hormonal responses due to the reduction in plasma volume.

Two seated 4hr protocols were employed as controls for the BR protocols. The seated control conditions were performed once with FL and once without. The orthostatic tests taking place before and after the 4hr seated period occurred at the same time of day as the Pre and Post LBNP testing in all BR models. This provided control for circadian effects in order to isolate and discern the BR effect.

Finally, a 4hr BR protocol was performed to discern the effect of short term BR without a change in PV. A comparison of this protocol with the 28hr protocols allowed separation of a BR effect from a combined BR and PV loss effect. Additionally, comparing this protocol to a seated control position clarified if any changes were due to the BR condition and not circadian effects.

2.1 Subjects and Preparation

This study was provided ethical clearance by the University Of Waterloo Office Of Research Ethics (ORE # 13928).

Participants were 9 healthy male non-smokers between 18 and 30 years of age with an average mass of 74 ± 2kg. The only criterion for exclusion, other than a health contraindication, was the inability to view the portal vein by ultrasound imaging. Prior to commencing the study, all subjects were brought into the lab for familiarization with equipment and procedures, particularly with the LBNP protocol. Subjects were briefly exposed to LBNP to ensure a relaxed and unstressed condition when testing began. At this point they filled out and signed informed consent forms and health questionnaires.

Subjects were asked to avoid caffeine, alcohol, and heavy exercise for at least 24 hours prior to testing. The night before and the morning of test days, subjects consumed 5ml/kg body weight of water in an attempt to normalize hydration status. Their recommended breakfast consisted of two pieces of toast with jam. If subjects were not able to consume this exact breakfast, a healthy light breakfast was consumed and kept consistent between protocols. Three-day food diaries were kept by the subjects in the days leading up to laboratory sessions. Suggestions to avoid very high salt foods were also included with the diaries and Pre-test information. After the first session, a copy of the food diary was given back to the subject and

they were asked to repeat it as closely as possible and record this three-day diet record again before each session.

All protocols commenced at the same time of day in order to control for any circadian effects in addition to BR. Subjects were asked to report to the laboratory at 0700hr on testing days. After collecting the subject's urine and allowing them to defecate if required, height and weight were measured and they proceeded to the bed where electrodes were placed for electrocardiogram (ECG) monitoring and impedance measurements. A venous catheter was then inserted into the antecubital vein of the right arm for measurement of both central venous pressure and blood sampling.

2.2 Blood Volume Measurement

A blood volume test occurred immediately following the insertion of the catheter and electrode placement. Once it was completed (after 25 minutes) the subject urinated again and then took his position in the LBNP chamber for test preparation. Total blood volume was determined using a modified version of the carbon monoxide (CO) rebreathe technique described by Burge and Skinner (Burge & Skinner, 1995). Briefly, subjects were seated and connected to an open breathing system for ten minutes, which was supplied with 100% oxygen in order to clear nitrogen from the blood. Next, the subjects breathed into a closed system until comfortable, at which time a priming dose of CO (~20mL) was injected into the system. After five minutes a venous blood sample was taken through the subject's catheter. Then, a second precisely measured test dose of CO (typically 70-90 mL depending on body size) was injected into the closed loop. After five minutes, another blood sample was taken. Blood samples were measured in triplicate by spectrophotometry in a phOX CO-Oximeter (Nova biomedical, Waltham, MA, U.S.A.), which gave the concentration of total hemoglobin, as well as the proportions of oxy and

deoxyhemoglobin, carboxyhemoglobin and methemoglobin. The co-oximeter was calibrated to 160g/L total hemoglobin concentration every morning and quality controls were run every 3-5 days. To calculate blood volume the following formula was used:

$$nCO = 1000 \times \left[\frac{\frac{P_B}{760} \times V_{CO}}{0.08206 \times (273 + T)} \right]$$

$$nHb = \frac{nCO \times 25}{\Delta HbCO}$$

$$V_{RBC} = \left[\frac{644 \times Hct}{[Hb]} \right] \times nHb$$

$$BV = \frac{V_{RBC} \times 100}{Hct \times F_{cell}\ ratio}$$

$$PV = BV - V_{RBC}$$

(Burge & Skinner, 1995)

Table 2.2.1: Variables involved in the calculation of total blood volume.

Variable	Explanation
nCO	Amount of CO added to system (mmol)
.022 Correction Factor	Corrects for CO left in rebreathe apparatus; therefore **$nCO = n_{injected}CO - n_{injected}CO(.022)$**
P_B	Barometric Pressure (mmHg)
V_{CO}	Volume of CO added to rebreathe system (L)
T	Room temperature (^oC)
nHb	Mass of Hb (mmol)
□HbCO	Difference in HbCO before and after rebreathing (%)
V_{RBC}	Volume of red blood cells (mL)
Hct	Hematocrit
[Hb]	Concentration of hemoglobin (g/L)
BV	Blood volume (mL)
PV	Plasma volume (mL)
F_{cell} ratio	Correction factor to account for difference in hematocrit between venous blood and the whole body.

Further changes in blood volume were calculated through changes in hematocrit, since the V_{RBC} was not expected to change to a significant extent:

$$BV = \frac{V_{RBC} \times 100}{Hct \times F_{cell}\ ratio}$$

2.3 Bed Rest and Seated Protocol Positioning

During seated protocols, the subjects were allowed to work, read, or use entertainment devices, so long as the seated position was maintained throughout the 4hr period. During BR,

subjects stayed in the 6-degree head down position with their head on one pillow, but were allowed to prop themselves up on one elbow to eat or urinate. When the subjects needed to defecate during the 28hr protocols, they were placed on a rolling cart in either a prone or supine position and taken to the washroom where they could quickly use the facilities as normal. They returned to the bed again on the cart in a horizontal position. This procedure took place at the subject's discretion; however, it never took place within 3hr of the LBNP Post-test session.

2.4 Food and Fluid Intake

During the first 1.5hr of the first protocol performed by each subject, an *ad libitum* fluid intake standard for this period was established. The standard achieved was subject to ±25% variability, depending on the subject's thirst in subsequent sessions. After the initial 4hr in a 28hr test, fluid intake was again *ad libitum* and this fluid intake was also used as a standard in the second 28hr BR. Once 24hr was reached, the subject consumed their 1.5hr standard again before the FL period began.

In addition to the individual's standard breakfast, food intake during protocols was standardized across all subjects and protocols. During both 4hr and 28hr test sessions, a turkey sandwich on whole wheat bread with lettuce, tomato, and cucumber was provided for the subject soon after taking the seated or BR position (within the first hour). During 28hr sessions caloric intake was 2500kcal per day with 4g of dietary sodium as in previous BR studies (Traon *et al.*, 1998;Iwasaki *et al.*, 2004;Waters *et al.*, 2005). Subjects received a granola bar and fruit juice box after 4hr. For dinner, subjects ate 2 servings of spaghetti with 250mL of tomato sauce and 6 meatballs. Gatorade (500mL) was consumed with dinner along with a fruit bottom yogurt cup. To achieve the ≥4g of sodium per day suggested by Waters *et al.* (2005), 2g (in capsules) of salt

(NaCl) was ingested with or after the dinner. One piece of fruit was ingested as a snack later in the evening before bed.

2.5 Fluid Loading

The NASA fluid loading protocol involves the ingestion of 15ml/kg of water taken with sodium chloride capsules equivalent to 1g per 125ml of water in the period of 0.5 to 2.5hr before re-entry to Earth's atmosphere (Waters *et al.*, 2005). Thus, the FL protocol took place between 1.5 and 3.5hr during the 4hr seated session and from 25.5 to 27.5hr during the 28hr session. The subjects consumed one 1g salt capsule with 125ml of water at even intervals according to the volume of water to be ingested. If the subjects could not consume the entire fluid load for any reason, this was noted and the session continued as normal.

2.6 Lower Body Negative Pressure

The LBNP protocol took place immediately after both the blood volume measurement and the BR and Seated periods. The LBNP chamber consisted of a sealed wooden box with an opening at one end into which the subject placed his lower body. A bicycle seat situated inside the box prevented the subjects from being pulled into the box and helped prevent unwanted muscle activity in the lower limbs. A skirt was fitted around the subject and attached to the opening of the box to seal the subject within the box as much as was comfortable. To induce a negative pressure, a conventional vacuum cleaner with an adjustable rheostat to control the level of suction was connected to the box and pressure was monitored via an electronic transducer. The box and supine subject were positioned on a padded horizontal platform which could be tilted axially in both directions for the purpose of ultrasound imaging.

The LBNP protocol consisted of five stages at progressively increasing negative pressures. After five minutes of baseline (0 mmHg LBNP), pressure was reduced to -10 mmHg

for three minutes, -20 mmHg for five minutes, -30 mmHg for three minutes, and finally, -40 mmHg for five minutes. Tests were terminated at the end of five minutes at -40mmHg, or if the subject experienced any presyncopal symptoms including dizziness, lightheadedness, nausea, sudden warmth or sweating, loss of peripheral vision, or if systolic pressure fell below 70mmHg.

2.7 Blood Sampling and Analysis

Blood sampling was performed by a qualified technician during both LBNP sessions at baseline, and 3.5min into each of the -20 mmHg and -40 mmHg LBNP stages. Blood was analyzed for plasma renin, angiotensin II, aldosterone, norepinephrine, epinephrine, dopamine, ANP, and AVP. Additional blood sampling was performed during the seated and BR periods in order to assess what changes were occurring due to BR. While the ingestion of the fluid load occurred for only two hours, the blood sampling during the protocols actually spanned ~2.5hr from the start of FL until the baseline period of the LBNP Post-test. Therefore, the FL period discussed hereafter refers to this longer ~2.5hr period. See *Appendix One* for a detailed blood sampling diagram.

The blood drawn was immediately added to anticoagulants and ice, or kept at room temperature as described in each assay below. Hematocrit was measured in triplicate micro capillary tubes which were centrifuged for 4 minutes and read on a hematocrit reader. The average of the triplicate results was taken as the hematocrit value.

Plasma renin was quantified in an immunoradiometric assay (Diagnostic Systems Laboratories Inc., Webster, TX, U.S.A.). Blood samples were mixed in 37.5µL EDTA by gentle inversion and spun at room temperature to avoid cryoactivation of prorenin (which would tend to give falsely high values). Samples were frozen at -80^{o}C until assay at a later date. Thawed

plasma samples (200µL) were added to anti-renin coated tubes along with 100µL of ^{125}I-anti-renin and vortexed gently. The tubes were incubated on a shaker for 3hr at room temperature. They were then washed with 2.0mL of distilled water three times, aspirated and counted for one minute on a gamma counter (Perkin Elmer Wizard 1470, Long Island Scientific, Long Island, NY, U.S.A.). Standards run with the assay provided a standard curve from which unknowns were interpolated. Intra-assay variation was 1.3%, inter-assay variation was 2.0% and sensitivity was 0.7pg/mL.

Angiotensin II concentrations were evaluated by an enzyme immunoassay (Angiotensin II EIA Kit. Societe de Pharmacologie et d'Immunologie – BIO, Montigny Le Bretonneux, France). Blood samples were mixed with EDTA and bestatin (to inhibit generation or degradation of angiotensin II) and kept on ice until centrifuged at 3000g for 20 minutes. Plasma samples were stored at -80^{o}C for future analysis. Angiotensin II peptides were extracted from plasma by purifying thawed samples through phenyl cartridges, which were pre-washed with 1.0mL of water and 1.0mL of methanol. The peptides were eluted from the cartridges with 0.5mL of methanol, which was then evaporated by vacuum centrifugation. Re-suspension was achieved by addition of 0.5mL EIA buffer and tubes were vortexed and centrifuged at 3000g for 10 minutes at 4^{o}C. Extracts (100µL) were added to the wells of the pre-washed anti-angiotensin II coated micro plate and incubated with gentle agitation for 1hr at room temperature. Next, 50µL of glutaraldehyde was added to the wells and incubated for 5 minutes to create amino bonds between the peptides and antibodies. To release the epitope from the antibody, 50µL of borane-trimethylamine (in 2N HCl) was added to each well. The plate was washed and 100µL of anti-angiotensin II tracer (bound to acetylcholinesterase) was added and the plate was incubated overnight at 4^{o}C. Following incubation, the plate was washed again and 200µL of Ellman's

reagent was added to the wells. The plate was incubated in the dark for one hour with gentle agitation. The Ellman's reagent created a yellow compound via the acetylcholinesterase that was then measured by a plate spectrometer (Spectramax Plus, Molecular Devices, Sunnyvale, CA, U.S.A.) at 414nm. A standard curve was generated with each plate using known angiotensin II concentrations and unknowns were then calculated. Inter and intra-assay variations were 6.3% and 9.1%, respectively and sensitivity was 2.0pg/mL.

Aldosterone levels in the serum were evaluated with a relatively simple coat-a-count competitive solid phase radioimmunoassay (Coat-a-count aldosterone RIA Kit, Diagnostic Products Corporation, Los Angeles, CA, U.S.A.). The serum samples taken during testing were frozen at -80^{o}C for future assay. Once thawed, 200µL of serum was incubated with 1.0 mL of ^{125}I-aldosterone in aldosterone Ab-coated tubes for 18hr at room temperature. After aspirating the tubes, they were counted for 1 minute in the gamma counter. Concentrations were calculated by interpolation on a standard curve created by known aldosterone concentrations. Intra and inter-assay variation were 3.3% and 8.4%, respectively and sensitivity was 11pg/mL.

Arginine vasopressin concentrations were measured using a double antibody competitive radioimmunoassay (ALPCO Diagnostics, Windham, NH, U.S.A.). Blood samples were mixed with EDTA to inhibit metalloprotease activity and plasma was stored at -80^{o}C until future assay. Samples (400µL), 250µL of phosphate buffer and 50µL of vasopressin antiserum were mixed in polystyrene tubes which were incubated at room temperature for 24hr. Following this, 100µL of ^{125}I-vasopressin tracer was added and the tubes were incubated for a second 24hr at room temperature. After incubation, 100µL of solid phase second antibody suspension was added to the tubes, which were then incubated for 20 minutes at room temperature. One mL of de-ionized water was added to each tube and the tubes were then centrifuged for 5 minutes at 1000g. The

supernatant was aspirated and the precipitate was counted for 1 minute in the gamma counter. Additional tubes with known concentrations of AVP were prepared simultaneously to generate a standard curve from which unknowns were calculated. Intra and inter-assay variations were 6.0% and 9.9%, respectively with a sensitivity of 0.75 pg/mL.

Plasma atrial natriuretic peptide was evaluated by radioimmunoassay (ALPCO Diagnostics, Windham, NH, U.S.A.). Blood samples were immediately mixed with 37.5mL of EDTA and aprotinin to inhibit protein degradation. Plasma was then stored at -80^{o}C until assay. Extraction began by pre-treating Seppak C-18 cartridges with 5mL 4% acetic acid in 86% ethanol, 5mL of methanol, 5mL distilled water and again with 5mL 4% acetic acid. Thawed plasma (500µL) acidified with 1.5mL of 4% acetic acid was then applied to the cartridge and this was washed twice with 3mL distilled water. The peptides were eluted from the cartridge with 3mL of acetic acid in 86% ethanol. The tubes were evaporated under a nitrogen stream for 1hr at 37^{o}C at which point ethanol (1mL) was added to the tubes and they were evaporated to dryness. The precipitate was then dissolved in 250µL of assay buffer (.2M borate + .2% BSA in 30mL water). Extracts (100µL) were added to tubes and vortexed with 200µL of sheep anti-ANP antibody and incubated for 18hr at 4^{o}C. The ^{125}I-ANP tracer (200µL) was then added to the tubes, which were incubated again overnight at 4^{o}C. After incubation, 1mL of precipitating reagent was added and the tubes were vortexed and incubated for 30 minutes at room temperature. After centrifugation, the supernatant was aspirated and the residue in the tubes was read for 1 minute on the gamma counter. Standard tubes run with the samples provided a standard curve of known concentrations from which the unknowns in the samples were calculated. ANP values were corrected for recovery of 90%. Intra-assay variation was 8.6%, inter-assay variation was 11.6% and sensitivity was 3.5pg/mL.

Plasma catecholamines, specifically, epinephrine, norepinephrine and dopamine were evaluated using high performance liquid chromatography. First, the thawed plasma sample (1mL) was purified using 10mg of acid washed alumina, mixed with 400µL of 2.0M Tris + 2% EDTA and 50µL of internal standard. The tubes were mixed by inversion for 30-60 minutes and the supernatant was discarded. The alumina was washed four times with 1.0mL of distilled water, then 100µL of .2M PCA was added and the tubes were vortexed for 2 minutes and centrifuged for 10 minutes at 4^{o}C. High performance liquid chromatography was performed by Marg Burnett at the Department of Kinesiology, University of Waterloo. Briefly, 50µL of extract was injected into the HPLC column (15cm, ^{18}C, 5µm particle size, Supelco Supelcosil (#58230U) and Waters Guard column module with Resolve C18 guard inserts (WAT085824)) with a non-polar mobile phase (50mM sodium acetate, 20mM citric acid, 2mM sodium octane sulfate, 1.mM Di-N-Butylamine, 100µM EDTA, 4% Methanol and 151.1mg KCl) at a flow rate of 1.2mL/min. Catecholamines eluted from the column at different times: Epinephrine – 5.26 minutes, Norepinephrine – 4.67 minutes, Dopamine – 10.31 minutes. The eluting compounds at the base of the column were measured by an electrochemical detector (Waters 2465, .6 volts) that detected the current produced by oxidation. The current produced at the specific times was proportional to the quantity of the catecholamine in the sample, which was referenced to working standards to calculate unknown concentrations.

Serum sodium was measured by sodium micro probe (Orion 9811BN Micro Sodium Electrode, Thermo Fisher Scientific, Inc., Waltham, MA, U.S.A). The probe was calibrated using duplicate 250µL standards of 0.01, 0.1, and 1M sodium chloride solution. Serum samples (250µL) were then measured in duplicate and the average taken as the serum sodium.

Serum osmolarity was measured by vapor pressure osmometry (Model 5100C, Wescor Inc., Logan, UT, U.S.A.). After calibration to 290 and 1000 mOsmol/kg, a small sample of serum (10μL) was pipetted onto a sample disc on the loading stage of the osmometer. The stage was pushed in and the chamber was tightened down. An osmolarity reading was given in approximately 1.5 minutes.

2.7 Urinalysis

All urine was collected and measured for volume. Two ~10mL samples from each urination were collected for measurement of urodilatin, sodium concentration, and osmolarity. Samples were stored in a freezer at -20^{o}C until assays are performed. Samples were collected before blood volume and before both LBNP sessions. Beyond this, urine samples were collected as required by the subject.

Urodilatin was measured by a radioimmunoassay kit (Phoenix Pharmaceuticals Inc., Burlingame, CA, U.S.A.). Briefly, 100μL of urine was mixed with 100μL of rabbit anti-urodilatin and vortexed. Tubes were then incubated for 16-24hr at 4^{o}C. After incubation, radioactive ^{125}I-urodilatin was added to each tube, vortexed, and incubated again for 16-24 hours at 4^{o}C. Subsequently, 100μL of goat anti-rabbit IGG and 100μL of rabbit serum were added to the tubes, which were then vortexed and incubated for 90 minutes at room temperature. An RIA buffer (500μL) was then added to each tube and they were centrifuged for 20 minutes at 3000g and 4^{o}C. The supernatant was aspirated and the tubes counted for 1 minute in the gamma counter. Standards run concurrently with the assay provided a standard curve from which to calculate the unknown urodilatin concentrations.

Urinary sodium was measured by a sodium probe (Orion 8411BN ROSS Sodium Combination Electrode, Thermo Fisher Scientific, Inc., Waltham, MA, U.S.A). Duplicate samples were diluted 1:11 for analysis to achieve an adequate volume for the probe. Dilution was achieved by addition of 18mL of water to 2mL of urine with an additional 2mL of ionic stabilizer solution (Thermo Fisher Scientific, Inc., Waltham, MA, U.S.A.). A standard curve was generated by three serial dilutions of a 1000ppm solution of sodium chloride. An average of the duplicates calculated from the standard curve was taken as the sodium concentration in the urine sample. Finally, urine osmolarity was measured in the same manner as serum osmolarity, in duplicate via the same vapour pressure osmometer.

2.8 Additional Equipment and Data Collection

During LBNP protocols, several cardiovascular variables were measured continuously. These included electrocardiogram, thoracic and pelvic impedance, arterial blood pressure, central venous pressure, brachial and aortic blood velocity, and cardiac output. While not integral to the scope of this study, measurement was occurring during blood sampling and data from these variables were useful in interpreting the changes observed in blood hormone levels during the orthostatic stress. Thus, the equipment and measures are discussed in brief.

All analog signals from the devices described below were sent to a 16 channel Powerlab Data Acquisition System (ADInstruments, Colorado Springs, CO, U.S.A.), which digitized these signals and sent them to a Dell laptop running Chart (v5.5.1) data collection software. Channels were calibrated in Chart before every test with the exception of central venous pressure, which was calibrated afterwards. Once data were collected, a macro was run in Chart to extract data beat-to-beat into a format suitable for Microsoft Excel 2003. This was accomplished by the

program creating intervals for all channels based upon the peak of each R-wave in the ECG channel.

The Colin Pilot (Colin Medical Instruments Corp., San Antonio, TX, U.S.A.) was used to monitor heart rate by ECG and central venous pressure. Two of the three lead ECG electrodes were placed just below the clavicle and outside the nipple line, with the third placed at the 5^{th} rib space. Central venous pressure was measured by a pressure transducer (TranStar, Medex Inc., Carlsbad, CA, U.S.A.) connected to the antecubital catheter via a saline filled line. The transducer was aligned to heart level with the aid of a laser guided level for accurate measure of cardiac filling pressure. Before testing, the pressure was zeroed to atmospheric pressure and the signal was calibrated to 4 different height levels (pressures) in Chart after the test was concluded.

The Finometer device (Finapres Medical Systems BV, Arnheim, The Netherlands) provided continuous measures of blood pressure (at 200Hz) and beat-to-beat estimates of cardiac output. Briefly, finger plethysmographic cuffs were wrapped around either the middle or ring finger of the subject, which were attached to a servo controlled pressure transducer. Pressure within the artery of the finger was matched in the cuff and this pressure was calibrated to brachial pressure via a return-to-flow operation using an arm cuff. Changes in pressure due to finger level were corrected to heart level by a height corrector. Via the Modelflow program, finger pressure waves were corrected to brachial waves and input into a three element windkessel model of the arterial tree. From the pressure wave and established relationships with age, height, weight, and sex (which are input into the Finometer) stroke volume was estimated and multiplied by the instantaneous heart rate to achieve cardiac output for each heart beat. The finometer output was calibrated in Chart before each test via a 0 and 100mmHg signal for the blood pressure channel and a 0 and 10L/min signal for the cardiac output channel.

The velocity of blood in both the aorta and the brachial artery were measured by the Neurovision Transcranial Doppler System (Model 500M, Multigon Industries Inc., Yonkers, NY, U.S.A.). This system uses the Doppler shift phenomenon to measure the velocity of red blood cells as they pass through the ultrasound signal sent from the probe. For the aorta, a 2MHz probe was placed in the sternal notch and set at a depth of ~4-6cm to measure velocity at the ascending aorta. Chart was calibrated using 0 and 200cm/s signals from the Multigon. The 4MHz brachial artery probe was placed several centimeters proximal and medial to the elbow joint and measured at a depth of ~1-3cm (placement was dependent on the ultrasound image location).

2.9 Statistical Analyses and Data Presentation

All data were analyzed with one or two-way ANOVA; however, due to orthostatic intolerance and/or technical issues, not all analyses could be performed with complete data for all nine subjects. The sample sizes used for each ANOVA are presented in *Appendix Two*. Additionally, some subjects were unable to complete the entire FL protocol due to nausea; however, data from these subjects are included in the analyses.

Blood volume as well as urinary volume, sodium, and osmolarity were compared using one-way ANOVA with "protocol" as the main effect. Serum sodium and osmolarity were analyzed by two-way ANOVA with "time" and "protocol" as main effects.

When analyzing the hormonal data, all Pre-test data were first compared using two-way ANOVA with "protocol" and "LBNP" as main effects to determine if any differences existed among Pre-tests on various days. Next, each individual Pre-test was compared with its respective Post-test to determine effects of the protocols with "Pre-Post" and "LBNP" as main effects. Comparison of the effects of the BR and Seated conditions was performed by lumping data from both 28hr BR protocols together and both 4hr Seated protocols together. Repeated measures

ANOVA were then performed to compare the hormonal changes from Pre-test baseline up to the beginning of FL.

Additionally, because we wanted to answer specific questions regarding the influence of FL on orthostatic responses and to minimize the confounding effects of other protocols unrelated to these questions, ANOVA were performed on the Post-tests of particular protocol subsets. These included comparisons of 4BR vs. 4NFLS, 4FLS vs. 4NFLS, 28FL vs. 4FLS, and 28FL vs. 28NFL vs. 4BR. The 4BR and 4NFLS contrasts were set up in order to discern any short term BR effects, which would likely be independent of plasma volume. The two seated controls (4FLS and 4NFLS) were contrasted to determine the basic effects of a fluid load on the responses to LBNP. The two FL protocols were contrasted to determine if BR changed the responses to orthostatic stress after a FL. Finally, we compared all of the BR protocols to examine the influences of PV, FL, and BR upon responses to orthostatic stress.

When significant differences were detected, Tukey's post-hoc test was used for identification. The SAS statistical package (v.9.1.3) was employed for all statistical analyses. Data are presented as mean ± SE in graphical form using Sigmaplot v10.0.

4.0 Results

4.1 Effects of Bed Rest

The 28hr bed rest protocols resulted in significant losses in plasma volume compared to 4hr protocols (Figure 4.1.1 - Top). Relative PV decreased between Pre-test and just prior to the start of fluid loading (FL Start) during 28FL and 28NFL, compared to no changes in PV during 4NFLS (-6.8 ±2.3 and -6.4 ±1.9% vs. +2.9 ± 1.4%, respectively; $p<.01$). The PV change up to FL Start in 4FLS (-1.0 ± 1.7%) was not significantly different from 4NFLS.

Serum sodium levels were unchanged over the course of both 28hr and 4hr protocols with mean concentrations of 135.3 ± 0.5 and 135.8 ± 0.6mmol/L before and after all protocols combined, respectively. Additionally, the FL period had no effect on serum sodium concentrations (FL Start = 136.5 ± 0.5 vs. 150min = 136.3 ± 0.6mmol/L). Serum osmolarity was generally lower after all protocols (Figure 4.1.2) (Main effect of time, $p<.05$). When data from the two 28hr BR protocols were grouped together and compared to the two 4hr Seated tests to examine effects of BR before the FL period, there was no change during 4hr protocols (Pre: 285.3 ± 1.5 and FL Start: 285.2 ±1.1mosmol/kg), but osmolarity was reduced in the 28hr BR protocols (Pre: 285.2 ± 1.32 and FL Start: 282.2 ± 0.8mosmol/kg, $p<.05$).

When comparing the time points during the two 28hr BR protocols, there were no differences between the 28FL and 28NFL protocols in the concentrations of renin, angiotensin II, or aldosterone, but there was a significant main effect suppression of the RAAS hormones in the initial phases of the BR (Figure 4.1.3). All three hormones were reduced compared to Pre-test baseline at 4hr, but the change from baseline reached statistical significance only with renin and aldosterone, which fell 40.1 ± 4.4% ($p<.05$) and 55.9 ± 4.7% ($p<.0001$), respectively (Main effect of time) while the change in angiotensin II was just 10.0 ± 14.9%. At 8hr, reductions were

still evident, but only aldosterone remained statistically different compared to baseline (Main effect of time, $p<.0001$). After 25.5hr of BR, renin and angiotensin II concentrations were statistically indistinguishable from baseline, though there was a trend for angiotensin II to be higher than baseline ($p=.0707$). Aldosterone remained suppressed compared to baseline at 25.5hr (Main effect of time, $p<.01$); however, the levels established at 25.5hr were greater than at 4hr and 8hr for all 3 hormones (Main effect of time, $p<.02$ for all).

Hormonal data was also grouped and 4hr Seated protocols compared to 28hr BR protocols at Pre-test baseline and FL Start (Table 4.1.1). There was a trend for renin to be generally increased at FL Start compared to baseline ($p=.0623$), though much of this effect may have been due to the larger increases during the Seated protocols. There was also an interaction trend for angiotensin II, such that in contrast to renin, concentrations tended to be higher at FL Start during the 28hr protocols ($p=.0662$). Additionally, there was a significant interaction effect on aldosterone, with lower concentrations at FL Start in the 28hr protocols and higher concentrations at FL Start in 4hr protocols compared to baseline ($p<.05$). There were also trends for norepinephrine to be elevated at FL Start ($p=.0606$), while the trend for interaction ($p=.0669$) indicated that most of the elevation occurred in the seated protocols. Finally, epinephrine was significantly elevated in both protocols at FL Start (Main effect of time, $p<.05$), with a tendency for greater increases in the 4hr protocols.

Table 4.1.1: Comparison of plasma renin, angiotensin, and aldosterone concentrations at baseline and FL Start in the 28hr and 4hr protocols.

Protocol	Time	28hr Protocols	4hr Protocols
Renin (pg/mL)	**Pre**	11.3 ± 2.3	8.9 ± 0.8
	FL Start	12.7 ± 1.6	13.8 ± 0.8
Angiotensin II (pg/mL)	**Pre**	8.4 ± 1.6	9.1 ± 2.0
	FL Start	14.2 ± 2.9	10.6 ± 1.8
Aldosterone (pg/mL)	**Pre**	72.5 ± 10.8 †	62.0 ± 6.1 †
	FL Start	53.3 ± 5.5 †	77.0 ± 14.2 †
Norepinephrine (pg/mL)	**Pre**	179.6 ± 23.3	208.2 ± 27.5
	FL Start	205.9 ± 37.3	307.3 ± 38.1
Epinephrine (pg/mL)	**Pre**	26.5 ± 4.5 *	24.8 ± 4.3 *
	FL Start	28.7 ± 3.0 *	36.0 ± 5.9 *

*Significant main effect of time from baseline to FL Start, $p<.05$; †Significant interaction effect between 28hr and 4hr protocols, $p<.05$.

In the Seated and BR protocols, the aldosterone:renin ratio was generally reduced from baseline to FL Start (Main effect of time, $p<.05$); however there were no differences between the 28hr and 4hr protocols.

Catecholamine levels remained unchanged throughout the 28hr protocols (Figure 4.1.4). Although there was a trend for an ANP increase of 69.4% ± 33.7% in the first 4hr of BR (Figure 4.1.5 - Top), values did not reach statistical significance, nor was it different at any other point during the BR period. Finally, AVP levels were unchanged throughout the BR periods (Figure 4.1.5 - Bottom).

Total urinary volume, osmolar, and sodium outputs were not different between the two 28hr protocols at any time (Table 4.1.2). Additionally, there were no differences between any of the urinary outputs during the overnight and morning period measured up until 24hr of BR (Pre2) and those measured over the same time period before the BR protocols began (Pre1).

Table 4.1.2: Cumulative urine outputs during 28hr BR protocols.

Protocol	28FL	28NFL
Pre 1		
Urine Volume (mL)	755 ± 165	673 ± 111
Urine Osmolar Output (mOsmoles)	310 ± 42	259 ± 56
Urine Sodium Output (mEq)	45 ± 9	40 ± 4
Day 1		
Urine Volume (mL)	2316 ± 262	2193 ± 337
Urine Osmolar Output (mOsmoles)	706 ± 46	678 ± 31
Urine Sodium Output (mEq)	172 ± 15	164 ± 18
Pre 2		
Urine Volume (mL)	732 ± 86	795 ± 140
Urine Osmolar Output (mOsmoles)	304 ± 24	286 ± 23
Urine Sodium Output (mEq)	48 ± 7	46 ± 3

Pre1 indicates outputs from all urine collected in the overnight and morning period before starting each protocol at 0hr, Day1 denotes the total outputs from the beginning of BR until sleep at ~13hr, and Pre2 represents the total outputs from the start of sleep until 24hr of BR (overlapping in time with Pre1).

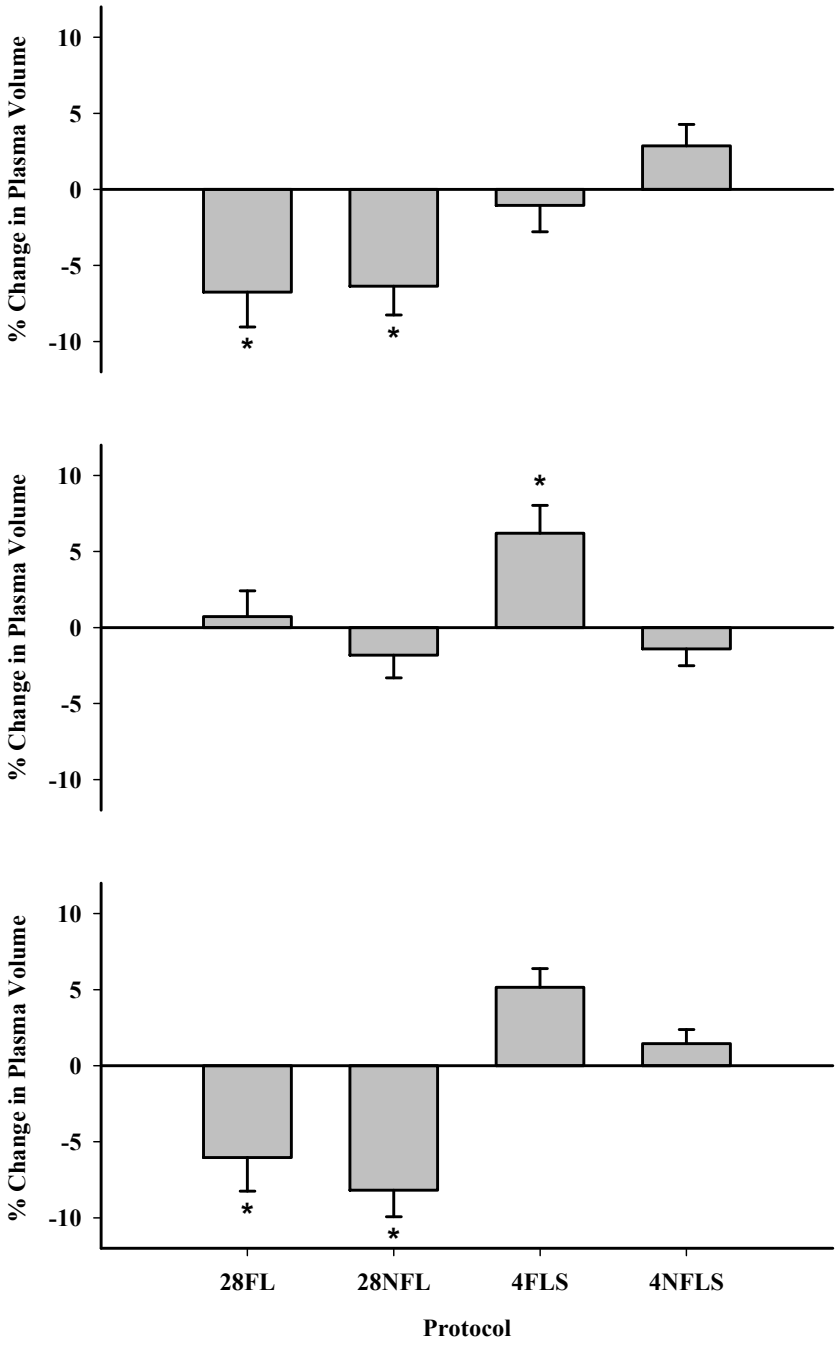


Figure 4.1.1: Relative plasma volume changes during protocols and the fluid loading period. **Top** – PV changes from Pre-test baseline until FL Start. Both the 28FL and 28NFL protocols showed significant PV decreases compared to the control condition (4NFLS) before the FL began. **Middle** – PV changes during the fluid loading period. Only the seated fluid loading (4FLS) condition showed a significant change in PV, which was positive, compared to control. **Bottom** – Overall PV changes from Pre-Post test. Both 28hr BR protocols had similarly reduced volumes and were markedly different from the slight elevation in volume in 4FLS, compared to the control condition. FL Start was 1.5hr and 25.5hr for the 4hr and 28hr protocols, respectively.*Significant difference compared to control (4NFLS), $p<.05$.

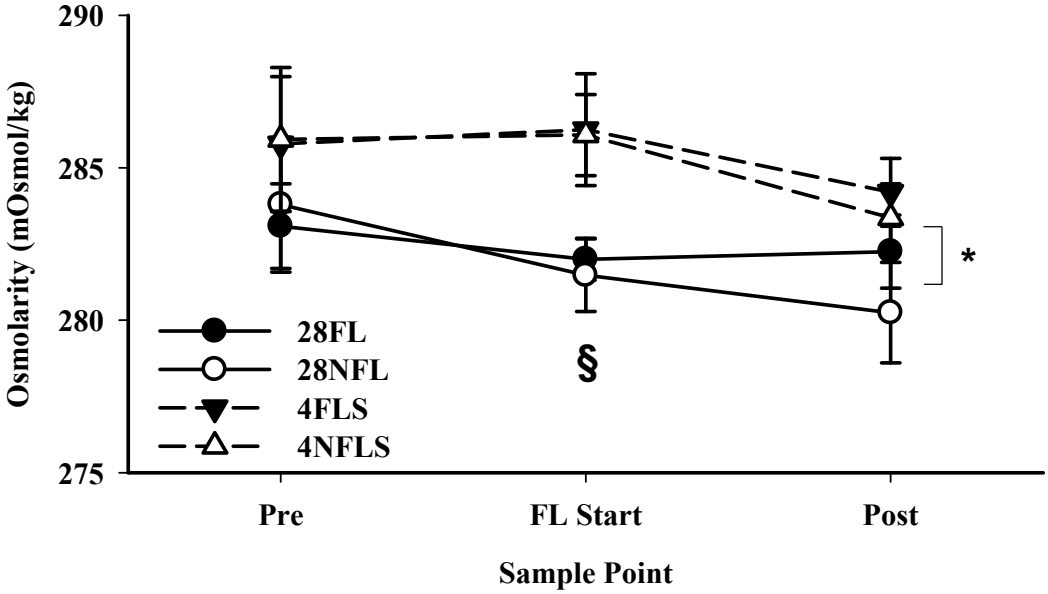


Figure 4.1.2: Serum osmolarity during 4hr Seated and 28hr BR protocols. There was a main effect reduction in osmolarity across all protocols from Pre-Post test. Osmolarity declined up to FL Start in the 28hr protocols compared to no change during the 4hr protocols. FL Start was 1.5hr and 25.5hr for the 4hr and 28hr protocols, respectively. *Significant main effect difference from Pre-Post test, $p<.05$; §Significant difference between grouped 4hr and 28hr protocols at FL Start, $p<.05$.

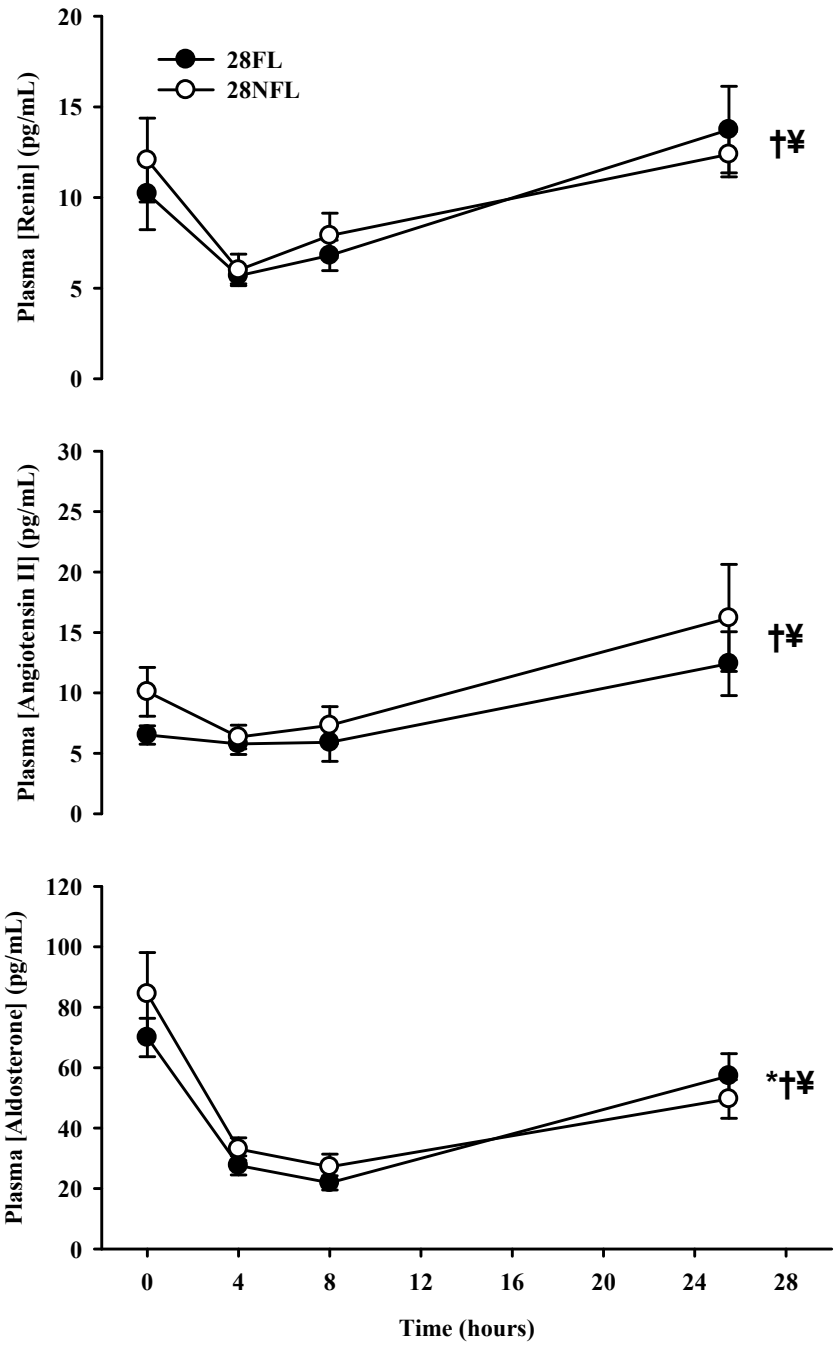


Figure 4.1.3: Plasma renin, angiotensin II, and aldosterone 28hr BR profiles. **Top –** Renin concentrations during the 28hr BR protocols up to FL Start (25.5hr). No differences between protocols were observed, but main effect decreases compared to baseline were observed at 4hr. At 25.5hr, concentrations were not different from baseline, but were significantly higher than at 4hr and 8hr. **Middle** – Angiotensin II concentrations during the 28hr BR protocols up to FL Start. No protocol differences were observed; however, levels at 25.5hr were not different from baseline, but higher than at 4hr and 8hr. **Bottom –** Aldosterone concentrations during 28hr protocols up to FL Start. No differences between protocols were detected, but aldosterone was suppressed at 4hr, 8hr, and 25.5hr compared to baseline. Concentrations at 25.5hr were higher than at 4hr or 8hr. *Significant main effect difference from baseline, $p<.05$; †Significant main effect difference compared to 4hr, $p<.01$; ¥Significant main effect difference compared to 8hr, $p<.05$.

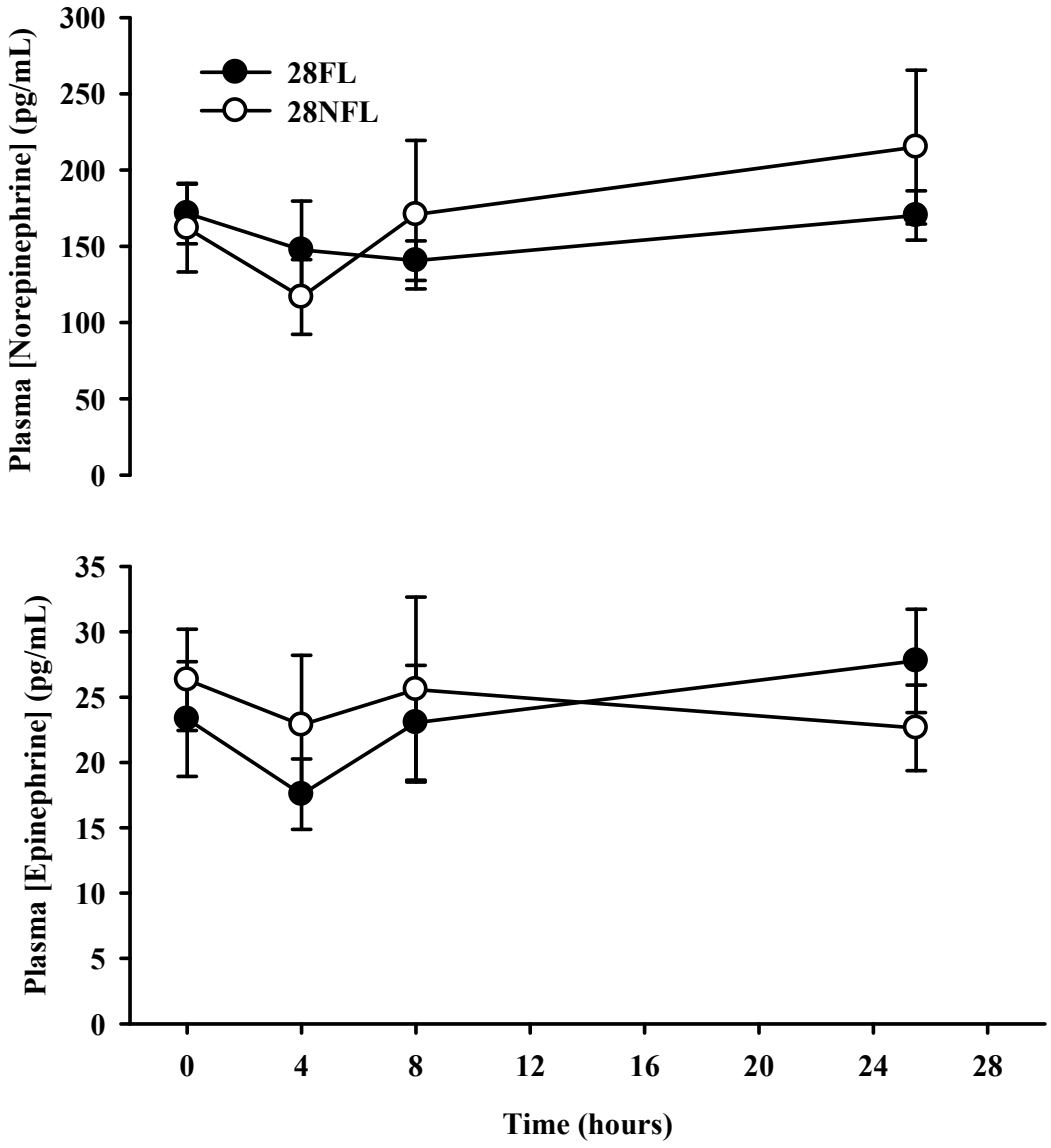


Figure 4.1.4: Plasma catecholamine 28hr BR profiles. **Top –** Plasma norepinephrine concentrations during 28hr BR protocols up to FL Start (25.5hr). **Bottom** – Plasma epinephrine concentrations during 28hr protocols up to FL Start. No differences between protocols or during protocols were detected.

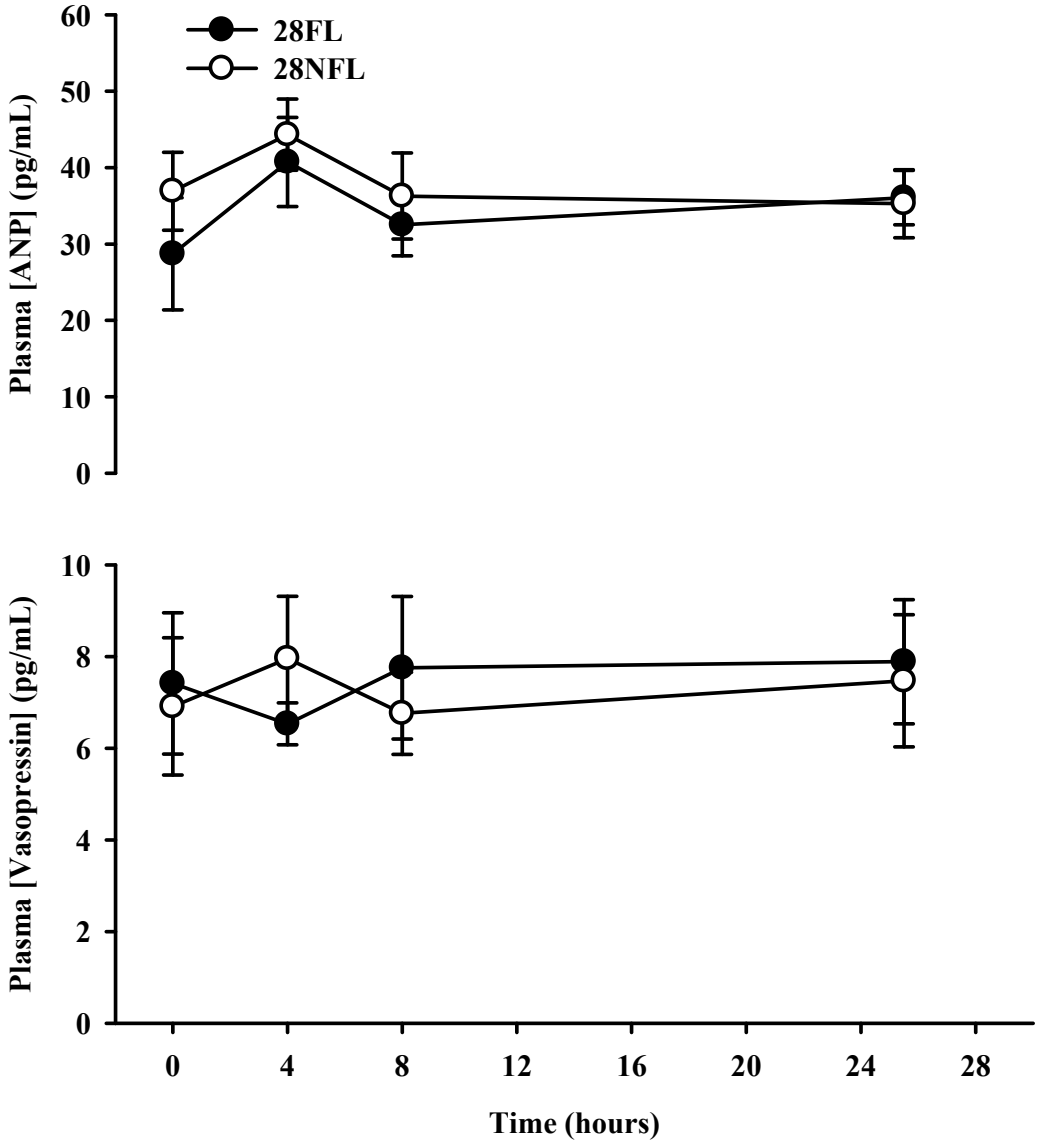


Figure 4.1.5: Plasma ANP and AVP 28hr BR profiles. **Top** – Plasma ANP concentrations during 28hr protocols up to FL Start (25.5hr). **Bottom** – Plasma AVP concentrations during 28hr protocols up to FL Start. No differences between protocols or during protocols were detected.

4.2 Fluid Loading Period

Blood volume during the FL period was unchanged except for 4FLS, during which a 6.2 ± 1.8% increase was observed ($p<.05$) (Figure 4.1.1 - Middle). This resulted in final protocol PV changes of -6.0 ± 2.2, -8.2 ± 1.7, +5.1 ± 1.2, +1.4 ± 0. 9, and 0.0 ± 0.0% for 28FL, 28NFL, 4FLS, 4NFLS and 4BR, respectively (Figure 4.1.1 – Bottom). During the FL period there was a trend toward reductions in the RAAS hormones. Main effect reductions in renin were observed after the first 90 minutes of the FL period for all protocols though only the changes in the 28FL and 4FLS protocols were significant, individually ($p<.05$ and $p<.01$, respectively). At the start of the Post-test LBNP protocol, ~150 minutes after FL Start, these reductions were even greater compared to FL Start ($p<.01$ and $p<.001$, respectively), though no changes compared to FL Start were observed in the NFL protocols. However, while renin continued to decline slightly after 90 minutes in 4NFLS, concentrations rose in 28NFL such that by the end of the FL period, renin levels were significantly higher than both FL protocols (150min: 28FL = 6.3 ± 0.7 vs. 4FLS = 8.25 ± 1.0 vs. 4NFLS = 9.1 ± 1.0 vs. 28NFL = 13.3 ± 2.4, $p<.05$) (Figure 4.2.1 - Top). Though it did not reach the same statistical significance, a similar trend was observed in angiotensin II over the course of the FL period. However, the 28NFL remained higher than all other protocols except for 4FLS (Main effect of protocol, $p<.05$ compared to 4NFLS and 28FL; $p=.0572$ compared to 4FLS), which was closest to 28NFL at FL Start, but which also saw the greatest reduction after 150 minutes (Figure 4.2.1 – Middle). Aldosterone levels were generally reduced by the end of the FL period ($p<.01$), and though 4FLS was the only individual protocol to show a significant reduction in aldosterone after 150 minutes ($p<.01$), 28FL also showed a trend toward reductions at 150min ($p=.0642$), whereas no changes were observed in the NFL protocols (Figure 4.2.1 – Bottom).

Norepinephrine also tended to decline as FL progressed, with the levels at 150 minutes being significantly lower than both FL Start and 90 minutes (Main effect of time, $p<.01$ and $p<.05$ respectively) (Figure 4.2.2 – Top). While both 4hr protocols tended to be higher than the 28hr protocols, only 4FLS was significantly different than 28FL (Main effect of protocol, $p<.05$), though there was a trend for 4FLS to be greater than 28NFL as well ($p=.0652$). Similarly, plasma epinephrine levels declined over the course of the FL period (Main effect of time, $p<.05$); however, no differences were detected between protocols (Figure 4.2.2 – Bottom).

Total urinary volume output over the last 4hrs and the Post-test session was not different in any condition; however, there was a trend for urine volume to be higher in 28FL compared to control (28FL = 1139 ± 149 vs. 4NFLS = 614 ± 147mL, $p =.0517$) (Figure 4.2.3 – Top). Urinary osmolar and sodium outputs were not different between protocols in this period (Figure 4.2.3 – Middle and Bottom, respectively).

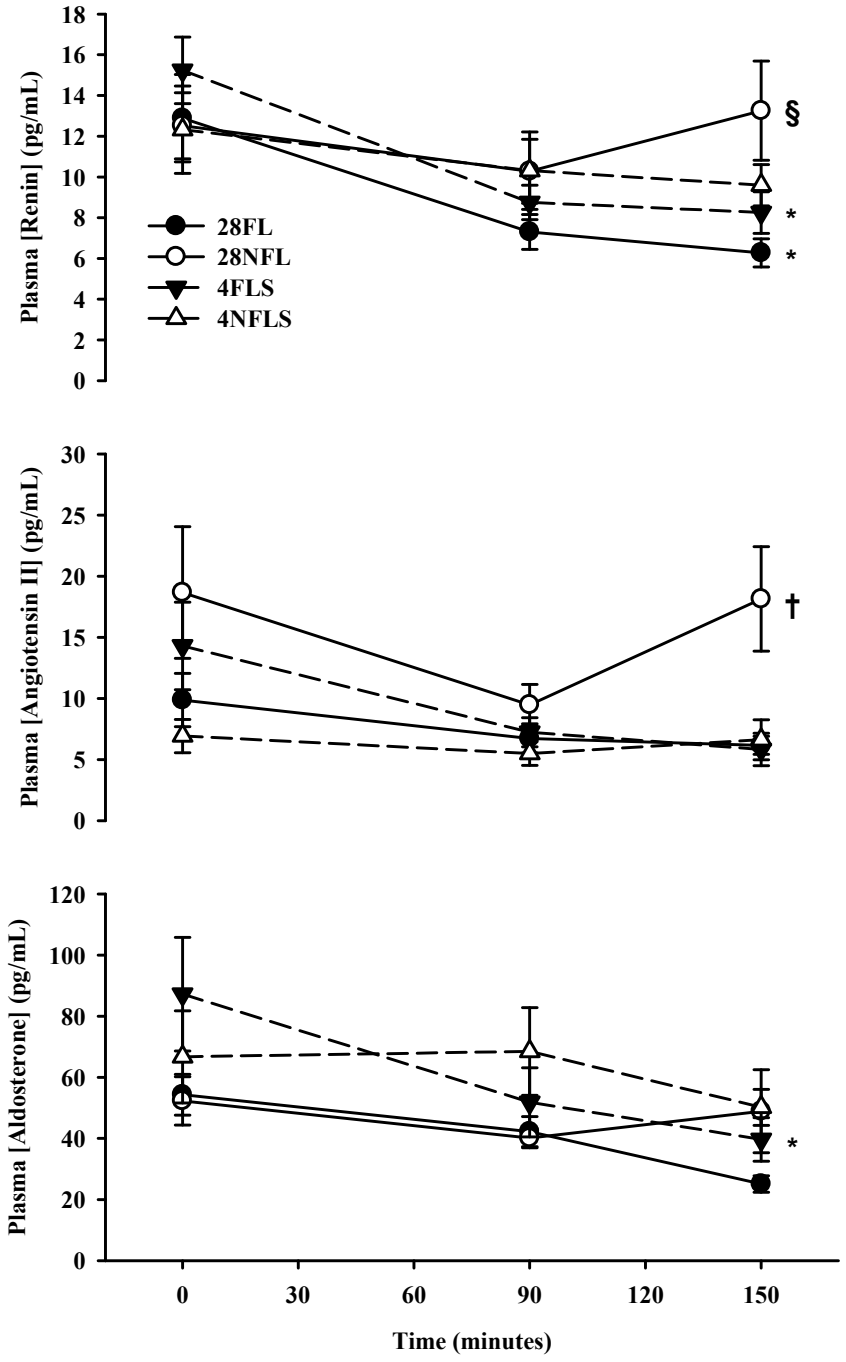


Figure 4.2.1: Plasma renin, angiotensin II, and aldosterone during the fluid loading period**. Top** – Plasma renin concentrations during the FL period. Renin decreased only during 4FLS and 28FL, with no changes observed in the 4NFLS or 28FL protocols. No differences were observed at FL Start, but after 150min, concentrations were elevated in 28NFL compared to 4FLS and 28FL, but were not different in 4NFLS. **Middle** – Angiotensin II concentrations during the FL Period. A protocol main effect was observed, with 28NFL being significantly higher than 4NFLS and 28FL. **Bottom** – Aldosterone concentrations during the FL period. There was a main effect of time with aldosterone levels being reduced at 150min; however 4FLS was the only individual protocol to show a significant reduction. *Significant within-protocol difference from FL Start, $p<.01$; †Significant protocol main effect; $p<.05$; §Significant point difference between protocols, $p<.05$.

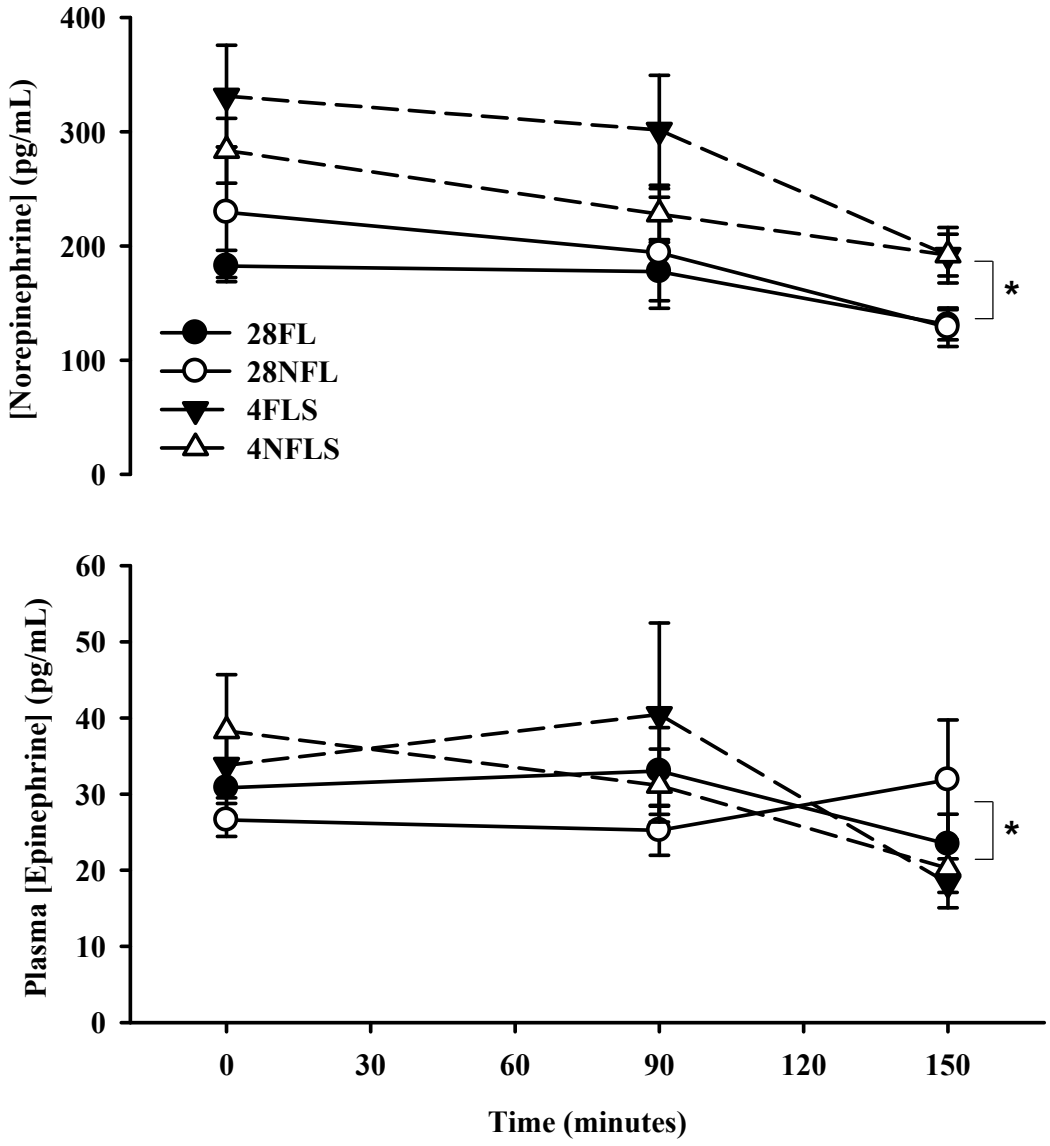


Figure 4.2.2: Plasma catecholamines during the fluid loading period. **Top** – Norepinephrine concentrations during the FL period. A main effect of time was detected, with concentrations declining over the course of the FL period. A main effect of protocol was also observed, though only the difference between 4FLS and 28FL was significant. **Bottom** – Epinephrine concentrations during the FL period. Only main effects were detected, with concentrations declining over the course of the FL period. *Significant difference from FL Start, $p<.05$. Brackets denote a main effect of time.

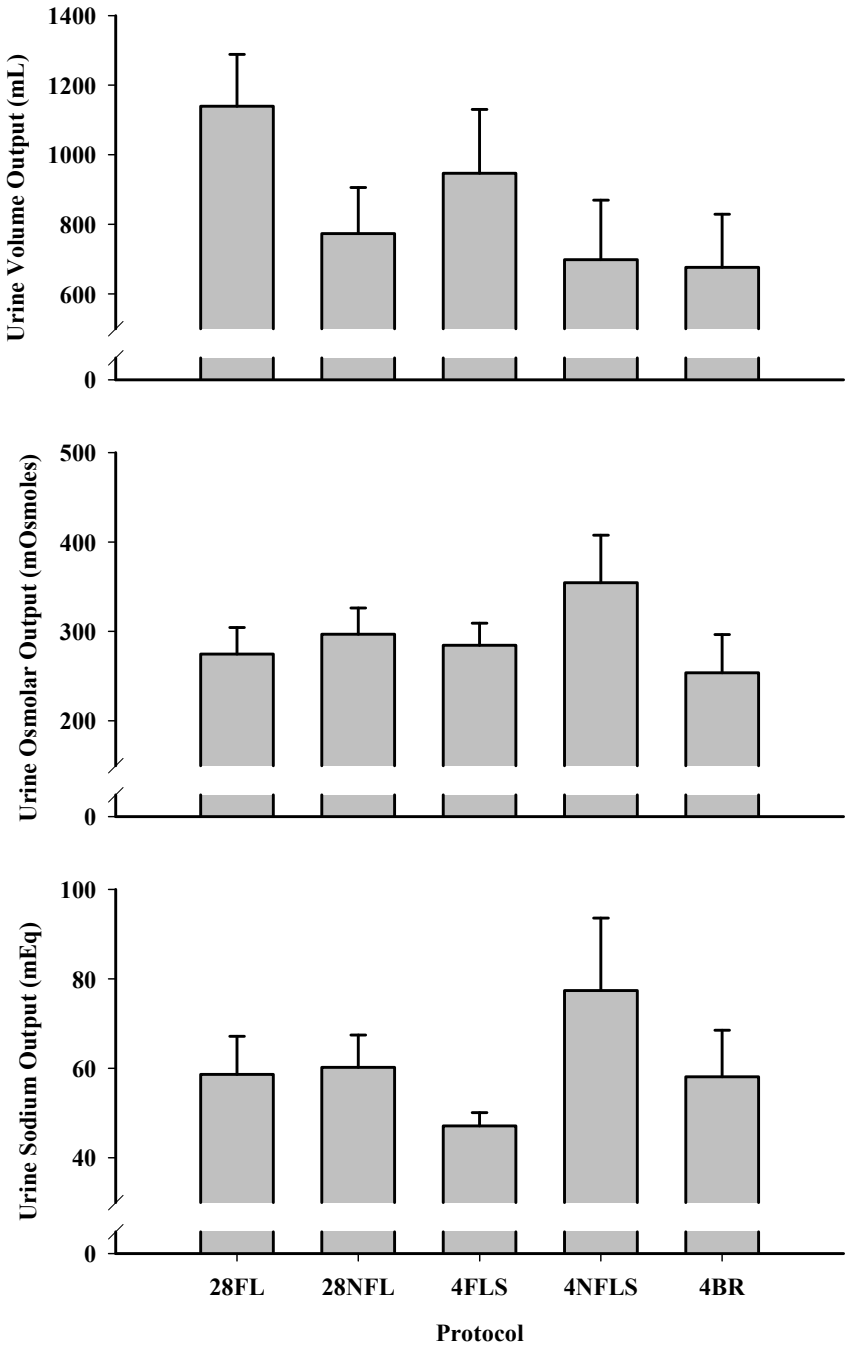


Figure 4.2.3: Cumulative urine outputs during the final 4hr of all protocols. **Top** - Urinary volume output; **Middle** – Urinary osmolar output; **Bottom** – Urinary sodium output. No differences were observed between protocols during this period; however, there was a trend for increased volume output during 28FL (p=.0576 compared to control)

4.3 Orthostatic Testing

Pre-tests for all 5 protocols were not statistically different for renin, angiotensin II, or aldosterone when analyzed with two-way ANOVA. The overall results of the Pre-tests showed significant main effect increases in renin concentrations ($p<.0001$), no differences in angiotensin II ($p=.1234$) and main effect decreases in aldosterone in response to increasing LBNP ($p<.01$) (Figure 4.3.1). Renin concentrations increased to a greater extent during 28NFL Post-tests than Pre-tests (-40mmHg: Pre = 12.4 ± 2.0 vs. Post = 37.6 ± 7.6 pg/mL; $p<.0001$) (Figure 4.3.2 – A); however, this effect was abrogated in the Post-test of 28FL and concentrations at -40mmHg were not different from Pre to Post-test (-40mmHg: Pre = 14.1 ± 3.0 vs. Post = 15.5 ± 3.7 pg/mL, $p=.8444$). Yet, concentrations were slightly, though not significantly, lower at 0mmHg (6.7 ± 0.6 vs. 10.2 ± 2.0, $p = .0928$) in the Post-test of 28FL, and therefore, still increased more than in Pre-tests (Figure 4.3.2 – B). Renin concentrations increased with LBNP in 4FLS (Main effect of LBNP, $p<.05$) with the Post-test concentrations being lower (Main effect of Pre-Post, $p<.05$), and the protocol attenuating increases in renin from the Pre to Post-test. Despite the reduction in renin levels in 28FL compared to 28NFL, concentrations were still greater at -40mmHg after 28FL as compared to 4FLS (-40mmHg: 28FL = 16.1 ± 4.7 pg/mL vs. 4FLS = 8.4 ± 1.0 pg/mL; $p<.05$) (Figure 4.3.2 – C). When comparing the three BR protocols, only the 28NFL Post-test showed a significant LBNP effect and concentrations at -40mmHg after this protocol were significantly higher than both 28FL and 4BR (-40mmHg: 28NFL = 37.6 ± 7.6 vs. 28FL = 15.5 ± 3.7 and 10.7 ± 1.4pg/mL; $p<.001$ and $p<.0001$, respectively) (Figure 4.3.2 – D). No changes in LBNP response were observed after the 4BR protocol, though renin concentrations were lower during the Post-test (Main effect of Pre-Post, $p<.05$) and there were no differences between 4BR and 4NFLS or 4FLS and 4NFLS Post-tests.

Angiotensin II was significantly higher in the Post-test of 28NFL (Main effect of protocol, $p<.05$) (Figure 4.3.3 – A). Similar to the renin response, this effect was abrogated by the 28FL protocol, with only a small rise in concentrations due to LBNP observed (Main effect of LNBP, $p<.05$) (Figure 4.3.3 – B). Also, somewhat similar to the renin response, there was a trend for angiotensin II to be lower during the Post-test after 4FLS ($p=.0515$); however, there were no differences observed between 4FLS and 4NFLS or 4BR and 4NFLS Post-tests. LBNP caused an increase in angiotensin II during 4BR tests with significantly lower concentrations observed across the LBNP levels of the Post-test (Main effect of Pre-Post, $p<.05$). Plasma angiotensin II was not different in the Post-tests of 4FLS and 28FL (Figure 4.3.3 – C). When the BR protocol Post-tests were compared, 28NFL was significantly higher than both 28FL and 4BR ($p<.05$, $p<.01$, respectively), which were not different from each other (Figure 4.3.3 – D).

After both 28FL and 28NFL, aldosterone levels were significantly lower at 0mmHg compared to Pre-test (0mmHg: Pre = 70.0 ± 6.3 vs. Post = 28.6 ± 3.5; $p<.0001$ and Pre = 84.4 ± 13.7 vs. Post = 48.15 ± 10.8 pg/mL; $p<.05$, respectively). However, during the 28NFL Post-test, aldosterone increased at -40mmHg to the same level as during the Pre-test (-40mmHg: Pre = 70.6 ±13.4 vs. Post = 79.1 ± 19.4 pg/mL), but this response was not seen in 28FL and aldosterone remained significantly below Pre-test values ($p<.01$) (Figure 4.3.4 – A/B). During the 4FLS protocols, Post-test baseline aldosterone was lower than Pre-test (0mmHg: Pre = 62.2 ± 10.9vs. Post = 36.3 ± 6.1 pg/mL, $p<.05$), but values were not different by the end of the test. Again, no effects were observed during the 4NFLS protocol, although aldosterone was generally lower during LBNP Post-tests after 4BR compared to Pre-tests (Main effect of Pre-Post, $p<.001$). Still, there were no differences between 4BR and 4NFLS or 4FLS and 4NFLS Post-tests. Concentrations of aldosterone were significantly lower across the LBNP levels in 28FL

compared to 4FLS Post-tests (Main effect of protocol, $p<.05$) (Figure 4.3.4 – C). In the comparison of all BR Post-tests, increasing aldosterone concentrations were observed after 28NFL (0mmHg = 48.2 ± 10.8 vs. -40mmHg = 79.1 ± 19.4 pg/mL, $p<.05$), but not 28FL or 4BR. At -40mmHg, aldosterone after the 28NFL protocol was significantly greater than after both 28FL and 4BR (28NFL = 79.1 ± 19.4 vs. 28FL = 30.5 ± 5.6 and 4BR = 45.1 ± 4.6 pg/mL; $p<.0001$ and $p<.01$, respectively) (Figure 4.3.4 –D).

Norepinephrine changes during Pre-tests were slightly different between protocols when all 5 Pre-tests were compared. Although a strong main effect of increasing norepinephrine with increasing LBNP was observed ($p<.0001$), individually, norepinephrine only rose significantly with LBNP in the two 28hr BR Pre-tests. However, when Pre-Post comparisons were made, norepinephrine increased with LBNP in all protocols (Main effect of LBNP, $p<.01$) with no differences detected between Pre and Post-tests. The small initial differences between protocols also resulted in protocol differences between Post-tests. Compared to 4FLS, the increase in norepinephrine during LBNP after 28FL was larger, though no differences could be detected between protocols at any level of LBNP (Interaction effect of protocol and LBNP, $p<.05$) (Figure 4.3.5 – C). Similarly, both 28hr protocols caused larger increases in norepinephrine during the Post-test compared to 4BR (Interaction effect of protocol and LBNP, $p<.05$) (Figure 4.3.5 – D). Epinephrine did not change during the Pre-test in any protocol. When comparisons were made Pre-Post within protocols, an LBNP main effect was only observed in the 28NFL tests and only at -40mmHg ($p<.05$) (Figure 4.3.6 – B). An LBNP main effect was also seen when comparing the Post-tests of the 3 BR protocols ($p<.01$), and 28NFL was generally higher across all LBNP levels (Main effect of protocol, $p<.05$) (Figure 4.3.6 – D).

ANP and AVP were not responsive to LBNP in any protocol, Pre or Post-test.

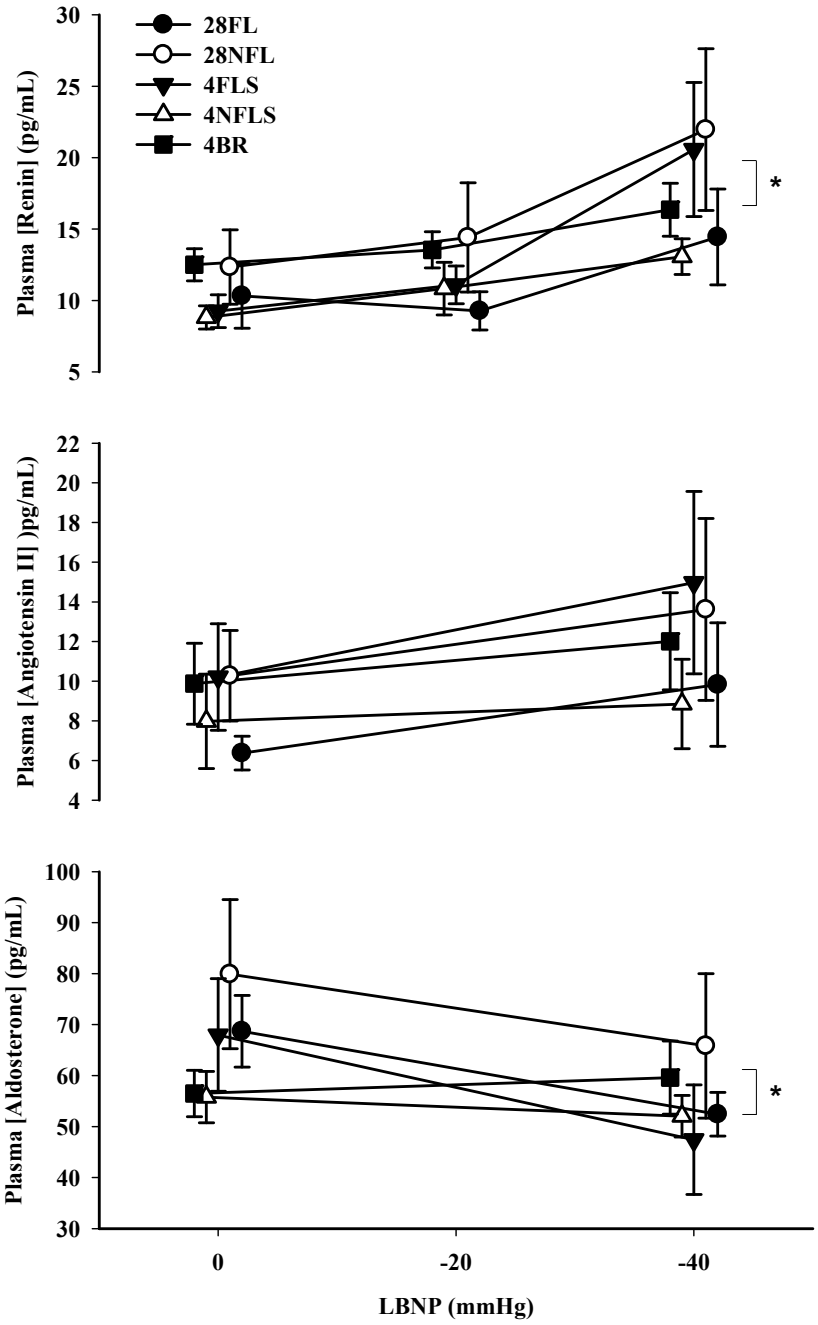


Figure 4.3.1: Plasma renin, angiotensin II, and aldosterone during LBNP Pre-tests. **Top –** renin. **Middle** – angiotensin II. **Bottom** – aldosterone. No protocol or interaction differences were detected; however, main effects for increasing renin and decreasing aldosterone were observed. No changes in angiotensin II were detected. *Significant difference from 0mmHg, $p<.05$. Brackets denote an LBNP main effect across all protocols. Individual protocols are offset for clarity.

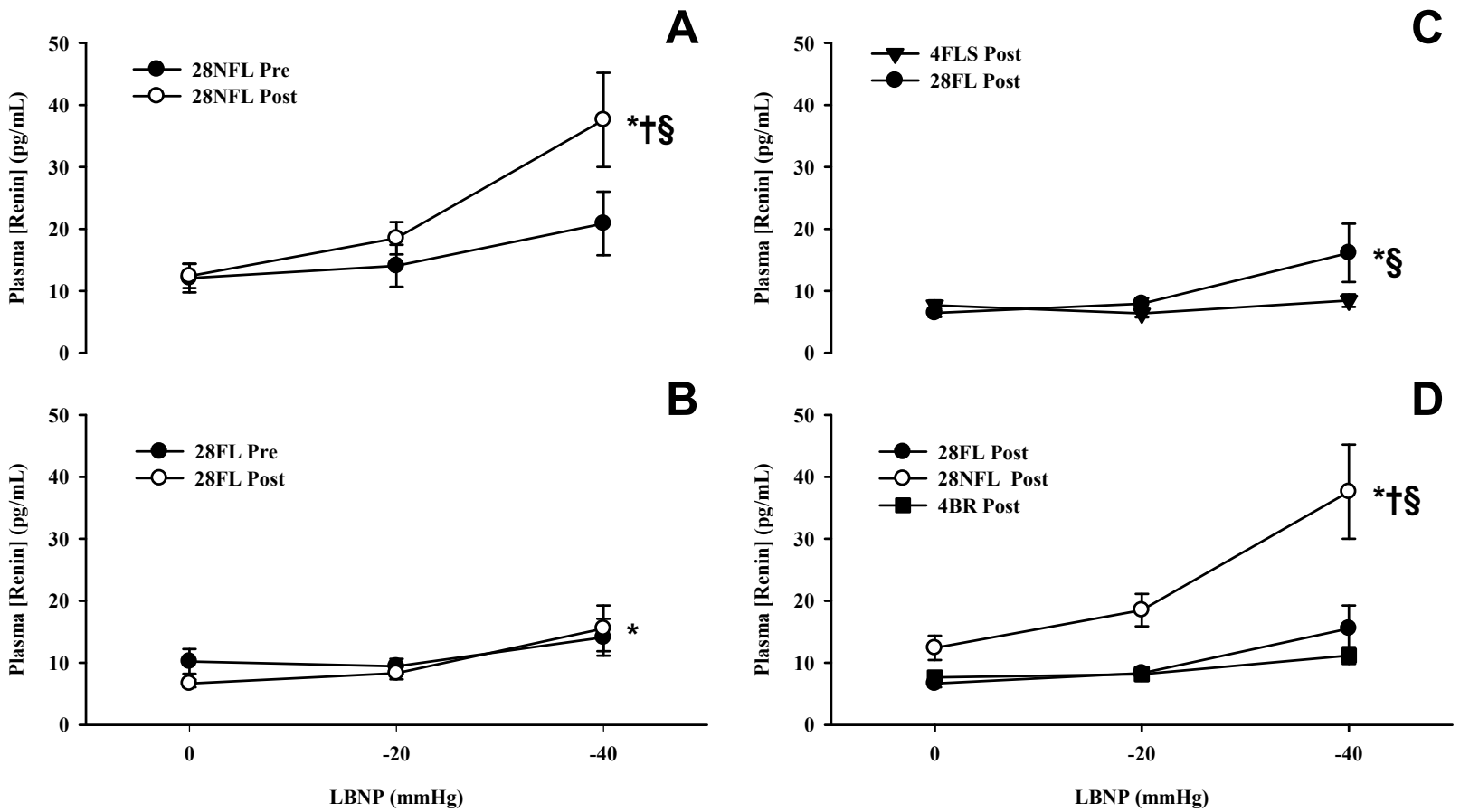


Figure 4.3.2: Comparison of renin responses to LBNP. **(A)** Plasma renin concentrations during Pre and Post-tests for 28NFL. A main effect of Pre-Post test was observed, with Post concentrations being higher; however, post-hoc analysis showed a significant point difference only at -40mmHg. **(B)** Plasma renin concentrations during Pre and Post-tests for 28FL. There was a small, though not significant, point difference at 0mmHg, which was no longer apparent at -20 or -40mmHg and concentration increases were greater in the Post-test where concentrations at -40mmHg were significantly higher than at 0mmHg. **(C)** Post-test comparison of 28FL and 4FLS. This comparison clarifies the interaction of BR and FL during the Post-test. Increases in renin were only observed with 28FL, which was significantly higher than 4FLS at -40mmHg. **(D)** Post-test comparison of all BR protocols: 28NFL, 28FL, and 4BR. This comparison examines the influence of BR length, FL, and PV on response to orthostasis. 4BR and 28FL were not different; however, renin concentrations during the 28NFL Post-test were generally higher, though a point difference was only detected at -40mmHg. *Significant difference from 0mmHg, $p<.05$; §Significant point difference between Pre and Post tests (A, B), or between protocols (C, D), $p<.05$; †Significant main effect difference from Pre-Post test (A, B) or between protocols (C, D), $p<.05$.

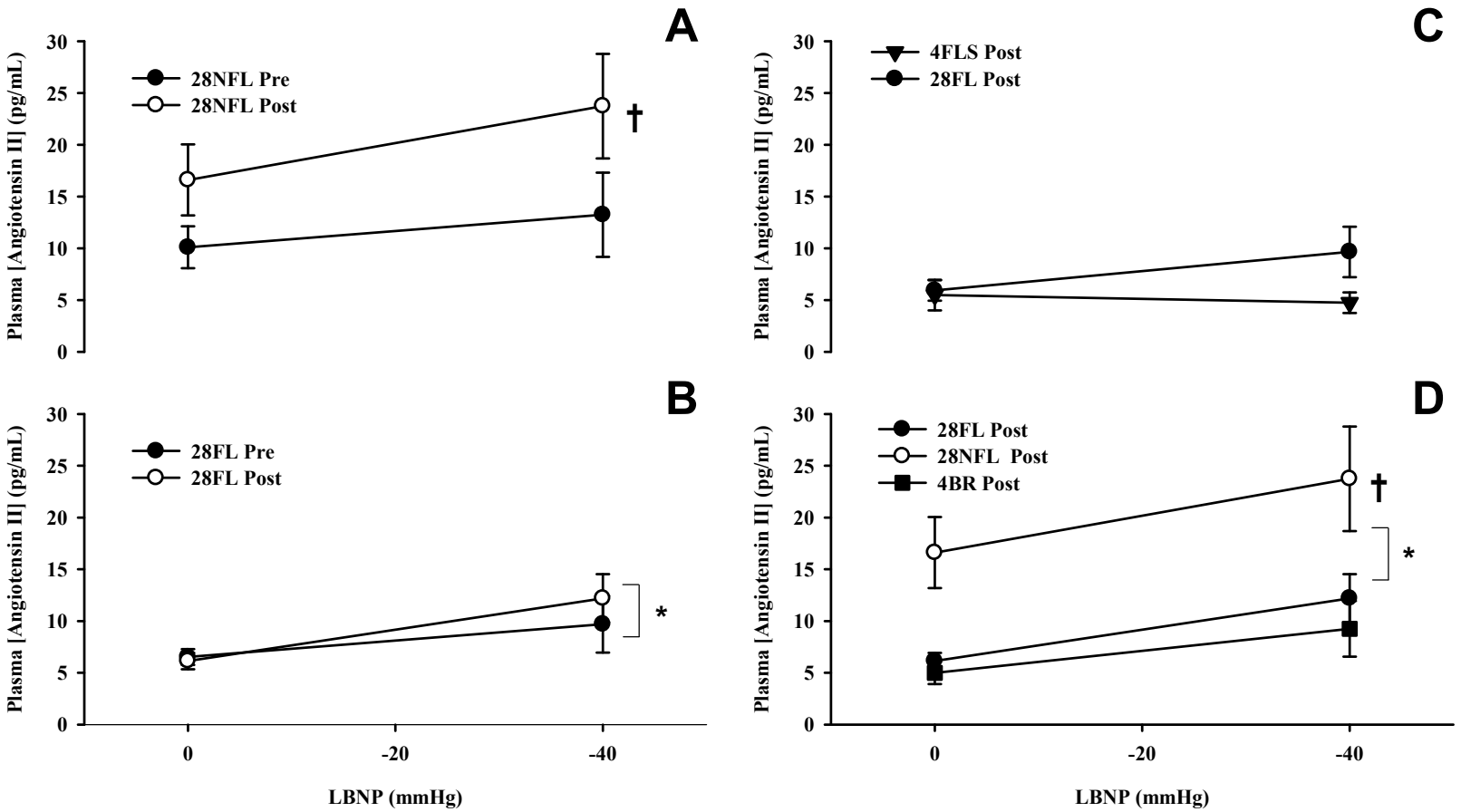


Figure 4.3.3: Comparison of angiotensin II responses to LBNP. **(A)** Plasma angiotensin II concentrations during Pre and Post-tests for 28NFL. Only a main effect of Pre-Post test was observed, with Post-test concentrations being higher. **(B)** Plasma angiotensin II concentrations during Pre and Post-tests for 28FL. An LBNP main effect was observed with no differences between Pre and Post-tests. **(C)** Post-test comparison of 28FL and 4FLS. This comparison clarifies the interaction of BR and FL during the Post-test. No differences were observed between the Post-test responses. **(D)** Post-test comparison of all BR protocols: 28NFL, 28FL, and 4BR. This comparison examines the influence of BR length, FL, and PV on response to orthostasis. A main effect of protocol was observed with 28NFL concentrations being generally higher than the other two protocols. Concentrations also increased with LBNP. *Significant difference from 0mmHg (B, D), $p<.05$; †Significant main effect difference from Pre-Post test (A) or between protocols (D), $p<.05$. Brackets denote an LBNP main effect.

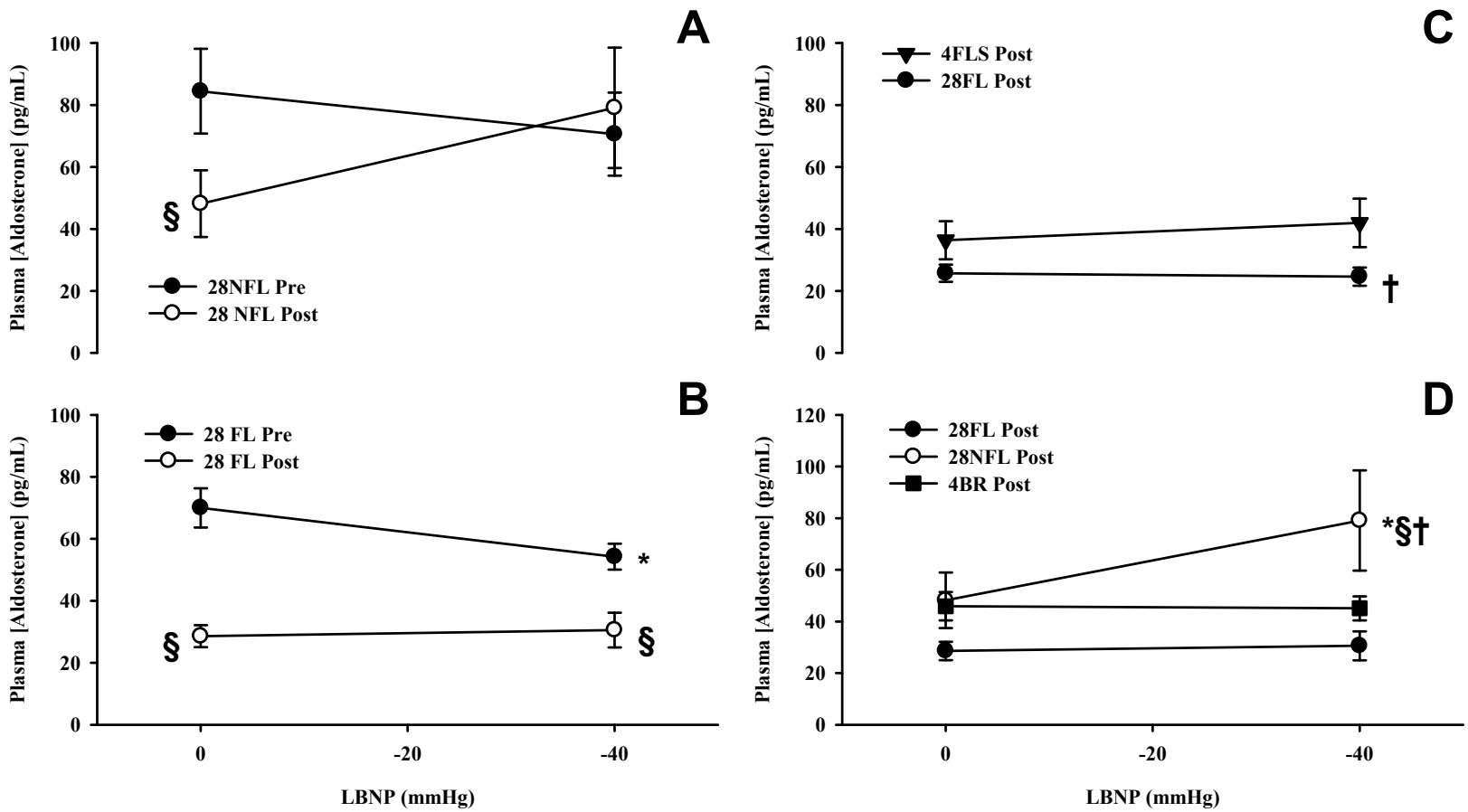


Figure 4.3.4: Comparison of aldosterone responses to LBNP. **(A)** Plasma aldosterone concentrations during Pre and Post-tests for 28NFL. Concentrations were lower at 0mmHg in the Post-test, but increased such that there were no differences at -40mmHg. **(B)** Plasma aldosterone concentrations during Pre and Post-tests for 28FL. Post-test aldosterone was lower at 0mmHg and remained lower at -40mmHg despite decreases during the Pre-test. **(C)** Post-test comparison of 28FL and 4FLS. This comparison clarifies the interaction of BR and FL during the Post-test. A main effect of protocol was observed such that aldosterone concentrations were lower during Post-tests in 28FL. **(D)** Post-test comparison of all BR protocols: 28NFL, 28FL, and 4BR. This comparison examines the influence of BR length, FL, and PV on response to orthostasis. A main effect of protocol was detected, with 28NFL being higher than 28FL, but not 4BR. While aldosterone was not different at 0mmHg, increases were observed only in the 28NFL protocol, with concentrations at -40mmHg being higher than at the same point in 28FL and 4BR. *Significant difference from 0mmHg (B, D), $p<.05$; †Significant main effect difference between protocols (D), $p<.05$; §Significant point difference between protocols (B, D), $p<.01$.

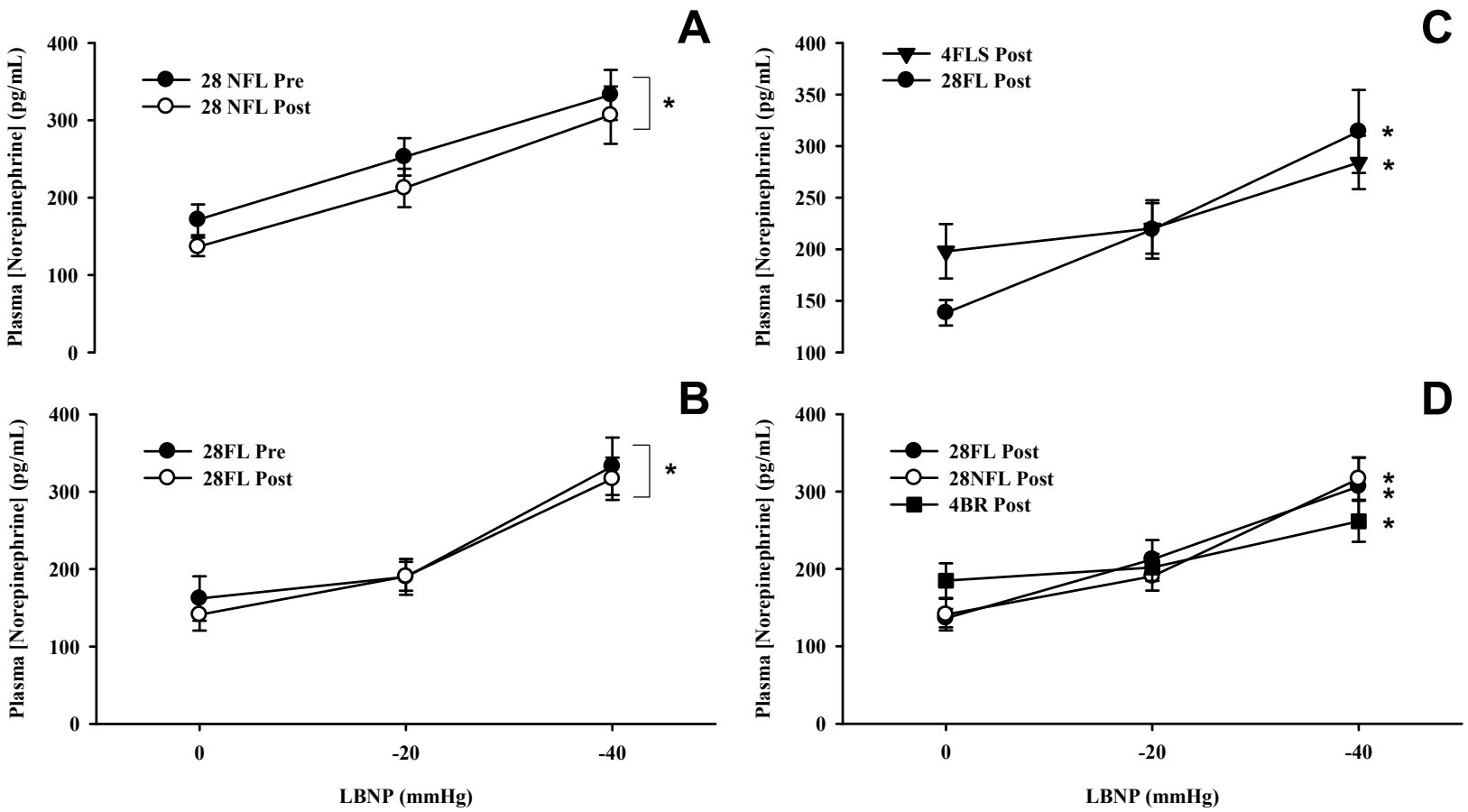


Figure 4.3.5: Comparison of norepinephrine responses to LBNP. **(A)** Plasma norepinephrine during Pre and Post-tests for 28NFL. A significant LBNP main effect was observed, but there were no differences from Pre-Post test. **(B)** Plasma norepinephrine concentrations during Pre and Post-tests for 28FL. Concentrations increased with LBNP, but there were no differences between Pre and Post tests. **(C)** Post-test comparison of 28FL and 4FLS. This comparison clarifies the interaction of BR and FL during the Post-test. Norepinephrine increased faster and to a greater degree in the 28FL Post-test; however, post-hoc analysis showed no differences between protocols at 0 and -40mmHg. **(D)** Post-test comparison of all BR protocols: 28NFL, 28FL, and 4BR. This comparison examines the influence of BR length, FL, and PV on response to orthostasis. Concentrations were not different at baseline or at -40mmHg, but were increased by -40mmHg in all protocols. *Significant difference from 0mmHg, $p<.05$. Brackets denote an LBNP main effect.

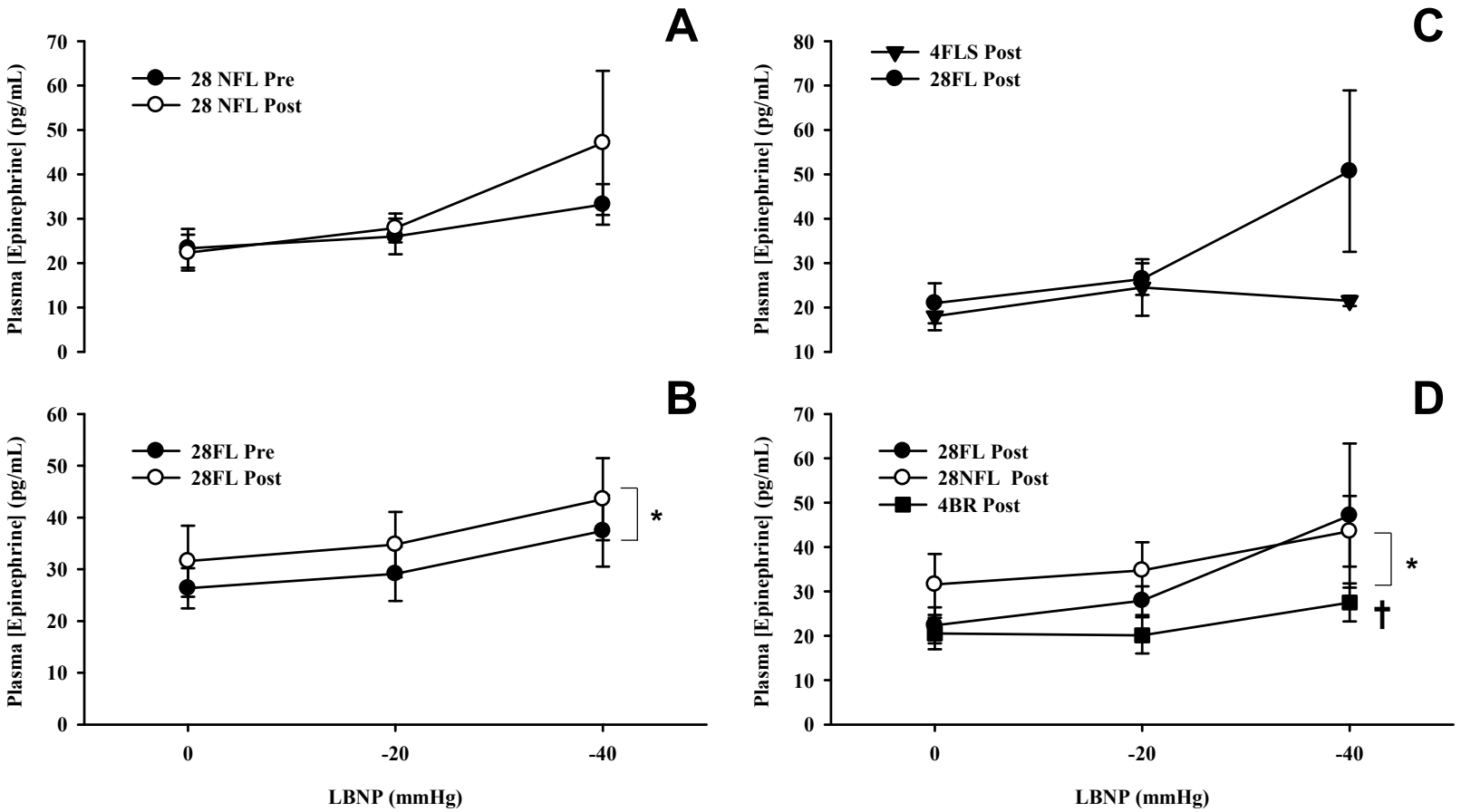


Figure 4.3.6: Comparison of epinephrine responses to LBNP. **(A)** Plasma epinephrine concentrations during Pre and Post-tests for 28NFL. No effects were observed. **(B)** Plasma epinephrine concentrations during Pre and Post-tests for 28FL. An LBNP effect was observed, but there were no differences between Pre and Post tests. **(C)** Post-test comparison of 28FL and 4FLS. This comparison clarifies the interaction of BR and FL during the Post-test. No effects were observed in this comparison. **(D)** Post-test comparison of all BR protocols: 28NFL, 28FL, and 4BR. This comparison examines the influence of BR length, FL, and PV on response to orthostasis. A protocol main effect was detected with 4BR epinephrine concentrations being lower than 28NFL, but not different from 28FL. An LBNP main effect was also observed, with higher concentrations at -40mmHg compared to 0mmHg. *Significant difference compared to 0mmHg, $p<.05$. †Significant protocol main effect, $p<.05$. Brackets denote an LBNP main effect.

5.0 Discussion

This study was the first to investigate the efficacy of the NASA fluid loading protocol using an appropriate control group design and our results expand upon several other investigations examining the role of plasma volume loss and restoration methods and the responses to orthostatic stress after bed rest. We tested several hypotheses: 1) that exposure to 28hr BR would result in a transient increase in blood volume and a hormonal compensation, followed by a reduction in blood volume toward a lower set point; 2) that the ingestion of an oral fluid load would not increase blood volume, but would be met with hormonal changes, which would induce removal of fluid from the vasculature and body; and 3) that after BR, hormonal responses to LBNP would be exaggerated, particularly after 28hr, with no benefit from the oral FL and no changes observed after seated controls.

Regarding these hypotheses, our results showed that after an initial period in which PV increased and hormonal levels were altered to compensate, a reduction in PV was observed after 25.5hr of BR. Second, after 25.5hr of BR, an oral water and salt tablet FL protocol did not restore plasma volume and this was markedly different from the increase in plasma volume observed when the same FL protocol was conducted during a seated control condition. Finally, hormonal responses were exaggerated after 25.5hr of BR, but not after 4hr of BR or the seated control conditions. Also, despite the lack of ability to restore plasma volume during BR, the FL had a significant impact on the activity of the renin-angiotensin-aldosterone system prior to and during orthostatic stress, which appeared to be dissociated from sympathetic activity. This indicated that the BR induced changes in the hormonal response to LBNP were perhaps not a primary result of hypovolemia *per se*, but of the prevailing neurohormonal conditions which were present leading up to and during orthostasis.

5.1 Initial BR Effects

Consistent with our first hypothesis, suppression of renin, angiotensin II, and aldosterone at 4hr and 8hr of BR were indicative of compensation for a transient increase in blood volume. While AVP, ANP, and catecholamines were statistically unchanged at any of our chosen sample times this is likely the result of peak levels occurring before 4hr. Several previous studies have shown no difference in catecholamines (Goldstein *et al.*, 1995) or ANP (Hughson *et al.*, 1995;Mauran *et al.*, 2003) after 4hr of BR and in the study of Hughson *et al.* this was revealed to be due to the response peaking at 2hr and returning to baseline by 4hr. Additionally, it is not uncommon to observe trends across this spectrum of hormones that simply do not achieve statistical significance, but which taken together imply a cephalad fluid shift and hormonal compensation (Nixon *et al.*, 1979;Annat *et al.*, 1986).

By the 25.5hr point in BR, renin and angiotensin II concentrations had returned to levels statistically indistinguishable from the acute supine position, while aldosterone remained below the Pre-test baseline. However, when comparing FL Start to baseline in the two 28hr protocols and also when the lumped 28hr protocols were compared to seated controls at FL Start, there were strong trends for BR to elevate angiotensin II ($p=.0707$ and $p=.0662$, respectively) (Table 4.1.1). Therefore, consistent with previous studies (Vernikos *et al.*, 1993;Waters *et al.*, 2005), we observed uncoupling of the RAAS by BR that appears to be occurring between angiotensin II and aldosterone. Similar resetting of hormone concentrations has been observed previously after relatively short-term BR (Nixon *et al.*, 1979;Gaffney *et al.*, 1985;Mauran *et al.*, 1999;Mauran *et al.*, 2003) and during longer term BR (Sigaudo *et al.*, 1998;Maillet *et al.*, 2000;Bestle *et al.*, 2001;Millet *et al.*, 2001). Like these previous studies, the reduced plasma volume, in concert with the resetting of RAAS hormones, likely indicate a reduction of set point for PV.

Plasma osmolarity during our protocols tended to decline from Pre to Post-test and during the 28hr protocols was significantly lower than the 4hr protocols at FL Start. This is in contrast to several studies showing either unchanged or increased osmolarity (Annat *et al.*, 1986;Goldstein *et al.*, 1995;Sigaudo *et al.*, 1996;Leach *et al.*, 1996;Millet *et al.*, 2001;Bestle *et al.*, 2001) and colloid osmotic pressure (Hsieh *et al.*, 1998) after periods of BR. It is unclear why our results contrast with previous studies, but it should be noted that while this difference was significant statistically, the absolute difference in osmolarity was only 3.0mOsmol/kg, which was unlikely to have a major impact on the production of AVP. This assertion is born out by the lack of change in AVP from Pre-test baseline to 25.5hr.

The unchanging serum sodium concentrations are not surprising. The human body is extremely efficient at buffering sodium loads and serum concentrations are generally kept in a narrow range even with large changes in sodium ingestion (Bie *et al.*, 2004), or during BR (Fortney *et al.*, 1991;Sigaudo *et al.*, 1998;Millet *et al.*, 2001). Also, it is normal for the initial day of BR to be accompanied by a pronounced diuresis and natriuresis (Vernikos *et al.*, 1993;Sigaudo *et al.*, 1998;Mauran *et al.*, 1999;Bestle *et al.*, 2001;Mauran *et al.*, 2003), but because we did not employ a control period prior to BR, or an entire seated control day, we cannot confirm any changes in urinary sodium output due to BR on the first day. However, sodium output was the same on the first day in both of the two 28hr protocols. Additionally, sodium output appeared to stabilize soon after the initial position change, since urinary sodium output during the overnight and morning hours of the BR (Pre2) was not statistically different from the same time period prior to BR (Pre1).

No significant changes in ANP were observed over the course of BR or during orthostatic stresses; however, the former observation may have been the result the time course of ANP

secretion, as mentioned earlier. While some studies have shown increased ANP after long term BR or spaceflight (Maillet *et al.*, 2000), this is not always observed (Sigaudo *et al.*, 1998;Millet *et al.*, 2001;Belin *et al.*, 2004). When any changes in ANP are observed, however, it is generally apparent after several days of adjustment to the new environment and so the lack of change after just 25.5hr is unsurprising. Although reductions in ANP were expected during LBNP, the lack of change is not inconsistent with some previous findings using a relatively short orthostatic stress (Hinghofer-Szalkay *et al.*, 1996;Engelke *et al.*, 1996;Freitas *et al.*, 2005) as was applied in our study.

Due to technical issues, the AVP data for the 4BR and 4NFLS protocols could not be used; however, based upon the results of the other protocols, no differences would have been expected. Similar to ANP, concentrations of AVP were unchanged during orthostatic stress and BR. Increases in AVP are not generally observed over short BR, but sometimes after several days of adaptation over longer term BR (Maillet *et al.*, 2000;Bestle *et al.*, 2001); therefore, unchanged AVP in this study was not unexpected. Furthermore, during LBNP it is certainly possible that the orthostatic stress applied was not severe enough or long enough to elicit significant changes in AVP, as has been previously observed (Waters *et al.*, 2005).

5.2 Hypovolemia and Plasma Volume Restoration

As hypothesized, we observed significant decreases in PV during the two 28hr protocols with PV falling ~6.5% before the start of FL. Additionally, the FL was not effective in restoring PV during BR, but resulted in an ~6% increase in PV during the seated FL protocol (4FLS), while no significant changes were observed in 4NFLS or 4BR. From these results it is clear that even after just one day of BR the set point for blood volume is reduced and attempts to restore PV are resisted by the body to a greater extent than in the seated position, despite a euvolemic

state while seated. Bestle *et al.* (2001) previously observed an augmented diuresis and natriuresis to saline infusion after one week of BR, while Norsk *et al.* (1995) showed that in-flight renal responses to saline infusion were slightly greater than in the ground based seated position. The latter result, in particular, is consistent with the trend for elevated cumulative urine volume measured in the final 4hr of 28FL compared to control. Additionally, our results agree with Convertino's set-point hypothesis that a major adaptation to BR is a new homeostatic blood volume that is appropriate for the prevailing hypokinesic and gravitational conditions (Convertino, 2007).

In further accordance with our second hypothesis, reductions in renin, angiotensin, and aldosterone levels were generally observed across the FL protocols, but not the NFL protocols. However, no significant differences could be detected between the Seated and BR conditions in the FL protocols. Therefore, in this study, we cannot point to any hormonal mechanism responsible for the large difference in PV changes between 28FL and 4FLS. Also, while the urine data show that volume output was slightly greater during 28FL than other protocols, output in 4FLS was statistically indistinguishable in comparison, and no differences in sodium output were observed.

Some caution may be warranted in interpreting the main effect difference between protocols in angiotensin II during FL, since 28NFL hormone levels were generally already the highest of the four protocols, though this may have been due to the effect of two outlier concentrations at 25.5hr (See *Appendix Three*). Additionally, similar to 28NFL, unchanging concentrations were observed during the 4NFLS protocol, though there were still notable differences between 28NFL and 4NFLS throughout the FL period.

Other studies examining the effects of water and salt tablets on plasma volume restoration and orthostatic tolerance (Vernikos & Convertino, 1994;Frey *et al.*, 1994;Shi *et al.*, 2004;Waters *et al.*, 2005) have been conflicting; however, our results supported the study of Vernikos and Convertino, which showed the ineffectiveness of saline ingestion on PV. The Waters *et al.* study appears to be at odds with much of the literature regarding oral PV restoration and orthostatic hypotension (Vernikos & Convertino, 1994;Buckey, Jr. *et al.*, 1996b;Fritsch-Yelle *et al.*, 1996;Shi *et al.*, 2004), although some studies have demonstrated clear effects of PV loss and restoration by rapid intravenous infusion on BR-induced baroreflex alterations (Iwasaki *et al.*, 2000;Iwasaki *et al.*, 2004) and even orthostatic tolerance (Takenaka *et al.*, 2002). Several explanations may account for the differences between the aforementioned study and our own. First, Waters *et al.* did not show a statistically significant loss of plasma volume compared to pre BR measures (2.80 ± 0.14L on Day 0 vs. 2.60 ± 0.12L on Day 12, p=.06) (Waters *et al.*, 2005). Additionally, it is unclear when the second measure of blood volume was taken. In that study it is conceivable that for a short time, plasma volume was raised enough to compensate for the small BR-induced loss, but was eliminated from the intravascular space by the time tilt-testing was performed. In our study, Hct measures were taken during the LBNP protocol and provide evidence that blood volume was not changed by FL during the orthostatic challenge.

5.3 Fluid Loading and Orthostatic Stress after Bed Rest

To investigate the interacting effects of FL and BR, we compared specific Post-test results in addition to examining Pre-Post test differences. These were set up to test the specifics of our third hypothesis regarding endpoint (Post-test) differences elicited by the various protocols. In contrast to the hypothesis that 4BR would elicit augmented responses to LBNP, we observed no differences between any of the Post-test hormonal responses after 4BR and 4NFLS,

despite concentrations in the Post-test of 4BR being generally lower than Pre-tests. However, as hypothesized, there were no differences between Post-test responses in 4FLS and 4NFLS, although unexpectedly we observed attenuated RAAS responses to LBNP in the 4FLS Post-test compared to Pre-test. Furthermore, our results confirm that after only 28hr of BR, RAAS responses to orthostatic stress are augmented compared to Pre-test; however, in contrast to our hypothesis, we observed no augmentation of the LBNP response after 28FL, despite unchanged PV due to FL. At the same time, both renin and aldosterone responses after 28FL were significantly augmented compared to 4FLS, suggesting a residual BR effect independent of the FL, but which may have been linked to the large difference in PV.

Interestingly, the results from the 4BR and 4NFLS comparison did not confirm the results of a previous study from our laboratory and part of our third hypothesis. While the observation of no PV changes after 4BR was replicated (Butler *et al.*, 1990;Fischer *et al.*, 2007), and Post-tests showed the same depressed RAAS activity at baseline, we did not observe an augmented hormonal response during LBNP as seen previously (Fischer *et al.*, 2007). Additionally, the RAAS activity across all levels of LBNP was slightly (though not significantly) lower after the 4NFLS protocols such that responses after 4BR and 4NFLS were not statistically different. Thus, we observed no effect of 4BR on the hormonal responses to orthostatic stress. The difference in results cannot currently be explained, particularly considering the very similar test conditions (meal, fluid intake) and the lower level of orthostatic stress applied in the previous study. It is possible in the study of Fischer *et al.* that the random LBNP protocol prior to the constant LBNP protocol in which blood was sampled affected the hormonal results of the following constant LBNP. However, this might be expected to bring those results more in line with the current

study, by increasing baseline RAAS hormone concentrations and therefore potentially attenuating any LBNP response.

The results of the RAAS response in the 4FLS protocol are also difficult to interpret, since they did not confirm the hypothesis that the LBNP response would be unchanged Pre-Post test, but when Post-tests were compared, there were no differences between 4FLS and 4NFLS. The lack of difference here may be explained by the fact that, by chance, the largest Pre-test responses occurred in 4FLS. Therefore, subjects appeared to be most susceptible to reductions in RAAS responses due to FL when undergoing this protocol. The significant increase in PV also observed during 4FLS supports this idea.

The effects of BR and FL on the RAAS and norepinephrine release represent major findings of the current study. We observed an augmentation of the renin and aldosterone responses to LBNP and angiotensin II was generally elevated after the 28NFL protocol; effects not observed after the seated control condition, 4NFLS. These augmented responses were also absent in comparisons to 28FL, 4BR and 4FLS Post-tests. At the same time, the norepinephrine responses to LBNP were unchanged Pre-Post test in any protocol. Although we observed differences between Post-tests in the norepinephrine responses it is likely not valid to say these were BR induced changes, since Pre-tests generally showed the 28hr protocols to have greater responses to LBNP compared to the 4hr protocols and no changes were observed from Pre-Post test in any protocol. This is also consistent with previous BR literature (Sigaudo *et al.*, 1998;Millet *et al.*, 2001;Kamiya *et al.*, 2003;Waters *et al.*, 2005), in which norepinephrine responses to orthostatic stress were unchanged by BR.

The activation of the RAAS has been implicated as a predictor of orthostatic tolerance in non bed rested subjects (Greenleaf *et al.*, 2000). Additionally, the inability to withstand orthostatic stress after spaceflight has been linked to the inability to mount an appropriate sympathetic vasoconstrictor response, with an exaggerated response compared to pre-flight being beneficial (Fritsch-Yelle *et al.*, 1996;Waters *et al.*, 2002;Meck *et al.*, 2004). However, while a generally elevated RAAS response has been observed in previous research (Millet *et al.*, 2001), enhanced responses are not present in men compared to women (Vernikos *et al.*, 1993;Millet *et al.*, 2001), or high compared to low tolerance individuals (Fritsch-Yelle *et al.*, 1996).

It is interesting, then, that there were no changes in norepinephrine responses during LBNP, while at the same time RAAS activity was modulated through BR and FL. This would seem to indicate a dissociation of the sympathetic nervous system and the RAAS during orthostatic stress. A dissociation of these systems has previously been observed in the resting head down position during long term BR (Leach *et al.*, 1996;Sigaudo *et al.*, 1998;Millet *et al.*, 2001) and during orthostatic stress after BR (Waters *et al.*, 2005). While Waters *et al.* commented upon the dissociation of these systems, they did so in the context of a restored PV and a renin activation that was greater than Pre-test levels, neither of which was observed after FL in our study. Thus, the clear abrogation of the augmented RAAS response to orthostatic stress after BR by oral FL, without apparent changes in sympathetic activity or plasma volume, appears to be a novel finding. Our results show that the RAAS hormones can be depressed by the natriuretic effects of a fluid load after BR and this is independent of whether or not that fluid load restores PV. This hypothesis gains more support when the cardiovascular data are examined. Blood pressure, heart rate, cardiac output, and forearm vascular resistance were not

different between 28FL and 28NFL (data not shown), indicating a change in the RAAS activation without concomitant improvements in cardiovascular functioning.

However, there are several possible explanations for the suppression of renin secretion in the absence of differences in norepinephrine. First, norepinephrine concentrations in samples taken from the antecubital vein are a general indicator of sympathetic activity and may not be representative of overall sympathetic activity, let alone renal sympathetic activity (RSNA), in particular (Esler, 1993). It is certainly possible that a small FL-induced increase in blood volume drove changes in renin secretion through low pressure baroreceptors, reducing RSNA with little or no effect on overall sympathetic activity. Another possibility is local suppression in the kidney by increased GFR and electrolyte changes at the macula densa. Suppression of the RAAS has been shown in the absence of changes in arterial pressure, or RSNA, which means a local mechanism in the kidney is likely the overriding controller of renin secretion when the body is faced with a large sodium load (Bie, 2009). In either case, it seems that the RAAS suppression effects of the fluid load were interacting with the baroreflex recruitment of the RAAS during orthostasis, leading to attenuated increases in circulating hormone concentrations. This hypothesis is further supported by the persistence of RAAS suppression for several hours after saline infusions during ground-based seated and BR protocols (Mauran *et al.*, 1999;Mauran *et al.*, 2003) as well as spaceflight (Norsk *et al.*, 1995).

Activation of the RAAS has been shown to be an important factor in the maintenance of blood pressure in the face of orthostatic stress (Greenleaf *et al.*, 2000); therefore it is important to understand whether attenuation of this system before and/or during orthostatic stress is beneficial. While depressing the system prior to orthostatic stress may in fact provide a benefit by providing additional headroom for larger increases in RAAS activity, this is speculation and

cannot be confirmed by the data presented in this study. Additionally, there is a lack of prior evidence supporting the hypothesis that a lower RAAS baseline and a subsequently larger response are beneficial to blood pressure control after BR. The studies of both Grenon *et al.* (2004) and Siguado *et al.* (1998) showed no differences between tolerant and intolerant subjects' baseline active renin concentrations after BR. Furthermore, one study (Millet *et al.*, 2001) observed a greater incidence of orthostatic intolerance in men compared to women after BR, while these men had greater increases in renin due to lower baseline concentrations. In our study, we observed no improvements in cardiovascular functioning in 28FL compared to 28NFL (data not shown). Therefore, while it would appear that FL 'improves' the response of the RAAS to orthostatic stress after BR, it is important to consider the interacting fluid volume and blood pressure regulating roles of this system and whether this improvement is functional in maintaining blood pressure.

While increases in renin and aldosterone were greater after BR with increasing levels of LBNP, angiotensin II concentrations did not follow the same pattern, but instead were generally elevated at baseline and remained so for the entirety of the orthostatic test. This is difficult to explain, particularly in the context of generally increasing aldosterone concentrations at -40mmHg LBNP. However, the differences in renin and aldosterone responses after 4FLS and 28FL seem to indicate that there is, in fact, a BR effect on this system that remains despite the implementation of FL, although the large PV differences between these protocols may account for this.

Finally, although we did not employ a prolonged BR period, or deliberately attempt to provoke orthostatic intolerance with LBNP, it should be noted that in 18 orthostatic tests after 28hr of BR, with FL and without, there was only one incident of presyncope and it was observed

after the 28FL protocol. This is similar to Waters *et al.* (2005) who observed no presyncopal episodes in 10 tests and while our orthostatic tests were shorter, our results highlight the importance of a control group when making conclusions regarding the efficacy of the FL intervention.

5.4 Limitations

The consumption of food approximately one hour prior to the FL period may have influenced some effects of the fluid load on hormone levels, as the timing of the lunch could have affected the rate of absorption of the fluid load from the stomach.

Also, we did not employ an extended control period prior to the start of each study day, so salt and water intake were not strictly controlled on entry into each protocol. We attempted to counteract day-to-day variability with consistent dietary recommendations and food diaries in addition to employing a standardized fluid intake which was administered in the 8-10hr prior to testing. However, some slight differences in hydration status and baseline hormone levels were present, as can be seen in the variation of the Pre-test responses, particularly norepinephrine.

Additionally, total urine outputs over the final stages of BR and the seated positions were used; therefore temporal resolution of the urine data during the final hours of our protocols is poor. Even so, our results are consistent with the changes in plasma volume that were observed over this period and our hypothesis of diuresis, though not natriuresis after FL.

While the 4hr seated control protocols (4FLS and 4NFLS) were time aligned with 28hr BR protocols, the length of the inactivity could have been a factor. Confinement and inactivity are able to produce some of the same hormonal effects as BR (Sigaudo *et al.*, 1996) and so we cannot be certain that comparison of the seated control and bed rest protocols represent a specific

head down tilt position effect. Also, while diet was strictly controlled in the 24hr prior to the FL period in the 28hr protocols, this was not the case in the 4hr protocols.

In addition, the change of position for the orthostatic challenges was relatively greater, as well as opposite in the Seated compared to BR protocols. Particularly since we were using Hct alone to track PV changes, we cannot discount changes in F-cell ratio (the ratio between whole body and systemic hematocrit) and hydrostatic gradient changes which could have influenced our estimates of PV change due to the FL. However, all comparisons were made in the supine position and subjects were in this position for at least 30 minutes before the LBNP protocol was performed and blood samples were taken. This may also explain the relatively large ~5% increase in PV that was observed in 4FLS. Fluid that was extravasated during the seated protocols may have been reintroduced to the circulation once the supine position was undertaken, "artificially" enhancing PV increases due to the FL period. However, Johansen *et al.* (1998) have shown previously that using Hct tends to underestimate PV changes due to postural change; therefore, we might expect even more dramatic differences between protocols had we employed a more accurate PV measurement method.

Finally, the reductions of aldosterone (in Pre-testing) and unchanged ANP (in both Pre and Post-testing) may indicate a poorly established baseline condition and/or an orthostatic stress that was of insufficient longevity and magnitude to observe the expected changes.

5.5 Practical Implications

This study has shown that fluid loading was not an effective strategy for restoring plasma volume after bed rest simulation of microgravity. However, caution should be taken in generalizing these results to astronauts prior to re-entry. Norsk *et al.* (2001) have suggested that the mechanisms involved in maintaining plasma volume in space are slightly different than during bed rest due to the complete removal of gravitational stress on the tissues, as well as possible central nervous system effects which lead to reduced voluntary fluid consumption. Infused fluid loads appear to be retained longer in space (Norsk *et al.*, 1995) though the results of ground based studies have shown augmented (Bestle *et al.*, 2001), attenuated (Mauran *et al.*, 1999;Mauran *et al.*, 2003) and unchanged (Drummer *et al.*, 1992) responses. Therefore, while our study may indicate that responses to FL are greater during BR than in the seated position, in agreement with Bestle *et al.*, these are both bed rest studies and should be interpreted as such. A properly controlled study of this protocol's effectiveness in astronauts during spaceflight and landing is warranted before any firm conclusions can be made.

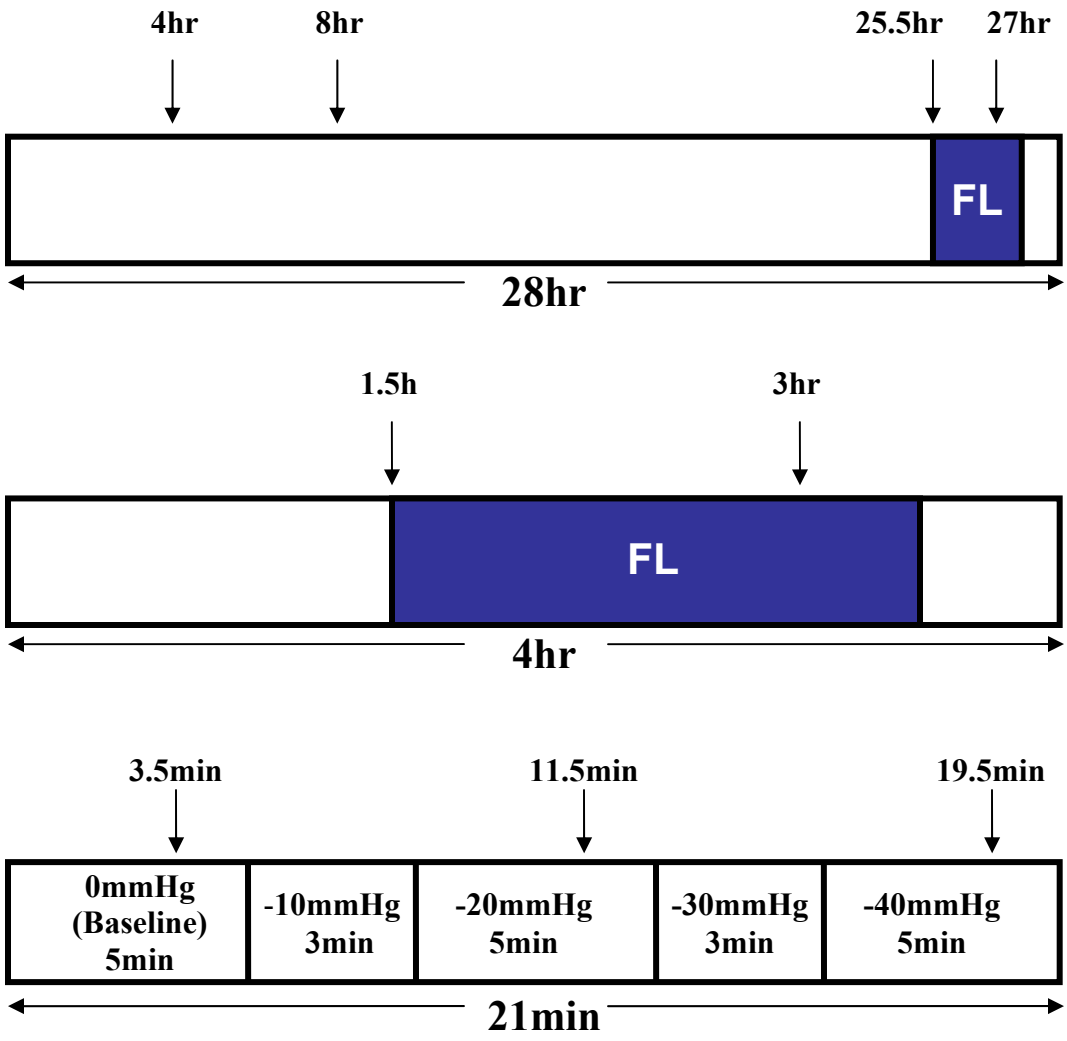
6.0 Conclusions

We conclude from this study that one day of bed rest resulted in reduced plasma volume, while hormone levels were reset after a transient depression early in BR. In confirmation of our second hypothesis, the FL protocol was met with depression of the RAAS and catecholamines in both the seated and BR positions, and while PV was unchanged during BR, the FL increased PV in the seated position. Comparisons of Post-test responses showed that, contrary to our third hypothesis, 4hr of BR did not result in an augmented hormonal response to LBNP and that while seated FL resulted in a Post-test response similar to seated control without FL, it was depressed compared to the Pre-test. Most importantly; however, we observed significant changes in the RAAS response to orthostatic stress after BR in accordance with our third hypothesis.

Unexpectedly though, these changes were moderated by FL such that hormonal orthostatic responses were attenuated to a level between 28hr BR without FL and the 4hr protocols, despite no improvements in cardiovascular functioning. Therefore, it appears that improvements in RAAS activation due to FL during orthostatic stress after BR are not, necessarily, a result of restored blood volume and improved cardiovascular functioning. Instead this is likely a reflection of the interacting forces of the residual diuretic and natriuretic responses to a fluid load and the blood pressure protection responses to orthostatic stress. Therefore, the current study shows that the oral salt and water countermeasure was ineffective in restoring blood volume after bed rest, but also highlights an important interaction between the plasma volume/sodium regulating and blood pressure regulating functions of the renin-angiotensin-aldosterone system.

Appendices

Appendix One: Blood sampling points



Blood sampling points during 28hr BR protocols **(Top)**, 4hr Seated protocols **(Middle)**, and the LBNP protocols **(Bottom)**. Hct measures were taken at the shown sample points, as well as every half hour during the FL period. During 4BR, blood was sampled for hormonal analysis only during the LBNP protocols; however, Hct was taken at 1hr, 2hr, and 3hr during this protocol. ↓ = Blood hormone sample point; FL = 2hr fluid ingestion period. Samples during the baseline of the LBNP Pre-test (at 0hr) were used as protocol baseline values and samples from the baseline of the LBNP Post-test (at ~4hr and ~28hr) were used as the end of the FL period (~150min).

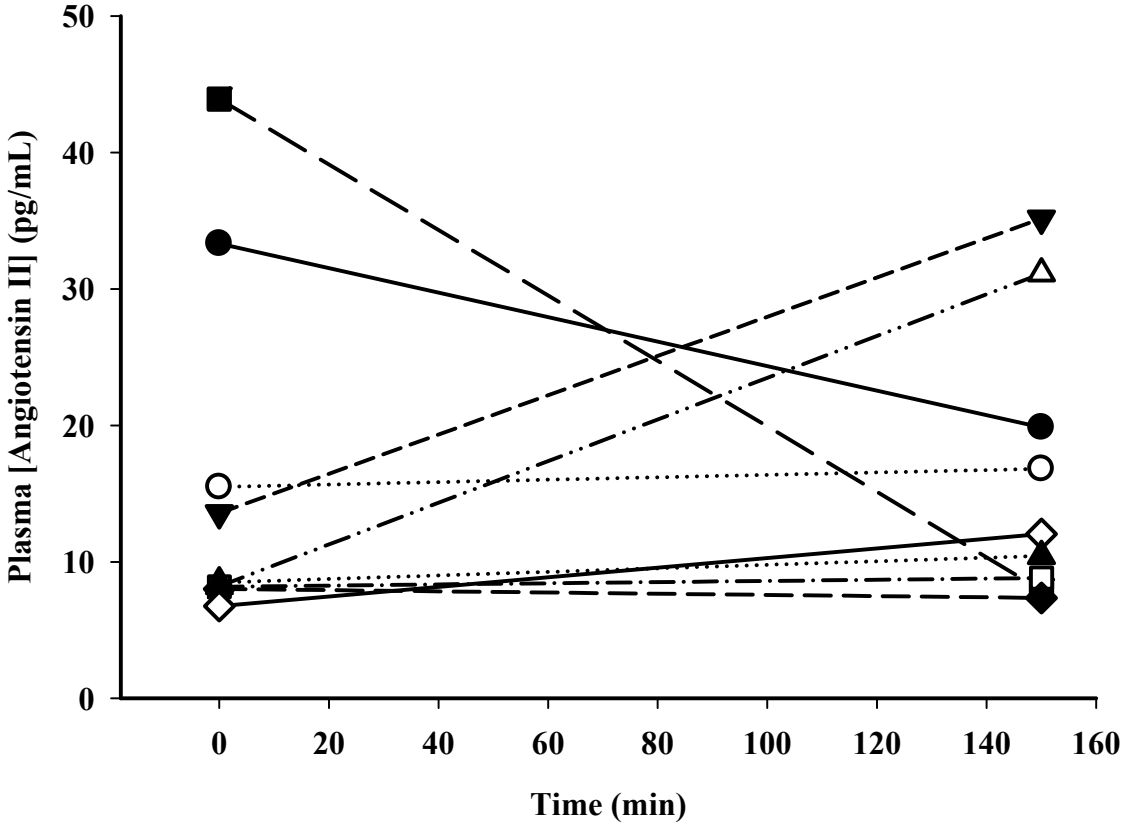
Appendix Two: Sample sizes

Sample sizes used for analyses of variance of blood hormone data.

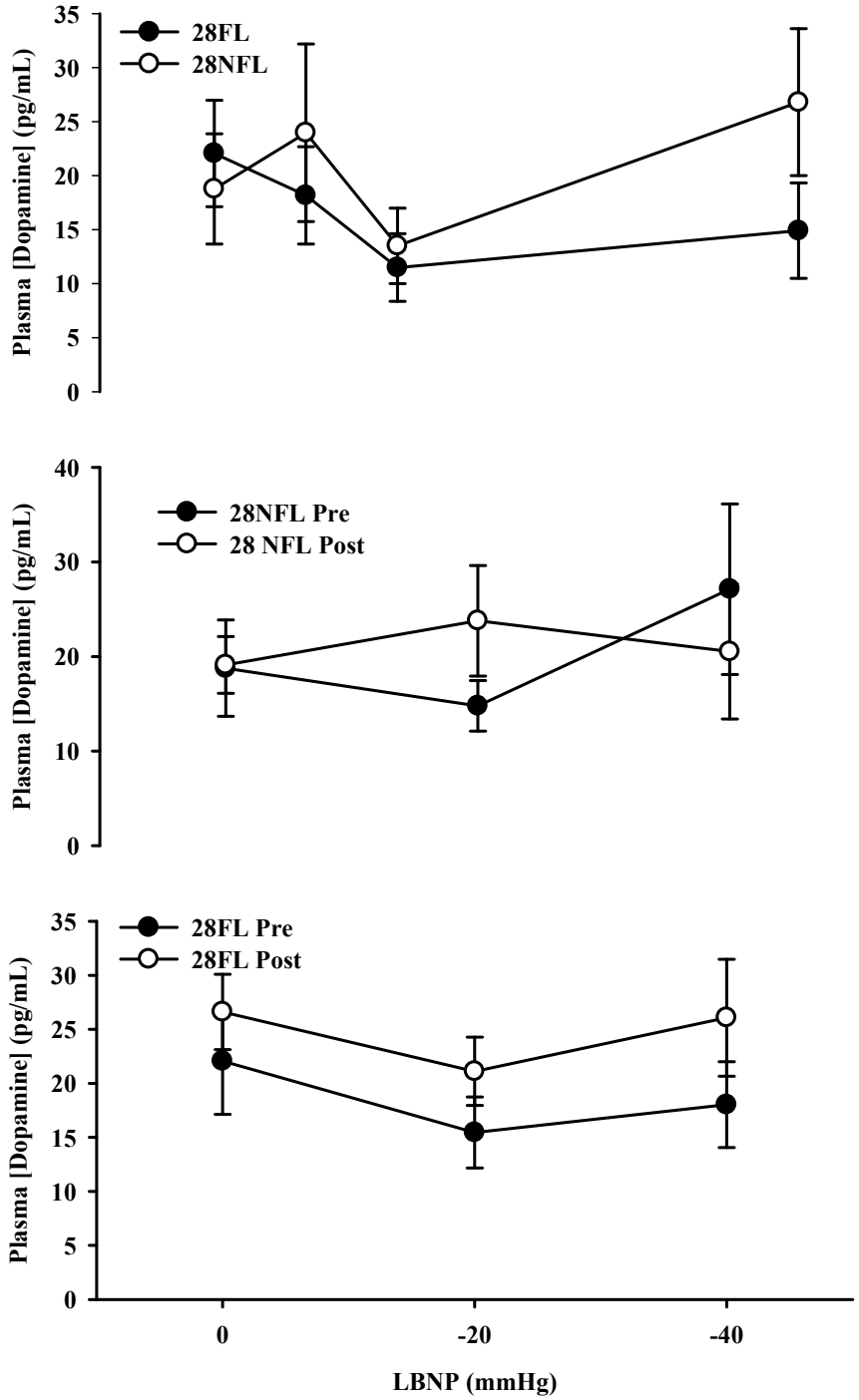
	Pre-Tests	Pre-Test vs. Post-Test					Post-Test Comparisons				During BR		
Hormone	**Pre-Tests**	**28FL**	**28NFL**	**4FLS**	**4NFLS**	**4BR**	**4BRvs4NFLS**	**4FLSvs4NFLS**	**4FLSvs28FL**	**28NFLvs28FLvs4BR**	**28hr**	**FL**	**4hrvs28hr**
Renin	8	9	9	7	8	9	8	6	7	9	9	7	7
ANGII	8	9	9	6	9	9	9	6	6	9	9	7	7
ALDO	8	9	9	7	8	9	8	6	7	9	9	7	7
AVP	7	9	8	7	X	X	X	X	7	8	9	5	X
ANP	7	7	7	6	5	7	5	4	6	7	7	7	7
NE	8	9	9	7	8	9	8	6	7	9	9	7	7
E	8	9	9	7	8	9	8	6	7	9	9	7	7

"X" indicates data which for technical reasons was not included in the analyses.

Appendix Three: Individual data during the 28NFL FL period



Appendix Four: Pilot dopamine data



Complete data sets for dopamine could only be obtained in five subjects in the 28hr protocols; therefore, it is presented as pilot data. **Top** – Dopamine concentrations were not significantly different at any point during the 28hr BR protocols; however, there appeared to be some reduction at the 8hr time point. Dopamine levels were also unchanged by LBNP or Pre-Post test in both 28NFL **(Middle)** and 28FL **(Bottom).**

Appendix Five: Pilot urodilatin data

Kits for urodilatin were generally unsuccessful, which may have been due to the length of time in storage. However, results from the 2 latest subjects are presented here as pilot data. Time periods are the same as for urine volume, sodium, and osmolarity.

Cumulative urodilatin output (ng) from two subjects.

Section	RV	ZS	Average
28FL			
Pre1	52.93	60.42	56.68
Day1	129.96	103.84	116.90
Pre2	56.78	27.15	41.97
Final 4hr	71.68	28.08	49.88
28NFL			
Pre1	31.64	29.25	30.44
Day1	83.01	192.15	137.58
Pre2	72.95	97.87	85.41
Final 4hr	27.98	36.81	32.40
4FLS			
Pre1	41.33	28.11	34.72
Final 4hr	49.03	104.10	76.57
4NFLS			
Pre1	X	93.78	93.78
Final 4hr	6.05	81.48	43.76
4BR			
Pre1	X	40.66	40.66
Final 4hr	7.09	31.60	19.34

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