Hormonal regulation and plasma volume during 60-days of head-down bed rest with exercise during artificial gravity

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 final revisions, as accepted by my examiners.

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Abstract

Reductions in plasma volume (PV), a hallmark of spaceflight and its analog head-down-bed rest (HDBR), trigger compensatory releases of renin and aldosterone to promote fluid retention. Artificial gravity (AG) and exercise have been proposed to counteract PV reductions during HDBR, but optimal protocols remain undefined. We investigated how simultaneous exercise and AG affects volume-regulating hormones and PV during HDBR compared to exercise or sedentary control groups. We hypothesized that exercise+AG would protect PV and maintain volume-regulating hormones at pre-HDBR levels, outperforming exercise and sedentary control groups. Twenty-four healthy males (29±6 yr) underwent 60 days of 6° HDBR and were assigned to sedentary control (n=8), exercise (n=8), or exercise+AG (n=8) groups. Exercise group participants performed near daily 30-minute supine moderate-to-high-intensity interval cycling throughout HDBR, while exercise+AG participants performed the same exercise during 30 minutes of short-arm centrifugation with head-to-foot gravitational profiles based on resting G tolerance tests. Changes in PV, fluid-regulating hormones, and erythropoietin were assessed using carbon monoxide rebreathing and biochemical assays. HDBR reduced PV (p<0.001), blood volume (p<0.001), and hemoglobin mass (p<0.001) in all groups, and reductions were inversely correlated with an increase in active renin (all p<0.05; r_{rm}=-0.615, r_{rm}=-0.553, r_{rm}=-0.426, respectively). Erythropoietin was reduced at HDBR day 3 (p<0.001) irrespective of group. Exercise+AG responses did not differ from the exercise or control groups, with countermeasures failing to maintain PV or blunt fluid-regulating hormone release. These results contrast work showing benefit of gravity-like exposure after exercise on PV and suggests that AG should not be applied exclusively during exercise.

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1.0 Literature Review

Long duration spaceflight is an extreme environment which disrupts multiple systems within the human body. While in orbit astronauts experience continuous free-fall, colloquially referred to as microgravity, where the human body is unloaded of Earth's gravitational forces. Skeletal muscles and bones are unloaded of normal mechanical loading from weight-bearing activities of daily living, while the cardiovascular system is unloaded of hydrostatic pressure gradients and orthostatic stress imposed by Earth's gravity (1–4). As a result, these physiological systems undergo adaptations which can be detrimental to human health both in space and upon return to Earth. The removal of the hydrostatic pressure gradient imposed by Earth's gravity results in a central fluid shift, facilitating cardiac filling while loading arterial and cardiopulmonary baroreceptors (5). Compensatory neuro-hormonal adjustments are stimulated to reduce circulating plasma volume by 10-15% in an attempt to regulate central pressures (2), while concurrent hemolysis reduces hemoglobin mass (Hbmass) (6). Upon return to Earth, astronauts often experience reduced cardiorespiratory fitness (7–9), and the inability to tolerate the upright posture (10–13) partially as a consequence of this reduction in blood volume.

As longer human missions are proposed to extend beyond low Earth orbit to the Moon and Mars, preserving optimal physiological functioning is critical for ensuring crew health upon return to Earth. Human research aboard the International Space Station (ISS) has many logistical considerations including high execution cost, small sample sizes and often takes years to complete. Evaluating underlying physiological mechanisms and studying potential countermeasures for improved astronaut health is critical for continued human presence in space. Therefore, ground based analogs such as head-down bed rest (HDBR) provide more accessible options to study the effects of spaceflight on the human body (14, 15). HDBR is a widely accepted ground-based

analog for spaceflight, as it mimics the hallmark physiological deconditioning that occurs during spaceflight (14). Lying down with the head below the level of the feet in a -6° head down tilt (HDT) position removes the normal head-to-foot gravitational vector and hydrostatic gradient (16). This posture induces a similar cephalad fluid shift, closely reproducing the compensatory hormonal response and volume changes to that experienced in microgravity (17–20).

1.2 Hormonal volume regulation

The renin-angiotensin-aldosterone system (RAAS) is a tightly controlled endocrine feedback loop which regulates extracellular fluid volume, arterial pressure, and systemic vascular resistance (21, 22). This system is constantly responding to changes in factors such as posture, physical activity, water intake, or variations in dietary intake to regulate fluid and sodium balance (22).

To contextualize the adaptations observed during HDBR and spaceflight, the following section describes how RAAS and erythropoietin (EPO) is regulated in ambulatory living. In response to renal hypotension and reduced sodium delivery to the distal tubule, plasma renin is released from the kidneys (23). Plasma renin is the rate-limiting enzyme within the system, as its primary function is to convert angiotensinogen into angiotensin I which initiates the rest of the negative feedback loop (21). Angiotensin I is then converted into the biologically active angiotensin II, directly interacting with peripheral vasculature inducing vasoconstriction to increase arterial blood pressure (24, 25). Angiotensin II also indirectly promotes fluid retention by stimulating the release of aldosterone and vasopressin (21, 26). Aldosterone, the primary hormone responsible for sodium resorption and water retention in the kidney, promotes the expansion of extracellular fluid volume (27). Vasopressin is released in parallel, regulated by both blood osmolarity and angiotensin II activity, enhancing water reabsorption, complementing aldosterone's volume-conserving effects

and ultimately returning the system to homeostasis and suppressing further renin release (28, 29). Conversely, in conditions of increased venous return, such as exercise or hypervolemia, mechanical stretch of the atrial walls stimulate the release of pro-atrial natriuretic peptide (pro-ANP) from cardiomyocytes, subsequently cleaved into its active form, atrial natriuretic peptide (ANP) (30). Reciprocal responses following increased ANP expression inhibit angiotensin-secreted stimulation of aldosterone (26, 31).

Dietary intake of minerals and electrolytes can result in hormonal fluctuations to maintain sodium balance. In particular, reductions in Na^+ intake can elevate plasma renin sufficiently to preserve blood pressure through Angiotensin II mediated vasoconstriction (32), while K^+ intake stimulates dose dependent aldosterone secretion (27, 33). Hormones within RAAS respond in tandem, while deviations from this coordinated response may present indicators of dysregulation (34). Disruptions in this balance, such as an elevated aldosterone-renin ratio (ARR) are associated with increased cardiovascular risk, correlated with the incidence of hypertension and increased arterial stiffness (35). Excess aldosterone relative to plasma renin may contribute to vascular reactivity, oxidative stress and tissue remodeling (36).

Erythropoietin (EPO) is a glycoprotein produced in the kidney, responsible for stimulating erythropoiesis, the production of erythrocytes (37). EPO is regulated by tissue oxygen availability, maintaining red blood cell mass and hemoglobin concentration [Hb] (g/dL) for adequate oxygen delivery. Conditions such as low arterial PO_2 and high-intensity exercise are known to rapidly increase EPO concentrations within hours, initiating erythropoiesis (37). Erythrocyte concentrations are typically increased days to weeks following the rise in EPO. As RAAS stimulates the increase in blood volume, EPO is directly enhanced through Angiotensin II to regulate Hct, maintaining balance between plasma volume and erythrocyte content (25, 37).

Hormonal fluid regulatory responses vary with the length of HDBR. Within the first few hours in the HDT position, the cephalad fluid shift stretches the atrial walls, triggering the release of ANP from cardiomyocytes (30, 38). Early HDBR is accompanied with a transient increase in circulating ANP concentrations (17, 39, 40), similarly observed during the initial phase of spaceflight (41). ANP release is involved in the suppression of plasma renin and subsequent release of aldosterone and vasopressin in the early hours of HDBR (17, 42). The combined suppression of RAAS and vasopressin facilitates natriuresis and water loss, contributing to a 10-15% reduction in plasma volume within the first 24-48 hours of HDBR (43).

The first days of HDBR are characterized by hemoconcentration, where the concentration of erythrocytes is greater relative to plasma volume, demonstrated as an increase in hematocrit (Hct) and [Hb] despite no increase in erythrocyte volume (44). Progressive decline in Hbmass occurs throughout HDBR through a combination of suppressed erythropoiesis and ongoing low-grade hemolysis, normalizing Hct despite persistent hypovolemia (44–46). During a 21 day HDBR study, greater [Hb] and Hct were reported after 10 days of HDBR, which were reduced closer to baseline levels by day 21 (47). Early research from spaceflight missions hypothesized that the hemoconcentration itself triggered the hemolysis and blunted EPO secretion as a homeostatic mechanism to rapidly normalize Hct and blood viscosity (2, 48).

Beyond 24-48 hours of HDBR, the continued plasma volume loss reduces atrial stretch lowering ANP concentrations and removing one of the key inhibitory influences on plasma renin release (18, 49). By day 7 of HDBR, both plasma renin activity and aldosterone concentrations are elevated, and this trend remains consistent across studies up to 90 days (19, 49, 50). Vasopressin concentrations remain largely unchanged throughout HDBR and recovery (18, 19). The persistent elevation in plasma renin and aldosterone promotes sodium retention and fluid conservation via

enhanced reabsorption and vasoconstriction, supporting the gradual restoration of intravascular volume (22). Evidence from a 12 day HDBR study demonstrated a reduction in ARR (51). Under normal conditions, aldosterone secretion is proportionally driven by renin-stimulated angiotensin II. Excess concentrations of aldosterone may contribute to the development of arterial stiffness (35) observed in some HDBR (52, 53) and spaceflight studies (54)

1.3 Role of blood volume

Blood volume is comprised of approximately 55% plasma volume, 45% erythrocytes, with less than 1% consisting of leukocytes and platelets. While plasma volume is critical for maintaining circulating volume for venous return and cardiac output ($\dot{Q}$), erythrocyte content determines oxygen-carrying capacity essential for sustaining aerobic metabolism in working muscles (55). Blood volume is dynamically regulated through the above hormonal mechanisms to maintain homeostatic levels during postural changes, exercise, and environmental challenges such as microgravity (41, 56).

Orthostatic tolerance (OT) is an individual's ability to tolerate upright posture and is often used as a tool to evaluate the baroreflex mechanism (57). In brief, during transitions into upright posture, gravity induces a rapid redistribution of blood volume into the lower limbs (58). Central hypovolemia triggers hypotensive unloading of both carotid and cardiopulmonary baroreceptors reducing their firing rate, resulting in tachycardia and peripheral vasoconstriction to maintain cardiac output and sustain arterial pressure (59). The inability to regulate this response, or sustain arterial pressure is referred to as orthostatic intolerance (OI), which can impair cerebral perfusion, leading to cognitive dysfunction or syncope, which can compromise functional performance (60).

When evaluating this relationship, individuals with greater plasma volume/lean body mass (mL/kg) had a superior OT, as measured by time to presyncope during a tilt-test (61). In another study, patients with diagnosed OI had a lower blood volume and suppressed plasma renin activity than healthy controls, impacting their ability to regulate blood pressure in response to the postural stress of the tilt test (62). Similarly, the use of methods such as lower body negative pressure (LBNP), which use a pressure differential to induce fluid redistribution to the lower extremities, demonstrate that progressive central hypovolemia reduce SV and accelerates symptoms of presyncope (63).

Fluid losses incurred during spaceflight exposure contribute to the well-established reduced OT upon re-ambulation on Earth (11–13, 64). Similarly, OT is reduced despite exercise countermeasures following 14 days up to 5 weeks of HDBR, (65–68). Some research has found no statistical link between reductions in plasma volume and OT following 14-days of HDBR (65), highlighting that plasma volume is only one component of this mechanism, while autonomic nervous system functioning and cardiovascular fitness also play important roles (69, 70).

Despite the asynchronous response between reductions in plasma volume and erythrocyte mass, both constituents contribute to an overall reduction in total blood volume during HDBR (44, 47). This decline has critical implications for aerobic fitness, as erythrocyte concentration, specifically hemoglobin content, directly determines the oxygen-carrying capacity required to sustain aerobic performance (71). $\dot{V}O_{2max}$, the maximal rate of oxygen consumption during exercise, is regulated by the capacity of the cardiovascular system to deliver oxygen and the ability of the skeletal muscle to extract and utilize it, as described by the Fick equation (Equation 1).

$$(1)\ \dot{VO}_{2max} = \dot{Q}\ x\ \dot{(}CaO_2 - CvO_2)$$

Where $\dot{Q}$; cardiac output, CaO_2; arterial oxygen content, primarily determined by the hemoglobin concentration (g/dL) and the degree of oxygen saturation, CvO_2; venous oxygen content. Higher Hbmass and thereby Hb concentration increases CaO_2, enhancing oxygen delivery to the muscle level and ultimately yielding higher $\dot{V}O_{2max}$ (71). $\dot{V}O_{2max}$ is a measure of oxygen consumption (L/min) during a plateau at maximal exercise, while $\dot{V}O_{2peak}$ reflects the highest rate of oxygen consumption in the absence of a plateau and is frequently reported as an index of maximal aerobic power (72, 73).

Hypovolemia during HDBR and spaceflight impairs aerobic capacity through reductions in circulating blood volume that limit $\dot{Q}$ and CaO_2. The initial decline in $\dot{V}O_{2peak}$ in microgravity does not recover despite aerobic exercise and the facilitated venous return, which preserves SV in the absence of gravitational pooling (8, 74). Upon re-entry into a 1-G environment, gravitational stress reduces SV and $\dot{Q}$ leading to a marked reduction in $\dot{V}O_{2peak}$ (8). Similarly, in HDBR models, cephalad fluid redistribution maintains SV during bed rest, while reambulation reveals a significant drop in SV due to reduced blood volume (4). Concurrent progressive decline in Hbmass and [Hb] impair arterial oxygen-carrying capacity, together contributing to a measurable decline in $\dot{V}O_{2max}$. A 14% reduction in $\dot{V}O_{2peak}$ and elevated submaximal HR was observed in control participants after 15 days of HDBR (75), similarly a 16% reduction in $\dot{V}O_{2peak}$ was observed following 30 days without exercise (76). The development of countermeasures that preserve blood volume to support OT and aerobic fitness during gravitational unloading are essential to protect astronaut health and expedite post-flight recovery.

1.4 Countermeasures

Current countermeasure for spaceflight aboard the ISS includes daily 2.5-hour aerobic and resistive exercise sessions to maintain pre-flight cardiorespiratory fitness, muscle and bone strength, though completed aerobic exercise time is only a fraction of the allotted time (54). Performing high-intensity exercise preserves cardiorespiratory fitness during spaceflight experiments (77, 78), however cannot maintain OT during return to Earth as a consequence of reduced blood volume (65–68).

Plasma volume expansion is well documented after both acute (43) and chronic exercise training (79). The expansion of blood volume achieved through an exercise training program is initially attributed to RAAS and vasopressin mediated plasma volume expansion followed by the upregulation of erythropoiesis to increase red blood cell mass and hemoglobin concentration (79, 80). While a single bout of exercise immediately following bedrest has been shown to acutely restore plasma volume to baseline levels, these changes do not influence Hct or [Hb] (g/dL) (43, 81). Normalizing circulating plasma volume through dextran infusion following 14 days of HDBR, along with daily 3x 30 minute sessions of supine cycling at 75% of maximal heart rate, has demonstrated the ability to preserve OT (67). However, acute plasma restoration may not have a meaningful protective effect for aerobic exercise capacity as oxygen delivering capacity remains impaired.

To date, exercise training throughout bedrest alone remains insufficient to preserve plasma volume. The combination of high-intensity aerobic, and resistance activities on upper and lower limbs (82) failed to preserve plasma volume following 14 days of HDBR in older adults, and both exercise and control groups experienced approximately a 500-600mL reduction in PV (65). Similarly, in another 14-day HDBR study, despite performing 3 daily sessions of 30 mins of supine

cycling at 75% their maximal, younger adults in both exercise and control groups experienced a reduction in both plasma volume and indexes of cardiac filling (67). The failure to restore plasma volume was a key contributing factor in the inability to maintain OT following HDBR (65–67).

Artificial gravity (AG) via short-arm centrifugation has been proposed as a multi-system countermeasure for spaceflight deconditioning through the reintroduction of a $1G_z$ gravitational force (83). The altered gravitational loading during spaceflight is associated with physiological adaptations of muscle atrophy, bone demineralization, reduced plasma volume, reduced exercise capability and OT (1–4). In theory, AG may help prevent these physiological adaptations induced by weightlessness by substituting terrestrial gravity with inertial forces produced via centrifugation. AG is not currently implemented aboard the ISS, and to-date research has only been conducted during Earth-based analogs (i.e. HDBR) (84, 85).

In the context of fluid regulation, AG would induce periodic central hypovolemia and reintroduce hydrostatic gradients similar to that experienced in upright posture on Earth (86). Vernikos et al. (1996) demonstrated that the reintroduction of "real gravity" through daily standing or walking for 4 hours blunted the reduction in plasma volume and $\dot{V}O_{2peak}$ in healthy males following 4-days of HDBR (51). In another study, Yajima et al 2001 found no change in Hct following 4 days of HDBR with 2 daily bouts of 30-minute centrifugation at $2G_z$ (87). The unchanged Hct suggest the daily AG use prevented hemoconcentration, suggesting that AG mitigated the hallmark reductions in plasma volume which were not measured (87). Later work opposed these findings, where neither daily 30-minutes nor daily 6x 5-minute intermittent bouts of $1G_z$ at the heart were effective in preventing a 10-15% reduction in plasma volume after 5 (85), or 60 (88, 89) days of HDBR. Similar to control, plasma renin concentrations remained elevated after these 60 days with no difference in the applied AG protocols (88, 89). Evaluating longer AG

exposure, Stenger et al 2012 reported that while 1-hour of continuous AG exposure resulted in equal reductions in plasma volume between AG and control, plasma renin levels were blunted with AG exposure (90). Blunted plasma renin levels suggest the periodic central hypovolemia experienced during 1-hour of AG unloaded atrial and arterial baroreceptor eliminating the stimulus for plasma renin release. Participants in this study were encouraged to perform ad-libitum heel raises and squats, estimated to be 70W for the entire AG protocol, to prevent hypotension and syncope. While AG exposure alone may not be sufficient to maintain plasma volume, its ability to influence hormonal responses highlights its potential when integrated with exercise to better sustain fluid balance during spaceflight and HDBR.

Given the limited efficacy of either exercise or AG alone in preserving fluid regulatory function, the combination of simultaneous exercise + AG has emerged as a promising synergistic countermeasure. In a 5-week HDBR study, near daily upright rowing and biweekly supine lower-body resistance training, provided 30 minutes of near-daily upright gravitational loading during the otherwise HDT protocol and partially attenuated plasma and blood volume losses compared to control (66). Additionally, exercise capacity was preserved in the exercising group highlighting the promising effect of a gravitational stimulus alongside dynamic exercise (66). This effect was replicated during 30 days of HDBR where participants underwent daily 40 minutes of supine treadmill running at 40-80% $\dot{V}O2_{peak}$ daily while experiencing 1.0-1.2 body weight of footward force through LBNP application (76). With the addition of 5-minutes of post exercise LBNP immediately following exercise the loss in OT was attenuated and intravascular fluid volume loss was prevented during HDBR (76). Similarly, though OT was not preserved, the loss in blood volume was reduced by the combination of 40 minutes of aerobic exercise in an LBNP chamber

followed by 10 minutes of resting LBNP during 60 days of HDBR compared to the control group (91).

While these results demonstrate the combined effect of a gravitational stimulus and exercise training, few studies to date have examined simultaneous short-arm centrifugation. The combination of simultaneous +1.2 G_z acceleration at heart level for 30 min and light intensity (40-60W) ergometric exercise training blunted the reduction in an estimate of plasma volume following 14 days of HDBR (92), while hormonal responses to the combined intervention were not assessed. The combination of human centrifuge and exercise has never been investigated in a longer duration HDBR trial, and the hormonal regulation of plasma volume following AG + exercise exposure is largely unknown.

2.0 Study Rationale

Prolonged exposure to microgravity during spaceflight and its Earth-based analog, head-down bed rest (HDBR), induce significant cardiovascular and fluid regulatory adaptations. Persistent 10-15% reduction in plasma volume and the gradual declines in hemoglobin mass result in a reduction in total blood volume and oxygen-carrying capacity, compromising OT and aerobic performance upon return to upright posture or Earth's gravity (64, 77, 93). Current exercise countermeasures are effective in preventing cardiovascular deconditioning (65, 66, 94), but remain insufficient to preserve plasma volume during HDBR (65, 67). Artificial gravity (AG), implemented through short-arm centrifugation, offers a complementary stimulus by restoring gravitational gradients. While AG alone has shown limited effectiveness in preserving blood volume during HDBR (85, 90), the integration of exercise and AG may offer synergistic benefits by simultaneously engaging volume-expanding and fluid-conserving mechanisms (75, 91). The BRACE (Bed Rest with Artificial gravity and Cycle Ergometer) was designed by the European Space Agency to investigate how exercise and combined exercise + AG influence plasma volume and key volume-regulating hormones during 60 days of HDBR. Understanding these responses is essential for optimizing countermeasure strategies to maintain circulatory homeostasis during prolonged gravitational unloading, with direct relevance for human spaceflight and clinical models of deconditioning.

3.0 Research Questions and Hypotheses

Research Questions

1. To what extent does exercise or combined exercise + AG countermeasures alter plasma volume (PV), blood volume (BV), hemoglobin mass (Hbmass), hematocrit (Hct), and hemoglobin concentration ([Hb]) at the end of 60 days of HDBR?
2. How do fluid regulatory hormone concentrations, specifically plasma renin, aldosterone, vasopressin, and arterial natriuretic peptide respond to exercise or combined exercise + AG countermeasures at the end of 60 days of HDBR?
3. To what extent does exercise or combined exercise + AG modulate erythropoietin (EPO) responses during HDBR, and how does EPO respond acutely following exercise or exercise + AG exposure?

Hypotheses

1. BV, PV, Hbmass, Hct, [Hb] will be better preserved relative to baseline in the exercise + AG group compared to exercise alone, with both interventions expected to attenuate losses more effectively than the control condition following 60 days of HDBR.
2. Plasma renin and aldosterone will remain closer to baseline in response to AG interventions compared to exercise alone or control following 60 days of HDBR.
3. Circulating EPO will be more strongly expressed in the exercise + AG group than in the exercise-only group, while evening post-intervention measurements will reveal higher EPO concentrations following exercise + AG exposure

4.0 Methods

4.1 Ethics

The study was registered as a clinical trial (NCT06544213) and performed in accordance with the declaration of Helsinki, following ethical approval from the Office of Research Ethics at the University of Waterloo (ORE #45090), the Swedish Ethical Review Authority (Dnr 2023-00164-01), the Comité de Protection des Personnes Ile-de France IV and the French Drug Agency (ANSM). All participants signed an informed consent form before participating in the study and were made aware of their right to withdraw at any time.

4.2 Participants

Candidates were recruited through a call on the MEDES and ESA websites and through the media (radio, social media, newspapers). Twenty-four young, healthy men (age: 29 ± 6 years; mass: 73.9 ± 7.7 kg; height: 1.76 ± 0.07 m) voluntarily participated in this 60-day, 6° HDBR study. Sample size for the BRACE study was based on the difference in muscle cross-sectional area following HDBR, with and without exercise, as the primary outcome measure. An additional sample size calculation was performed to ensure sufficient power for the changes in plasma volume following HDBR. Based on previous HDBR studies with exercise and exercise + LBNP (67, 91), a sample size of 8 participants per group is 87% powered to detect changes in plasma volume with a p-value of 0.017 to account for 3 post-hoc comparisons (control vs. exercise, control vs. exercise, and exercise vs. exercise + AG).

Participants were screened to be free of cardiovascular, psychiatric, or musculoskeletal conditions contraindicated for HDBR or AG exposures and were recruited based on these criteria

established by the European Space Agency (ESA) and Centre National d'Études Spatiales (CNES), as outlined in Appendix 1.

4.3 Experimental Protocol

Two campaigns were conducted in successive years, both consisting of twelve participants. All participants remained in the Institut de Médecine et de Physiologie Spatiales (MEDES, Toulouse, France) for 88 days including 14 days of pre-bedrest baseline data collection (BDC-14 to BDC-1), 60 days of -6° HDBR (HDBR1 to HDBR60) and 14 days of recovery (R+1 to R+14). Throughout the BDC and post-HDBR phases, participants were permitted to move freely within the MEDES facility, engage in normal daily activities. During BDC, participants performed individualized daily exercise under MEDES supervision, comprising of treadmill running, cycling, and walking, to match their habitual physical activity levels. During HDBR, participants were continuously supervised and performed all activities in a strict -6° head-down position, with pillow permitted. Following HDBR, participants performed daily 20–30-minute exercise sessions under the supervision of a rehabilitation specialist.

4.4 Countermeasure Protocol

Participants were randomly assigned to one of three groups: control, exercise (*section 4.4.1*), or exercise + AG (*section 4.4.2*). The control group remained inactive during all 60 days of HDBR and received only passive manual therapy.

4.4.1 Cycle ergometer

A dedicated supine $\dot{V}O2_{peak}$ test was performed during BDC with a supine cycle ergometer (Angio Imaging, Lode B.V., Groningen, The Netherlands) to determine individual work rate

loading profile. The exercise group performed 30 min of daily supine interval cycling, beginning with a 5-minute warm-up at 40% of peak oxygen uptake ($\dot{V}O2_{peak}$), followed by 2-minute intervals at 65%, 70%, 80%, 80%, 70%, and 65% of $\dot{V}O2_{peak}$, interspersed with 2-minute recovery intervals at 40% $\dot{V}O2_{peak}$, and concluding with a 3-minute cooldown at 40% $\dot{V}O2_{peak}$ (Figure 8). If the participant was unable to perform 80% $\dot{V}O2_{peak}$, a backup protocol would be executed with 2-minute higher intensity intervals at 65% $\dot{V}O2_{peak}$followed by four 2-minutes intervals of 70% $\dot{V}O2_{peak}$interspersed with 2-minute low-intensity intervals at 40% $\dot{V}O2_{peak}$. This backup program was performed until the volunteer felt better and managed to execute the nominal one.

4.4.2 Cycle ergometer + Artificial gravity

The exercise + AG group followed the same cycling protocol as the exercise group, described above, while simultaneously undergoing centrifugation on a short-arm human centrifuge (2.8m radius, 3.5g max). The AG stimulus was applied in the head-to-foot direction ($+G_z$) and individualized based on participants' baseline orthostatic response determined during a preparatory test session. The level of AG that elicited a 20% increase in HR from baseline and a corresponding 20% reduction in calf oxygenated hemoglobin measured by near-infrared spectroscopy (Δ20%) was identified. Participants were continuously monitored through a 5-lead ECG. (Intellivue, Philips) and finger photoplethysmography (Finapres). The AG protocol was stopped if the participant experienced pre-syncopal symptoms or showed signs that cardiac output had dropped. The participant would then be asked to do a heel raises to increase blood flow to the brain while they were returned to baseline (0 Gz). Pre-syncopal signs include a sudden drop in systolic BP ≥ 25mmHg/min, diastolic BP ≥ 15 mmHg/min, or a drop in HR by ≥ 15bpm. Symptoms of presyncope include pallor, nausea, diaphoresis, or clammy skin.

During the countermeasure sessions, the initial AG stimulus was set to the previously defined Δ20% level-0.15Gz, increasing stepwise by 0.15+G_z every 4 minutes in parallel with ergometer workload up to a maximum of 70% of the difference between the Δ20% level and presyncope (PS) (70%[PS– Δ20% level] + Δ20% level) (95). AG was then decreased by 0.15 +G_z every 4 minutes until the 30-minute session was complete (Figure 8.B). This individualized, graded AG-exercise protocol ensured consistent cardiovascular stress across participants by scaling both G-load and workload to each participant's $\dot{V}O2_{peak}$ and Δ20% level, while minimizing the risk of presyncope. Centrifuge rotation direction alternated between clockwise and counterclockwise across sessions to avoid vestibular habituation (96).

4.5 Dietary Intake

Water intake was ad libitum but maintained between 35 and 50 mL/kg/day, considering both beverage and food-derived water. Dietary intake was standardized across participants based on individual basal metabolic rate and followed a standardized 10-day menu harmonized with experimental time points. Macronutrient targets were set at 1.0–1.2 g/kg body weight/day of protein, 30–35% of total energy expenditure from fat, and the remaining caloric intake from carbohydrates. Daily electrolyte intake was controlled, with sodium (Na^+) maintained between 3000–4500 mg, potassium (K^+) between 3500–5000 mg, and chloride (Cl^-) between 5500–7500 mg. Meals were obtained frozen from Sysco, who provided nutritional values for each meal. Dietary intakes were quantified by measuring the weight of each component of the meal before and after (in the case of leftovers) each meal, and by calculating the intakes for each nutrient.

4.6 Lean Body Mass

Whole-body Dual Energy X-ray Absorptiometry (DEXA) scans were completed on BDC-2, HDBR14, HDBR28, HDBR42, and HDBR58. Whole body lean mass (kg) was derived according to the standard techniques described in the DEXA Operator's manual. Data from all time-points will be published elsewhere, and only BDC-2 and HDBR58 will be included for the purposes of this project.

4.6 Blood volume and related variables

Carbon monoxide (CO) rebreathing was performed on BDC-5, BDC-4, HDBR55 and HDBR59. Tests were performed in the early morning before any upright activities during BDC, following 20 minutes of rest in HDT posture. Total hemoglobin mass (Hbmass) was determined using the protocol of Schmidt and Prommer (2005) (97). Briefly, a rebreathing assembly consisting of a mouthpiece and a carbon dioxide absorber at one end and a 3-liter rebreathing bag at the other was used (Bloodtec, Germany). The system was filled with 100 % oxygen. The participant, wearing a nose clip, started by rapidly exhaling to residual volume and then rapidly inhaling to capacity. During the inhalation, an operator injected 0.9 ml/kg body mass of pure CO into the system, and the participant held their breath for 10 s, followed by 1 min and 50 s of rebreathing, and ending by exhaling to residual volume into the rebreathing system. After 4 min, end-tidal CO was determined with a CO analyzer (Pac 7000, Dräger, Germany). The CO remaining in the rebreathing system was also determined with same analyzer and a spirometer. Venous blood samples were obtained before and 7 min after the onset of rebreathing and analyzed with a blood gas analyzer (ABL 800, Radiometer, Denmark) for carboxyhemoglobin fraction (%COHb), hemoglobin concentration [Hb] and hematocrit (Hct). After compensation for the CO remaining in the lungs and rebreathing system and for losses between min 2 and 7, Hbmass was calculated

based on the net volume of CO uptake, the resulting increase in %COHb, and Hufner's number (the binding capacity of Hb for CO) as previously specified (Schmidt and Prommer, 2005)

Blood volume (BV) and PV were computed from Hbmass and Hb and Hct. The compensation for a postulated CO uptake to myoglobin proposed by Schmidt and Prommer (2005) was not applied, since it would not have impacted the paired comparisons between BDC and late HDT.

4.7 Blood processing and analysis

Blood samples for fluid regulatory hormones were taken during Pre-bedrest (BDC-5 and BDC-4) and late HDBR (HDBR55 and HDBR59), and were analyzed for total renin, plasma renin, aldosterone, vasopressin, and ANP concentrations. Samples were collected by MEDES staff, centrifuged and stored at -80°C prior to shipping to Waterloo for analysis by the candidate. For erythropoietin (EPO) blood samples were taken on BDC-4, HDBR3, HDBR55 and HDBR60, with repeat samples taken in the evenings of HDBR3 and HDBR60, at least 2 hours after countermeasure sessions in the exercise and exercise + AG groups. The EPO analyses were performed at the Karolinska University Hospital Lab, Stockholm, Sweden.

Total and plasma renin were determined in duplicate using immunometric assays (DREN00 Biotechne, Minneapolis, MN and EIA-5125R; RayBiotech, Peachtree Corners, GA) based on the sandwich ELISA principle. Aldosterone was determined in duplicate using an inhibition assay (ADI-900-173; RayBiotech, Peachtree Corners, GA) based on the competitive ELISA principle. The ratio of serum aldosterone to plasma renin concentration (ARR) was calculated as plasma aldosterone concentration/plasma renin concentration (36). Vasopressin and ANP were determined in duplicate using inhibition assays (EIA-VAS-1 and EIA-ANP-1,

RayBiotech, Peachtree Corners, GA) based on the competitive ELISA principle. Peptide concentrations in samples were measured by absorbance using a microplate reader (Synergy, BioTek, Waterloo, ON), with measurements calibrated against standard curves.

Erythropoietin (EPO) was measured using the IMMULITE 2000 XPi Immunoassay System, which employs an enzyme-labeled chemiluminescent immunometric assay with a solid-phase bead coated with streptavidin-derived anti-ligand. In the liquid phase, ligand-labeled monoclonal mouse anti-EPO antibody and alkaline phosphatase–conjugated monoclonal mouse anti-EPO antibody formed a sandwich complex with EPO in the sample. The measuring range was 1–750 U/L, with a reference range of 3.1–17.9 U/L.

4.8 Statistical Analyses

Linear mixed models were used to assess the effects of HDBR and group on hormone peptide concentrations and derived blood volume outcomes. The models included fixed effects for HDBR (BDC vs. HDBR55) and group (control vs. exercise vs. exercise + AG), with participant as a random effect [R library: lmerTest (98)]. Linear mixed models with fixed effects of HDBR (BDC vs. HDBR3 vs. HDBR55 vs. HDBR60) and group, and a random effect of participant, were used to evaluate differences in EPO concentrations. To evaluate within-day changes in EPO concentrations during HDBR, data from HDBR3 and HDBR60 were pooled and analyzed using a linear mixed model with fixed effects of group and time of day (morning vs. evening), and a random effect of participant. Post hoc testing was performed with pairwise comparisons of the estimated marginal means against BDC when indicated, using the Holm correction for multiple comparisons [R library: emmeans (99)]. A repeated-measures correlation analysis was performed to evaluate the presence of a shared linear relationship between two

variables across all participants. This method estimates a common slope while accounting for within-participant dependencies [R library: rmcorr (100, 101)]. Differences in dietary intake of Na^+, Cl^- and K^+ between days of the same phase (i.e., BDC or HDBR) or campaigns were tested using paired and independent t-tests, respectively. Day-to-day variability for repeated measurements of hematological and hormone variables was assessed through the coefficient of variation (CV). As will be further explained in the Discussion, hormonal and hematological variables from HDBR59 were excluded from all analyses due to variability in diet between HDBR54 and HDBR58. All statistical analyses were conducted in R Studio (v. 4.4.1). Statistical significance was set to $p < 0.05$, and data are reported as means ± standard deviation

5.0 Revised manuscript (Revisions submitted to JAPPL on 01/15/2026)

5.1 Introduction

Prolonged exposure to microgravity during spaceflight and its Earth-based analog, head-down bed rest (HDBR), induce cardiovascular and fluid regulatory adaptations. The absence of a normal head-to-foot gravitational axis induces a cephalad fluid shift, increasing central blood volume and pressure (38). This triggers compensatory hormonal responses (17–20) including release of atrial natriuretic peptide (ANP) and suppression of the renin-angiotensin-aldosterone (RAAS) system, causing diuresis and hemoconcentration during early HDBR. A sustained reduction in plasma volume leads to a compensatory upregulation of the RAAS promoting fluid retention (17–19). This 10-15% reduction in plasma volume contributes to the mechanisms underlying orthostatic intolerance and cardiovascular deconditioning upon re-exposure to gravity (4, 10) or upright posture (65, 102).

Plasma volume can be expanded during HDBR through a single bout of maximal exercise (43, 81), or saline infusion (67). Exercise countermeasures play a central role in expanding plasma volume (79), and maintenance of aerobic fitness (65, 66, 94). Alone they remain insufficient to completely preserve plasma volume and orthostatic tolerance during HDBR (65, 67); however, the combination of exercise during HDBR and fluid loading at the end of HDBR reversed plasma volume loss and protected orthostatic tolerance (66, 67).

Artificial gravity (AG) via short-arm centrifugation has been proposed as a countermeasure for spaceflight deconditioning as AG targets physiological systems through the reintroduction of head-to-foot acceleration normally experienced during upright posture (103). By replicating gravitational loading, AG may help prevent reductions in plasma volume by inducing periodic central fluid shifts (86). While AG attenuates impairment of orthostatic

tolerance after HDBR (85, 88, 90), studies have demonstrated a null effect of AG alone to prevent plasma volume loss during HDBR (85, 88, 90) although 1 hour per day of AG did blunt the effect of HDBR on active renin levels (90). Combining exercise with AG may better sustain fluid balance during spaceflight and HDBR than exercise or AG alone. One study found the combination of cycling exercise + AG attenuated reductions in plasma volume estimated from changes in hematocrit during 14 days of HDBR and reduced the effect of HDBR on cardiovascular control during head-up tilt (92). Similarly, brief application of lower body negative pressure, another gravity-like stimulus, after supine treadmill exercise protected plasma volume during 30 days of HDBR in men (104) and 60 days of HDBR in women (91). However, cycling exercise during 3 G_z has been shown to promote venous return and to result in elevated arterial pressure (105), which may negate the orthostatic stress required for fluid maintenance. Accordingly, investigations examining the influence of combined countermeasures on fluid regulatory mechanisms are needed during long-duration HDBR.

Therefore, to evaluate simultaneous exercise + AG as a potential time-efficient countermeasure to fluid loss during prolonged HDBR, the European Space Agency conducted the BRACE (Bed Rest with Artificial gravity and Cycle Ergometer) project. The objectives of the present study were to examine the changes in plasma volume and fluid regulating hormones during 60 days of HDBR in participants who performed near daily exercise with or without simultaneous AG, as well as participants who remained inactive for the duration of HDBR. This study provided the opportunity to test the hypothesis that incorporating simultaneous moderate-to-high-intensity interval cycling during AG by short-arm centrifugation would protect plasma volume and maintain fluid-regulating hormones, outperforming exercise and sedentary control groups.

5.2 Methods

The study was registered as a clinical trial (NCT06544213) and performed in accordance with the declaration of Helsinki, following ethical approval from the Office of Research Ethics at the University of Waterloo (ORE #45090), the Swedish Ethical Review Authority (Dnr 2023-00164-01), the Comité de Protection des Personnes Ile-de France IV and the French Drug Agency (ANSM). All participants signed an informed consent form before participating in the study and were made aware of their right to withdraw at any time.

Twenty-four healthy males (29 ± 6 years; 73.9 ± 7.7 kg; 1.76 ± 0.06 m) voluntarily participated in this 60-day, -6° HDBR study. Sample size for the BRACE study was predetermined by the European Space Agency (ESA), with their sample size calculation based on anticipated large effect sizes for the effects of exercise and exercise + AG on musculoskeletal outcomes. Participants were screened to be free of cardiovascular, psychiatric, or musculoskeletal conditions contraindicated for HDBR or AG exposure and were recruited based on the criteria established by ESA and Centre National d'Études Spatiales (CNES).

Two campaigns were conducted in successive years, both including twelve participants. All participants remained in the Institut de Médecine et de Physiologie Spatiales (MEDES, Toulouse, France) for 88 days including 14 days of pre-bedrest baseline data collection (BDC-14 to BDC-1), 60 days of -6° HDBR (HDBR1 to HDBR60) and 14 days of recovery. During the pre- and post-HDBR phases, participants were permitted to move freely within the MEDES facility, engage in normal daily activities, and were required to complete prescribed exercise or rehabilitation sessions to remain physically active. During HDBR, participants were continuously supervised and performed all activities in a strict -6° head-down position, and

pillows were generally not permitted, with the exception of when participants were lying on their side to eat or for short periods for medical reasons when participants had neck/shoulder pain. Lean body mass was measured through dual energy x-ray absorptiometry (QDR-4500C, Hologic Inc., Bedford, USA) on BDC-2 and HDBR56. Water intake was ad libitum but maintained between 35 and 50 mL/kg/day, considering both beverage and food-derived water. Dietary intake was standardized based on individual basal metabolic rate, with macronutrient targets set at 1.0–1.2 g/kg body weight/day of protein, 30–35% of total energy expenditure from fat, and the remaining caloric intake from carbohydrates. Daily electrolyte intake was controlled, with sodium (Na^+) maintained at 3000–4500 mg, potassium (K^+) at 3500–5000 mg, calcium (Ca^{2+}) at 1000–1200 mg, and chloride (Cl^-) at 5500–7500 mg.

Participants were randomly assigned to one of three groups: control, exercise, or exercise + AG. The control group remained inactive, receiving only passive manual therapy. Countermeasure sessions for exercise and exercise +AG groups were performed on 49 of 60 days during HDBR. On BDC-8, participants performed an incremental cycling test to exhaustion (3 min baseline at 50 W followed by 25 W steps each min) on a supine cycle ergometer (Angio Imaging, Lode B.V., Groningen, The Netherlands) to determine supine peak oxygen uptake ($\dot{V}O_{2peak}$) and individual work rate loading profiles, as previously described (106). The exercise group performed 30 min of daily supine interval cycling, beginning with a 5-minute warm-up at a work rate determined to be 40% of the pre-HDBR supine cycling $\dot{V}O_{2peak}$ followed by 2-minute intervals at 65%, 70%, 80%, 80%, 70%, and 65% of $\dot{V}O_{2peak}$, interspersed with 2-minute recovery intervals at 40% $\dot{V}O_{2peak}$, and concluding with a 3-minute cooldown at 40% $\dot{V}O_{2peak}$.

The exercise + AG group followed the same 30-minute cycling protocol as the exercise group, while simultaneously undergoing centrifugation in a short-arm human centrifuge (2.8 m

radius). A preparatory AG test (0.6 G_z for 10 min followed by 0.1 G_z steps every 3 min) was performed to determine the G_z levels at participants' center of gravity that induced pre-syncope (PS) and a 20% increase in baseline heart rate along with a reduction in calf oxygenated hemoglobin measured by near-infrared spectroscopy (Δ20%) (107). For the countermeasure sessions, AG started at 0.15G_z below the identified Δ20% level and then AG increased in a stepwise manner at minutes 5, 9, and 13 to reach the protocol's maximum AG level of (70%·[PS – Δ20% level] + Δ20% level), followed by 3 stepwise reductions at minutes 19, 23, and 27 to finish the protocol back at the initial AG level. Peak G_z measured at the center of gravity during countermeasure sessions ranged from 1.02 - 1.52 G in the exercise + AG group, with that level being sustained for 6 min, as previously described (106). Data pertaining to the completion of the exercise or exercise + AG program and individual workloads and G_z levels have been previously reported (106).

Blood Volume and related variables

Carbon monoxide (CO) rebreathing was performed on BDC-5, BDC-4, HDBR55 and HDBR59. Tests were performed in the early morning before any upright activities during BDC, following 20 minutes of rest in head-down tilt posture (108). Total hemoglobin mass (Hbmass) was determined using the protocol of Schmidt and Prommer (97), without correction for estimated CO uptake by myoglobin. Briefly, a rebreathing assembly consisting of a mouthpiece and a carbon dioxide absorber at one end and a 3-liter rebreathing bag at the other was used (Blood Tec GmbH, Bayreuth, Germany). The system was filled with 100 % oxygen. The participant, wearing a nose clip, started by rapidly exhaling to residual volume and then rapidly inhaling to capacity. During the inhalation, an operator injected 0.9 mL/kg of body mass of pure

CO into the system, and the participant held their breath for 10 s, followed by 1 min and 50 s of rebreathing, and ended by exhaling to residual volume into the rebreathing system. After 4 mins, end-tidal CO was determined with a CO analyzer (Pac 7000, Dräger Safety AG & Co., Lübeck, Germany). The CO remaining in the rebreathing system was also determined with the same CO analyzer and a spirometer. Venous blood samples were obtained before and 7 min after the onset of rebreathing and analyzed with a blood gas analyzer (ABL 800, Radiometer Medical ApS, Brønshøj, Denmark) for carboxyhemoglobin fraction (%COHb), hemoglobin concentration ([Hb]) and hematocrit (Hct). After compensation for the CO remaining in the lungs and rebreathing system and for losses between min 2 and 7, Hbmass was calculated based on the net CO uptake, the resulting increase in %COHb, and Hufner's number (the binding capacity of Hb for CO), as previously specified (97). Blood volume and plasma volume were computed from Hbmass, [Hb] and Hct.

Blood processing and analysis

Blood samples for fluid regulatory hormones were taken first thing in the morning after an overnight fast and prior to any upright posture, ambulation, meal or fluid consumption in the supine position on BDC-5 and BDC-4, as well as in 6° head-down tilt on HDBR55 and HDBR59, and were analyzed for total renin, active renin, aldosterone, vasopressin, and ANP concentrations. For erythropoietin (EPO), blood samples were taken on BDC-4, HDBR3, HDBR55 and HDBR60, with repeat samples taken in the evenings of HDBR3 and HDBR60, at least 2 hours after countermeasure sessions in the exercise and exercise + AG groups.

Total and active renin were determined in duplicate using immunometric assays (DREN00 Biotechne, Minneapolis, MN and EIA-5125R; RayBiotech, Peachtree Corners, GA)

based on the sandwich ELISA principle. Aldosterone was determined in duplicate using an inhibition assay (ADI-900-173; RayBiotech, Peachtree Corners, GA) based on the competitive ELISA principle. The ratio of serum aldosterone to active renin concentration (ARR) was calculated as plasma aldosterone concentration/active renin concentration (36). Vasopressin and ANP were determined in duplicate using inhibition assays (EIA-VAS-1 and EIA-ANP-1, RayBiotech, Peachtree Corners, GA) based on the competitive ELISA principle. Peptide concentrations in samples were measured by absorbance using a microplate reader (Synergy, BioTek, Waterloo, ON), with measurements calibrated against standard curves.

EPO was measured using the IMMULITE 2000 XPi Immunoassay System (Siemens Healthcare GmbH, Erlangen, Germany), which employs an enzyme-labeled chemiluminescent immunometric assay with a solid-phase bead coated with streptavidin-derived anti-ligand. In the liquid phase, ligand-labeled monoclonal mouse anti-EPO antibody and alkaline phosphatase–conjugated monoclonal mouse anti-EPO antibody formed a sandwich complex with EPO in the sample. The measuring range was 1–750 IU/L, with a reference range of 3.1–17.9 IU/L.

Statistics

Linear mixed models were used to assess the effects of HDBR and group on hormone peptide concentrations and derived blood volume outcomes. The models included fixed effects for HDBR (BDC vs. HDBR55) and group (control vs. exercise vs. exercise + AG), with participant as a random effect [R library: lmerTest (98)]. Linear mixed models with fixed effects of HDBR (BDC vs. HDBR3 vs. HDBR55 vs. HDBR60) and group, and a random effect of participant, were used to evaluate differences in EPO concentrations. To evaluate within-day changes in EPO concentrations during HDBR, data from HDBR3 and HDBR60 were pooled and

analyzed using a linear mixed model with fixed effects of group and time of day (morning vs. evening), and a random effect of participant. Post hoc testing was performed with pairwise comparisons of the estimated marginal means against BDC when indicated, using the Holm correction for multiple comparisons [R library: emmeans (99)]. A repeated-measures correlation analysis was performed to evaluate the presence of a shared linear relationship between two variables across all participants. This method estimates a common slope while accounting for within-participant dependencies [R library: rmcorr (100, 101)]. Differences in dietary intake of Na^+, Cl^- and K^+ between days of the same phase (i.e., BDC or HDBR) or campaigns were tested using paired and independent t-tests, respectively. Day-to-day variability for repeated measurements of hematological and hormone variables was assessed through the coefficient of variation (CV). As will be further explained in the Discussion, hormonal and hematological variables from HDBR59 were excluded from all analyses due to variability in diet between HDBR54 and HDBR58. All statistical analyses were conducted in R Studio (v. 4.4.1). Statistical significance was set to $p < 0.05$, and data are reported as means ± standard deviation

5.3 Results

Lean Mass

Control (54.8 ± 6.9 vs. 52.2 ± 6.8 kg), exercise (59.1 ± 4.9 vs. 56.8 ± 4.1 kg), and exercise + AG (56.6 ± 5.1 vs. 55.0 ± 4.9 kg) groups all experienced reductions in whole body lean mass from BDC to HDBR56 ($p < 0.001$), with no main effect of group ($p = 0.297$) or HDBR × group interaction ($p = 0.126$).

Blood Volume and related variables

Intravascular volume outcomes and comparisons between BDC and late HDBR are presented in Figure 1. HDBR induced reductions in plasma volume ($p < 0.001$), blood volume ($p < 0.001$), and Hbmass ($p < 0.001$) in all groups, while Hct was unchanged ($p = 0.09$). Across all variables, there were no main effects of group (all $p > 0.05$). A HDBR × group interaction ($p = 0.030$) was observed for [Hb], such that [Hb] increased in both the control ($p = 0.043$) and exercise ($p = 0.043$) groups during HDBR, but was unchanged in the exercise + AG group ($p = 0.366$).

CV of hematological variables between BDC-5 and BDC-4 were plasma volume, 6.5 ± 5.9%; blood volume, 5.1 ± 3.9%; Hbmass, 3.7 ± 4.0%; [Hb], 2.7 ± 3.4%; Hct, 3.0 ± 3.5%. Between HDBR55 and HDBR59 CV were plasma volume, 7.5 ± 5.6%; blood volume, 6.4 ± 4.8%; Hbmass, 5.4 ± 4.4%; [Hb], 1.8 ± 1.4%; Hct, 1.9 ± 1.8%.

Erythropoietin

There was a main effect of HDBR on EPO ($p < 0.001$), while no main effect of group ($p = 0.301$) or HDBR × group interaction ($p = 0.604$) were observed. Across all groups, EPO levels decreased from BDC to HDBR3 ($p < 0.001$) but were not different between BDC and HDBR55 ($p = 0.105$) or HDBR60 ($p = 0.065$; Figure 2). Data from HDBR3 and HDBR60 were pooled and used to assess changes in EPO from morning to evening during HDBR (Figure 3). Across all groups, EPO levels increased from morning to the evening ($p < 0.001$), without a main effect of group ($p = 0.180$) or a group × time of day interaction ($p = 0.059$).

Fluid Regulating Hormones

Fluid-regulating hormone concentrations and comparisons are presented in Table 1. Both active renin ($p < 0.001$) and total renin ($p < 0.022$) concentrations were increased in all groups during late-HDBR, increasing the relative amount of active to total renin concentrations ($p < 0.001$), while aldosterone ($p = 0.363$) and vasopressin ($p = 0.791$) remained unchanged. As a result, ARR was reduced at late-HDBR ($p = 0.003$). Notably – regardless of group – 11 of 12 participants in the first campaign had a decrease in aldosterone with HDBR ($\Delta = -38.6 \pm 27.8$ pg/mL), while aldosterone increased with HDBR in 8 of 12 participants in the second campaign ($\Delta = 21.6 \pm 35.1$ pg/mL). No other hormone or blood volume outcome displayed this apparent campaign effect. Across all hormones, there were no main effects of group ($p > 0.05$). A HDBR × group interaction was observed for ANP ($p = 0.016$), with ANP increasing during HDBR in the exercise group ($p = 0.025$) but not changing in the control ($p = 0.251$) and exercise + AG ($p = 0.451$) groups.

Hormone concentrations showed high CV between BDC-4 and BDC-5 for active renin, 35.6 ± 26.1%; aldosterone, 29.7 ± 20.8%; vasopressin, 16.0 ± 14.2%; ANP, 24.1 ± 16.9%, while total renin was stable, 3.5 ± 3.6%. High day-to-day variability was also observed between HDBR55 and HDBR59 for active renin, 26.8 ± 19.3%; total renin, 6.3 ± 4.2%; aldosterone, 27.2 ± 21.1%; vasopressin, 12.7 ± 12.4%; ANP, 24.1 ± 14.5%.

Relationship between blood volume outcomes and fluid regulating hormones

Repeated measures correlations across all participants revealed that the elevated active renin levels were associated with the reductions in plasma volume [r_{rm} (95%CI) = -0.615 (-0.785, -0.448), $p = 0.001$], blood volume [r_{rm} (95%CI) = -0.553 (-0.756, -0.368), $p = 0.004$], and Hbmass [r_{rm} (95%CI) = -0.426 (-0.669, -0.189), $p = 0.034$], while positively correlated with [Hb]

[r_{rm} (95%CI) = 0.603 (0.354, 0.831), p = 0.001] and Hct [r_{rm} (95%CI) = 0.526 (0.168, 0.806), p = 0.007]. While no relationships were observed between aldosterone and hematological variables ($p > 0.05$), the reduced ARR was associated with the reductions in plasma volume [r_{rm} (95%CI) = 0.493 (0.319, 0.791), p = 0.012], blood volume [r_{rm} (95%CI) = 0.506 (0.264, 0.779), p = 0.001], and Hbmass [r_{rm} (95%CI) = 0.497 (0.200, 0.776), p = 0.012]. No relationships were observed between vasopressin or EPO levels and hematological variables ($p > 0.05$). ANP levels were positively related with [Hb] [r_{rm} (95%CI) = 0.400 (-0.047, 0.712), p = 0.048].

Dietary Intake

Variations in dietary intake of Na^+, Cl^-, and K^+ preceding blood draws and corresponding variability in active renin and aldosterone levels during BDC and late-HDBR and are presented in Table 2. Na^+, Cl^-, and K^+ intakes were different between days preceding both baseline (BDC-6 compared to BDC-5) ($p < 0.001$; $p < 0.001$; p = 0.002, respectively) and late-HDBR (HDBR54 compared to HDBR58) ($p < 0.001$; p = 0.010; $p < 0.001$) fasting blood draws. Nutrient intake normalized to body mass for the days preceding fasting blood draws for the two campaigns are presented in Table 4. Na^+, Cl^-, and K^+ intake did not differ between campaigns ($p > 0.05$).

5.4 Discussion

This study aimed to determine whether exercise in combination with AG could prevent plasma volume loss during 60 days of HDBR. Contrary to the hypothesis of the BRACE study, exercise during simultaneous short-arm centrifugation failed to blunt the activation of the RAAS or the corresponding loss in plasma volume. We observed a reduction in plasma and blood volume in all groups, a reduction in lean mass in all groups, an increase in [Hb] in control and

exercise only, a suppression of EPO in early HDBR, as well as an upregulation in active renin in all groups at the end of 60 days of HDBR. The combination of exercise + AG did not blunt or accentuate the degree of fluid loss or hormonal responses when compared to exercise only or sedentary control groups.

Both continuous and intermittent AG protocols without exercise applied over 5 and 60 days have failed to mitigate plasma volume loss or alter fluid regulatory responses (85, 88), suggesting that 30 minutes of AG alone, whether during brief or prolonged HDBR periods, does not sufficiently counteract hypovolemia. Though 3 weeks of 1 hour per day of 1 G_z centrifugation at the heart level with light lower-body exercise did not prevent reduction in plasma volume, it did nullify the increase in active renin activity (90). In another 14-day HDBR study, daily exposure to 1.2 G_z and leg cycling exercise partially preserved estimated plasma volume (92) but fluid regulatory hormones were not measured. While exercise is known to expand plasma volume (79) and stimulate EPO production (109–111)*, it* has not been sufficient on its own to prevent circulating fluid volume loss during HDBR (65–67); however, protection of aerobic exercise fitness with high-intensity exercise underscores its functional benefits (66, 67, 102).

Simultaneous supine cycling and AG exposure was proposed to have multi-system benefits and oppose multi-system deconditioning following long-term HDBR (112). *However, when AG is combined simultaneously with exercise, the action of the skeletal muscle pump facilitates* venous return which opposes the caudal fluid shift induced by AG (105), limiting baroreflex unloading and reducing the simulated orthostatic stress experienced by participants that is necessary to stimulate fluid-conserving adaptations. Future studies may consider continuing AG exposure during post-exercise recovery to better replicate the cardiovascular

challenge required for fluid maintenance. A similar concept was accomplished by having participants perform supine exercise on a treadmill inside a lower-body negative pressure chamber and then extending the negative pressure exposure beyond the end of exercise (76, 91, 104). Incorporation of post-exercise orthostatic-like stress prevented intravascular fluid volume loss and attenuated loss of orthostatic tolerance following 30 days of HDBR (76, 104). This may be a more optimal protocol, since the current step-wise simultaneous exercise + AG did not confer additional benefit for these hematological outcomes, nor orthostatic tolerance, $\dot{V}O_{2peak}$, muscle atrophy, or fat accumulation, as reported from this multi-investigator project (106, 113).

While RAAS is blunted during the initial hours following a transition from ambulatory conditions into head-down tilt (17–20), the duration or magnitude of a gravitational stimulus required to elicit endocrine suppression has not been evaluated. AG exposures exceeding one hour are thought to be necessary to protect fluid maintenance (85, 90), though longer protocols may reduce adherence due to increased participant burden. This highlights a potential antagonistic relationship between the transient nature of AG-induced orthostatic stress and the sustained central fluid shifts associated with prolonged HDBR. With shorter sessions, such as the present 30 min of daily continuous exposure, adherence to the exercise + AG protocol during HDBR was high, with only one missed session reported in a single participant (106), but the desired effect was not produced.

By HDBR3 reductions in morning EPO concentrations were observed irrespective of group. Early HDBR is characterized by a transient reduction in EPO concentrations driven by increased central venous pressure that promotes diuresis resulting in hemoconcentration (44, 47) signaling a temporary suppression of erythropoiesis to re-establish homeostatic balance (114). Erythrocyte loss acts to normalize hematocrit when there are sustained reductions in plasma

volume (47), as was reflected by the observed reduction in Hbmass irrespective of group; by late HDBR, [Hb] was elevated in control and exercise only groups and Hct was not different from pre-HDBR. High-intensity normoxic exercise training stimulates EPO production through a homeostatic drive to maintain red blood cell concentration within a narrow physiological range (110). Importantly, the physiological stress imposed by intermittent and moderate-to-high intensity exercise in the current study of 60-days of HDBR did not affect EPO dynamics compared to sedentary control, likely owing to the inability of exercise or exercise + AG during HDBR to protect plasma volume. EPO concentrations were consistently higher in the evening irrespective of group at both HDBR3 and HDBR60, consistent with its known circadian rhythm in ambulatory individuals (115), although evidence for circadian variation in EPO during HDBR is limited and highly individualized (45). This rhythm is believed to be transcriptionally regulated and not directly influenced by diurnal fluctuations in oxygen tension (116).

During both the BDC and HDBR periods, there was variation in hormone concentrations between the repeated measurements. In the BDC, active renin increased 42.5% and aldosterone increased 47.3% from BDC-5 to BDC-4. Likewise, near the end of HDBR, active renin increased 30.4% and aldosterone increased 43.0% between days HDBR55 and HDBR59 (Table 3). Although Na^+ intake was maintained overall, there were variations between days associated with the different menu items in the 10-day meal cycle (Table 3). For both the BDC and HDBR repeated samples, the Na^+ intake on the days prior to the blood sample with higher renin and aldosterone was greater than the days with lower concentrations. An average of BDC-5 and BDC-4 was taken, however further complicating the HDBR55 with HDBR59 comparison was the insertion of an ad libitum lunch on HDBR57 associated with an unrelated investigation of the regulation of food intake and energy balance. Reductions in Na^+ intake can elevate active renin

sufficiently to preserve blood pressure through angiotensin II-mediated vasoconstriction (32), while K^+ intake stimulates dose dependent aldosterone secretion (27, 33). Measured fluctuations in active renin and aldosterone between HDBR55 and HDBR59 may be attributed in part to the unknown dietary intake on HDBR57. Additionally, four participants received 1000 mg of paracetamol at the discretion of study physicians between HDBR55 and HDBR59, which may have also confounded aldosterone concentrations (117). To preserve the integrity of the analyses, data from HDBR59 were excluded due to the confounding influence of an unknown ad libitum diet on hormonal concentrations and hematological variables. Our observation of highly variable fluid regulating hormones, which may be linked to relatively small changes in dietary intake, is important as the magnitude of change could mask or enhance the observed effect of the experimental intervention, and would not have been discovered if duplicate sampling had not occurred. Some of the between day differences in blood and plasma volume in the BDC-5 and BDC-4 samples appear to be related to the ~1500mg difference in between day Na^+ intake (Table 3) as identified by repeated measures correlation analysis (both plasma volume [r_{rm} (95%CI) = -0.701 (-0.867, -0.576), $p < 0.001$] and blood volume [r_{rm} (95%CI) = -0.701 (-0.866, -0.462), $p < 0.001$]); however no relationship was identified between HDBR55 and HDBR59, where Na^+ fluctuations were largely kept within ~500mg.

Data collection for the BRACE study was conducted in two successive campaigns, one year apart. Having the capacity to accommodate only 12 participants for the duration of the three-month study, the MEDES facility identically repeated the protocol the following year to achieve the target sample size of 24. Dietary intake within the standardized 10-day menu was harmonized with experimental time points and Na^+, Cl^-, and K^+ intakes were kept consistent across both campaigns (Table 4). Despite consistent increases in active renin activity during

HDBR for both campaigns, aldosterone primarily decreased in participants from campaign 1 but increased in most participants of campaign 2 with HDBR. While the underlying mechanisms remain unclear to explain this variability in the present study, these observations highlight the complex and variable endocrine adaptations that occur in long-duration bed rest. The coordination of a HDBR study is complex, balancing the examination of interacting physiological responses. Careful scheduling is essential to minimize confounding influence, particularly those related to dietary intake and hormonal regulation and should be a central consideration in the planning and execution of such protocols.

An important limitation of this study was the failure to include female participants. While female participants tend to have broadly similar responses for fluid-regulating hormones (19) and plasma volume changes over the course of HDBR to males (18, 118), sex-specific differences in response to exercise + AG remain insufficiently characterized.

5.5 Conclusion

This study provided novel insights into the hormonal and fluid regulatory adaptations during 60 days of HDBR while evaluating how exercise or exercise with simultaneous short-arm centrifugation during HDBR affects fluid loss. Contrary to the BRACE study hypothesis, exercise + AG failed to prevent reductions in plasma volume or change the response of fluid regulating hormones to HDBR, which may be attributable to the action of the muscle pump during exercise minimizing the AG-induced caudal fluid shift. Furthermore, our observed variability for repeated measurements taken on sequential days within the same phase of the study (e.g., BDC) at the same time of day in a highly controlled environment, which may be linked to small differences in dietary intake, highlights the need for strict dietary control in

investigations of fluid regulating hormones. Overall, these results suggest that 30 mins of moderate-to-high intensity interval exercise, with or without simultaneous short-arm centrifugation, does not protect plasma volume and reinforces the need for precisely controlled, non-overlapping investigator protocols in future bed rest and spaceflight studies.

6.0 Additional Results

Fluid Regulatory Hormones: Campaign Effect

The first set of aldosterone plates show a main effect of time ($p < 0.001$), main effect of campaign ($p < 0.001$), and a time x campaign interaction ($p < 0.001$). Aldosterone was reduced following HDBR in campaign 1 ($p = 0.001$) and increased following HDBR in campaign 2 ($p < 0.001$). The repeated aldosterone plate also showed a main effect of time ($p = 0.003$), no main effect of campaign ($p = 0.42$), and a time x campaign interaction ($p < 0.001$). Aldosterone was non-significantly reduced following HDBR in campaign 1 ($p = 0.07$) and increased in campaign 2 ($p = 0.001$), as shown in Figure 4. No other hormone or BV outcome displayed a campaign effect or interaction effect ($p > 0.05$). As discussed, no clear explanation for this campaign effect has been identified.

Repeatability of Aldosterone Plates

Figure 5 shows the differences in aldosterone between the repeated ELISA plate performed in the Bland–Altman plot, the mean differences and standard deviations were -17.52±39.43%. While the CV of the repeated aldosterone ELISA plates was high, 26.52±18.28%, the trends between campaign remained.

7.0 Discussion

This study aimed to determine the effect of daily cycling exercise, or simultaneous cycling and short-arm centrifugation, on plasma volume and fluid regulating hormones during 60 days of HDBR (17–19). While the submitted manuscript, pending revisions, above discusses the major outcomes and considerations of this study, this discussion section aims to provide additional information on the study, as well as its limitations. Contrary to the hypothesis of the BRACE study, neither exercise alone nor exercise during simultaneous short-arm centrifugation influenced RAAS or the corresponding loss in blood volume. All groups, irrespective of countermeasure, experienced a suppression of EPO at HDBR3, a reduction in lean mass, a reduction in plasma and blood volume and a corresponding upregulation in active renin in all groups at the end of 60 days of HDBR. Neither exercise nor the combination of exercise + AG altered the degree of fluid loss, hormonal responses, or changes in whole body lean mass when compared to control.

Consistent with previous HDBR studies, the exercise protocol within the BRACE study was insufficient to prevent the decline in blood volume and related variables. Despite near daily sessions of moderate-to-high-intensity ramped supine cycling up to 80% participants plasma volume, blood volume, and Hbmass were reduced following 60 days of HDBR, and no differences were observed between the exercise and control group. The lack of preservation in plasma volume can be explained by the unaltered circulating concentrations of fluid regulating hormones and EPO between control and exercise. These findings are aligned with previous 14 day HDBR studies, where neither the aerobic nor resistance training programs were able to preserve plasma volume in either young or older adults (65, 67). Despite well documented plasma volume expansion following exercise training mediated through both RAAS and vasopressin activity, and

erythropoiesis (119), results from this study imply this exercise stimulus was insufficient to overcome the adaptive response to reduce blood volume.

Similarly, and contrary to the hypothesis of the BRACE study, the combination of this same exercise protocol with simultaneous AG by short-arm centrifugation was also not different from exercise alone or control. The lack of hormonal differentiation among groups supports the notion that neither exercise nor exercise + AG stimuli were sufficient to meaningfully modulate baroreceptor or volume sensitive endocrine pathways. These findings from the BRACE project highlight the need for the optimized implementation of AG, as the current simultaneous protocol with supine cycling exercise did not prove effective for preserving plasma volume or orthostatic tolerance (106). Further research is needed to validate individualized levels of AG applied during HDBR and to evaluate the benefit of AG exposure during post-exercise rest prior to implementation during spaceflight.

While short-arm centrifugation remains technically feasible within HDBR studies, its translation to operational spaceflight will prove challenging. The ISS is scheduled for decommission in 2030 and is anticipated to be replaced by commercially operated stations, such as VAST or Axiom. While still in development, VAST aims to create a commercial artificial gravity space station, spinning in space to generate centrifugal forces throughout the entire station. While the whole-station AG would similarly reintroduce hydrostatic gradients to the current short-arm centrifuge model, the degree of exposure and potential benefit to human cardiovascular and musculoskeletal health would need to be validated.

A limitation of the BRACE study was the exclusion of female participants, a decision made by ESA based on the logistical and statistical constraints of having a mixed sex design. A statement made by ESA highlighted their previous experience from the AGBRESA bed-rest study, indicated

that statistical power was too low with a sex balanced design with 8 participants per group. They also state that recruiting women for prolonged HDBR remains exceptionally challenging. While valid concerns, the inclusion of female participants remains crucial to clarify sex-dependent mechanisms of deconditioning and ensure that countermeasures are appropriately designed for the increasingly diverse astronaut or commercially traveling population.

Evidence from prior HDBR suggest that reductions in plasma volume tend to be lower in female compared to male participants, though this relationship is not clear due to variations in HDBR duration, countermeasure, and inter-individual variability. Fortney et al. demonstrated a ~10% reduction in female vs. a ~15% reduction in males after 13 days of HDBR (118). Whereas, a comparable ~9% reduction in males and female participants were observed following 7 days of HDBR (120). Volume regulatory hormones demonstrate a very similar response between male and female participants following 7 days of HDBR (19). The WISE-2005 study demonstrated that daily LBNP and treadmill running in female participants attenuated the reduction of blood volume, though likely attributed to the inclusion of 10 minutes of post-exercise exposure rather than sex-specific physiological responses (91). Given the present study's failure to preserve plasma volume or modify hormonal regulation in males, similar findings would be expected in females.

As highlighted in the manuscript discussion, data collection for the BRACE study was conducted in two successive campaigns, one year apart. Having the capacity to accommodate only 12 participants for the duration of the three-month study, the MEDES facility identically repeated the protocol the following year to achieve the target sample size of 24. Campaign 1 ran from April to July 2023, while campaign 2 ran between February - May 2024. Timing of all study protocols, interventions, and standard measures were kept identical between campaigns.

When examining campaigns separately, aldosterone was reduced in campaign 1 while increased in campaign 2 following HDBR. Despite these differing responses, neither campaign showed an effect of countermeasure or interaction. All hormones were assayed from the same blood samples, which were centrifuged and stored at -80°C prior to shipping to Waterloo for both campaigns, and thus the potential thawing or contamination of samples would not explain these results. To verify the validity of these results, the aldosterone ELISA was run in duplicate. The second ELISA plate confirmed the campaign specific trends as shown in Figure 4, posing the question; what was different between campaign 1 and 2?

While both campaigns demonstrated a similar rise in active and total renin concentrations at the end of 60 days of HDBR, aldosterone in campaign 2 did not respond to these elevations as anticipated. Potential confounding factors in the regulation of aldosterone did not provide a sufficient explanation for this dichotomy. As mentioned throughout, dietary intake and water intake was standardized and consistent between campaign and experimental time points. Pain medication use was controlled and administered as needed by MEDES physicians. Paracetamol (acetaminophen) increases aldosterone production with a dose dependent response, and may confounded aldosterone concentrations depending on participant intake (117). During BDC, four participants received 1000 mg of paracetamol for pain related to chest, biopsy, and shoulder pain at the discretion of study physicians between BDC-10 and BDC-5. Of these participants, one was from campaign 1, who exhibited a decrease in aldosterone following HDBR, while the remaining three were from campaign 2, and all demonstrated an increase in aldosterone. During HDBR, paracetamol was given to three participants between HDBR 55 and HDBR59 for pain related to muscle biopsy pain, headaches, pack pain, and right leg pain. Each of these participants showed an increase in aldosterone production. These results do not suggest a causal role of paracetamol,

as similar aldosterone trends were observed in participants who did not receive any medication near their blood sampling days. Drug usage during HDBR is unlikely to account for the variability in aldosterone concentrations during either BDC or HDBR. Prior research has demonstrated a blunted aldosterone response in chronic hypovolemia due to adrenal insensitivity to angiotensin II or Na^+ retention (121), though adrenal sensitivity in these participants seems highly unlikely. While both plasma renin and aldosterone concentrations fluctuate diurnally, demonstrating their peak in the early hours of the morning and a nadir in the evening, these diurnal fluctuations remain consistent following 11 days of HDBR (122). Blood draws were taken first thing in the morning prior to ambulation during BDC, or other activities during HDBR which were consistent between campaign to avoid these fluctuations within the circadian rhythm. These results remain unexplained in the present study but highlight the potential for confounding results when coordinating a multi-year project.

Another limitation of this study is the reproducibility of the CO rebreathing method. We report poor reproducibility of Hbmass with a typical error of 6%. Research implementing the same CO rebreathing technique demonstrate typical error below 2% (97, 123). This discrepancy is likely due to the imprecision in the analysis of %COHb in blood, which were analyzed as single values before and after rebreathing in the present study. Other groups perform duplicate (123) or quadruple (97) analysis of each sample. The variability of single measurements for the %ΔCOHb, Hb, and Hct compound in the calculation of Hbmass. The analysis of quadruple samples from venous blood would not require additional blood from participants and should be performed to improve confidence in these outcomes. Another potential source of error lies in the volume of CO within the syringe, which is measured to 0.9 ml/kg body weight. CO was filled into plastic syringes in a shed outside MEDES to avoid possible contamination of the atmosphere by 7 different

physicians over the course of both campaigns. The use of glass syringes with mechanical stops would limit gas expansion with heat changes, and inter-operator variability (123). Overall, there exist areas of variability within the administered CO rebreathing performed at MEDES which should be revised for future studies.

8.0 Conclusion

This study provided the first opportunity to examine hormonal and fluid regulatory adaptations during 60 days of HDBR with short-arm centrifugation. Contrary to the BRACE study hypothesis, neither exercise nor exercise + AG prevented reductions in plasma volume or changed the response of fluid regulating hormones to HDBR. The BRACE study was a complex effort incorporating 14 international research teams, coordinated over 2 years. Secondary findings from this project highlight the sensitive nature of repeated measures for both hematological and hormonal variables. Confounding influence of other dietary interventions during otherwise standard intakes emphasizes the importance of careful scheduling and consideration in HDBR protocols to avoid biological variation in outcomes. The coordination of a HDBR study is a complex undertaking, and balancing the examination of interacting physiological responses requires careful consideration but remains critical for understanding these systems.

9.0 Tables

Table 1: Baseline participant characteristics

Characteristic	Control (n = 8)	Exercise (n = 8)	Exercise + AG (n = 8)
Age (year)	29 ± 7	30 ± 5	30 ± 6
Height (m)	1.73 ± 0.07	1.78 ± 0.04	1.77 ± 0.07
Mass (kg)	71.9 ± 8.0	76.1 ± 7.8	73.7 ± 7.9
Lean mass (kg)	53.68 ± 6.89	58.03 ± 4.38	55.91 ± 4.95
Peak oxygen uptake (mL/min/kg)	50.1 ± 7.1	46.3 ± 5.5	46.0 ± 5.2
AG at pre-syncope (G_z)	1.41 ± 0.22	1.47 ± 0.17	1.46 ± 0.22

Table 2: Summary of pre-bedrest baseline and day 55 of head-down bed rest blood volume outcomes and fluid regulating hormones

	Control (*n* = 8)		Exercise (*n* = 8)		Exercise + Artificial Gravity (*n* = 8)		Linear Mixed Model *(p-value)*		
Hormones	**BDC**	**HDBR**	**BDC**	**HDBR**	**BDC**	**HDBR**	**Time**	**Group**	**Interaction**
Active Renin (pg/mL)	15.1 ± 19.8	25.1 ± 25.2	13.4 ± 9.2	22.9 ± 14.7	9.7 ± 5.3	13.9 ± 8.1	<0.001	0.516	0.242
Renin (pg/mL)	888.2 ± 254.2	959.8 ± 274.6	905.3 ± 181.0	906.1 ± 202.2	1027.5 ± 410.7	1120.4 ± 437.0	0.022	0.491	0.235
Aldosterone (pg/mL)	119.9 ± 44.8	100.6 ± 35.9	91.7 ± 31.0	92.4 ± 40.9	93.9 ± 40.5	87.1 ± 35.5	0.363	0.387	0.671
ARR ([pg/mL]/[pg/mL])	21.2 ± 20.1	7.0 ± 4.7	8.7 ± 3.4	5.2 ± 3.3	16.4 ± 19.3	8.4 ± 6.8	0.003	0.366	0.254
Vasopressin (pg/mL)	3.2 ± 0.8	3.3 ± 1.0	3.2 ± 1.2	3.5 ± 0.7	3.1 ± 0.8	2.9 ± 1.8	0.791	0.720	0.863
ANP (pg/mL)	63.3 ± 35.1	50.6 ± 25.1	49.0 ± 19.8	72.2 ± 42.7*	55.3 ± 36.7	61.4 ± 40.1	0.242	0.975	0.016

Means ± SD. BDC, pre-head-down bed rest; HDBR, day 55 of head-down bed rest; ARR, aldosterone to active renin ratio; ANP, atrial natriuretic peptide. *, different ($p < 0.05$) from BDC within a given group. Statistical analysis shows comparison of time points in the study, group and their interactions in a linear mixed model analysis.

Table 3. Daily sodium, chloride and potassium intake and corresponding active renin and aldosterone concentrations measured after an overnight fast during pre-bed rest baseline and late head-down bed rest.

n = 24	Na + Intake (mg)	Cl - Intake (mg)	K+ Intake (mg)	n = 24	Active Renin (pg/mL)	Aldosterone (pg/mL)
BDC-7	4174 ± 388	7327 ± 725	2913 ± 220			
BDC-6	4886 ± 380	6865 ± 535	3900 ± 415	**BDC-5**	10.5 ± 10.7	82.4 ± 30.7
BDC-5	3371 ± 269*	5746 ± 393*	4153 ± 243*	**BDC-4**	15.0 ± 15.1*	121.3 ± 57.9*
HDBR53	3615 ± 346	5851 ± 573	4272 ± 560			
HDBR54	4084 ± 389	7265 ± 661	3840 ± 452	**HDBR55**	20.6 ± 17.4	93.3 ± 36.3
HDBR55	4697 ± 460	6882 ± 646	3932 ± 477			
HDBR56	4010 ± 408	6921 ± 669	4215 ± 518			
HDBR57	N/A	N/A	N/A			
HDBR58	4488 ± 451*	7543 ± 794*	5102 ± 741*	**HDBR59**	26.9 ± 15.7*	133.4 ± 47.3*

Means ± SD. BDC, pre-head-down bed rest; HDBR, late head-down bed rest; Na^+, sodium; Cl^-, chloride; K^+, potassium. Complete data are unavailable for HDBR57 due to an ad libitum lunch. Paired t tests were used to compare dietary intake (BDC-6 vs. BDC-5, and HDBR54 vs. HDBR58) and blood hormone levels (BDC-5 vs. BDC-4, and HDBR55 vs. HDBR59) on days specific to blood sampling. *, different ($p < 0.05$) from the other stated day of BDC or HDBR.

Table 4. Comparison of daily sodium, chloride, and potassium intake normalized to body mass on BDC-6, BDC-5, HDBR54 and HDBR58 between campaigns.

Na^+ Intake (mg/kg)	**Campaign 1 (n=12)**	**Campaign 2 (n=12)**	***p*-value**
BDC-6	67.17 ± 5.57	66.01 ± 2.51	0.257
BDC-5	46.85 ± 5.90	45.29 ± 2.49	0.204
HDBR54	55.77 ± 4.96	58.39 ± 3.01	0.066
HDBR58	62.68 ± 3.38	62.53 ± 2.00	0.450
Cl^- Intake (mg/kg)			
BDC-6	94.32 ± 8.12	92.93 ± 5.47	0.314
BDC-5	80.08 ± 8.15	76.98 ± 4.03	0.125
HDBR54	99.61 ± 9.27	103.60 ± 5.89	0.111
HDBR58	104.9 ± 7.48	105.53 ± 3.35	0.396
K^+ Intake (mg/kg)			
BDC-6	53.09 ± 5.16	53.50 ± 7.71	0.440
BDC-5	57.88 ± 5.74	55.72 ± 3.36	0.136
HDBR54	53.91 ± 4.48	53.45 ± 6.37	0.420
HDBR58	70.32 ± 8.86	72.17 ± 9.17	0.310

Means ± SD. BDC, pre-head-down bed rest; HDBR, late head-down bed rest; Na^+, sodium; Cl^-, chloride; K^+, potassium. Dietary intake normalized to body mass on the days prior to blood sampling was compared between campaigns using independent t-tests.

10.0 Figures

Figure 1: Changes in intravascular variables from pre-head-down bed rest (BDC) to late head-down bed rest (HDBR) across three intervention groups: control, exercise, and exercise + AG. Boxplots represent the median, interquartile range, and individual data points, with colored lines indicating individual trajectories. (A) Plasma volume (mL) (B) Total blood volume (mL) (C) Hemoglobin mass (g) (D) Hemoglobin concentration (g/dL) (E) Hematocrit (%). †, significant main effect of time ($p<0.001$) in A, B, C. ‡, significant interaction of time * group in D with post-hoc comparisons shown by * ($p < 0.05$).

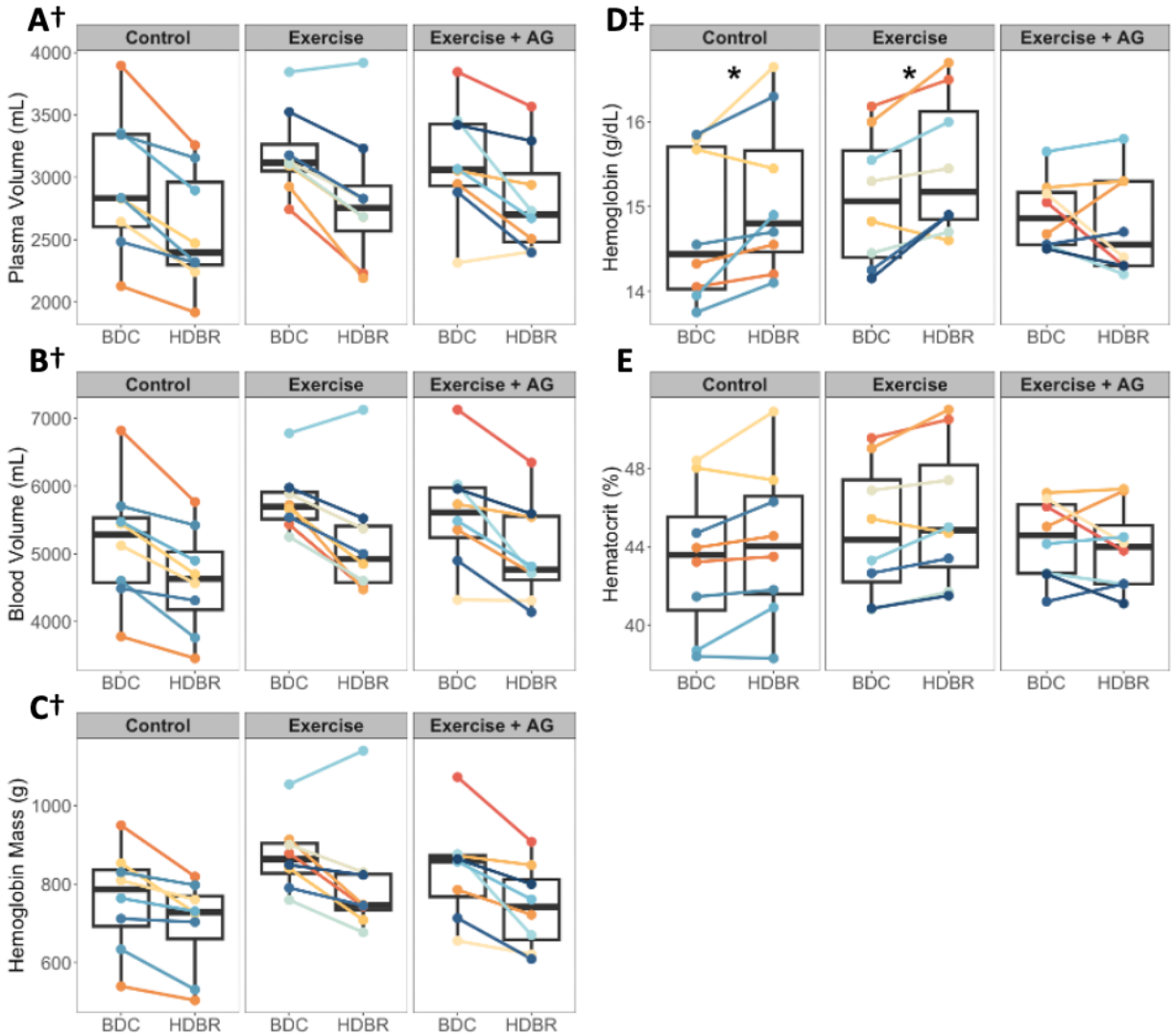


Figure 2: Erythropoietin concentrations across four time points: pre-bed rest (BDC-4), early head-down bed rest (HDBR3), and late head-down bed rest (HDBR55, HDBR60). Boxplots display the median, interquartile range, and individual data points, with colored lines representing individual trajectories within each group. Groups include control (yellow), exercise (green), and exercise + AG (blue). *, different ($p < 0.05$) from BDC-4.

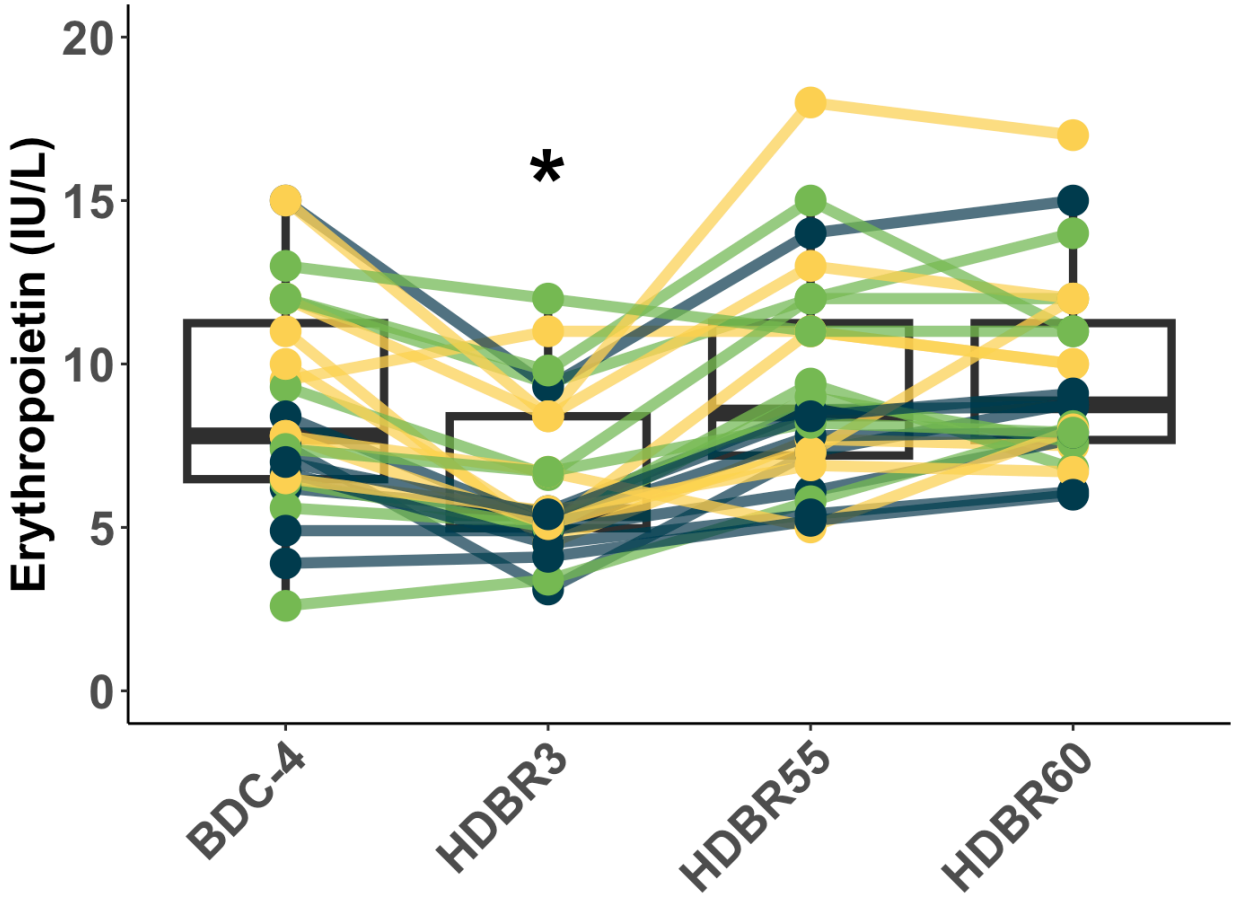


Figure 3: Erythropoietin concentrations in the morning and evening across two time points during head-down bed rest: early (HDBR3) and late (HDBR60). Boxplots display the median, interquartile range, and individual data points, with colored lines representing individual trajectories within each group. Groups include control (yellow), exercise (green), and exercise + AG (blue). Data from HDBR3 and HDBR60 were pooled for statistical analysis.

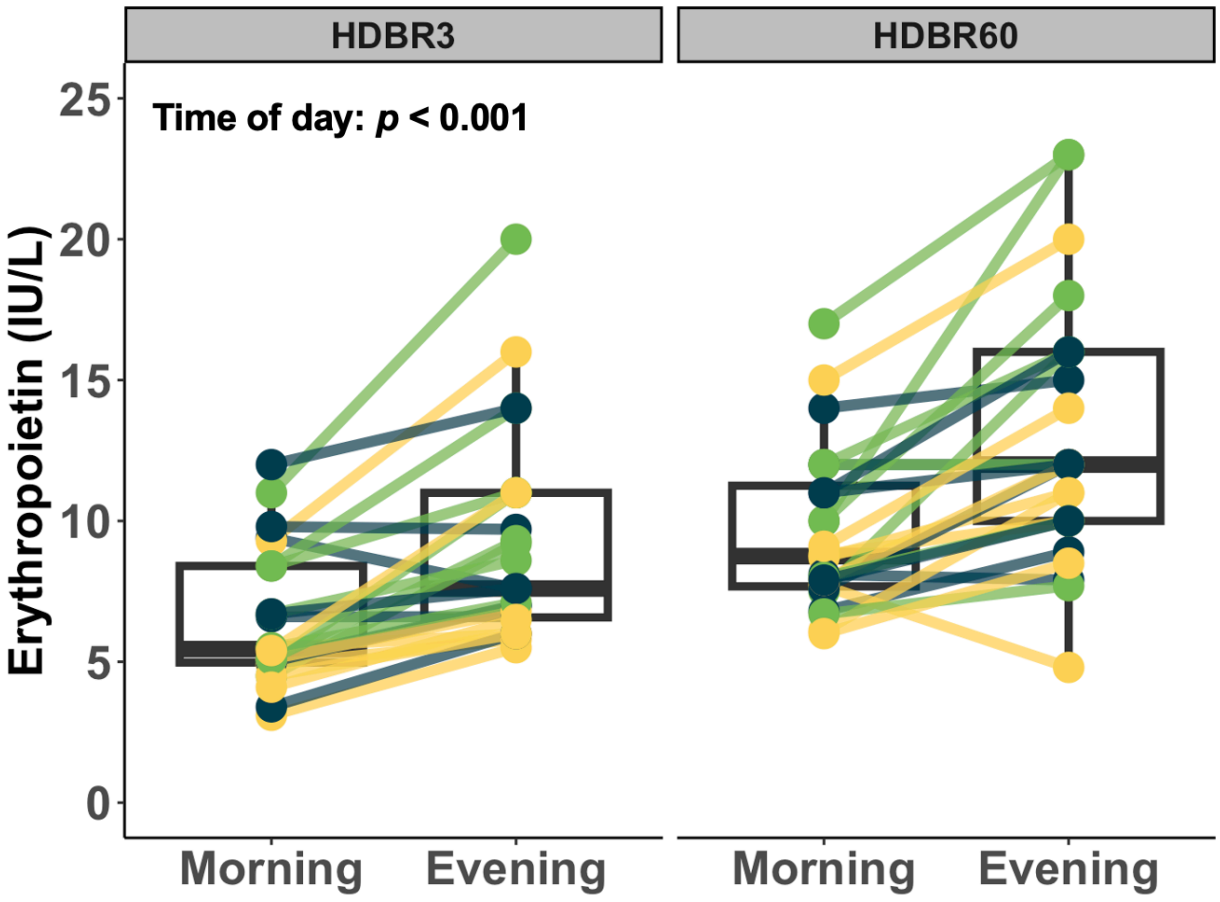


Figure 4: Aldosterone concentrations (pg/mL) from baseline (BDC) and late-head down bed rest (HDBR) from campaign 1 and campaign 2 from repeated biochemical assays. A: Plate 1, B: Plate 2. ‡, significant interaction of time * campaign in A, B with post-hoc comparisons shown by * ($p < 0.05$).

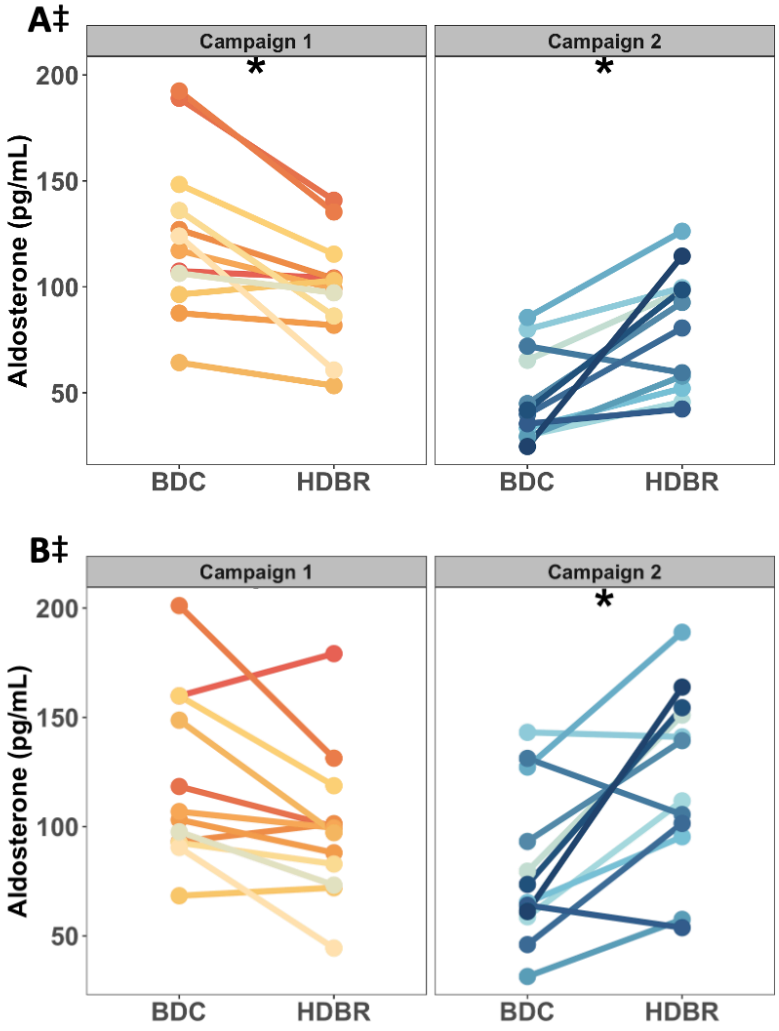


Figure 5: Bland-Altman bias plot between repeated ELISA plates for the difference in aldosterone concentrations (pg/mL). The middle horizontal line represents the overall mean difference (-17.52pg/mL) between the plates and the other two dashed red lines represent the 95% limits of agreement (-94.81 to 59.77pg/mL)

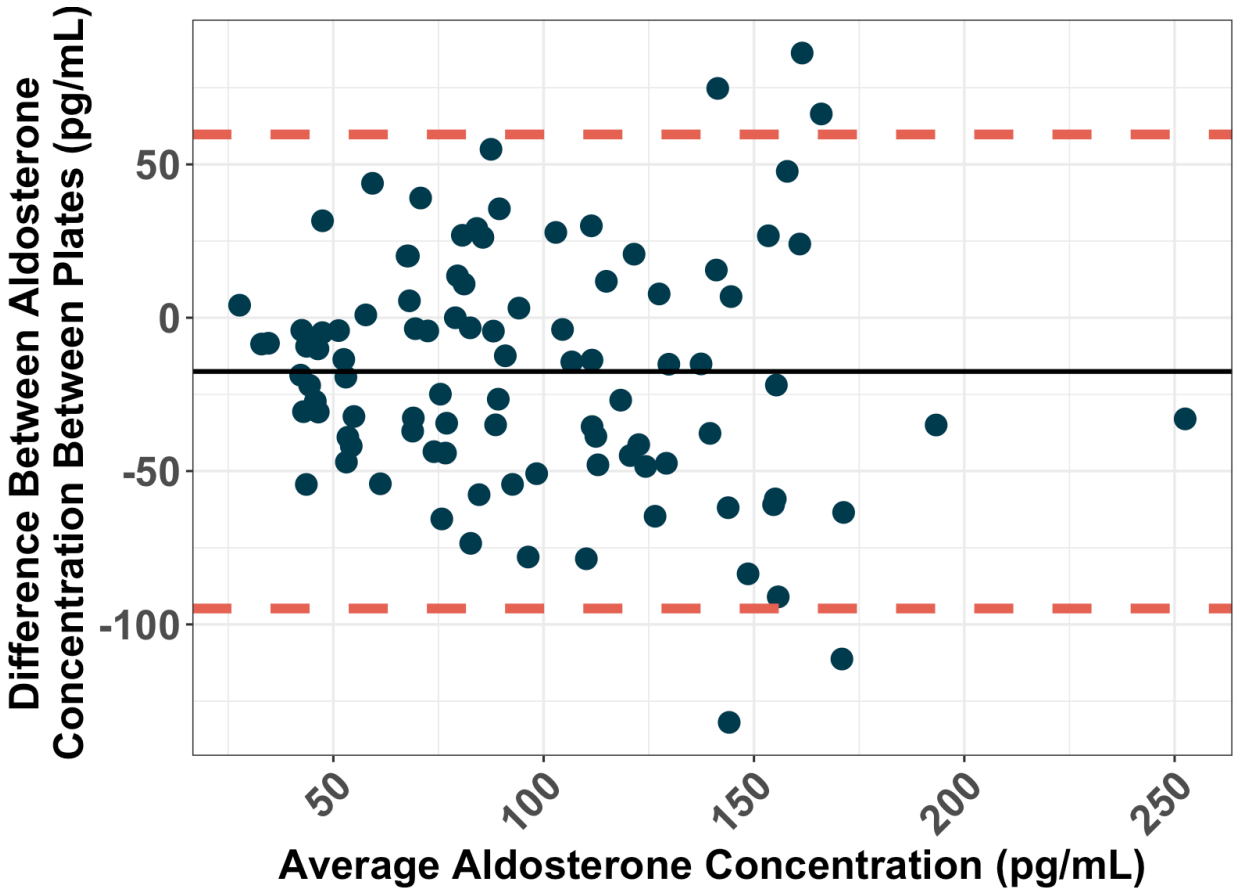


Figure 6: Electrolyte intake of sodium (Na^+), chloride (Cl^-), and potassium (K^+) within the standardized 10-day menu harmonized with experimental time points. Electrolytes include Cl^- (yellow), K^+ (green), and Na^+ (blue)

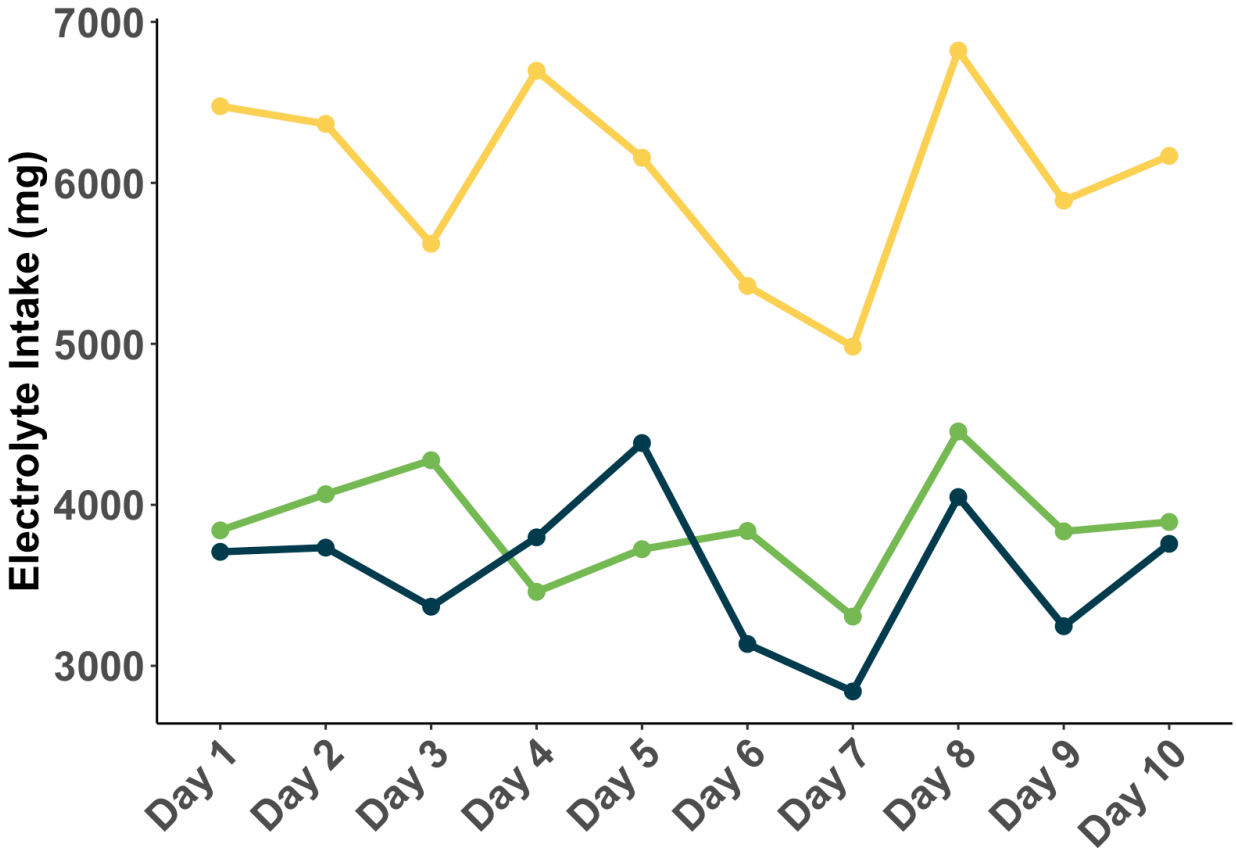


Figure 7: Repeated measures correlation between active renin concentrations (pg/mL) and daily sodium intake across repeated blood draw days. Individual data points represent paired measurements from BDC-5 (triangle) and BDC-4 (circle).

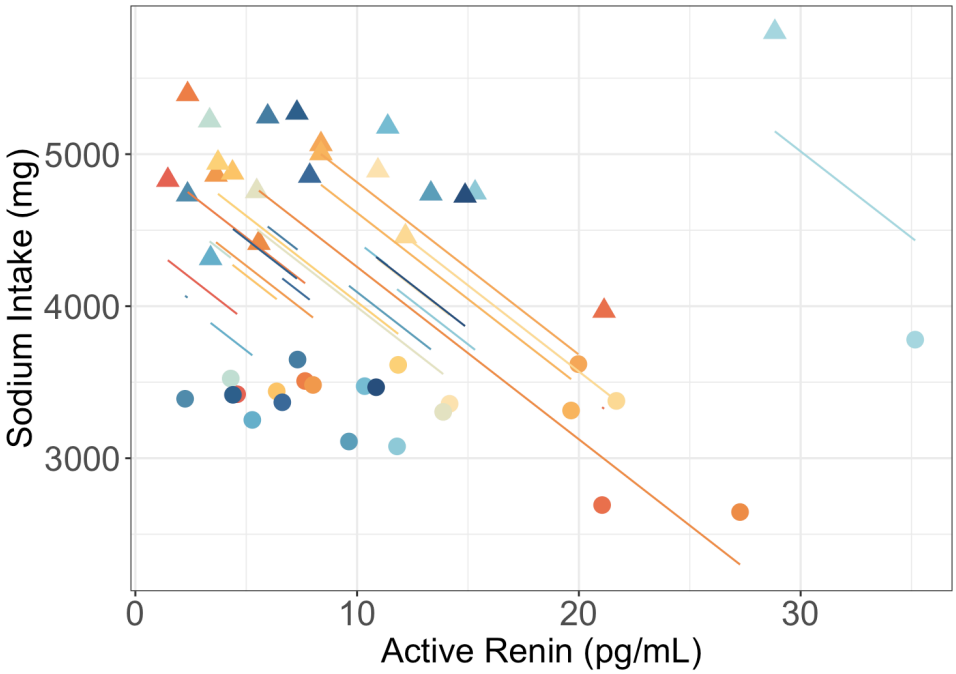
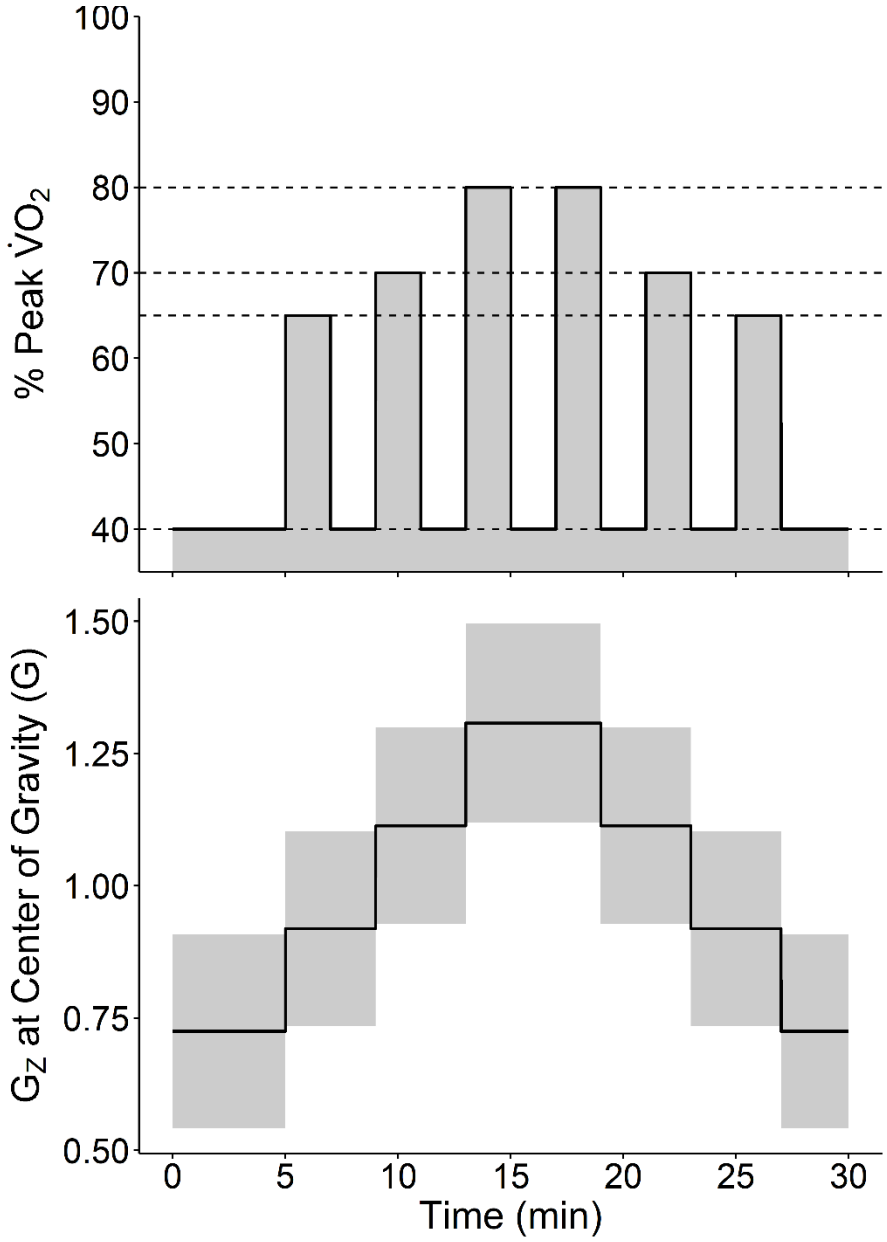


Figure 8. A: Reference diagram taken from Hedge et al. (100) of the timing and target % peak VO2max of the daily supine high-intensity interval exercise protocol performed by participants in the exercise and exercise + AG countermeasure groups. **B:** Reference diagram of the timing and target G_z at the center of gravity experienced by participants during daily simultaneous exercise + AG countermeasure. The solid line represents the group mean (n = 8), while grey shaded area represents ± 1 standard deviation (106)



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Appendix

Table 1: Inclusion and exclusion criteria for participant selection as outlined by ESA

Inclusion Criteria	**Exclusion Criteria**
Healthy male volunteer	History or presence of clinically relevant cardiovascular, neurological or ENT disease, any chronic disease; any acute infectious disease. ▪ Symptomatic orthostatic hypotension whatever the decrease in blood pressure, or asymptomatic postural hypotension defined by a decrease in SBP equal to or greater than 20 mmHg within 3 minutes when changing from the supine to the standing position, ▪ Cardiac rhythm disorders, ▪ Hypertension, ▪ Chronic back pain, ▪ Vertebral fracture, scoliosis, or herniated disc, ▪ Glaucoma, ▪ Self-reported hearing problems, ▪ Vestibular disorders ▪ History of migraines, ▪ History of hiatus hernia or gastro-esophageal reflux, ▪ History of thyroid dysfunction, renal stones, diabetes, ▪ History of head trauma, ▪ Abnormal result for lower limbs echo-doppler, ▪ History of genetic muscle and bone diseases of any kind, ▪ Past records of thrombophlebitis, family history of thrombosis or positive response in thrombosis screening procedure (anti thrombin III, S-protein, C-protein, factor V Leiden mutation and the mutation 20210 of the prothrombin gene),
Aged 20 to 45 years	Bone mineral density: T-score ≤ -1.5,
No overweight nor excessive thinness with BMI (weight kg/ height m2) between 20 and 27 kg/m2	Poor tolerance to blood sampling,
Height between 158 and 190 cm	Having given whole blood (more than 7mL/kg) in a period of 8 weeks or less before the start of the experiment, or having given whole blood more than 2 times in the past year,
Certified as healthy by a comprehensive clinical assessment (detailed medical history and complete physical examination): in particular, free from any chronic disease or any acute infectious disease or cardiovascular, neurological, ENT (especially orthostatic hypotension and vestibular disorders), orthopaedic or musculoskeletal disorders	Significant history of allergy, especially no dermatological or food allergy,
Good mental health: tests and psychological interview will be held by a specialist in extreme environment	Significant anomaly detected in the biological analysis,
Fitness level assessment: 35 mL/min/kg < VO2max < 55mL/min/kg	Positive reaction to any of the following tests: HVA IgM (hepatitis A), HBs antigen (hepatitis B), anti-HVC antibodies (hepatitis C), anti-HIV1+2 antibodies,
Non active smokers	Vegetarian or vegan,
No alcohol or drug dependence, and no medical treatment	Refusal to give permission to contact his general practitioner,

Covered by a Health Insurance System	Participant who, in the judgment of the investigator, is likely to be non-compliant during the study, or unable to cooperate because of a language problem or poor mental development,
Having signed the information consent	Subject already participating or in the exclusion period of a clinical research,
Free of any engagement during the study	Subject who has received more than 4500 Euros within 12 months for being a research subject,
	Subject who cannot be contacted in case of emergency,
	Subject who had the two knees injured
	MRI contraindications ▪ History or active claustrophobia ▪ Osteosynthesis material, presence of metallic implants or any other contra-indication for MRI
	Vulnerable persons according to law “Code de la Santé Publique” (L1121-5 to L1121-8): ▪ Persons deprived of their liberty by an administrative or judicial decision ▪ Persons under involuntary psychiatric care ▪ Persons admitted in a health or social establishment for purposes other than research ▪ Minors ▪ Adults subject to legal protection (subject under guardianship or trusteeship) or unable to express their consent