Sex Differences in Hormonal and Extracellular Vesicle Responses to Military-Based Physiological Stress and Exercise

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Military personnel undergo training with both positive and negative implications for military readiness. Regular exercise training promotes fitness improvements, which translates to heightened combat readiness. In contrast, rigorous field training characterized by prolonged physical work, sleep disruption or restriction, and/or undernutrition results in physical deterioration and reduced readiness. The balance between this dichotomy of exercise and field training with bifurcating endpoints may be improved via the use of objective markers of physiological adaptation and maladaptation. There are known physiological differences between men and women, yet how they respond to military training stress is largely unknown. This begs the question: *Does the physiological response differ between men and women during military-based stress and exercise?* This question is timely given the recent inclusion of women into direct ground combat roles in the United States military following long held policies of their exclusion in such positions. The collection of studies in this document examined physiological response patterns in men and women undergoing simulated military operational stress and exercise. Specifically, applied metrics of soldier performance were assessed in conjunction with conventional measures of circulating stress and anabolic hormones using established methods. Sophisticated techniques were also employed to measure extracellular vesicles in a high-throughput manner, yielding multiparametric information about their features. The results indicate that men and women have similar responses in physical performance to simulated

military operational stress, but circulating factors underpinning physiological changes during stress and exercise training display sex dimorphism. Given that biomarkers serve as early warning indicators of phenotypic change, results from this thesis suggest potential downstream implications for readiness and identify novel targets for monitoring sex-specific differences in military-based stress and training.

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Preface

As an active-duty service member, the end of a three year period typically signifies the end of a tour at a duty station and time for another military move. These past three years have meant much more. My mission coming into this doctoral program was to grow personally and professionally—a mission that I have completed. Or, as we say in the military, "Charlie Mike". However, I could not have envisioned how truly enriching this experience would be. For that, I have many people I would like to thank for their support throughout this process.

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have crossed paths with such an eclectic and remarkable group of people. I can honestly say that I have no doubt each of you will go on to extraordinary work in your respective fields. I am grateful to have each of you as friends, colleagues, and future collaborators. To my "battle buddy", Meaghan Beckner, I would not have been able to pursue such a formidable research direction had you not been there with me to learn and troubleshoot all of the newness that came our way. Thank you for being on the same page, same line, same word.

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Finally, to my family, I am grateful for the love and support you have shown me over the years and for instilling in me a strong moral compass and sense of work ethic. Thank you for the support during the losses and your presence to celebrate the wins.

1.0 Introduction

"War is hell". These words, famously uttered by American Civil War General William T. Sherman regarding military conflict, still ring true today and accurately convey the rigors of intense military operations. Modern-day warfare is unforgiving and exposes military service members to *operational stressors* including prolonged physical work, disrupted or restricted sleep, inadequate caloric intake, environmental extremes, and high cognitive burden (Figure 1). Extended exposure to military operational stress, combined with the inability to recover, results in deterioration of individual preparedness and unit readiness. Though the effects of operational stress are evident in nearly every military occupation, they are perhaps most apparent in the more physically demanding military occupations, such as direct ground close combat roles.

Figure 1. Characteristics of military operational stress.

Direct ground close combat roles are military occupations that engage in direct enemy combat using lethal force. Examples include infantry, artillery, armor, and special forces. These tactical occupations are some of the most physically and mentally demanding in the military and involve exposure to intense operational stress. Women have only recently been integrated into ground close combat employment in many militaries (1). As such, much of our knowledge pertaining to the impact of intense military operational stress and training exists predominately in men, with far less data in women. The 1994 Defense Women's Health Research Program provided $40M in congressional appropriations to study issues related to female service members (2). The results were improvements in training, doctrine, and equipment that benefited both men and women alike. The decision to allow women into previously restricted military occupations requires further re-evaluation of the training doctrine employed to optimize readiness and performance.

One means to counteract the inevitable declines in physiological status and physical performance is through attainment of optimal physical performance prior to operational stress exposure. Peak physical performance is established through a delicate balance of physical training and recovery. According to the theory proposed by Hans Selye, positive adaptations occur when hormetic stressors are matched with adequate resources such as rest and nutrition, whereas maladaptive responses (i.e., overreaching or overtraining) emerge when stress exceeds the capacity to recover (Figure 2) (3). Many of the adaptive responses that occur with physical training are mediated through tissue crosstalk (4) via circulating factors such as "myokines", defined as biomolecules released from muscle during contraction (5, 6). Though physical readiness is a critical aspect of occupational job performance and a standard by which military service members are evaluated, their trade also entails intense field training, which, paradoxically, may cause a decline in fitness when training stress overwhelms physiological adaptive capabilities. As such, a

paradigm exists whereby precise measures of physiological status are desirable for objectively and quantifiably monitoring health, performance, and recovery during physiological stress.

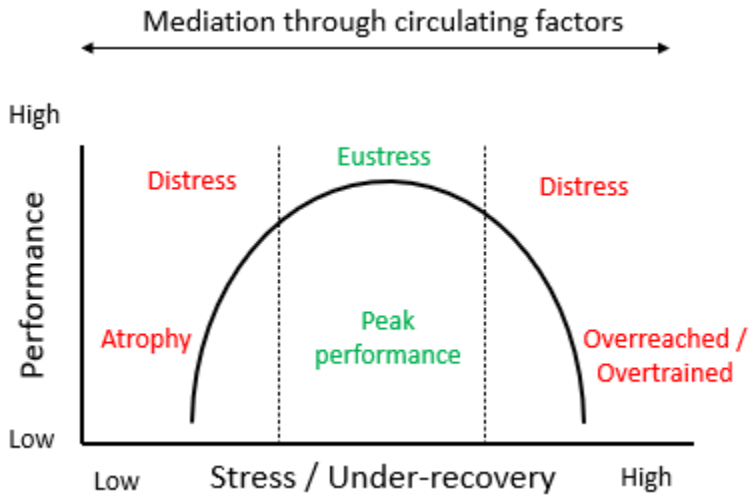


Figure 2. Stress and performance continuum.

Blood biomarkers have long been studied during military training to objectively monitor physiological status. Seminal studies in US Army Ranger school highlight this point. Following 8-weeks of rigorous training, Soldiers experienced significant declines in anabolic markers such as testosterone (−70% to 83%) and insulin-like growth factor-I (IGF-I) (−39% to 55%); metabolic markers including triiodothyronine (T3) (−25%), thyroxine (T4) (−20%), and brain-derived neurotrophic factor (BDNF) (−33%); whereas hormones related to stress and inflammation such as cortisol (+32%), c-reactive protein (+1520%), and interleukin-6 (+217%) increased (7–9). Simultaneously, Soldiers experienced precipitous drops in physical performance, including vertical jump height (−16%), lower-body power output (−21%), maximal lifting capacity (−20%), speed and agility (−15%), anaerobic capacity (−7%), and aerobic endurance (−24%) from pre- to post-training (7, 10). Interestingly, alterations in blood chemistries corresponded with declines in physical performance and preceded phenotypic changes such as body composition during the recovery period after training (8, 9), highlighting the usefulness of blood biomarkers as early

indicators of changes in health and physical readiness and their function in mediating physiological changes.

Nonetheless, the use of traditionally measured circulating hormones to monitor physiological status during training is somewhat limited. As noted by Lee et al. (11), commonly studied biomarkers pose a number of challenges including providing a relatively narrow range of information from a single biomarker, varying sensitivity, a lack of reference ranges for specific populations such as athletes or military personnel, and large inter-individual variances. As a result, there is a need for more holistic monitoring of physiological status that goes beyond commonly measured circulating proteins and metabolites.

Extracellular vesicles (EVs) have emerged as novel biomarkers for studying an array of pathological and non-pathological states with high sensitivity and specificity (12). *Extracellular vesicles* are membrane-bound, nano-sized particles that transfer functional biological materials, such as *proteins, lipids,* and *nucleic acids*, from host to target cells and impart downstream physiological change (13). Extracellular vesicles represent an evolutionarily conserved mechanism of intercellular communication in complex organisms (14). Study of their cargo provides robust information pertaining to tissue microenvironments, and, more broadly, the systemic status of biological organisms (15–18). Their biophysical characteristics (e.g., small size, stability in circulation, etc.) and presence in essentially all biofluids makes them easily accessible targets to study a wide array outcomes (15). Extracellular vesicles have been proposed as important mediators of systemic adaptation during exercise and may serve as biomarkers in the context of military stress physiology (19).

A largely understudied area in biomedical sciences research (20, 21) and military stress physiology (22) pertains to sex-based differences, despite well-established physiological

differences between men and women. Published data have demonstrated that men have significantly larger muscle cross-sectional area, larger type I and type II muscle fibers (23, 24), and larger blood volume, heart, and lungs compared to women (25). These physiological differences translate to greater physical performance in men compared to women. Specifically, men are 48% stronger in the upper body and 34% stronger in the lower body than women; have 30-40% greater $\dot{V}O2_{max}$ compared to equally trained women (25); have 48-55% greater anaerobic capacity than women (26, 27); and have 6-12% less body fat than women across the lifespan (28). In contrast, women are more resistant to fatigue in certain tasks such as isometric and intermittent elbow flexion and knee extension when performed at the same relative intensity (29).

The divergence in physical performance capacity between sexes begins during puberty when testosterone increases in men and estrogen increases in women (30). Beery and Zucker (21) sampled articles from 2009, and only 30% and 58% of studies in human physiology and endocrinology, respectively, analyzed outcomes by sex. Previous data has demonstrated that growth hormone peaks higher and earlier in women compared to men following both aerobic (31, 32) and resistance exercise (33). Women also experience a more substantial decline in insulin-like growth factor-I during operational stress compared to men, which is primarily driven by energy deficit (34, 35). Reports by Shill et al. (36) and Lansford et al. (37) have also noted declines in EV concentration following aerobic exercise in women, whereas there were no changes in men. Taken together, there are well-established differences endocrine signaling between men and women. Exclusion of sex as a factor during analyses can lead to misinterpretation of results and inhibit tailored approaches to training and recovery or therapeutic interventions (38, 39).

1.1 Definition of the Problem

Data predominately collected in men have demonstrated that intense military operational stress causes a decline in physical readiness and alters mediating blood constituents. Optimized exercise training programs can be part of a strategy to offset the inevitable declines in physical performance associated with operational stress exposure. Likely due to the historical exclusion of women from direct ground close combat positions, few data exist directly comparing biomarker responses in men and women undergoing the same training stress paradigms. Blood biomarkers are useful biological indicators of adaptation and maladaptation during physiological stress and may give insights into mechanistic underpinnings of adaptive processes. Based on sex-specific biological and physiological differences, it is reasonable to believe that men and women may respond differently to common military operations stressors such as physical exertion, sleep restriction, and underfeeding, as well as acute resistance exercise training.

1.2 Purpose

The overarching purpose of these studies is to identify sex-specific response patterns in circulating factors and their relationship to physical performance outcomes with the goal of elucidating characteristics of blood biomarkers that may be targeted for better monitoring physiological status and optimizing training strategies in men and women. The central hypothesis is that men and women will display unique signatures of circulating milieu during simulated military operations and exercise training, and such circulating factors will be related to physical performance outcomes.

1.3 Specific Aims and Hypotheses

Specific Aim 1.1: To examine sex differences in physical performance and hormonal responses related to anabolic status (GH, IGF-I), metabolism (IGF-I, BDNF), and stress (cortisol) from pre- to post-exertion at baseline.

Specific Aim 1.2: To examine sex differences in physical performance and hormonal responses related to anabolic status (GH, IGF-I), metabolism (IGF-I, BDNF), and stress (cortisol) from pre- to post-exertion following 48 h of moderate sleep and caloric restriction (i.e., peak stress).

Specific Aim 1.3: To examine the relationship between hormonal responses and physical performance.

Hypothesis 1.1: The magnitude of GH response to exercise will be greater in women than men, whereas BDNF response will be greater in men than women. There will be no difference between sexes in IGF-I or cortisol responses from pre- to post-exertion.

Hypothesis 1.2: Women will experience a greater decline in IGF-I concentrations compared to men following exposure to operational stress. There will be no differences in changes of GH, BDNF, or cortisol between men and women.

Hypothesis 1.3: Blood biomarkers associated with anabolic status, metabolism, and/or stress will be related to physical performance across the simulated military operational stress protocol.

Specific Aim 2.1: To examine sex differences in size, concentration, and surface proteins of EVs from pre- to post-exertion at baseline.

Specific Aim 2.2: To examine sex differences in size, concentration, and surface proteins of EVs from pre- to post-exertion following 48 h of moderate sleep and caloric restriction (i.e., peak stress).

Hypothesis 2.1: Women, but not men, will experience a decline in EV concentration from pre- to post-exertion at baseline. Mean size will remain stable from pre- to post-exercise with no differences in response according to sex. EV surface proteins will increase from pre- to post-exercise with no differences according to sex.

Hypothesis 2.2: EV concentration will decline following sleep and caloric restriction, but mean size will increase with no differences between men and women. EV surface proteins will remain stable in men and women from baseline to peak stress.

Specific Aim 3: To examine sex differences in size, concentration, and surface proteins of EVs before and after an acute bout of resistance exercise.

Hypothesis 3: EV concentration will increase similarly in men and women following resistance exercise, and there will be no differences in EV size in either sex. EV surface proteins will significantly increase following an acute bout of resistance exercise compared to pre-exercise, and there will be no difference in response according to sex.

1.4 Study Significance

Published literature has demonstrated the utility of circulating hormones in objectively and quantifiably monitoring physiological status during exposure to military training. Unfortunately, much of what we know about biochemical responses and their relationship to phenotypic outcomes in the context of operational stress and training exists predominately in men. This study will be one of the first to directly compare hormonal and physical performance responses in men and women undergoing the same acute training stress scenario. It will also be the first study to thoroughly describe EV profiles pre- and post-stress, thereby extending our understanding of sex

differences in the military training paradigm. Identification of sex-specific alterations in blood biomarkers attributed to multi-factorial operational and exercise training stress would give greater insight into sex-specific response patterns present during military operational stress and may better inform training and recovery strategies.

2.0 Literature Review

Much of what is known pertaining to the effects of military training on readiness is derived from data in men, and its impact on women is understudied. Well-established biological differences between men and women raises the question: *Does military training affect men and women differently?* This chapter will provide justification for the proposed studies by: (1) summarizing and synthesizing pertinent literature related to physiological variability between sexes, (2) detailing the effects of military operational stress on physical performance and physiology to include any sex-specific responses, and (3) highlighting studies demonstrating the importance of human performance optimization programs, which may be used as a strategy to offset the inevitable declines in physical readiness following operational stress exposure. Novel biomarkers with a functional role in intercellular communication will also be introduced and proposed as information-rich targets for studying sexually dimorphic physiological changes that occur during stress with possible phenotypic implications.

2.1 Physiological Differences Between Men and Women

"Every cell has a sex"—this phrase, published in a document by the US Institute of Medicine, succinctly sums up the point that men and women are biologically different beginning at the level of the cell (40). With the additional consideration of hormonal differences, sexually dimorphic anthropometric and physiological characteristics become apparent. These differences—

genetic and biochemical—are largely responsible for physical differences between men and women.

2.1.1 Strength and Power

Many of the physiological differences between men and women emerge during puberty when muscle mass and strength begin to increase in both sexes, though more so in men than women (30, 41). Data from Miller et al. (42) demonstrated that women were 48% and 34% weaker than men in upper and lower body strength, respectively. Further, women had 25-45% smaller muscle cross-sectional areas in upper and lower body muscles compared to men. Men also had larger type I (4597 *vs.* 3483 μm^2) and type II fiber (7700 *vs.* 4040 μm^2) areas than women (42), although women have proportionally more type I fibers relative to type II fibers compared with men (43). Though the strength to cross-sectional area ratio varied by body region, the authors concluded that differences in strength between men and women is attributed to muscle fiber characteristics (i.e., larger muscle cross-sectional area, larger type I and II muscle fiber areas) (42), which is at least partly explained by rising concentrations of reproductive hormones during early adolescents (41).

In a study of physically active men (n = 48) and women (n = 55), peak torque in the upper (shoulder flexion) and lower (knee extension) body limbs was greater in men and women (44). However, strength disparities diminished after adjusting for lean body mass and limb girth, indicating that differences between men and women in upper and lower body strength may be a function of lean body mass and skeletal mass distribution. These findings were corroborated by Bishop et al. (45) who reported that 97% of the variance in strength between sexes could be explained by fat-free mass and forearm and thigh fat-free cross-sectional areas in both athletes and

non-athletes. These results provide evidence that training strategies to increase strength, particularly in the upper body, may reduce some of the gap in strength between men and women.

Men also have a greater proportion of type II muscle fibers, but women have more type I fiber area as a percentage of total fibers than men (43). Individuals with more type II fibers are able to generate more power due to the higher proportion of myosin heavy chain and faster calcium kinetics in type II fibers (29). However, type II fibers are more glycolytically demanding and fatigue quicker, offering women a potential advantage in terms of muscle fatiguability (29, 46).

2.1.2 Aerobic and Anaerobic Fitness

Cardiorespiratory fitness depends on how well oxygen can be delivered to cells and extracted for use in generating energy. Men have an advantage in their ability for oxygen exchange largely due to the size differences between men and women. Men have larger hearts, greater left ventricular mass (47), more blood volume (70 *vs.* 80 mL/kg body mass), and greater lung capacity (25). Additionally, women have less hemoglobin and are more at risk for iron deficiency anemia due to menstruation (48). The summation of these factors results in greater stroke volume, oxygen delivery, and better performance in men. Available data shows that men have 30% to 40% higher $\dot{V}O2_{peak}$ compared to equally trained women (25). However, women appear to have an advantage in terms of their ability to oxidize more fat and preserve glycogen stores during aerobic exercise, whereas men utilize a higher proportion of glycogen and amino acids as a substrate for gluconeogenesis (49, 50). Differences in lipid oxidation rates between sexes appear to be at least partially mediated by estrogen (51). This may offer a long-term protective effect on lean body mass for women and give a slight edge in terms of long duration aerobic activity which are

common during military operations. Nonetheless, in absolute terms, men have superior cardiorespiratory fitness.

Similarly, anaerobic capacity is greater in men during maximal effort (25). Studies have demonstrated that men are able generate greater peak power during the Wingate test (i.e., 30 s maximum effort cycle ergometer test) (26, 27); however, sex differences in power generation were reduced after adjusting for body mass and fat-free mass (27). Interestingly, lactate thresholds are similar between men and women during an incremental exercise protocol, though women accumulate plasma lactate slower than men (52). This is likely due to differences in substrate utilization during exercise with women utilizing more fat whereas men use more on carbohydrate at a given intensity. Estrogen concentrations in women are thought to drive the higher proportion of lipid oxidation (51) and may be advantageous relative to endurance and recoverability.

2.1.3 Body Composition

Women generally have more fat mass relative to body mass compared to men (28). This sexual dimorphism begins as early as birth (53) and extends into puberty, primarily as female sex hormones begin to increase (54). In a study of body composition using whole body magnetic resonance imaging in men (n = 268) and women (n = 200) ranging from 18-88 years old, men had significantly more lean body mass than women in absolute (33 *vs.* 21 kg) and relative (38.4 *vs.* 30.6%) terms (28). Further, these differences were more evident in upper body (40%) compared to lower body (33%) (28). Data from the National Health and Nutrition Examination Survey provides further insight whereby, within any age group ranging from 12-80 years old, women have 6-12% higher body fat than men (55).

Beyond total fat and fat-free mass, there are also differences in tissue type distribution. Men typically carry more fat in the abdominal region as visceral fat, whereas women have more fat in the gluteofemoral region and have less visceral and more subcutaneous fat (56). Central adiposity is associated with poor long-term health outcomes as compared to having a more gynoid distribution (57). Further, sex-differences in anthropometry have implications on biomechanics, affecting performance and injury risk (58).

2.1.4 Fatiguability

Sex-based differences in fatiguability are task-specific and depend on factors such as intensity, musculature, dynamic versus isometric exercises, and time course of the contraction (i.e., sustained *vs.* intermittent) (43). Fatiguability, or fatigue-resistance, is reduction in force and power over the time course of a contract at a relative intensity (29). Women are generally more fatigue-resistant than men at lower intensity contractions, whereas, sex differences become less apparent as intensity increases (59, 60). Muscle region is an important factor as well with men becoming fatigued more quickly than women in isometric and intermittent contractions of the knee extensors (42), dorsiflexors, back extensors, finger flexors (61), elbow flexors (62, 63), and adductor pollicis (64). Interestingly, when men and women are strength-matched, sex differences dissipate for sustained isometric contractions (65) but remain for intermittent isometric contractions (66), suggesting different mechanisms for sex-dimorphisms based on work-rest cycles.

Variations in tissue perfusion, muscle substrate utilization, enzyme concentrations, proportions of type I versus type II muscle fibers make up some of the possible mechanisms for sex-based differences in fatiguability. Men have larger muscle mass which causes greater blood flow restriction and quicker accumulation of lactic acid during muscle contraction (67, 68). Blood

flow restriction due to larger muscle mass in men compared to women is less of a factor for causing muscle fatigue during intermittent contractions when muscles can re-perfuse. Sex differences in energy substrate utilization may also have an effect on performance fatiguability. Women oxidize more fat and less carbohydrate than men when working at the same relative intensity (51). Compared with glycolysis, fatty acid oxidation results in less accumulation of acidic by-products of metabolism, which serve as signaling molecules to the central nervous system to reduce exercise intensity (69). The higher rates of fatty acid oxidation in women is likely influenced by estradiol (38); however, fluctuations in female sex hormones across the menstrual cycle have a minor influence on performance fatiguability, whereas susceptibility to fatigue increases after menopause (43). As mentioned in a previous section, women also have a greater percentage of type I fiber surface area relatively to type II fibers, the former being more fatigue resistant.

In summary, men have less fat mass, more lean muscle mass, larger muscle fibers, more type II fibers, and larger organs than women which translates to greater absolute strength, power, aerobic and anaerobic capacity. Women, on the other hand, possess metabolic and anthropometric characteristics, such as greater fatty acid oxidation and less blood vessel occlusion during muscle contraction, which may result in greater fatigue resistance or quicker recovery than men. Physical attributes of either sex should be viewed in the context of a particular task.

2.2 Military Operational Stress

Intense military operational stress negatively impacts physical performance and alters circulating hormonal and metabolic milieu (70). However, the majority of data are in men. The following sections will highlight the effects of military operational stress on physical performance

and endocrine signaling in men, followed by studies that directly compare men and women undergoing the same training stress.

2.2.1 Data in Men Undergoing Military Field Training

Post-World War II, Dr. Ancel Keys published a two-volume tome (71), now referred to as the "Minnesota Starvation Study", detailing the effects of semistarvation conditions and physical labor on fitness and psychological health, and providing one of the first comprehensive glimpses into what occurs when men are severely underfed (72). The study was funded by the US Department of Defense with the aim of understanding the effects of starvation on prisoners of war and how to best refeed them upon return from captivity. Thirty-four conscientious objectors were recruited and fed a eucaloric diet (~3200 kcal·day^{-1}) for three months, followed by a six-month period on a hypocaloric diet (~1800 kcal·day^{-1}) combined with regular physical work to expend ~3000 kcal·day^{-1}. Throughout the protocol, body mass, fitness, and health were meticulously measured (71). Following the six-month interventional period, subjects had lost over 25% of their body mass, and $\dot{V}O2_{max}$ declined by 42%, while grip and back strength fell by 30% compared to pre-starvation, suggesting that aerobic fitness was more compromised than strength (73). There was also a significant increase in depressive symptoms and apathy, an obsessed preoccupation with food, and overwhelming lethargy, highlighting the relationship between psychological state and underfeeding (73).

One of the pioneers in military stress physiology, P.K. Opstad, later demonstrated the effects of multi-stressor conditions on endocrine status using an intense 5-day military field training exercise as a model of operational stress (74–79). Training was characterized by near constant physical work, environmental extremes (e.g., cold, darkness), underfeeding (12% to 17%

of measured expenditure), and 0-3 h of sleep per night. Under these conditions, androgen concentrations (testosterone, dihydrotestosterine, androstenedione) declined by 50% to 80%, metabolic hormones (triiodothyronine, thyroxine) decreased by 23% to 33%, and bimodal responses were observed for human growth hormone, noradrenaline, adrenaline, and cortisol (74–79). These data were the first to demonstrate changes in physiological status indicative of overreaching or overtraining syndrome during intense military training (80).

A lineage of military researchers went on to expand these early reports using military field training exercises and simulated lab-based scenarios to model the multi-stressor conditions that occur during military operations and study their effects on physical performance and endocrine signaling. For example, after three days of sustained operations characterized by a ~3,000 $kcal \cdot d^{-1}$ energy deficit and limited sleep (3.6 h total), Nindl et al. (81) reported a 9% decline in lower-body ballistic power and a 7% increase in obstacle course completion time. Following one week of Norwegian Special Forces training, candidates experienced 20% and 28% reductions in lower-body strength and jump height, respectively (82). Over the same period, an in cortisol (+154%) and a decrease in testosterone (−70%) and insulin-like growth factor-I (IGF-I) (−51%) was observed (82). Physical performance remained depressed two weeks after training whereas anabolic status (i.e., IGF-I and testosterone) had returned to or surpassed baseline concentrations while cortisol remained higher than pre-training levels (82). After 21-days of intense field training, Ojanen et al. (83) reported a 6% decrease in jump performance and declines in muscular endurance as measured by the number of sit-ups (−9%) and push-ups (−15%) in male conscripts. Using a comprehensive battery of tests, Conkright et al. (10) reported a significant decline in physical performance that persisted for six weeks following 61-days of Army Ranger training in events measuring speed and agility (−15%), anaerobic capacity (−7%), core strength (−27%), and aerobic

endurance (−24%). Henning et al. (9) reported corresponding alterations in testosterone (−70%), total and free IGF-I (−39% and −41%, respectively), brain-derived neurotropic factor (−33%), and cortisol (+25%). All hormones, except triiodothyronine, had returned to baseline concentrations within 2-6 weeks after training. In the same Army Ranger training, Friedl et al. (84) demonstrated that the addition of 400 kcal per day during negative energy balance resulted in recovery of triiodothyronine, testosterone, and IGF-I despite no changes in any other operational stressors (e.g., sleep restriction, physical and psychological stress, environmental extremes), suggesting the reliability of these hormones as biomarkers of acute energy deficiency in the context of other stressors. Taken together, short- and long-term studies of military training designed to mimic the stressors of combat demonstrate a substantial deterioration in physical ability, which is often preceded by alterations in circulating hormones, *in men*. These data emphasize the utility blood biomarkers as early indicators preceding declines in physical performance.

2.2.2 Data in Men and Women Undergoing the Same Military Field Training

Men and women experience differential responses in certain physiological outcomes. Hoyt et al (49) reported significant differences in the amount of fat-mass and fat-free mass lost by men and women following intense military training. After seven days of semi-starved conditions (i.e., <100 kcal·d^{-1} intake) juxtaposed with total energy expenditure exceeding 5,000 kcal·d^{-1}, both men and women lost a significant amount of body mass (−7.7 ± 1.1 and −5.9 ± 1.1 kg, respectively). Men and women lost a significant amount of body mass (−7.7 ± 1.1 *vs.* −5.9 ± 1.1 kg), fat-free mass (−4.0 kg *vs.* −2.5 kg), and fat-mass (−3.5 *vs.* −3.4 kg). However, women lost more fat-mass and less fat-free mass relative to total body mass compared to men. Consistent with the changes in body composition, women oxidized 40% more fat as a proportion of total energy expenditure

during training compared to men. In a recently published study, Vikmoen et al. (85) directly compared physical performance changes in men and women enduring a 5.5-day Norwegian special operations selection exercise. Both sexes experienced similar declines in countermovement jump (CMJ) height immediately post-training (−18% to −19%), but women recovered more quickly than men at 3 days (women: −14% *vs.* men: −24%) and 2 weeks (women: −9% *vs.* men: −17%) post-training when compared to baseline. Peak lower-body power followed a similar pattern, although only women had fully recovered by two weeks (men: −11%). Despite differences in recovery between sexes, men had a higher CMJ height and produced more power than women at all time points. In contrast to CMJ, performance in the casualty evacuation drag and 10-kg medicine ball toss deteriorated and recovered similarly in men and women. The differences in recovery between men and women in some, but not all events may be explained by sex-specific variations in energy utilization, neuromuscular drive, or muscle morphology such that women are more fatigue resistant in some tasks but not others (86). The U.S. Marine Corps completed a field study to compare performance between male-only and sex-integrated squads during operational training scenarios (87). After a 12-month integration process, researchers concluded that women were able to perform physically demanding tasks, but not at the same level as their male counterparts. The integrated squads performed at a lower level than the all-male squads in task completion, movement under load, and timely mission completion, including 3% to 35% slower 7-km ruck march times, 5% to 159% slower casualty evacuation times, and 10% to 60% slower employment of select weapon systems. While this field study gives insight into the effects of simulated combat missions with good ecological validity, results from this study should be interpreted with caution due to the lack of adequate control measures, including varying leadership experience and different numbers of female marines per squad.

Women are able to perform many of the same occupational tasks as men but experience greater physiological strain when doing so. A field study by Blacker et al. (88) demonstrated that when military men and women perform similar tasks, men operated at a lower heart rate reserve (HRR) than both women in the same platoon (24 ± 2% *vs.* 32 ± 2% HRR) and men in a male-only platoon (24 ± 2 *vs.* 33 ± 2 %HRR). In contrast, women had a similar heart rate in the mixed-sex and female-only platoons (33 ± 2 *vs.* 33 ± 3 %HRR). These findings have been corroborated in different trials at the same training unit (89, 90), reporting that men performed 12% to 13% more physical work and expended more energy during the first two weeks (16.8 ± 2.6 *vs.* 11.9 ± 1.4 $MJ \cdot d^{-1}$, $p < 0.001$) and the last two weeks (17.8 ± 2.3 *vs.* 14.2 ± 1.4 $MJ \cdot d^{-1}$, $p < 0.001$) of training compared with women but with no difference in %HRR (89, 90) or rating of perceived exertion (89). The relationship between cardiovascular fitness and %HRR in each of these studies suggests that cardiovascular strain may be explained by fitness rather than sex (88–90). O'Leary et al. (91) reported similar findings in male and female basic trainees completing a paced, 90 min 9.7 km loaded march carrying similar loads (15 or 20 kg, depending on occupation); heart rate was higher in women compared with men ($b \cdot min^{-1}$: 9% and %HRR: 15%). Despite women carrying a greater relative load than men (25% *vs.* 22% body mass and 36% *vs.* 28% fat-free mass), men had a greater decline in knee extensor maximal voluntary contraction force than women (−9 ± 13% *vs.* −12 ± 9%), but similar reductions in vertical jump height (−5 ± 6% *vs.* −5 ± 11%) suggesting that fatiguability due to load carriage may affect single-joint, slow contractions of the knee extensor more so than multi-joint, explosive vertical jumps.

2.3 Physical Training for Performance Optimization

Physical performance gaps between men and women, as well as declines resulting from operational stress exposure, may be offset through exercise training. In a comprehensive review, Varley-Campbell et al. (92) reported significant fitness improvements in male basic trainees, but greater absolute improvements in their female counterparts. Specifically, aerobic fitness increased by 7.4% in women and 4.0% in men; run times increased by 10.4% and 5.7%; Wingate performance improved by 3.7% and 0.1%; whole-body strength increased by 13.5% and 9.3%; upper-body strength went up by 13.0% and 6.9%; lower-body strength improved by 10.5% and 7.0%; and muscle endurance went up by 27.2% and 19.6%. Greater gains in fitness performance in women compared with men were likely due to women having lower baseline fitness. Interestingly, power declined by −13.3% in men and −17.9% for women. A recent meta-analysis by Roberts et al. (93) examined the sex-dependent effects of resistance training and concluded that men and women respond similarly in hypertrophy (effect size = 0.07 ± 0.06; $p = 0.31$; $I^2 = 0$) and lower-body strength (effect size = -0.21 ± 0.16; $p = 0.20$; $I^2 = 74.7$), but women had a better response to training than men in upper body strength (effect size = -0.60 ± 0.16; $p = 0.002$; $I^2 = 72.1$).

Targeted training programs in place of the general fitness programs, typical of basic military training, may facilitate optimized physical performance in women. Long-duration (*e.g.*, >4-6 months), multi-modal exercise programs have produced significant increases in muscle hypertrophy and force-generating type II muscle fibers in military-aged women (94–98). These physiological changes translate into substantial improvements in fitness and military occupational task performance, including an increase of 16% to 19% in manual material handling capability (97), 30% to 47% in maximal occupational lifting capacity (96), 18% to 32% in repetitive lifting

capacity, 24% in load carriage ability, and 14% in aerobic endurance (95). These data suggest improvements in physical capabilities can be achieved through general and, more so, targeted exercise programs which may reduce the physiological strain in women relative to men when performing a given task and offset the unavoidable decrements in physical performance when exposed to intense military operational stress.

Taken together, although women experience greater physiological strain than men at a given effort, women are able to complete many of the same physical and occupational tasks as men. Targeted physical training programs may reduce the fitness gap between sexes and offset inevitable declines experienced during exposure to operational stress. The studies presented here lay a foundation for future work to investigate sex differences in the context of operational stress and to develop training strategies for optimal performance of sex-integrated units.

2.4 Biomarkers

Circulating blood factors mediate many of the physiological changes occurring during metabolic stress, including operational (dis)stress, where health and fitness generally decline, and exercise training (eu)stress, where performance is expected to increase assuming adequate recovery (4). Two categories of circulating biomarkers include hormones and EVs. Each biomarker type will be described below, with particular focus on the more novel EVs.

2.4.1 Hormones

Hormones are functional elements of the endocrine system which are predominately controlled through hypothalamic-pituitary regulation (99). Hypothalamic-pituitary control ensures integrated coordination between the endocrine and central nervous systems. Hormones control a wide-range of biological functions including growth, development, metabolism, electrolyte balance, and reproduction, and exert their effects by entering cells and binding to internal cell receptors (i.e., steroids and amino acids) or through ligand-receptor activation of intracellular signaling pathways (i.e., polypeptides) (99).

2.4.1.1 Growth Hormone (GH)

GH consists of a complex group of molecular isoforms ranging in size from 5 kDa to upwards of 66 kDa, with the 191-amino acid, 22 kDa isoform being the most studied (100). GH is secreted in a pulsatile manner with notable peaks occurring around midnight, mid-day (~1100-1200 h, and evening (~1600-1800 h) (101). GH receptors in tissues throughout the body are evidence of its ubiquitous and vast role in cellular growth. GH exerts its effects on cellular growth directly, via the MAPK/ERK pathway, and indirectly through stimulation of IGF-I (102). GH also mediates energy provision by decreasing lipoprotein lipase and increasing hormone sensitivity lipase and lipolysis (103). Exercise produces a robust release of GH in an intensity-dependent manner, making it particularly attractive for study of acute bouts of exercise, with higher intensity and short rest periods resulting in a greater magnitude of response (100). Previous data demonstrate sexually dimorphic patterns of GH during exercise with women experiencing a greater increase and an earlier peak in GH concentration during exercise compared to men (31, 32). This sex-related difference in release, however, disappears after ~30 min.

2.4.1.2 Insulin-Like Growth Factor-I (IGF-I)

IGF-I is a polypeptide hormone produced primarily in the liver in response to growth hormone signaling, and also locally in tissues such as skeletal muscle (104). The majority of IGF-I existing in circulation is derived from the liver with smaller amounts found in interstitial fluid, transdermal body fluid, and directly in tissues such as muscle, bone, and nerve tissue (105). IGF-I exists in many forms including free circulating (<2%), bound in a binary complex to one of six IGF-I binding proteins (IGFBP) (20-25%), or in a ternary form bound to IGF-I binding protein-3 (IGFBP-3) or IGF-I binding protein-5 (IGFBP-5) plus acid-labile subunit (>75%) (105). The binding proteins extend the life of IGF-I in circulation and mediate its activity at the cellular level. IGF-I binding proteins also have functions independent of IGF-I, such as the promotion of cell migration, adhesion, and differentiation (106). IGF-I plays a role in metabolism, growth, and tissue repair and has been shown to be sensitive to changes in energy balance and body mass (107), making it a useful biomarker in military research. Data from both intense short-term (i.e., 5-days) field training (85) and longer-term (i.e., 4 months) initial entry training (108) military studies have noted differential responses in IGF-I concentrations in women compared with men across the stress.

2.4.1.3 Cortisol

Cortisol is the primary glucocorticoid present in the body and is regulated through a negative feedback loop involving the hypothalamus, corticotropic releasing hormone, and adrenocorticotropic hormone (99). Cortisol is produced by the adrenal cortex (109) and is often associated with the response to stress (9, 110) with peak concentrations being reached approximately 30 min after stress exposure (111). Exercise stress causes an intensity-dependent release of cortisol with moderate to high intensity (i.e., >60% $\dot{V}O2_{max}$), but not low intensity (i.e.,

<40% $\dot{V}O2_{max}$), exercise causing a significant increase in circulating concentrations (112). Cortisol plays a role in energy homeostasis via upregulation of gluconeogenesis and protein and lipid breakdown. Acutely, this response is beneficial by making nutrients available for use during stress but becomes detrimental to health if concentrations remain high for extended periods (i.e., days or months). Under normal conditions, cortisol is highest in the morning and tapers off throughout the day, but this diurnal pattern can become dysregulated with prolonged intense stress or disrupted sleep-wake cycles (113).

2.4.1.4 Brain-Derived Neurotrophic Factor (BDNF)

Primarily known for its role in neural growth and plasticity, BDNF also mediates energy metabolism by decreasing appetite and enhancing lipid oxidation during glucose deprivation via activation of the AMP-activated protein kinase – a master regulator of cellular energy metabolism (114). BDNF has a bimodal response with short-term stress, such as energy restriction, causing an increase in BDNF following the switch from glucose- to ketone-dependent energy metabolism (115), and declining concentrations associated with longer-term stress (9). A large meta-analysis by Dinoff et al. (116) which included 55 studies and 1180 participants (75.4% men), concluded that men, but not women, had a significant increase in BDNF concentration following exercise. Higher aerobic fitness and greater muscle mass are both associated with increased BDNF response after exercise (116). Therefore, greater aerobic fitness and muscle mass in men compared to women may explain the differences in BDNF response following exercise. Furthermore, sex-specific response patterns emerging from the meta-analysis should be interpreted with caution as there were five times more men than women in the studies included in the meta-analysis. Thus, there may have been an effect of exercise on BDNF concentration in women if more women were included in the studies.

2.4.2 Extracellular Vesicles

Complex organisms maintain homeostasis through an evolutionarily conserved mechanism of intercellular communication via transmission of lipid bilayer membrane enclosed nanoparticles from host to target cells (13). These nanoparticles, dubbed extracellular vesicles, are secreted by all cells and are found in every biofluid where they have been studied, including blood, urine, breast milk, saliva, cerebrospinal fluid, and sweat (117). The ubiquitous presence of EVs in multiple biocompartments suggests their vast influence in long-range cellular crosstalk (13), including inter-species transfer (118) and host-pathogen interactions (119). Extracellular vesicles promote physiological change in pathological and non-pathological states through delivery of the cargo, which includes proteins, lipids, and nucleic acids (Figure 3) (120, 121).

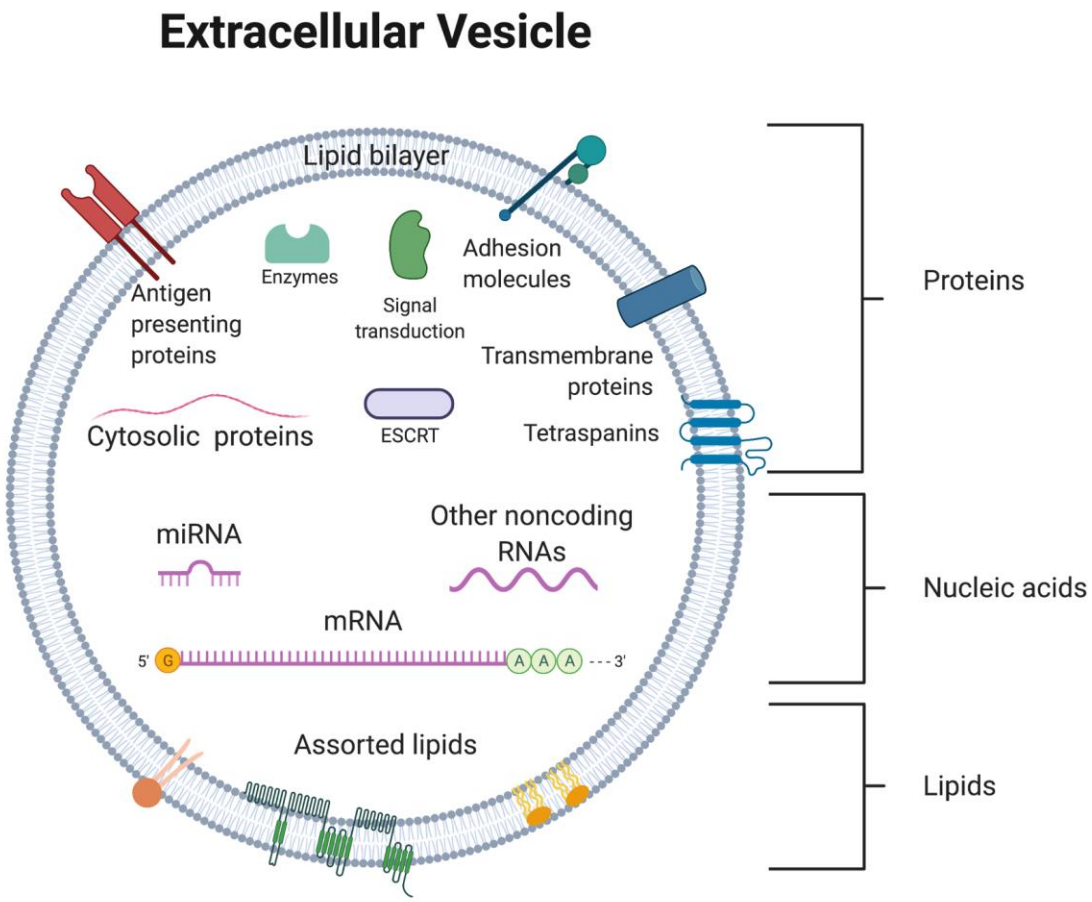


Figure 3. Extracellular vesicle illustration.

Extracellular vesicles contain proteins, nucleic acids, and lipids protected by a lipid bilayer membrane. Figure was created using BioRender.com.

2.4.2.1 Extracellular Vesicle Subtypes

The term "extracellular vesicle" is broadly used to a heterogenous groups of nanoparticles released from a host cell without the ability to replicate (122). According to the International Society of Extracellular Vesicles, EVs can be further classified based on descriptive characteristics, including size (e.g., small, or <200 nm, *vs.* medium/large, or >200 nm), molecular characteristics (e.g., CD63+/CD9- EVs), or by condition or host cell type (e.g., hypoxic EVs,

skeletal muscle-derived EVs) (122). Extracellular vesicles may also be denoted by their biogenesis pathway, which can be classified as being of either endosomal (e.g., exosomes) or ectosomal (e.g., microvesicles, apoptotic bodies) origin.

Plasma membrane-derived EVs, or ectosomes, include apoptotic bodies (ApoBDs) and microvesicles (MVs), whereas exosomes (EXO) are of endosomal origin (Figure 4). Ectosomes are generally larger relative to EXO with ApoBDs ranging in size from 50-5000 nm and MVs from 100-1000 nm, whereas EXOs are typically considered to be <150 nm (117). While there is clear overlap between EV subtypes, there is no definitive stance on these size ranges, and the upper end of the size range of EVs is yet to be determined, although most studies focus on sub-micron size EVs (123).

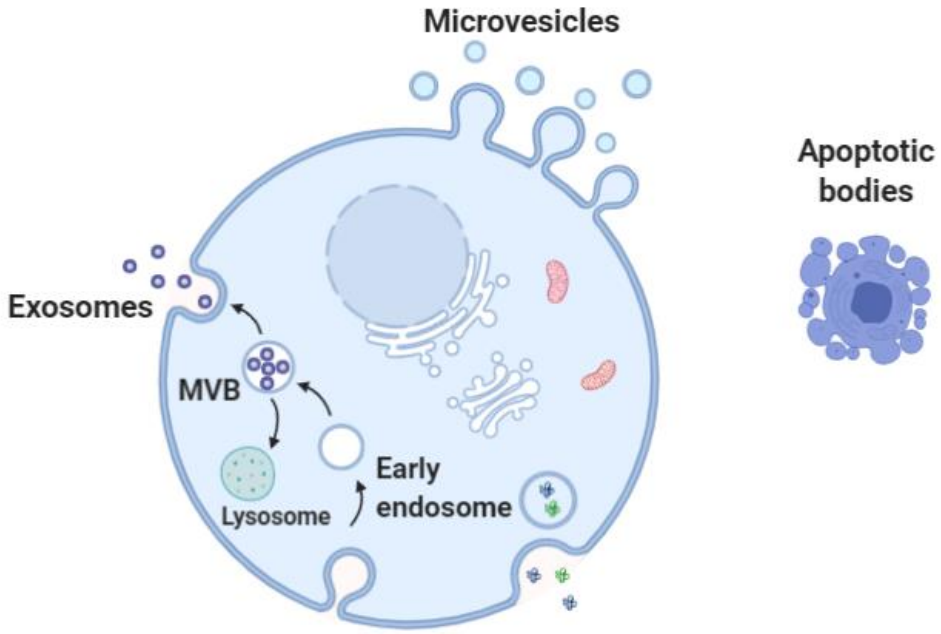


Figure 4. Extracellular vesicle subtypes and biogenesis pathways.

Figure was created using BioRender.com.

2.4.2.2 Biogenesis, Composition, and Tissue Crosstalk

A distinguishing characteristic of EV subtypes is their biogenesis pathway. Exosomes undergo an invagination process from the cellular membrane, forming multivesicular bodies which contain intraluminal vesicles (Figure 4) (117). This process of formation and sorting is coordinated through an ensemble of proteins and includes both endosomal sorting complex required for transport (ESCRT)-dependent (124) and -independent mechanisms (125). Multivesicular bodies are then either directed to undergo lysosomal degradation or to the plasma membrane for release into the extracellular environment (117). Multivesicular body fate is poorly understood, though it may be connected to vesicular attributes such as cholesterol (126) and major histocompatibility complex class II content (127). Upon release, intraluminal vesicles are considered exosomes in the extracellular space. During the biogenesis process, exosomes are typically enriched with specific cargo, including tetraspanin markers such as CD63, CD81, and CD9 (15). These tetraspanins are commonly used to identify the exosome subpopulation, but have also been observed in microvesicles and apoptotic bodies (128). In contrast, ectosomes (i.e., microvesicles and apoptotic bodies) are products of plasma membrane budding or shedding; unlike exosomal biogenesis, microvesicle formation is less well understood, but is thought to involve contractile machineries such as actin and myosin in combination with lateral pressures caused by protein-protein interactions, causing an outward enrichment and budding (129). Apoptotic bodies undergo a similar shedding process, though under different circumstances (i.e., programmed cell death) (130). Apoptotic bodies play a key role in maintaining homeostasis through clearance of cellular by-products and immune signaling (130). Similar to exosomes, there are no exclusive markers for ectosomes, although ADP-ribosylation factor 6 (ARF6) and vesicle-associated membrane protein

3 (VAMP3) are commonly associated with microvesicles (131, 132), and annexin-V, thrombospondin-1 (THSD1), and C3b are related to apoptotic body formation (132, 133).

It is still unclear how EVs target specific recipient cells, though some evidence points to canonical ligand-protein interactions (134). For example, Saunderson et al. (135) reported enrichment of the adhesion molecule CD169 in exosomes resulted in uptake by the spleen and lymph nodes. In the same study, EV uptake was inhibited in the lymph system in CD169 knockout mice. Other studies have demonstrated similar targeting mechanisms between EVs and recipient cells (134). EV uptake in target cells occurs through macropinocytosis, fusion, or phagocytosis. The method of uptake likely depends on the biophysical properties of the recipient cell (134).

Extracellular vesicles are enriched with membrane and cytosolic proteins, lipids, and nucleic acids which then get transferred to recipient cells and induce a diverse range of physiological change (15, 136) providing a means of tissue crosstalk (Figure 5) (18, 137). For example, following 1 h of cycling, quantitative proteomics analysis of 5,359 EV-related proteins revealed alterations in over 300 proteins, including selective enrichment of proteins associated with small EVs (e.g., CD63, CD81, TSG101, etc.) (137). Intravital imaging demonstrated EV uptake in a range of tissues but mostly in the liver (137). Exercise training also causes alterations in nucleic acid content of EVs (18, 138). In an eloquent series of experiments, Castaño et al. (18) observed significant upregulation of six different miRNA including, a 11-fold increase of miR-133b-3p, and downregulation of one miRNA following 5-weeks of high intensity interval training. Network pathway analysis and subsequent experiments revealed that miR-133-3p targets the forkhead box O1 (FoxO1) pathway, resulting in improved insulin sensitivity and glucose tolerance. Guescini et al. (138) reported a 4-fold increase in miR-206 following 40 min of vigorous intensity (80% $\dot{V}O2_{max}$) exercise. Using a different mode of exercise, blood flow restriction training, Just et

al. (139) observed significant alterations in 12 miRNAs involved in skeletal muscle turnover processes such as mTOR signaling, AKT/mTOR pathway, NFκB activation, and more. Importantly, neither size or concentration changed from pre- to post-blood flow restriction exercise, demonstrating that EV cargo changes can occur independent of characteristics such as size and concentration. Quantitative and stoichiometric analysis demonstrated that, on average, less than one copy of miRNA was present per small EV (140), suggesting that EVs are selectively enriched, likely based on the imposed demands on the cellular microenvironment. In this way, EVs are information-rich biomarkers and may be considered a *biological thumbprint*.

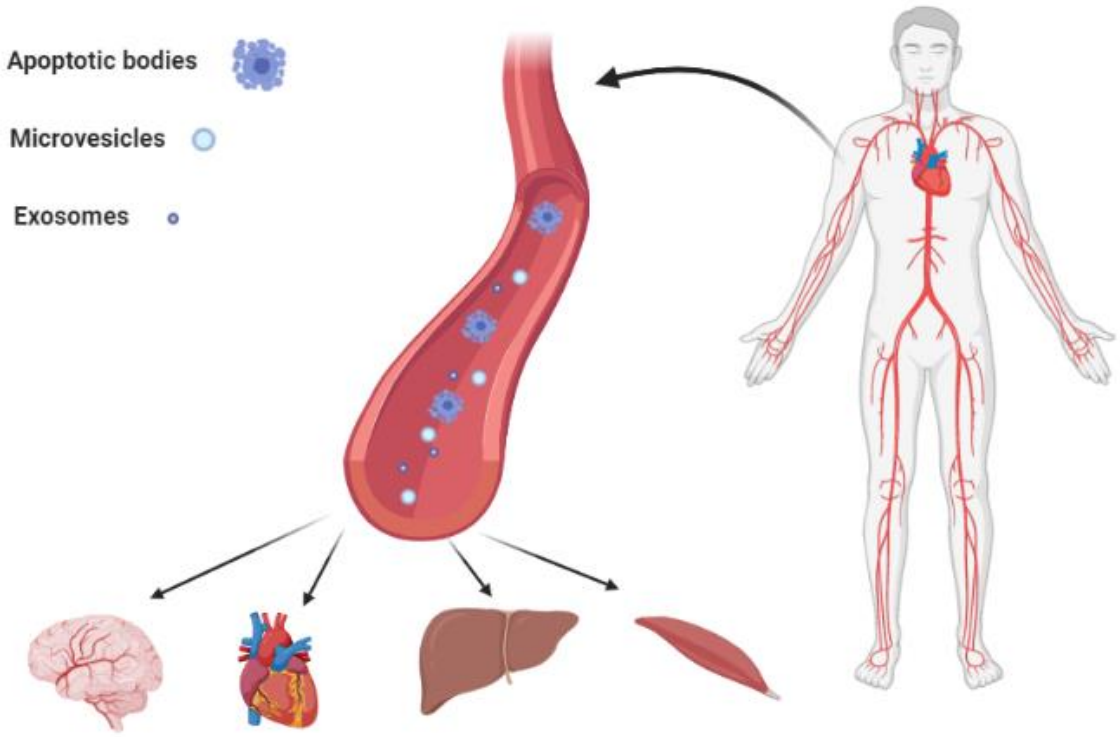


Figure 5. Extracellular vesicles facilitate tissue crosstalk.

Figure was created using BioRender.com.

Extracellular vesicles are rapidly released into circulation during exercise and return to baseline within 60-90 min (18, 137, 141, 142). More specifically, Frühbeis et al. (141) examined

EV kinetics during an incremental cycling exercise protocol to exhaustion (16 ± 5 min total time). Blood was collected every 3 min after exercise initiation for 90 min total. Their analysis revealed an increase in circulating EVs above baseline at 9 min into the exercise bout, which peaked between 15-21 min, followed by a steady return to baseline around 90 min after exercise was initiated.

Extracellular vesicle secretion may be sex- and exercise intensity-dependent. Using a randomized cross-over design, Shill et al. (36) examined the effects of sex and exercise intensity on circulating EV concentrations using two different exercise protocols: (1) moderate intensity exercise at 65% $\dot{V}O2_{max}$ and (2) high intensity interval training consisting of 10 x 1 min intervals at 90% to 100% of $\dot{V}O2_{max}$ with 75 s of recovery. Each exercise session was matched for energy expenditure. Moderate intensity exercise resulted in an 18% decrease in endothelial-derived (CD62+) EVs from baseline to post-exercise, whereas high intensity interval training had no effect in CD62+ EV concentration. In women, but not men, CD62+ EVs by decreased 19% at post-exercise compared with baseline during the moderate intensity session. There were no differences in results for women after controlling for menstrual cycle phase. Further, Rigamonti et al. (143) reported a greater increase in moderate to large sized EVs (130-700 nm) in women compared with men, but a lower release of small EVs (30-130 nm) immediately after moderate intensity exercise (60% $\dot{V}O2_{max}$) for 30 min or until volitional exhaustion. Taken together, physiological stress, such as exercise, causes substantial alterations in EV cargo which may be sex- and intensity-dependent. Of note, few studies have examined the effects of resistance exercise (i.e., weight training) on EV properties (139, 144, 145).

3.0 Methods

3.1 Experimental Design

Data for Specific Aims 1 and 2 were derived from a parent study titled “Impact of Military Operational Stressors on Cognitive Performance” (CORES; Department of Defense Award # W81XWH-17-2-0070) aimed at understanding characteristics and predictors of cognitive and physical resilience during simulated military operational stress. Data for Specific Aim 3 were derived from the parent study titled “Soldiers Performance and Readiness as Tactical Athletes” (SPARTA; United Kingdom Ministry of Defense Award # WGCC 5.5.6 - Task 0107) aimed at optimizing physical readiness and training for military occupational performance in men and women. Both parent studies utilized a within-subjects, repeated measures prospective cohort design.

3.2 Participant Recruitment

3.2.1 Participant Recruitment for Specific Aims 1 and 2

Subjects were recruited using electronic correspondence, flyers, and in-person briefings. Interested personnel contacted the study staff directly and were pre-screened telephonically to verify eligibility. Eligible participants provided written, informed consent prior to participation. Participation was voluntary, and subjects could withdraw at any point in time. For aim 3,

3.2.2 Participant Recruitment for Specific Aim 3

Subjects were recruited via email, listservs, electronic mailing list, flyers, social media, new stations, online advertisement (Pitt+Me), and in-person communications. Following a telephonic pre-screen, eligible participants provided written, informed consent. Participation was voluntary, and subjects could withdraw at any point in time.

3.3 Participant Characteristics

Specific Aims 1 and 2 consisted of male and female military service members. A subgroup of 10 men and 10 women were selected for analysis in Specific Aim 2. Specific Aim 3 was comprised of a subgroup of 10 men and 10 women that were healthy and recreationally active.

3.3.1 Inclusion Criteria

3.3.1.1 Inclusion Criteria for Specific Aims 1 and 2

Individuals qualified for the study if they a) were between the ages of 18-41 years old and currently serving or recently (within 2 years) separated in either Active or National Guard/Reserve components of the United States Army, Marines, Navy, Air Force, or senior Reserve Officer Training Corps cadets b) had an absence of any current (within 1 year) mental health, mood, anxiety, or addictive disorders or conditions; c) had passed their most recent service-specific physical fitness test; and d) had qualified within the last year or had a high level of comfort with shooting a M4/M16.

3.3.1.2 Inclusion Criteria for Specific Aim 3

Healthy recreationally active men and women between the ages of 18-36 years old qualified for the study. Participants had to be weight stable (i.e., <5-10 lbs fluctuation) for at least six months. Subjects were free of any musculoskeletal conditions or conditions that could influence the results of the study.

3.3.2 Exclusion Criteria

3.3.2.1 Exclusion Criteria for Specific Aims 1 and 2

Individuals were excluded if they self-reported: a) using any prescribed or over-the-counter medications known to affect sleep, or cognitive performance (e.g., hypnotics, benzodiazepines, antidepressants, beta blockers, corticosteroids, etc.); b) having an active substance abuse disorder; c) having past (< 6 months) or current suicidal or homicidal ideation; d) having past or current psychotic or bipolar disorder or significant sub-threshold symptoms of psychotic or bipolar disorders; e) being previously diagnosed with traumatic brain injury (TBI) with current chronic post-injury symptoms; f) being injured or had a recent (≤ 3 months) injury that would prevent participation in sport or military deployment; g) taking hormone replacement therapy (except birth control); h) having a history of neurological or seizure disorder; i) having a history of falls (<1 year); j) being pregnant; k) having untreated or unstable medical condition(s); l) having an apnea hypopnea index of greater than or equal to 15 as determined by an in-lab apnea screen prior to baseline assessments; or m) being unable to commit to duration of investigation (5-consecutive nights) or unwillingness to attempt all daytime activities. Additionally, recently separated individuals that gained more than 10% of body weight from time of discharge and currently exercising less than 150 minutes per week during separated status were also excluded.

3.3.2.2 Exclusion Criteria for Specific Aim 3

Individuals were excluded from the study if they were training for a competitive sporting event, had a ≥ 10 lbs weight fluctuation within the previous two months (self-report), had a medical condition precluding them from participating in intense physical activity, had a heart condition or high blood pressure, experienced chest pain at rest or during exercise, experienced dizziness or loss of consciousness in the previous 12 months, were diagnosed with a chronic medical condition, had been advised by a physician to only participate in physical activity under supervision, had an injury that prevented physical activity for more than one month in the previous two years, had any psychological disorders, were currently pregnant or planning to become pregnant during the study, or were unwilling to perform any of the testing procedures.

3.4 Power Analysis

All sample size calculations were performed using GPower 3.1.9.2 (Franz Faul, Universität Kiel, Germany).

For Specific Aim 1, based on a previous investigation reporting differences between men (n = 23) and women (n = 12) in countermovement jump changes following military field training (85), a sample size of 24 was determined to be adequate based on an α error probability of 0.05, 80% power, and an effect size of 0.46.

Based on a previous investigation able to detect differential responses between men and women in CD62E+ EVs following exercise (36), sample sizes of 16 and 20 were determined to be adequate for Specific Aims 2 and 3, respectively, based on an α error probability of 0.05, 80% power, and an effect size of 0.60.

3.5 Instrumentation

3.5.1 Profile of Mood States

The Profile of Mood States (POMS) questionnaire is a 65-item survey used to assess mood (146). Mood is assessed across six different domains, including vigor, tension, depression, anger, fatigue, confusion. Responses are rated using a 5-point Likert scale ranging from 0 (not at all) to 4 (extremely). Responses from each domain are summed (vigor was reverse scored) to derive a total mood disturbance score with a lower score corresponding to a more positive overall mood state and a higher score indicating a more negative overall mood state. The POMS scale has been demonstrated as a valid and reliable measure with internal consistency (alpha) coefficients for subscales ranging from 0.84 to 0.95 and test-retest reliability coefficients ranging from 0.65 to 0.74 (146). The POMS survey has previously been used in athlete (147) and military training studies (81, 148, 149) and is associated with cognitive and physical performance outcomes.

3.5.2 Borg Scale

The Borg Scale is a reliable indicator of overall effort and fatigue, or perceived exertion (150). The original self-report measure uses a scale ranging from 6-20 with a 6 indicating little to no effort and a 20 indicating maximal effort (used in Specific Aims 1 and 2). The original scale was designed to correspond with heart rate (e.g., RPE of 15 corresponds to a heart rate of 150 beats per minute) (151). The modified scale ranges from 1-10 where 0 is an effort of “nothing at all” and 10 is “very, very strong”.

3.5.3 Enzyme-Linked Immunosorbent Assays (ELISA)

For Specific Aim 1, biomarkers related to stress (cortisol), anabolic status (GH and IGF-I), and energy metabolism (IGF-I and BDNF) were measured using standard enzyme-linked immunoassay or magnetic bead-based (Luminex® / xMAP® technology; BDNF only) procedures. Serum was used for GH, IGF-I, and cortisol and plasma was used for BDNF. Estradiol and progesterone (Alpco) were measured in women from blood serum drawn in a fasted state (~0800 h) on day 0. Assay sensitivities were 0.5 ng/mL for GH, 0.09 ng/mL for IGF-I, 0.4 μg/dL for cortisol, 2.5 pg/mL for BDNF, 10 pg/mL for estradiol and 0.1 ng/mL for progesterone. All measures were performed in duplicate with intra-assay coefficients of variation of ≤ 10%.

For Specific Aim 2, serum myoglobin, creatine kinase, estrogen, and progesterone concentrations were measured using enzyme-linked immunoassays according to each manufacturer's protocol. Myoglobin (Alpco 25-MYOHU-E01; Salem, NH, USA) assays had a range of 25-1000 ng/mL and sensitivity of 5 ng/mL. Creatine Kinase MM (LSBio LS-F20706-1; Seattle, WA, USA) assays had a range of 1.563-100 ng/mL and sensitivity of 0.94 ng/mL. Estradiol (Alpco 11-ESTHU-E01; Salem, NH, USA) assays had a range of 20-3200 pg/mL and sensitivity of 10 pg/mL. Progesterone (Alpco 11-PROHU-E01; Salem, NH, USA) assays had a range of 0.3-60 ng/mL and sensitivity of 0.1 ng/mL. Each analyte was measured in duplicate with coefficients of variation of ≤ 10%.

For Specific Aim 3, estradiol and myoglobin were measured from serum using standard enzyme-linked immunoassay procedures. The range and sensitivity for estradiol assays (Alpco 11-ESTHU-E01; Salem, NH, USA) were 20-3200 pg/mL and 10 pg/mL, respectively. Myoglobin assays (Alpco 25-MYOHU-E01; Salem, NH, USA) had a range of 25-1000 ng/mL and sensitivity

of 5 ng/mL. Intra-assay coefficients of variation were ≤9.3% and ≤6.6% for estradiol and myoglobin, respectively. All samples were measured in duplicate.

3.5.4 Size Exclusion Chromatography

For Specific Aims 2 and 3, EVs were isolated from plasma using size exclusion chromatography (SEC) (qEV 70 nm Original; IZON, Medford, ME, USA) according to manufacturer's guidelines. Stored SEC columns were flushed with 10 mL of 0.22 μm filtered phosphate buffer solution (PBS) before adding 450 μl of plasma. The first 3 mL of eluate was discarded, and the next 1.5 mL (EV fraction) was collected for analysis. Isolated EV samples were aliquoted and stored at –80ºC until analysis. Size exclusion chromatography is a method of isolation based on size, shape, and molecular weight of particles, with larger particles eluting first, followed by smaller particles. qEV Original 70 nm SEC columns (IZON, Medford, ME) have an optimal isolation range of 70-1000 nm (152) and results in high purity isolation of EVs using a small volume of input and at low cost relative to other methods (153).

3.5.5 Nanoparticle Tracking Analysis

For Specific Aim 2, EV size and concentration (particles/mL) were determined using nanoparticle tracking analysis (NanoSight NS300, Malvern Panalytical Ltd, Malvern, UK) equipped with a 532 nm (green) laser. Samples were analyzed by a single, trained user. Ten microliters from each EV sample were diluted 1:100 in type 1 EV-free water and infused into the flow cell using a syringe pump (Harvard Apparatus 98-4730). Three 45-second videos were recorded for each sample with the camera level set to 14. The flow cell was flushed with 1 mL of

type 1 water between each sample. All samples were batch analyzed using computer software (NTA 3.4, build 3.4.003). Samples from each within-subject time point were assessed on the same day. The remaining isolated EV sample was divided into 150 µl aliquots and stored at −80°C for later analysis.

3.5.6 Vesicle Flow Cytometry (vFC™)

For Specific Aim 3, EV size and concentration were measured using the Vesicle Flow Cytometry™ assay (Cellarcus Biosciences, San Diego, CA, USA) on a CytoFLEX™ flow cytometer (Beckman Coulter, Brea, CA, USA) according to manufacturer's recommendations. Preliminary experiments were run to determine the optimal dilution for subsequent staining and analysis (Figure 6). Samples were stained using a membrane dye (1:100; vFRed™, Cellarcus Biosciences) and incubated for one hour in the dark at ambient temperature. Data was acquired on the CytoFLEX™ using the following settings: forward scatter (488 nm), side scatter (488 nm), and violet side scatter (405 nm) gains at 100; fluorescence gains at 1000 (all channels); primary trigger threshold using violet side scatter (405 nm); manual threshold of fluorescence above background at 1500; and width channel to 690/50. Data was collected for 120 seconds for each sample using the fast setting (60 µL/min) and analyzed using FCS Express 7 software (De Novo, Pasadena, CA, USA).

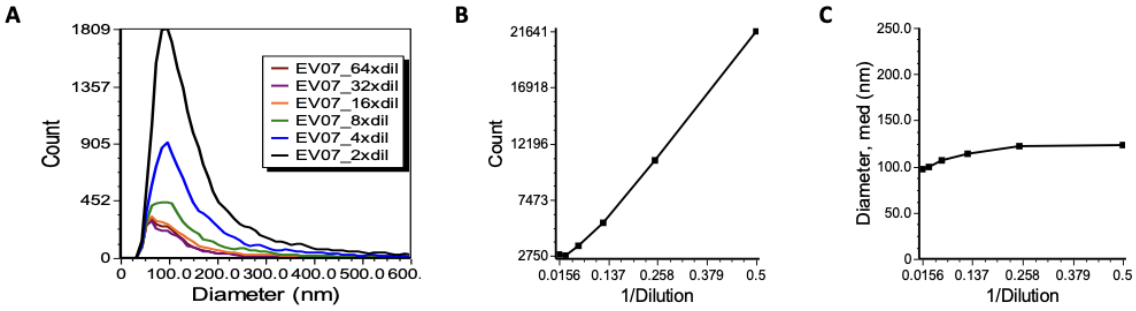


Figure 6. Vesicle Flow Cytometry dilution series experiment.

Samples were serially diluted to determine the optimal concentration for sample measurement. (A) Histogram of sample concentration at dilutions ranging from 2x to 64x. (B) Concentration decreased in a linear manner with each subsequent dilution. (C) Vesicle diameter was stable across dilution series demonstrating absence of swarm detection.

3.5.7 Imaging Flow Cytometry

For Specific Aims 2 and 3, isolated EV samples were fixed for 10 min using equal parts (140 µl each) of sample and 4% paraformaldehyde. Fixed samples were then centrifuged for 30 min at 16,000 x *g* at 4°C before removing 140 µl of supernatant and subsequently adding 140 µl of blocking buffer (3% bovine serum albumin, 0.1% Triton-X), then incubating on a rocker for 1 h at ambient temperature. Following the 1 h incubation, samples were centrifuged for 30 min at 16,000 x *g* at 4°C and 140 µl of supernatant was removed. Samples were then stained with the following antibodies: 0.5 µL anti-human CD63 Alex Fluor® 700 (1:280, NBP2-42225AF700, Novus Biologicals, CO, USA), 0.5 µL anti-human vesicle-associated membrane-3 (VAMP3) Alex Fluor® 405 (1:280, NBP1-97948AF405, Novus Biologicals, CO, USA), 1.4 µL anti-human thrombospondin-1 (THSD1) Alex Fluor® 594 (1:100, FAB5178T-100UG, Novus Biologicals,

CO, USA), and 0.35 µL anti-human alpha-sarcoglycan (SGCA) FITC (1:400 dilution, orb29665, Biorbyt, MO, USA). Stained samples incubated overnight at 4ºC. The following day, samples were centrifuged for 30 min at 16,000 x *g* at 4°C before removing 60 µL and adding 20 µL for a final volume of 100 µL.

Single-stained compensation controls (UltraComp eBeads™ Plus; Invitrogen) were used to correct for fluorescence carryover between channels. Fluorescence minus one (FMO) controls were used to establish gates for each spectral channel. Compensation and FMO controls followed the same staining protocol as outlined above with the exception that antibody volumes were half that of the volume used in samples.

Imaging flow cytometry (ImageStream®X Mark II, EMD, Millipore Sigma, Seattle, WA, USA) combines conventional flow cytometry capabilities, including forward and side scatter detection, with up to 10 fluorescent channels and two channels of high-resolution microscopy of up to 60x magnification in a high-throughput manner (154). Data was collected using INSPIRE™ software with the following settings: objective 60x, slow speed, high sensitivity, 7µm core size, auto-focus and auto-centering. Samples were run using normal gain mode for Specific aim 2 and high gain mode for Specific Aim 3. High gain mode enables better detection of small, dim particles on the ImageStream®X Mark II instrument compared to normal gain mode (155). Lasers were set to maximum voltage as follows: 405 nm 175 mW, 488 nm 200 mW, 561 nm 200 mW, 642 nm 150 mW, and SSC 70 mW. Data was collected for 3 min for all EV samples, and 2,000 events were collected for compensation and FMO controls. Samples from the same subject point were stained and analyzed on imaging flow cytometry on the same day to control for potential day to day variability. Intensity is used to measure protein expression (e.g., SGCA, THSD1, VAMP3, CD63) and estimated fluorescence activity.

3.6 Testing Procedures

3.6.1 Specific Aims 1 and 2: Simulated Military Operational Stress Protocol

Eligible male and female service members underwent a 5-day simulated military operational stress protocol characterized by frequent cognitive testing, simulated marksmanship, physical exertion, energy deficit, and sleep restriction (Figure 7). Participants arrived on day -1 (reception) and completed a series of screening and history questionnaires addressing psychological health and wellness parameters, military training and deployment history, and exercise training and injury history. Menstrual cycle and birth control data was collected on female participants. On day 0, participants completed body composition and physical fitness assessments and were familiarized to all study tasks. Body fat, fat-free mass, and estimated resting metabolic rate will determined using air-displacement plethysmography (Bod Pod® Body Composition System; Life Measurement Instruments, Concord, CA). The validity and test-retest reliability (ICC = 0.99) have previously been reported (156, 157). Estimated total daily energy expenditure (TDEE) was derived using the predicted resting metabolic rate and applying an "active" physical activity factor of 1.6. Relative peak oxygen consumption ($\dot{V}O2_{peak}$) was measured directly using a metabolic cart (Parvo TrueOne® 2400; Salt Lake City, UT) during an incremental Bruce protocol (158) until volitional exhaustion on a treadmill (Woodway; Waukesha, WI). Days 1-4 had a similar schedule except that sleep and caloric intake was restricted. On unrestricted days (days 1 and 4), participants were afforded 8 h of sleep opportunity from 2300-0700 h and 100% of their estimated caloric needs. On the nights leading into days 2 and 3, sleep was restricted to two 2-hr blocks (0100-0300 h and 0500-0700 h) and separated by cognitive testing. Caloric intake was reduced to 50% of estimated energy needs on days 2 and 3. Caloric intake was controlled using a standardized

breakfast and the remaining meal(s) consisted of Meals, Ready to Eat (MRE) individualized to each participant's estimated caloric needs based on calculated TDEE as noted above. Breakfast was consumed at ~0830 h, lunch at ~1500 h, and dinner at ~2100 h on unrestricted days. Participants only received breakfast and lunch on restricted days (days 2 and 3). Meals were consumed at the same times each day. Body mass was measured each morning (~0730 h) in a fasted state using a digital scale (Health-o-meter®, model 349KLX, Pelstar® LLC, McCook, IL). Sleep was measured using polysomnography (159).

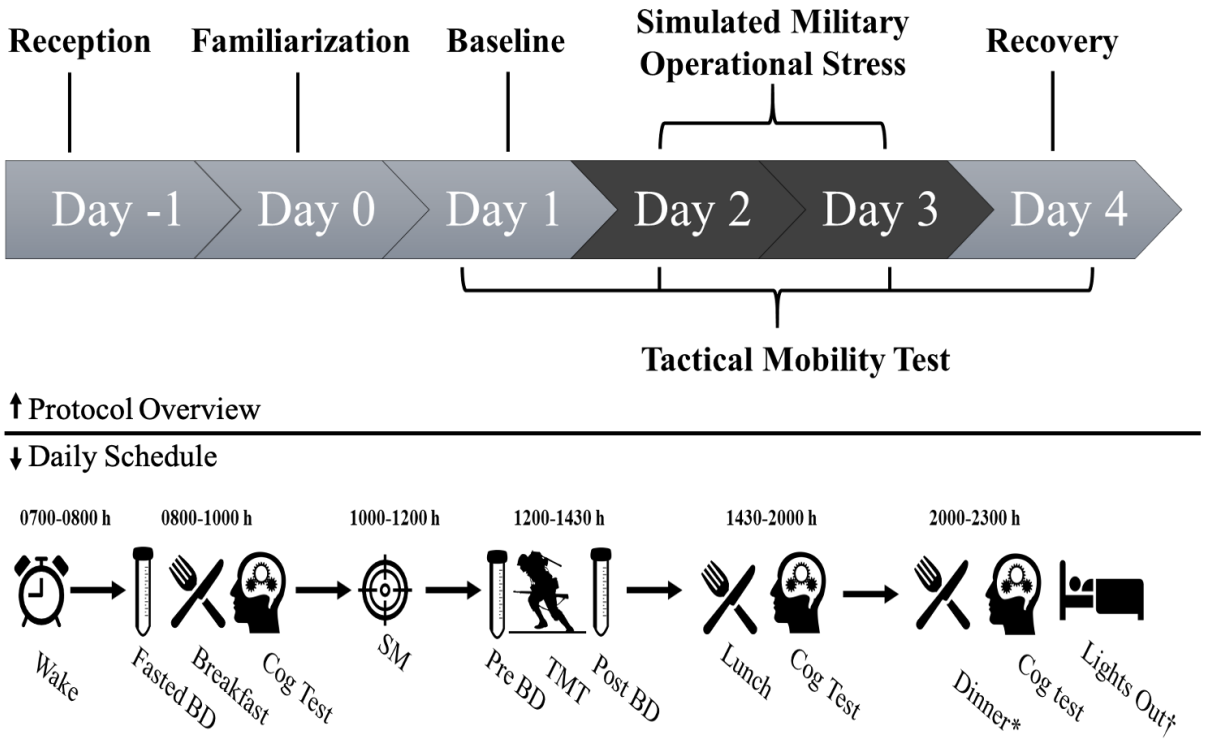


Figure 7. Five day simulated military operational stress protocol overview and daily schedule.

BD = blood draw. Cog = cognitive. SM = simulated marksmanship. TMT = tactical mobility test. *Dinner not provided during caloric restriction (days 2 and 3). †Sleep hours duirng nights leading into days 2 and 3 were from 0100-0300 h and 0500-0700 h.

3.6.2 Specific Aims 1 and 2: Tactical Mobility Test

Participants completed an occupationally-relevant physical exertion protocol called the Tactical Mobility Test (TMT) each day at approximately 1200 h. An overview of the TMT layout is presented in Figure 8. The testing battery consisted of seven unique events conducted in a series with minimal rest and took approximately 90 min to complete. All tasks were performed in a climate-controlled, indoor sports facility on a turf field and in the order as described below. Subjects wore their service-specific duty uniform and boots or shoes. Rating of perceived exertion (RPE) was recorded before and after each event using a 6-20 Borg scale (150). Participants received verbal encouragement from graders during testing.

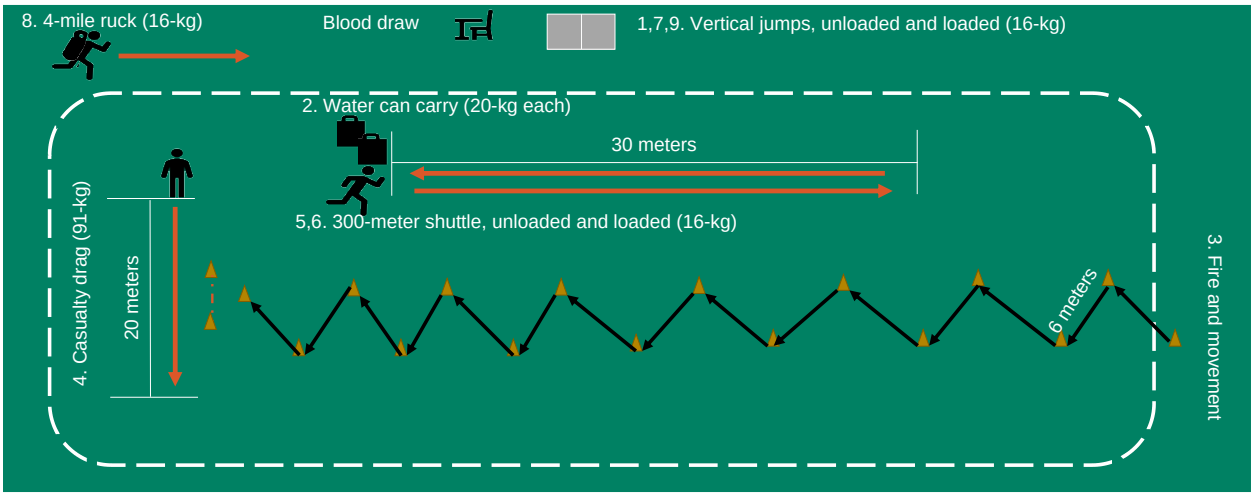


Figure 8. Tactical Mobility Test (TMT) Layout.

3.6.2.1 Vertical Jump (VJ)

Jump performance was assessed at three different time points during the TMT – before all other TMT tasks (VJ-PRE), following the loaded 300-m shuttle run (VJ-MID), and at the conclusion of the ruck march (VJ-POST). Participants stood on two force plates (Kistler

Instrument Corp., Novi, MI) with one foot positioned on each plate and performed a countermovement jump in an unloaded condition. Data was saved in BioWare® files and converted to text files to be processed using a customized script in MATLAB (MathWorks, version 2018b, Natick, MA). Primary outcomes for this event were jump height (cm) calculated using flight time and maximum force prior to takeoff (MFPTT) (N).

3.6.2.2 Water Can Carry (WCC)

The WCC was used to evaluate the ability to hand-carry moderate weight for prolonged periods. The event replicates military tasks such as a litter carry or movement of supplies (160). Participants carried two 20-kg weighted water cans (one in each hand) continuously up and down a 30 m course for 2 min while wearing 12-kg of external load (Army Combat Helmet, body armour, and mock weapon). The task ended after 2 min or if a water can touched the ground. Distance travelled at the completion of the task was the primary outcome. To account for participants that could not complete the carry for two-minutes, each participant's value was converted to $m \cdot s^{-1}$ by dividing the distance travelled by the total time carried.

3.6.2.3 Fire & Movement/Casualty Drag (FM/CD)

To assess operational performance of a simulated combat scenario, participants completed a modified "move under direct fire" drill first reported by Foulis et al. (161), immediately followed by a 20-m casualty drag. Participants wore a 12-kg fighting load (Army Combat Helmet, body armour, and weapon) and started in a prone unsupported position at one end of a marked course. When told to begin, participants stood up and sprinted 6.6-m to the first marker, quickly assumed a kneeling fighting position for 5 seconds, and then sprinted to the next marker on command. Participants continued sprinting between markers, changing between two kneeling and one prone

position through the entire course (16 movements, six in the prone position). After a familiarization trial, the task demonstrates excellent reliability (ICC=0.93, 95% CI: 0.88-0.96; 95% Limit of Agreement = 0.16) (161).

Upon rising from the final cone, participants sprinted through a gate marked by two cones and dragged a 91-kg Rescue Randy dummy (Dummies Unlimited, Pomona, CA), outfitted with a weighted vest for hand grips, across a 20-m distance to replicate a casualty drag. The FM and CD were run as one continuous movement but were scored as two separate events with the FM time ending and the CD time beginning when the participant passed through the gate at the end of the FM course. Time to complete FM and CD were primary outcomes.

3.6.2.4 300-meter Shuttle Run

Three minutes after completing CD, participants performed 10 shuttle sprints covering a 30 m distance in an unloaded condition (300US), followed by another three-minute rest, and then repeated the same 300-m shuttle run in a loaded condition with a 16-kg vest (300LS). Participants had to touch each end line with one hand at each turn. Time to complete the 300-m shuttle run in both unloaded and loaded conditions were primary outcomes.

3.6.2.5 Ruck march

Participants were allotted a 10 min rest period after the VJ-MID and before beginning the ruck march event. The 4-mile ruck march consisted of a 2-mile march paced at 6.0 $km \cdot hr^{-1}$ (3.73 mph), followed by a 30 s rest period, then a 2-mile march for an individual's best time. Participants wore an Army Combat Helmet and carried a ruck sack and a weapon (total load of 16-kg). An administrator facilitated pacing during the first two miles by instructing participants to speed up

or slow down as necessary. Time to complete the 2-mile best effort ruck march was a primary outcome.

3.6.3 Specific Aim 3: Acute Heavy Resistance Exercise Test (AHRET)

The AHRET was performed as part of a longitudinal study (SPARTA; United Kingdom Ministry of Defense Award # WGCC 5.5.6 - Task 0107) aimed at optimizing physical readiness and training for military occupational performance in men and women. AHRET is a well-established resistance exercise protocol for inducing a hormonal stress response (Kraemer et al., 2006). Testing was performed at the Neuromuscular Research Laboratory / Warrior Human Performance Center, Pittsburgh, PA and occurred over multiple testing sessions (Figure 9). Familiarization to testing procedures and baseline measurements of aerobic fitness, strength, and body composition were completed prior to the AHRET. Beginning with testing Day A, body composition was determined using dual-energy x-ray absorptiometry (Lunar iDXA, GE, Wauwatosa, WI, USA), and aerobic fitness ($V\dot{O}2_{peak}$) was assessed on a treadmill (Woodway; Waukesha, WI) using the Bruce protocol (158). One-repetition maximum (1-RM) assessments of the back squat, bench press, and deadlift were performed according to National Strength and Conditioning Association testing guidelines (162) on testing Day B. A 1-RM was considered the highest weight an individual could lift with each respective exercise using proper technique. Testing Day C consisted of measurements of performance on a military-style obstacle course (data not used in this study). The AHRET was performed on test Day D. Participants arrived to the testing facility between 0500-1000 h having withheld from food for 10 h, caffeine for 8 h, non-steroidal anti-inflammatory drugs for 72 h, and exercise for 72 h. Prior to performing the AHRET, participants competed a dynamic warmup consisting of the following: fire hydrants (10 each side),

knee circles (10 each side), thoracic rotations (10 each side), Y's, T's, and W's (both arms at same time; 10 reps), external shoulder rotations (2.5lb plates; 10 reps), forward leg swings (10 each leg), lateral leg swings (10 each leg), hip hinges (15 reps), body squats (15 reps), and stationary lunges (step back) into single leg jump (10 reps each leg). Following the warmup, subjects competed the AHRET which consisted of six sets of 10 repetitions of back squats using 75% of their 1-RM as determined at a prior visit (Day B). Squats were performed on a smith machine rack (Star Trac, Max Rack) which allowed for movement along the x- and y-axes. An adjustable box was positioned to standardize squat depth so that the subject's thighs were parallel to the ground at the end range of flexion. Subjects were instructed not to bounce off the box. If, despite his/her best effort, a subject was unable to perform 10 repetitions, the weight was adjusted to allow for completion of all repetitions. Participants were allotted two minutes of rest between each set. Rating of perceived exertion was recorded before and after each set using the 1-10 Borg ratio scale (150), where 0 is an effort of "nothing at all" and 10 is "very, very strong". Trained staff provided spotting and monitored completion of all sets and repetitions during the AHRET. All sessions were supervised by a Certified Strength and Conditioning Specialist®.

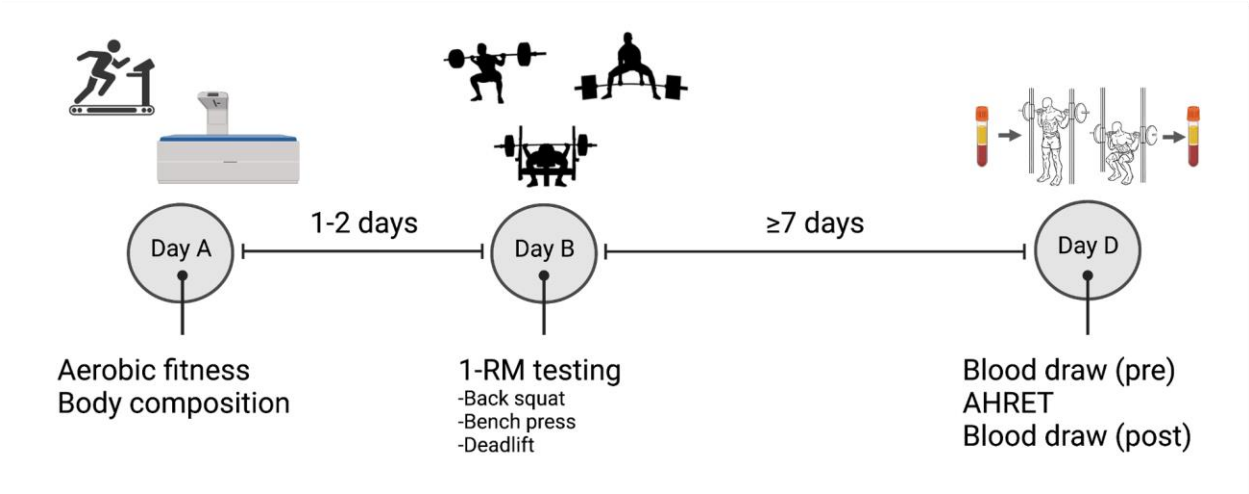


Figure 9. Overview of Soldiers Performance and Readiness as Tactical Athletes (SPARTA) testing procedures. On testing Day A, aerobic fitness ($\dot{V}O2_{peak}$) was assessed using the treadmill Bruce protocol, and body composition was determined by dual energy x-ray absorptiometry scan. On Day B, participants completed one repetition maximum (1-RM) testing on the back squat, bench press, and deadlift. On Day D, participants completed the acute heavy resistance exercise test (AHRET) bout consisting of six sets of 10 repetitions of back squats at 75% 1-RM; each set was separated by two minutes. Blood was drawn before and immediately after the AHRET. Testing days A and B were separated by 1-2 days, and testing days B and D were separated by at least seven days. Figure was created using BioRender.com.

3.7 Blood Processing

3.7.1 Blood Processing for Specific Aims 1 and 2

Blood was drawn from an upper arm vein before and immediately after the physical exertion protocol using a 21-guage or 23-guage butterfly needle (Becton, Dickinson and Company Vacutainer, Franklin Lakes, NJ, USA). Ethylenediaminetetraacetic acid (EDTA)-lined tubes were immediately placed on ice, and serum tubes were allowed to clot for 30 min at ambient

temperature. Within 60 min of blood draw, samples were centrifuged at 1500 x *g* at 4°C for 15 min. Platelet-poor plasma and serum supernatants were aliquoted and stored at −80°C for later analysis.

3.7.2 Blood Processing for Specific Aim 3

Venous blood was drawn shortly before exercise in a rested and seated or supine position and again immediately after exercise using a 21-guage or 23-guage needle (Becton, Dickinson and Company Vacutainer, Franklin Lakes, NJ, USA). Blood was collected into 10 mL serum and 6 mL EDTA plasma tubes. Serum was allowed to clot for 30 min at ambient temperature and subsequently centrifuged at 1500 x *g* for 15 min at 4ºC. Plasma was centrifuged immediately after blood draw using the same settings as serum. Serum and plasma were aliquoted and stored at –80ºC until analysis.

3.8 Data Reduction

3.8.1 IDEAS®

Imaging flow cytometry samples were analyzed using the IDEAS® 6.2 software. An overview of gating procedures is presented in Figure 10. SpeedBeads® were gated out during data collection by plotting a histogram for channel 6 (side scatter) and collecting events less than the high side scatter peak indicative of SpeedBeads® (i.e., <1e+5 channel 6 intensity x-axis). Positive events in each EV subpopulation were manually gated using the following procedures. Scatterplots

were made by plotting each stained fluorescence marker (y-axis) against its neighboring channel (x-axis). Events with a positive intensity were gated for each fluorescence channel. The file was then saved as a template, and gates were adjusted using each FMO control. Samples were batch processed using the template containing the final adjusted gates so that all samples run on the same day were gated in the same manner. Features exported for analysis included objects per mL, percentage of total events, and intensity of channels 2 (SGCA), 4 (THSD1), 7 (VAMP3), and 11 (CD63). *Intensity* is the sum of fluorescence within the defined pixel region for each EV after correction for background pixel values.

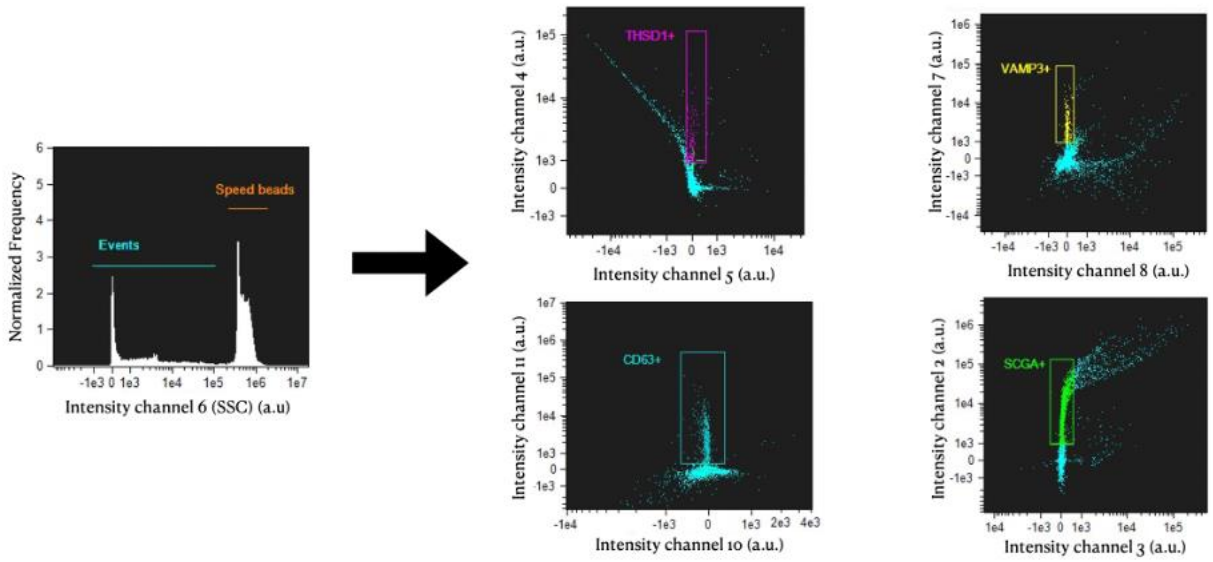


Figure 10. Imaging flow cytometry gating procedures.

3.9 Statistical Analysis

3.9.1 Statistical Analysis for Specific Aim 1

Data were screened for assumptions prior to conducting analyses. Normality of standardized residuals was assessed using Shapiro-Wilk tests. The Levene's test was used to determine homogeneity of variances, and examination of Mauchly's test was used to determine sphericity. Independent variables for each TMT event were sex and day (days 1-4) with baseline, stress onset, peak stress, and recovery defined as day 1, day 2, day 3, and day 4, respectively. There was an additional factor of time (i.e., pre- and post-TMT) for all blood biomarker outcomes, and blood was only measured on days 1, 3, and 4. Separate two-way mixed analysis of variance (ANOVA) were used to test for the effects of day, sex, and the interaction of day by sex on each TMT event. For biomarker analysis, separate three-way mixed ANOVAs were used to analyze the effect of sex, day, and time and the interaction of sex by day by time on each biomarker. Significant interactions were followed by simple main effects and subsequent estimated marginal means as appropriate. Absent an interaction effect, the main effects of each independent variable were assessed, followed by Bonferroni adjusted pairwise comparisons as appropriate. Data transformations were performed for the CD, 300US, 300LS, ruck march, GH, and cortisol due to assumption violations. Nonparametric tests were used for BDNF following unsuccessful data transformations. Raw data is presented in tables and figures for all variables for the purposes of reader interpretation. Effect sizes from the ANOVAs are expressed as partial eta squared (η_p^2). Pearson's r (r) or Spearman's rho (r_s) were used to determine the relationship between change scores from baseline (day 1) to peak stress (day 3) in TMT events and POMS domains, as well as TMT events and biomarker response (i.e., delta pre- to post-TMT). Group sample sizes

approximated the proportion of men (85%) and women (15%) in the military. Statistical tests were performed using IBM SPSS Statistics for Windows, Version 25.0 (Armonk, NY: IBM Corp) and significance was set at $p < 0.05$ (two-tailed) *a priori*.

3.9.2 Statistical Analysis for Specific Aim 2

Data were tested for assumptions prior to inferential statistical analysis. Normality was assessed using Shapiro-Wilk, and Levene's test was used to determine homogeneity of variance. Data violating assumptions were transformed using natural log, square root, or reciprocal transformations for analysis; raw data is presented for reader interpretability. Independent variables included sex (men, women), time (pre-, post-exercise), and day (day 1, or baseline; day 3, or peak stress). Primary outcomes included: a) total EV mean size, b) total EV mean concentration, c) objects per mL for each subpopulation (i.e., CD63+, THSD1+, VAMP3+, SGCA+ EVs), d) percentage of each subpopulation as a proportion of total EVs, e) sum, mean, and median fluorescence intensity within each subpopulation of EVs. Total EV size and concentration measurements consisted of all vesicles assessed using nanoparticle tracking analysis. Subpopulation assessments were only particles within each respective subpopulation gate (i.e., CD63+, VAMP3+, THSD1+, SGCA+ EVs) as measured using imaging flow cytometry. The percentage of each subpopulation as a proportion of total EVs was defined as the number of gated particles within a subpopulation divided by the total number of particles collected during the 3-min run time on imaging flow cytometry. Secondary outcomes included sum, mean, and median fluorescence intensity of CD63, THSD1, and VAMP3 within the SGCA+ EV subpopulation as a measure of change in EV subtype tetraspanin expression within the skeletal muscle-derived EVs. Changes in intensity were measured to assess changes in overall tetraspanin expression within

each subpopulation and as an estimate of tetraspanin surface density when normalized to particle count. A separate three-way analysis of variance was used to examine interactions or main effects of sex, time, or day on each of the primary outcomes followed by Bonferroni adjusted pairwise comparisons when appropriate. Subject characteristics were analyzed using independent samples *t-tests*. Partial eta squared (η_p^2) and Cohen's *d* effect sizes were calculated for analysis of variance and *t*-tests, respectively. Significance was set at $p < 0.05$ (two-tailed). Statistical analyses were performed using SPSS, version 27 (IBM, Armonk, NY).

3.9.3 Statistical Analysis for Specific Aim 3

Subject characteristics were analyzed using independent samples *t-tests*. The interaction and main effects of sex and time were analyzed using a two-way mixed analysis of variance with Bonferroni *post hoc* adjustments, as appropriate. Data were natural log, square root, or reciprocal transformed and used for analysis when assumptions of normality were not met. If assumptions were still not met following data transformations, Wilcoxon signed-rank exact tests were used to compare pre- and post-exercise, split by sex. Dependent variables included: total EV concentration as measured by vesicle flow cytometry assay, concentration (objects per mL) of each EV subpopulation (CD63+, VAMP3+, THSD1+, SGCA+ EVs), proportion of each subpopulation as a percentage of total EVs, and median fluorescence intensity (normalized to count) (MFI) for each subpopulation. Raw data are presented as mean ± standard deviation or estimated marginal means ± *95%CI* for pairwise comparisons following a significant omnibus test for all outcomes. Effect sizes were calculated using partial eta squared (η_p^2) for analysis of variance, Cohen's *d* for paired samples *t*-tests, and Cohen's *r* for Wilcoxon signed-rank tests (163). Statistical significance was

set at $p < 0.05$ (two-tailed). Analysis was performed using SPSS, version 27.1 (SPSS, IBM Corp., Armonk, NY, USA).

4.0 Manuscript 1: Neuromuscular Performance and Hormonal Responses to Military Operational Stress in Men and Women

All content for chapter 4 has been previously published (34) and is reprinted here with permissions from Wolters Kluwer Health Inc.

4.1 Abstract

Women have recently been integrated into ground close combat positions; however, there is limited data in women in these roles. We aimed to test the hypothesis that there would be no sex-specific neuromuscular responses but hormonal signaling would be differentially impact when exposed to simulated military operational stress (SMOS). Neuromuscular performance was assessed daily using a tactical mobility test (TMT) in 54 male and 15 female military members. Blood was drawn pre-/post-TMT. Mood states were assessed each morning. Unloaded 300-m shuttle time increased 6% in both sexes and remained 7% higher following one day of recovery compared to baseline ($p < 0.05$ for both), whereas performance was maintained in other TMT events ($p > 0.05$). GH increased in men, but not women, pre- to post-TMT ($p < 0.001$ vs. $p = 0.086$). Women experienced a greater decline in IGF-I across days compared to men ($\eta_p^2 = 0.778$ vs. 0.209, respectively, $p < 0.001$). Brain-derived neurotrophic factor increased significantly in men only from pre- to post-TMT on day 1 (men: +107% vs. women: +10%) but no difference on days 3 or 4. Cortisol increased 69% from pre- to post-TMT when averaged by sex and day. Negative mood states (depression, tension, anger) and altered hormonal concentrations were

associated with poorer TMT performance. Acute SMOS differentially impacted circulating hormonal milieu in men and women, but no differences in physical performance responses. Unloaded 300-m shuttle was negatively impacted while other fitness domains were maintained. Relationships between performance and mood/endocrine signaling highlight the potential for self-report measures and biomarkers to serve as indicators of performance change.

4.2 Introduction

Military field operations can provide a unique model to study subsequent physiological responses and deleterious effects on body composition, neuromuscular performance, endocrine/metabolic function, and cognitive capacity (8, 83, 110). Common characteristics of military operational stress include prolonged periods of physical exertion combined with cognitively demanding tasks which are oftentimes performed in the context of sleep restriction or disruption and underfeeding. Both laboratory and military field training settings lasting from days to months in duration have consistently shown declines in physical performance with concomitant alterations in endocrine signaling when training stress exceeds the ability to recover (81, 83, 164). For example, after three days of sustained operations characterized by a ~3,000 $kcal \cdot d^{-1}$ energy deficit and limited sleep (3.6 h total), Nindl et al. (81) reported a 9% decline in lower-body ballistic power and a 7% increase in obstacle course completion time in conjunction with a 65% decrease and 54% to 544% increase in positive and negative mood states, respectively. Though the relationship between mood and performance outcomes was not statistically analyzed in the Nindl et al. study, previous data have demonstrated a significant correlation between mood states and athletic performance (147). Following one week of Norwegian Special Forces training, candidates

experienced 20% and 28% reductions in lower-body strength and jump height, respectively, and a corresponding increase in cortisol (+154%) and decrease in testosterone (−70%) and insulin-like growth factor-I (IGF-I) (−51%) over the same period (82). Physical performance remained depressed two weeks after training whereas anabolic status (i.e., IGF-I and testosterone) had returned to or surpassed baseline concentrations while cortisol remained higher than pre-training levels (82). After 21-days of intense field training, Ojanen et al. (83) reported a 6% decrease in jump performance and declines in muscular endurance as measured by the sit-up (9%) and push-ups (15%) in male conscripts. Finally, using a comprehensive battery of tests, Conkright et al. (10) reported a significant decline in physical performance that persisted for six weeks following 61-days of Army Ranger training in events measuring speed and agility (−15%), anaerobic capacity (−7%), core strength (−27%), and aerobic endurance (−24%). Similar studies with Army Rangers have reported corresponding alterations in testosterone, IGF-I, and cortisol with recovery of those hormone concentrations noted prior to that seen in performance (9, 165). Taken together, short- and long-term studies of military training designed to mimic the stressors of combat demonstrate a substantial deterioration in physical ability which is often preceded by alterations in qualitative and quantitative measures such as mood and circulating hormones, respectively. These data emphasize the utility of mood and blood biomarkers as early indicators preceding declines in physical performance. Of note, resting hormone concentrations have been the focus of previous research in military stress physiology, and there is limited data characterizing acute endocrine signaling following physical exertion in the context of common military operational stressors. Evidence from studies in athletes suggest that investigations of acute biomarker responses may give additional insight into important readiness outcomes such as overreaching or overtraining status (166, 167). More specifically, studies of acute responses versus resting biomarker

concentrations may be of particular interest in a military training setting to better understand hormonal modulation (i.e., growth hormone, insulin-like growth factor-I, cortisol, and brain-derived neurotrophic factor) related to anabolic/catabolic status and energy metabolism.

Despite the well-documented and consistent evidence that military operational stress has a negative effect on endocrine- and performance-related outcomes, most of the studies consist of men only (8–10, 82, 83, 110, 164, 165), and of the few studies that do contain men and women, a small number directly assess the effect of sex when undergoing the same type of training stress (49, 168). This disparity of data between men and women in operational settings is highly relevant given the repeal of the combat exclusion rule in 2013, which opened previously restricted ground close combat positions to women. In one of the few instances where men and women were directly compared, Hoyt et al. (49) reported significant differences in the amount of fat-mass and fat-free mass lost by men and women following intense military training. After seven days of semi-starved conditions (i.e., <100 kcal·d^{-1} intake) juxtaposed with total energy expenditure exceeding 5,000 kcal·d^{-1}, both men and women lost a significant amount of body mass (−7.7 ± 1.1 and −5.9 ± 1.1 kg, respectively). However, men lost more fat-free mass (−4.0 kg) than women (−2.5 kg), but there was no difference between sexes in fat-mass decline (−3.5 vs. −3.4 kg, respectively) indicating a greater proportion of total body mass lost was due to fat-mass in women whereas men lost more fat-free mass. Consistent with the changes in body composition, women oxidized 40% more fat as a proportion of total energy expenditure during training compared to men. In a recently published study, Vikmoen et al. (168) reported similar differential changes in body composition as well as biochemical indicators of stress (i.e., cortisol) and anabolic status (i.e., IGF-I) among male and female conscripts following 5.5 days of near-identical intense field training. Interestingly, both sexes had similar declines in measures of upper- (−11% for both) and lower-body (−18% to −19%)

explosive power and anaerobic capacity (−55% vs −47% in men and women, respectively) after training, but women experienced better recovery in countermovement jump compared to men two weeks after training (−9% vs. −17%, respectively, comparted to baseline). These studies provide reason to believe that men and women may respond differently to similar types of operational training stress. Yet, there remains a substantial gap in the literature which must be addressed.

Therefore, the purpose of this study was to compare physical performance and hormonal trajectories in men and women undergoing simulated military operational stress (SMOS) lasting five days. Secondarily, we aimed to characterize the relationship between self-report (mood) and objective (biomarkers) indicators of allostasis with physical performance. We hypothesized that there would be no difference between men and women in physical performance changes across the stressor, but that men and women would experience differing response patterns in hormones related to anabolic status, energy metabolism, and stress. We further hypothesized that there would be significant correlations between physical performance outcomes, mood, and hormone concentrations.

4.3 Methods

4.3.1 Experimental Approach to the Problem

Data presented herein are part of a larger study (US Department of Defense award # W81XWH-17-2-0070). A prospective cohort study design was used to characterize the effects of SMOS exposure on occupationally-relevant physical performance and hormonal responses in male and female military members.

4.3.2 Subjects

Sixty-nine healthy men (N = 54) and women (N = 15) service members completed the 5-day SMOS protocol. Eligible participants were currently serving in either Active or National Guard/Reserve components of the United States Army, Marines, Navy, Air Force, or senior Reserve Officer Training Corps cadets. Participants qualified for the study if they met their service-specific physical and medical requirement standards or, if recently separated, had not gained more than 10% of body mass since time of discharge and were currently exercising at least 150 minutes per week. Participants were excluded if they were pregnant or had an apnea hypopnea index of ≥15 as determined by an in-lab apnea screen prior to baseline assessments. Volunteers were between 18-41 years old with a mean ± standard deviation age of 26.4 ± 5.3 years for men and 25.6 ± 5.6 years for women. Frequencies (percentage) of branch of service of subjects were as follows: 3 (4.3%) Air Force, 56 (81.2%) Army, 6 (8.7%) Marines, and 4 (5.8%) ROTC. Anthropometric and fitness parameters for participants are presented in Table 1.

The study received Institutional Review Board approval through the University of Pittsburgh and is compliant with the Human Research Protection Office. Subjects were informed about the study using electronic correspondence, flyers, and in-person briefings. Interested personnel contacted the study staff directly and were pre-screened telephonically to verify eligibility. Eligible participants were informed of the benefits and risks of the investigation before signing the institutionally approved informed consent. Participation was voluntary, and subjects could withdraw at any point in time.

Table 1. Participant anthropometric and fitness characteristics.

	Men (n = 54) Mean ± *SD*	Women (n = 15) Mean ± *SD*
Height (cm)	177.9 ± 6.5	164.6 ± 8.5*
Body mass (kg)	85.2 ± 14.0	67.0 ± 9*
% Body fat	20.2 ± 7.1	27.4 ± 7.2*
$VO2_{peak}$ ($mL \cdot kg \cdot min^{-1}$)	47.8 ± 7.6	40.5 ± 5.0*
Average KE MVC (N)	1221.6 ± 376.8	796.6 ± 266.9*

KE MVC = knee extension maximal voluntary contraction.

***Significant difference from men. Data are presented as mean ± standard deviation.**

Significance was set at $p < 0.05$.

4.3.3 Procedures

Participants underwent a 5-day SMOS protocol utilizing a within-subjects repeated measures design characterized by frequent cognitive testing, simulated marksmanship, physical exertion, energy deficit, and sleep restriction (Figure 11). Participants arrived on day −1 (reception) and completed a series of screening and history questionnaires addressing psychological health and wellness parameters, military training and deployment history, and exercise training and injury history. Menstrual cycle and birth control data was collected on female participants. On day 0, participants completed body composition and physical fitness assessments and were familiarized to all study tasks. Body fat, fat-free mass, and estimated resting metabolic rate were determined using air-displacement plethysmography (Bod Pod® Body Composition System; Life Measurement Instruments, Concord, CA). The validity and test-retest reliability (ICC

= 0.99) have previously been reported (156). Estimated total daily energy expenditure (TDEE) was derived using the predicted resting metabolic rate and applying an "active" physical activity factor of 1.6. Relative peak oxygen consumption ($\dot{V}O_{2peak}$) was directly measured using a metabolic cart (Parvo TrueOne® 2400; Salt Lake City, UT) during an incremental Bruce protocol (158) until volitional exhaustion on a treadmill (Woodway; Waukesha, WI). Days 1-4 consisted of a similar schedule each day except that sleep and caloric intake were manipulated on days 2 and 3. On unrestricted days (days 1 and 4), participants were afforded 8 h of sleep from 2300-0700 h and 100% of their estimated caloric needs. In contrast, on the nights leading into days 2 and 3, sleep was restricted to two 2-h blocks (0100-0300 h and 0500-0700 h) and separated by cognitive testing, and energy intake was reduced to 50% of estimated energy needs (Figure 11). Sleep was measured using polysomnography. Caloric intake was controlled using a standardized breakfast and the remaining meal(s) consisted of Meals, Ready to Eat (MRE) individualized to each participant's estimated caloric needs based on calculated TDEE as noted above. Breakfast was consumed at ~0830 h, lunch at ~1500 h, and dinner at ~2100 h on unrestricted days. Participants received only breakfast and lunch on restricted days (days 2 and 3), and meals were consumed at the same times each day. Body mass was measured each morning (~0730) in a fasted state using a digital scale (Health-o-meter®, model 349KLX, Pelstar® LLC, McCook, IL).

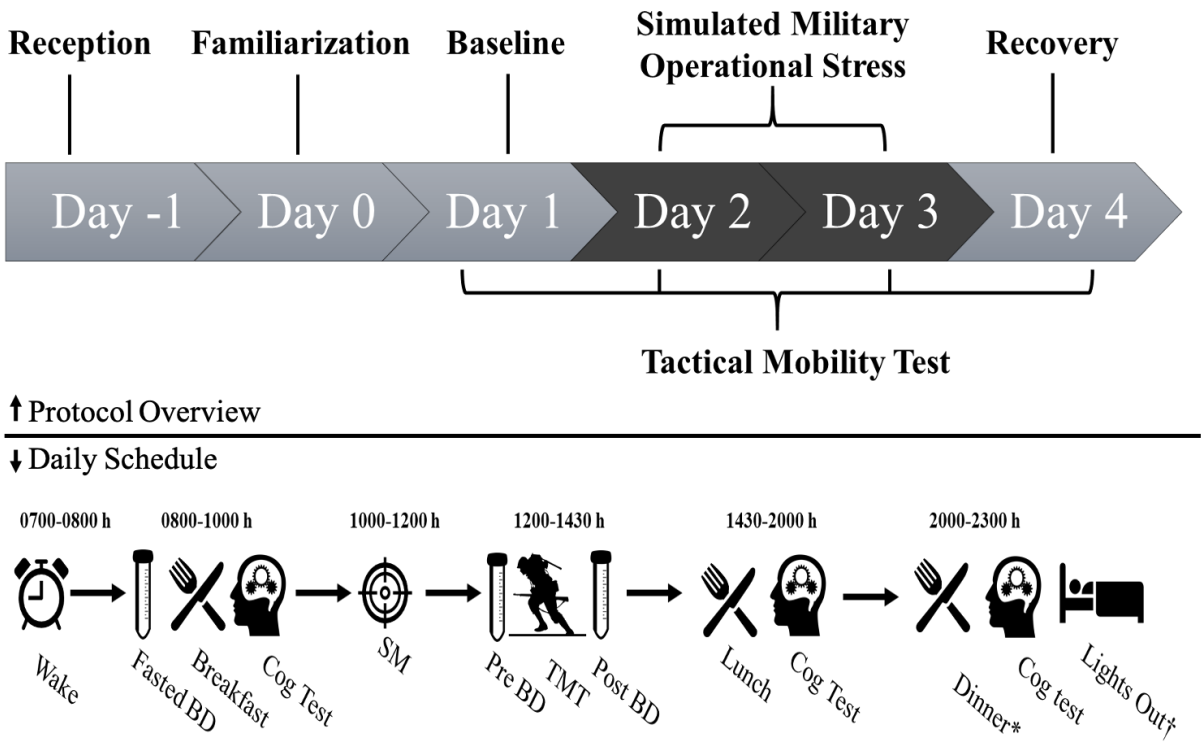


Figure 11. Overview of the simulated military operational stress protocol (top) and daily schedule for days 1-4 of the study (bottom).

BD = blood draw. Cog Test = cognitive testing. SM = simulated marksmanship. TMT = tactical mobility test. †Dinner not provided during caloric restriction on days 2 and 3. ‡Sleep hours on nights leading into unrestricted days were from 2300-0700 h. Sleep hours on nights during restricted days (days 2 and 3) were from 0100-0300 h and 0500-0700 h with cognitive testing from 0300-0500 h.

Mood was self-reported each day using the profile of mood states (POMS) questionnaire (146). Participants completed the 65-item survey at approximately the same time each morning (~0830 h), responding to a series of questions falling into six domains – vigor, tension, depression, anger, fatigue, confusion – on a 5-point Likert scale ranging from 0 (not at all) to 4 (extremely). Responses from each domain were summed (vigor was reverse scored) to derive a total mood disturbance score with a lower score corresponding to a more positive overall mood state and a higher score indicating a more negative overall mood state. The POMS survey has previously been

used in military training studies as an associative measure corresponding to changes in physical performance outcomes (81).

Maximum strength of the lower limbs was assessed daily via bilateral isometric knee extensions using an S-type load cell sampling at 2000 Hz (SSM-AJ-500, Interface Inc., Scottsdale, AZ, USA). Participants were seated with ankle-, knee- and hip joint angle fixed at 90º and performed a series of four 3-5s maximal voluntary contractions (MVC) with at least one minute of rest between sets. Verbal encouragement was provided and trials displaying excessive postural deviations were excluded from analysis. MVCs were averaged between legs, and only the best of the four trials was retained for analysis.

4.3.3.1 Tactical Mobility Test (TMT)

Each day at approximately 1200 h, subjects completed an occupationally-relevant physical exertion protocol called the Tactical Mobility Test (TMT). An overview of the TMT layout is presented in Appendix A Figure 1. The testing battery consisted of seven unique events conducted in a series with minimal rest and lasted ~90 min in total. Familiarization to all tasks took place on day 0. All tasks were performed in a climate-controlled, indoor sports facility on a turf field and in the order as described below. Subjects wore their service-specific duty uniform and boots or shoes. Footwear was kept consistent throughout the protocol. Rating of perceived exertion (RPE) was recorded before and after each event using a 6-20 Borg scale. Participants received verbal encouragement from graders during testing.

4.3.3.1.1 Vertical Jump (VJ)

Jump performance was assessed daily at three different time points during the TMT – before all other TMT tasks (VJ-PRE), following the loaded 300-m shuttle run (VJ-MID), and at the conclusion of the ruck march (VJ-POST). Participants stood on two force plates (Kistler Instrument Corp., Novi, MI) with one foot positioned on each plate and performed a countermovement jump in an unloaded condition. Data saved in BioWare® files were converted to text files and processed using a customized script in MATLAB (MathWorks, version 2018b, Natick, MA). Primary outcomes for this event were jump height (cm) calculated using flight time and maximum force prior to takeoff (MFPTT) (N).

4.3.3.1.2 Water Can Carry (WCC)

The WCC evaluates the ability to hand-carry moderate weight for prolonged periods and replicates military tasks such as a litter carry or movement of supplies. Participants carried two 20-kg weighted water cans (one in each hand) continuously up and down a 30 m course for 2 min while wearing 12-kg of external load (Army Combat Helmet, body armour, and mock weapon). A "hook grip" was prohibited. The task ended after 2 min or if a water can touched the ground. Distance travelled at the completion of the task was the primary outcome. To account for participants that could not complete the carry for two-minutes, each participant's value was converted to $m \cdot s^{-1}$ by dividing the distance travelled by the total time carried. Values are presented in the results as $m \cdot s^{-1}$.

4.3.3.1.3 Fire and Movement / Casualty Drag (FM/CD)

To assess operational performance of a simulated combat scenario, participants completed a modified "move under direct fire" drill first reported by Foulis et al. (161), immediately followed by a 20-m casualty drag (CD). Both events have excellent test retest reliability (ICC = 0.90 for both) (161). Participants wore a 12-kg fighting load (Army Combat Helmet, body armour, and weapon) and started in a prone unsupported position at one end of a marked course. When told to begin, participants stood up and sprinted 6.6 meters to the first marker, quickly assumed a kneeling fighting position for 5 seconds, and then sprinted to the next marker on command. Participants continued sprinting between markers, changing between two kneeling and one prone position through the entire course (16 movements, six in the prone position). After a familiarization trial, the task demonstrates excellent reliability (ICC=0.93, 95% CI: 0.88-0.96; 95% Limit of Agreement = 0.16) (161).

Upon rising from the final cone, participants sprinted through a gate marked by two cones and dragged a 91-kg Rescue Randy dummy (Dummies Unlimited, Pomona, CA), outfitted with a vest containing shoulder straps for hand grips, across a 20-m distance to replicate a casualty drag. The FM and CD were run as one continuous movement but scored as two separate events with the FM time ending and the CD time beginning when the participant passed through the gate at the end of the FM course. Time to complete FM and CD were primary outcomes.

4.3.3.1.4 300-meter Shuttle Run

Three minutes after completing CD, participants performed 10 shuttle sprints covering a 30 m distance in an unloaded condition (300US), followed by another three-minute rest, and then repeating the same 300-m shuttle run in a loaded condition with a 16-kg vest (300LS). Participants touched each end line with one hand at each turn and were reminded throughout the test of the remaining distance. Time to complete the 300-m shuttle run in both unloaded and loaded conditions were each primary outcomes.

4.3.3.1.5 Ruck March

Participants were allotted a 10 min rest period after the VJ-MID and before beginning the ruck march event. The 4-mile ruck march comprised a 2-mile march paced at 6.0 $km \cdot hr^{-1}$ (3.73 mph), followed by a 30 s rest, then a 2-mile march for an individual's best time. Participants wore a ruck sack and Army Combat Helmet and had a weapon (total load of 16-kg). An administrator facilitated pacing during the first two miles by instructing participants to speed up or slow down as necessary. Time to complete the 2-mile best effort ruck march was a primary outcome.

4.3.4 Blood Collection and Biomarker Analysis

Blood was drawn from an upper extremity vein using a 21G/23G butterfly needle (Becton, Dickinson and Company Vacutainer, Franklin Lakes, NJ, USA) and collected into one each of 6 mL serum and EDTA plasma tubes. Blood was analyzed for GH, IGF-I, cortisol (Alpco), and

BDNF (Millipore Sigma) and was drawn immediately before and after completing all TMT events. Serum was allowed to clot for 30 min and plasma tubes were placed on ice before being transported to the lab facility for processing. Blood was centrifuged at 1500 x *g* at 4°C for 15 min within 30-45 min of being drawn. Blood supernatant was aliquoted into tubes and then frozen at −80°C until analysis. Biomarkers related to stress (cortisol), anabolic status (GH and IGF-I), energy metabolism (IGF-I and BDNF) were measured using standard enzyme-linked immunoassay or magnetic bead-based (Luminex® / xMAP® technology; BDNF only) procedures. Serum was used for GH, IGF-I, and cortisol and plasma was used for BDNF. Estradiol and progesterone (Alpco) were measured in women from blood serum drawn in a fasted state (~0800 h) on day 0. Assay sensitivities were 0.5 ng/mL for GH, 0.09 ng/mL for IGF-I, 0.4 μg/dL for cortisol, 2.5 pg/mL for BDNF, 10 pg/mL for estradiol and 0.1 ng/mL for progesterone. All measures were performed in duplicate with intra-assay coefficients of variation of 10% or less.

4.3.5 Statistical Analysis

Data were screened for assumptions prior to conducting analyses. Normality of standardized residuals was assessed using Shapiro-Wilk tests. The Levene's test was used to determine homogeneity of variances, and examination of Mauchly's test was used to determine sphericity. Independent variables for each TMT event were sex and day (days 1-4) with baseline, stress onset, peak stress, and recovery defined as day 1, day 2, day 3, and day 4, respectively. There was an additional factor of time (i.e., pre- and post-TMT) for all blood biomarker outcomes, and blood was only measured on days 1, 3, and 4. Separate two-way mixed analysis of variance (ANOVA) were used to test for the effects of day, sex, and the interaction of day by sex on each TMT event. For biomarker analysis, separate three-way mixed ANOVAs were used to analyze the

effect of sex, day, and time and the interaction of sex by day by time on each biomarker. Significant interactions were followed by simple main effects and subsequent estimated marginal means as appropriate. Absent an interaction effect, the main effects of each independent variable were assessed, followed by Bonferroni adjusted pairwise comparisons as appropriate. Data transformations were performed for the CD, 300US, 300LS, ruck march, GH, and cortisol due to assumption violations. Nonparametric tests were used for BDNF following unsuccessful data transformations. Raw data is presented in tables and figures for all variables for the purposes of reader interpretation. Effect sizes from the ANOVAs are expressed as partial eta squared (η_p^2). Pearson's r (r) or Spearman's rho (r_s) were used to determine the relationship between change scores from baseline (day 1) to peak stress (day 3) in TMT events and POMS domains, as well as TMT events and biomarker response (i.e., delta pre- to post-TMT). Group sample sizes approximated the proportion of men (85%) and women (15%) in the military. Statistical tests were performed using IBM SPSS Statistics for Windows, Version 25.0 (Armonk, NY: IBM Corp) and significance was set at 0.05 (two-tailed) *a priori*.

4.4 Results

4.4.1 Sleep and Caloric Intake

Subjects consumed an average of 2390 ± 478 kcal·d^{-1} (31% FAT, 57% CHO, 12% PRO) on unrestricted days and 1480 ± 273 kcal·d^{-1} (29%FAT, 59%CHO, 12%PRO) during the restricted period (i.e., days 2-3). Body mass declined by ~1% over the study period as a result of caloric restriction and daily physical exertion. Subjects slept 7.4 ± 0.3 h and 7.6 ± 0.3 h on baseline and

recovery nights, respectively, when provided 8 h of opportunity to sleep and 3.8 ± 0.1 h on sleep restriction nights divided into ~2 h sleep periods with two hours of cognitive testing between each sleep period.

4.4.2 Profile of Mood States

With the exception of anger and depression, all negative mood state domains, including tension, fatigue, and confusion, increased significantly (all p-values < 0.01) while vigor decreased ($p < 0.001$) from baseline (day 1) to peak stress (day 3) and recovered on the final day (day 4) (Figure 12). Total mood disturbance increased by 62% from baseline (day 1) to peak stress (day 3) ($p < 0.001$; 95%CI: −26.475, −8.383) with a return to near baseline (day 1) values by recovery (day 4) ($p > 0.05$). There were no differences between sexes by domain or for total mood disturbance ($p > 0.05$).

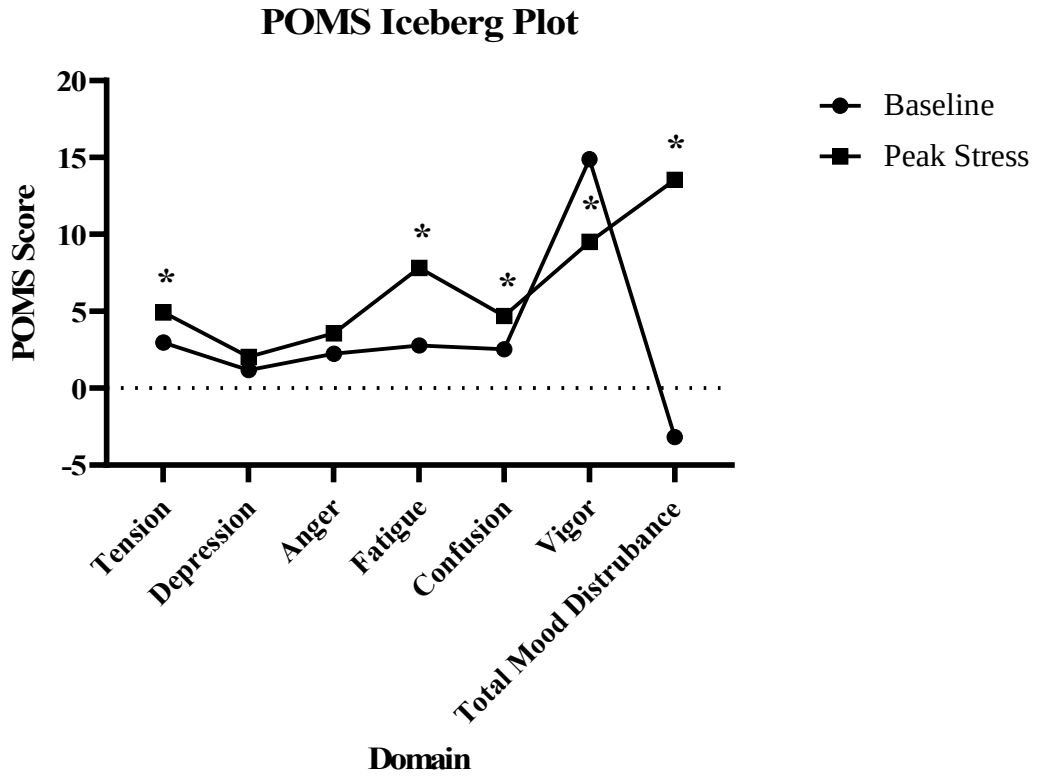


Figure 12. Profile of mood states (POMS) iceberg plot for baseline (day 1) and peak stress (day 3). Tension, depression, anger, fatigue, confusion, and vigor are individual mood domains and were summed (vigor was reversed scored) to derive a total mood disturbance score with a lower score corresponding to a more positive overall mood state and a higher score indicating a more negative overall mood state. *Denotes a significant difference from baseline. Data are presented as means. Significance was set at $p < 0.05$.

4.4.3 Tactical Mobility Test

Results for TMT events are presented in Tables 2 and 3. There were no significant interactions between sex and day for any TMT event (all *p*-values > 0.05). However, average 300US time increased by 6% from baseline (day 1) to peak stress (day 3) and remained 7% higher than baseline after one night of sleep recovery (day 4). When averaged across sex and day, jump

height decreased by 6% from PRE compared to MID and POST time points, and MFPTT increased by 6% and 8% at MID and POST time points compared to PRE, respectively. When examined as a ratio of MFPTT to jump height, these data suggest that more force was required to achieve a given jump height as fatigue accumulated over the course of the TMT protocol. Performance values were significantly higher for men than women in nearly every TMT event including jump height (p = 0.002, 95%CI: 2.486, 9.967), MFPTT (p < 0.001, 95%CI: 317.203, 843.005), WCC $m \cdot s^{-1}$ (p < 0.001, 95%CI: 0.332, 0.708), FM (p = 0.020, 95%CI: −18.582, −1.676), CD (p < 0.001, 95%CI: −42.153, −22.960), and 300US (p = 0.017, 95%CI: −22.461, −1.199).

Table 2. Tactical Mobility Test (TMT) events during five days of simulated military operational stress.

Event	Group	Day 1	Day 2	Day 3	Day 4	Main effect of day p-value (η_p^2)	Main effect of sex p-value (η_p^2)
		Baseline	Stress Onset	Peak Stress	Recovery		
WCC ($m \cdot s^{-1}$)	Men	1.53 ± 0.38	1.56 ± 0.32	1.50 ± 0.39	1.50 ± 0.33	0.173	<0.001
	Women	0.98 ± 0.35	1.07 ± 0.42	0.96 ± 0.31	1.00 ± 0.37	(0.027)	(0.327)
FM (s)	Men	145.6 ± 13.1	148.8 ± 16.1	146.5 ± 16.0	148.8 ± 16.6	0.119	0.020
	Women	152.5 ± 13.3	157.4 ± 19.2	150.7 ± 17.0	145.7 ± 15.2	(0.032)	(0.083)
CD (s)	Men	41.5 ± 23.5	40.0 ± 13.7	39.8 ± 14.2	43.6 ± 11.3	0.129	<0.001
	Women	82.0 ± 32.2	72.7 ± 23.1	67.5 ± 11.0	67.1 ± 15.3	(0.030)	(0.374)
300US (s)	Men	95.5 ± 18.5	98.3 ± 19.1	102.0 ± 21.7*	102.5 ± 18.4*	0.001	0.017
	Women	109.1 ± 14.8	109.8 ± 15.7	113.3 ± 16.7*	113.5 ± 17.9*	(0.084)	(0.087)
300LS (s)	Men	117.5 ± 25.8	117.1 ± 28.5	117.7 ± 28.5	119.5 ± 25.8	0.440	0.130
	Women	128.9 ± 19.8	128.9 ± 21.4	133.5 ± 23.7	130.3 ± 18.8	(0.015)	(0.038)
RM (s)	Men	1661.4 ± 385.2	1617.2 ± 313.5	1631.0 ± 322.3	1545.2 ± 323.5	0.127	0.881
	Women	1659.9 ± 149.8	1599.4 ± 138.6	1645.8 ± 252.5	1604.7 ± 146.5	(0.036)	(0.000)

WCC = water can carry. FM = fire and movement. CD = casualty drag. 300US = 300-m unloaded shuttle run. 300LS = 300-m loaded shuttle run. RM = ruck march. *Significantly different than baseline. Data are presented as mean ± standard deviation. Significance was set at $p < 0.05$.

Table 3. Jump height and maximum force prior to takeoff for men (n = 34) and women (n = 12) at PRE, MID, and POST time points in the unloaded condition.

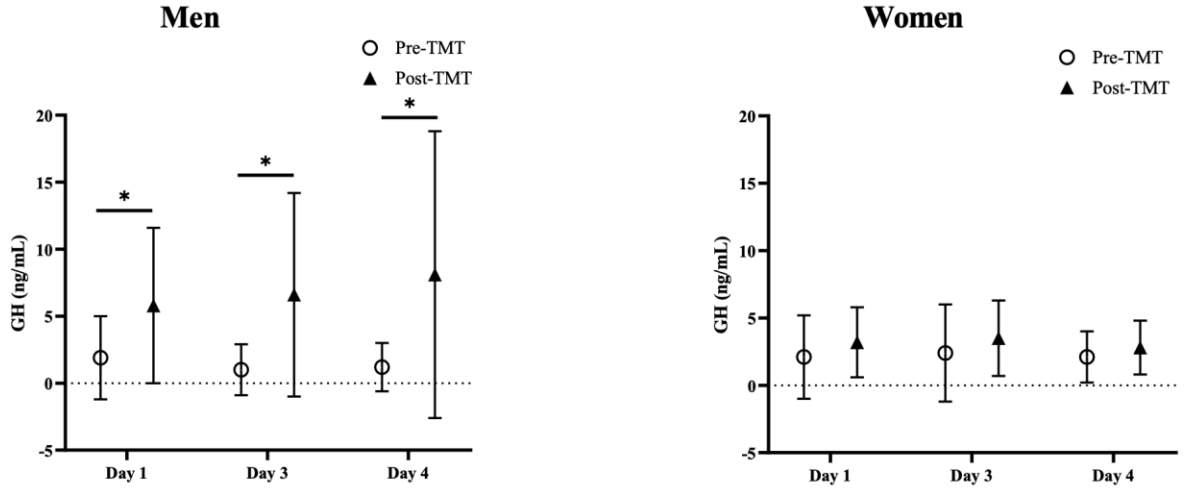
Event	Time	Group	Day 1	Day 2	Day 3	Day 4	Main effect of day p-value (η_p^2)	Main effect of time p-value (η_p^2)	Main effect of sex p-value (η_p^2)
			Baseline	Stress Onset	Peak Stress	Recovery			
JH (cm)	PRE	Men	26.9 ± 5.3	25.8 ± 5.5	26.4 ± 5.6	26.4 ± 5.9	0.645 (0.010)	0.001 (0.143)	0.002 (0.204)
		Women	21.0 ± 4.6	19.4 ± 4.4	19.6 ± 4.5	19.3 ± 4.2			
	MID*	Men	24.4 ± 6.7	24.8 ± 6.6	24.5 ± 6.7	24.3 ± 6.7			
		Women	19.2 ± 4.6	19.0 ± 4.5	18.6 ± 5.1	18.9 ± 5.5			
	POST*	Men	25.9 ± 7.6	25.2 ± 7.1	24.7 ± 7.4	24.8 ± 7.5			
		Women	18.2 ± 5.2	19.1 ± 4.4	17.9 ± 5.5	18.3 ± 5.1			
MFPTT (N)	PRE	Men	2102 ± 439	2114 ± 416	2127 ± 409	2151 ± 437	0.237 (0.031)	<0.001 (0.396)	<0.001 (0.310)
		Women	1519 ± 240	1523 ± 204	1572 ± 247	1609 ± 254			
	MID*	Men	2223 ± 437	2251 ± 483	2208 ± 488	2256 ± 468			
		Women	1642 ± 228	1678 ± 255	1694 ± 240	1691 ± 228			
	POST*	Men	2288 ± 467	2320 ± 502	2280 ± 446	2304 ± 471			
		Women	1661 ± 197	1713 ± 237	1642 ± 340	1718 ± 226			

JH = jump height. MFPTT = maximum force prior to takeoff. PRE = before any other event. MID = after water can carry, fire and movement, casualty drag unloaded 300-m shuttle run, and loaded 300-m shuttle run. POST = after 4-mile ruck march. *Significantly different than PRE. Data are presented as mean ± standard deviation. Significance was set at $p < 0.05$.

4.4.4 Blood Biomarkers

Hormone concentrations for GH, IGF-I, cortisol, and BDNF are displayed in Figure 13. The pattern of change was significantly different between sexes from pre- to post-TMT for logarithmic transformed GH (p = 0.017, partial η^2 = 0.115) such that men, but not women, experienced a significant increase in GH post-TMT on days 1, 3, and 4 ($p < 0.001$ for each day, partial η^2 = 0.371, 0.659, 0.568, respectively). IGF-I was also differentially expressed in men and women across days with women ($p < 0.001$, partial η^2 = 0.778) having a much steeper decline than men (p = 0.001, partial η^2 = 0.209). There was also a statistically significant increase in IGF-I concentrations from pre- to post-TMT (p = 0.046, partial η^2 = 0.082) when averaged across sex and day. There were no interaction effects for logarithmic transformed cortisol; however, there was a 69% increase from pre- to post-TMT when averaged across sex and day ($p < 0.001$, partial η^2 = 0.571). Results from nonparametric comparisons of pre- to post-TMT BDNF concentrations at each level of sex and day revealed a significant increase of BDNF in men, but not women, on day 1 (men: p = 0.001, r = -0.393; women: p = 0.129, r = -0.377) but no difference on day 3 (men: p = 0.087, r = -0.215; women: p = 0.910, r = -0.042) or day 4 (men: p = 0.524, r = -0.082; women: $p > 0.999$, r = -0.014).

A



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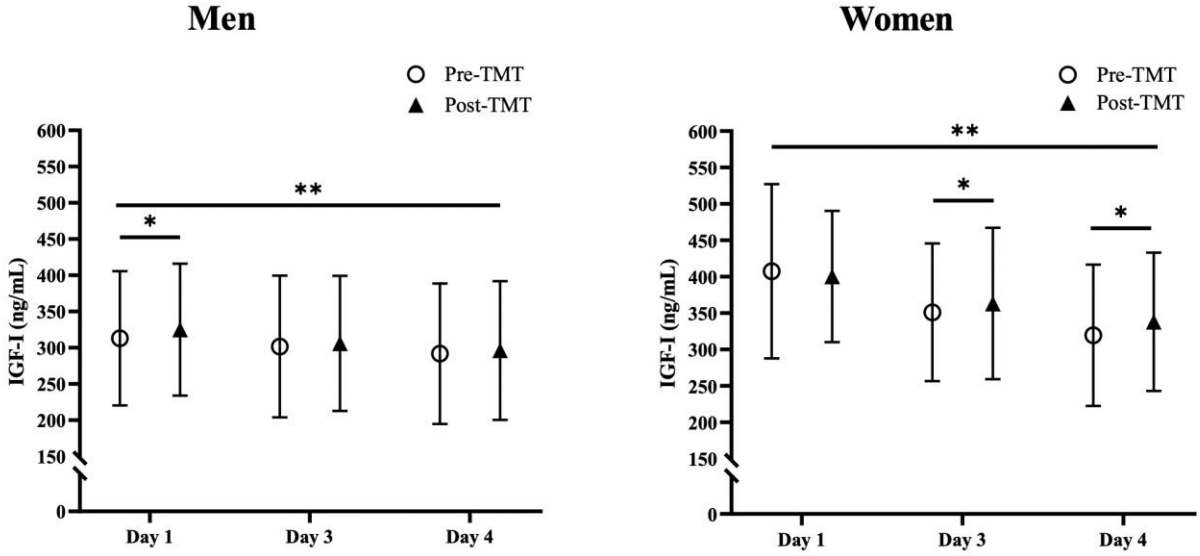
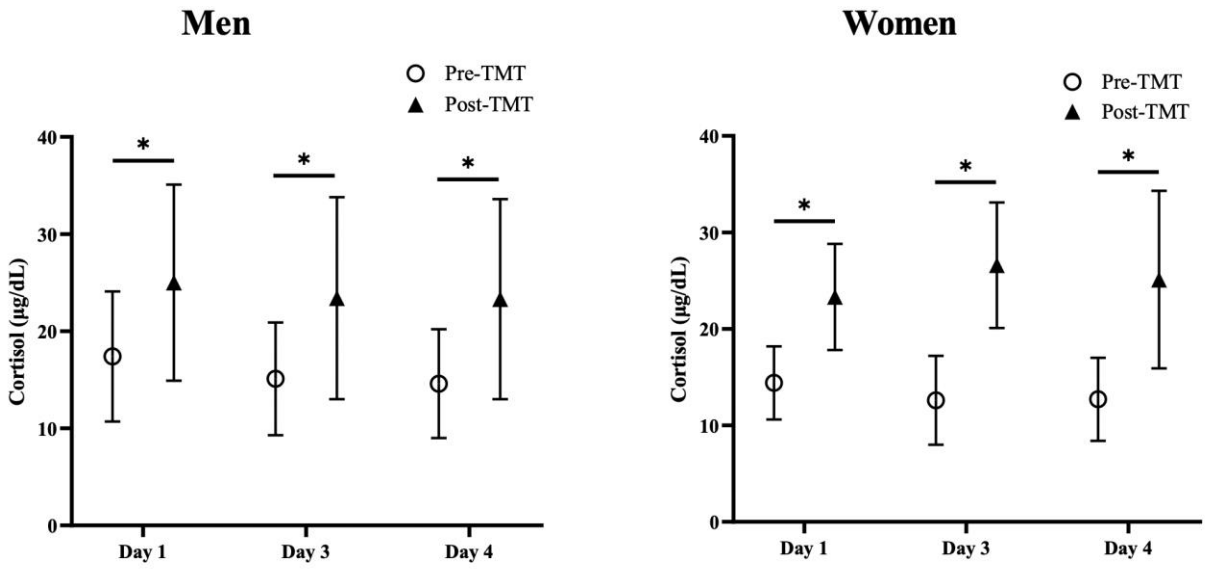


Figure 13. Concentrations of growth hormone (GH) (A) and insulin-like growth factor-I (IGF-I) (B) before and immediately after the tactical mobility test (TMT) on baseline (day 1), peak stress (day 3), recovery (day 4) in men and women.

***Denotes a significant difference between pre- and post-TMT. **Denotes a main effect of day. Data are presented as mean ± standard deviation. Significance was set at $p < 0.05$.**

C



D

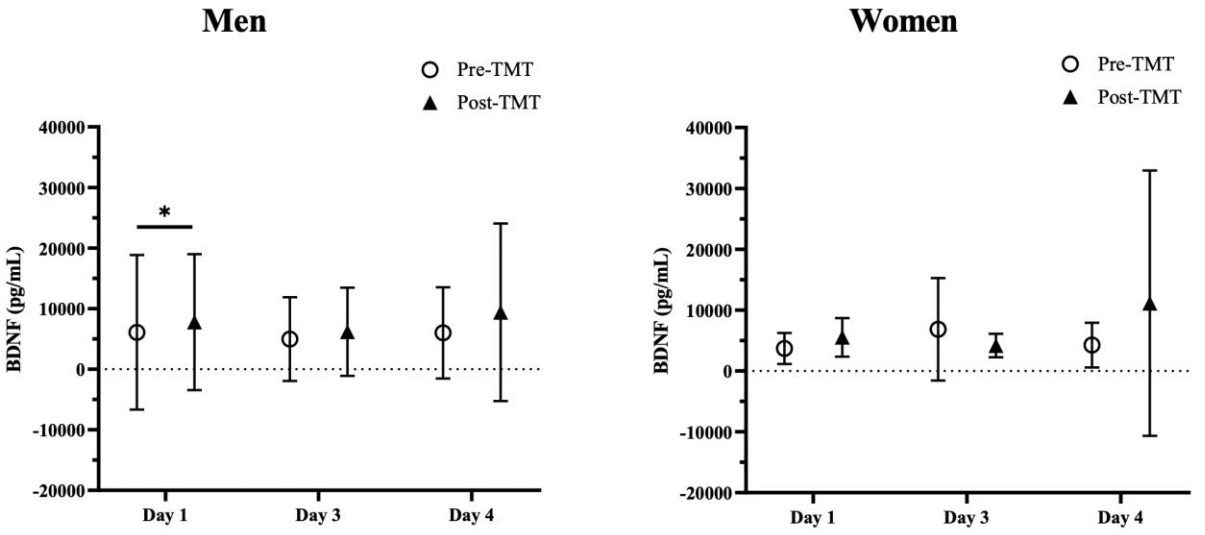


Figure 13 cont'd. Concentrations of cortisol (C) and brain-derived neurotrophic factor (BDNF) (D) before and immediately after the tactical mobility test (TMT) on baseline (day 1), peak stress (day 3), recovery (day 4) in men and women.

***Denotes a significant difference between pre- and post-TMT. **Denotes a main effect of day. Data are presented as mean ± standard deviation. Significance was set at $p < 0.05$.**

4.4.5 Associations Between TMT Performance, Mood, and Biomarkers

Changes in mood and hormone concentrations, respectively, were associated with changes in TMT performance from baseline (day 1) to peak stress (day 3). Specifically, depression (r = 0.270, p = 0.029) and tension (r = 0.277, p = 0.026) change scores were positively associated with the difference in 300US completion times from day 1 to day 3. A similar relationship was present between changes in MFPTT and tension (r_s = 0.297, p = 0.025) along with ruck march time and anger (r = 0.260, p = 0.043). Together these data demonstrate a positive association between negative mood states and degradation in performance.

Day 1 to day 3 hormonal response (i.e., delta of pre- to post-TMT) change scores were also related to TMT performance. Here, the magnitude of release in circulating cortisol was positively correlated with an increase in jump height (r = 0.413, p = 0.005), and a greater decline in IGF-I response from baseline (day 1) to peak stress (day 3) was associated with an increase in casualty drag time (r = −0.384, p = 0.006). Change in BDNF response was negatively correlated with changes in MFPTT (r_s = −0.377, p = 0.020) and positively correlated with casualty drag time (r_s = 0.340, p = 0.027).

4.4.6 Female Sex Hormones

Menstrual cycle and contraception data were collected for 12 of 15 female participants. Seven women (58%) reported currently using a form of birth control including: etonogestrel implant (3 women), levonorgestrel-releasing intrauterine device (2 women), and medroxyprogesterone acetate injection (1 woman). One woman did not report the type of contraception used. Five women (42%) had not used birth control for at least six months prior to

study enrollment. All but one subject using birth control reported having irregular menstrual periods, while 4 out of 5 women not on birth control reported having regular menstrual periods. Mean ± standard deviation estradiol and progesterone concentrations were 228.3 ± 235.9 pg/mL and 4.2 ± 5.4 ng/mL, respectively.

4.5 Discussion

This study investigated the effects of 5-days of SMOS on a dynamic range of military occupationally-relevant physical tasks and blood biomarkers measured before and after physical exertion in military men and women. Simulated military operational stress was achieved through caloric and sleep restriction concomitant with daily physical exertion (i.e., TMT). The results from this study reveal that tactical mobility was affected similarly in men and women with speed and anaerobic capacity (i.e., 300US) being most negatively impacted while other performance metrics were maintained over the course of five days. As hypothesized, men achieved higher values than women across the protocol in most events including the VJ, WCC, FM, CD and 300US. Furthermore, we report differential response patterns between men and women over time (GH and BDNF) and day (IGF-I) with no differences in patterns in cortisol but rather a significant increase in men and women following intense physical activity.

This study reveals several novel aspects relevant to military operations. First, we measured blood hormones related to anabolic status, energy metabolism, and stress *before* and *after* physical exertion as well as across days. Previous military training studies have only characterized hormonal signaling under resting conditions. Thus, data reported in this study offer a more complete description of endocrine signaling patterns during military operational stress. Another

novel approach in this study was the use of multiple within-subjects time points (i.e., VJ-PRE, VJ-MID, and VJ-POST) to measure vertical jump performance. Previous studies indicate that VJ is a sensitive marker of neuromuscular fatigue during sustained military operations (169). Data presented here highlight the deterioration in lower body explosiveness following high-intensity anaerobic movements with no further decline after a loaded aerobic event (i.e., ruck march). Finally, only one other recently published study has directly compared physical and biochemical outcomes in men and women undergoing *similar* intense military training (168). In contrast, participants from this study performed the exact *same* tasks. These findings are important given the lack of published data comparing men and women in operational scenarios and is highly relevant with an increasing number of militaries world-wide integrating women into previously restricted military units and occupations. The results presented here highlight the fact that men and women have similar response patterns in physical performance and mood during SMOS but exhibit differential biochemical responses.

4.5.1 Tactical Mobility Performance

Vertical jump height and force production have been previously shown to be sensitive indicators of declines in physical performance following military training (164, 169). We observed a 6% reduction in jump height and a corresponding 6-8% increase in MFPTT after a series of high intensity tasks but no further decline following a loaded endurance event (i.e., loaded ruck march). The increase in MFPTT to jump height ratio may be interpreted as more effort being required to achieve a given jump height and thereby an indicator of fatigue. However, this concept would need to be tested in future studies. Further, we found no difference in jump performance across days when averaged across time (i.e., VJ-PRE, VJ-MID, and VJ-POST time points) and sex. This is in

contrast to a recently published study by Vikmoen et al. (168) where they reported a significant decline in countermovement jump performance in men (−19%) and women (−18%) following an intense 6-day military field training exercise and differential recovery rates in men versus women two weeks following training (−17% and −9%, respectively, compared to baseline). The differences between our findings and those reported by Vikmoen et al. (168) can be explained by a more substantial training stress in their training paradigm as evidenced by an energy deficit of approximately 5,500-6,600 kcal·day^{-1} compared to 48 h of moderate (50%) reduction in sleep and caloric intake in this study. Taken together, our data extends that of previously published studies (164, 169) which show a decline in lower-body explosiveness across days by highlighting the detrimental effects of military operational stress on acute jump performance.

Participants in this study experienced a 6% increase in 300US time from baseline (day 1) to peak stress (day 3) which correlated with an increase in ratings of depression and tension. Previously published meta-analyses have reported similar relationships between mood states and athletic performance and achievement (147). Further, 300US time remained 7% slower than baseline (day 1) following one day of recovery (day 4). These differences in 300US performance correspond with other studies which have reported similar declines in sprint performance following both shorter- and longer-term military operational training scenarios in men (10, 81). Our results add to existing literature, which has only reported data on men undergoing military operational stress, by demonstrating similar response patterns in women. Speed and anaerobic capacity are critical factors in combat, and a decrease in these fitness attributes may increase a Warfighter's probability of being injured or killed on the battlefield (170, 171). Such decrements have serious implications on core Warfighter tasks such as bounding and moving to cover under fire. Interestingly, we did not observe a difference in 300LS times across the 5-day SMOS. This

may be explained by a pacing or testing order effect whereby participants were, consciously or subconsciously, conserving energy for the subsequent 4-mile ruck march (172, 173).

Men performed significantly better than women in the WCC event. Although not directly measured, the WCC test requires a high degree of grip strength. Our results from the WCC event align with previous reports comparing grip strength between sexes (174, 175). In a study by Leyk et al. (175) including over 2,100 untrained men and women and elite female athletes participating in sports requiring substantial hand grip, untrained men had significantly greater grip strength than nearly all untrained women and the majority of trained women. Differences remained after adjusting for lean body mass and were not influenced by hand dimensions. Grip strength is an important attribute for a variety of military tasks such as a litter carry or carrying equipment.

Similarly, men outperformed women by an average of 33 seconds on the CD event. This task also heavily relies on grip strength with the addition of lower body power and total body strength. Previous data has demonstrated that both absolute and relative lower body strength, as measured by a hexagonal trap bar deadlift 1RM, explains ~44% of the variation in a standard 91-kg and 75-kg dummy drag after controlling for sex (176). In this study, men were 35% stronger than women in lower body strength as measured by the KE MVC (day 0), which may explain some of the difference in CD time. Other studies report similar findings whereby lower body peak force and lean body mass contribute significantly to better dummy drag performance (177, 178). Thus, a training program focusing on building lower body strength/power may be of importance, especially for women, in casualty drag performance.

Though men had significantly greater physical performance measures than women in many of the TMT events, previous studies have indicated that women can achieve similar training adaptations as men, thus reducing the sex performance gap (95–97). Specifically, a holistic

strength and endurance training program lasting at least six months can result in significant increases in strength and power in women which translates to improved military task-performance (97). Data from this study suggest grip strength should be prioritized in addition to overall strength and power for an optimal training strategy as women transition into sex-integrated units.

4.5.2 Hormonal Responses to Physical Exertion During Simulated Military Operational Stress

In this study, we measured blood biomarkers related to anabolic signaling (GH, IGF-I), stress (cortisol), and energy metabolism (IGF-I, BDNF). Changes in endocrine status typically precede phenotypic outcomes and are useful for monitoring physiological status and training adaptations (179). We observed a significant sex by session interaction for GH with men experiencing a 394% increase in GH from pre- to post-TMT whereas GH concentration in women was not significantly different. Interestingly, previous studies have reported a greater increase and an earlier peak in GH concentration during exercise in women compared to men (31, 32). However, these sex differences disappeared after ~30 min. The differences observed here may be due to the lag time in GH release between sexes with women clearing GH sooner than men, thereby resulting in seemingly greater elevations by the end of the ~90 min TMT protocol.

We also observed a more dramatic decline in IGF-I concentrations across the protocol in women compared to men (−19% vs. −8%, respectively, from day 1 to day 4). Our findings corroborate those of Vikmoen et al. (168) which showed a greater percent decline in IGF-I concentration in women (−43%) than men (−28%) following 6 days of intense field training. Interestingly, Nindl et al. (108) reported no difference in change of IGF-I concentrations between men and women after a four-month gender-integrated Israeli basic training course. However,

women may experience a more pronounced decline in IGF-I compared to men in the acute phase of training stress (i.e., days) followed by an adaptation or stabilization period such that sex differences disappear over longer periods. It should be noted that despite the more precipitous drop across days, IGF-I concentrations in women remained higher than men. We also observed that IGF-I concentrations remained depressed in both men and women despite one day of recovery. IGF-I has been described by others (180) as a sensitive marker during energy restriction, and previous data has indicated that recovery may take at least one week (9, 168). The lack of rebound in IGF-I concentrations may be further explained by the fact that subjects were sleep but not nutritionally recovered on the last day of the protocol because the blood draw time points occurred before their recovery meal.

We also observed a significant increase in BDNF concentrations in men, but not women, following intense physical exertion on day 1 but not days 3 or 4. The differential response pattern between sexes corresponds with a large meta-analysis including 55 studies and 1180 participants, whereby men had an increase in BDNF following exercise, but women did not (116). Primarily known for its function as a mediator of neuroplasticity and neurogenesis, BDNF also mediates energy metabolism by decreasing appetite and enhancing lipid oxidation during glucose deprivation via activation of the AMP-activated protein kinase—a master regulator of cellular energy metabolism (114). Though we did not measure blood glucose in this study, it is reasonable to believe that completion of the TMT resulted in an exercise-induced hypoglycemic state. Therefore, the increase in circulating BDNF observed in men, but not women, following exercise may indicate the role of BDNF in differential metabolic signaling important for liberating energy stores. Further, women oxidize fat at a higher proportion than men at the same relative working intensity, which may contribute to the difference in circulating BDNF observed in the present

study (181). Thus, elevation of BDNF may help liberate fat stores for oxidation in men when glucose supply is low and there is a metabolic shift to utilize fat stores. However, this is speculative and warrants further investigation.

4.6 Conclusion

Following 5-days of SMOS, men and women military service members experienced similar changes in occupationally-relevant tactical mobility performance. Specifically, 300US performance deteriorated following 48 h of sleep and caloric restriction and remained depressed following one day of recovery. Though other TMT outcomes appeared to be unaltered, the absence of a longer familiarization period may have led to training effects and subsequently masked deterioration of physical performance in other events. In contrast, hormones associated with anabolic status and energy metabolism were differentially expressed between sexes. In absolute terms, men performed better than women on events requiring a high degree of strength, explosive power, and speed, indicating the need for optimized training to reduce the gap between physical abilities. Data presented here also demonstrate that changes in mood and hormone concentrations are associated with physical performance and may be useful indicators of compromised physical readiness. As women continue to integrate into previously restricted direct combat units and occupations, there will be a greater need to demonstrate similarities and differences in physical, cognitive, and biochemical differences between men and women to better plan for training and recovery goals, both in garrison and combat settings alike.

4.7 Practical Applications

As the US Army's Holistic Health and Fitness initiative transforms training for optimal physical performance and readiness, the tactical strength and conditioning professional would be well served to focus on aspects of soldier performance that are most sensitive to decline during military operational stress scenarios. Exercise training programs aimed at maximizing physical performance should be prioritized to offset the inevitable declines in physical capacity experienced during operational stress exposure. Given that one day of recovery was inadequate to return some, but not all, events requiring speed and anaerobic capacity (e.g., 300US) to baseline levels, military leaders should give consideration to adequate recovery periods during mission planning.

As expected, men outperformed women in most physical events. Targeted training strategies have been observed to reduce the sex performance gap (95–97) and should be prioritized to achieve occupational standards, particularly in the more demanding occupations such as direct ground combat roles.

Given that hormonal signaling often precedes phenotypic outcomes, the differential hormonal changes presented here should be further explored to determine if longer and/or more intense training results in differences in physical performance responses between men and women. Our results showed a relationship between mood and hormonal changes and physical performance changes from baseline (day 1) to peak stress (day 3). These measures may be useful as early warning indicators of subsequent declines in physical performance during intense training.

5.0 Manuscript 2: Men and Women Display Distinct Extracellular Vesicle Signatures in Response to Military Operational Stress

5.1 Abstract

Extracellular vesicles (EVs) are mediators of physiological changes that occur during exercise. Little is known about the interaction of exercise and other common stressors such as sleep and caloric restriction on EV characteristics in men and women. The purpose of this study was to examine the effects of sex and stress condition on EV size, concentration, and surface proteins. Twenty participants (10 men) completed a 5-day simulated military operational stress protocol. Subjects performed daily physical exercise. On day 1, subjects received 100% of caloric needs and 8 h of sleep. On days 2 and 3, intake was reduced to 50% of needs and sleep was restricted to 4 h. Blood was drawn before and immediately after exercise on days 1 and 3. Total EV size and concentration were assessed using nanoparticle tracking analysis. EVs were probed for markers associated with exosomes (CD63), microvesicles (VAMP3), apoptotic bodies (THSD1), and skeletal muscle-derived EVs (SGCA) and analyzed using imaging flow cytometry. Three-way ANOVAs were used to assess interactive and main effects of sex, day, and time on EV features. EV mean size significantly increased in men and women from pre- to post-exercise (+13%; $p < 0.001$) and from day 1 to day 3 (+8%; $p = 0.002$), whereas total EV concentration declined from pre- to post-exercise in women on day 1 (-37%; $p = 0.014$) and day 3 (-38%; $p = 0.003$) but was stable in men. The single stress condition of exercise (day 1) resulted in no change in CD63+ EVs, but the combination of exercise plus sleep and caloric restriction caused a significant increase in CD63+ EV concentration (+43%), proportion of total EVs (+76%), and

CD63 surface protein expression (+55%) regardless of sex ($p < 0.05$ each). The proportion of skeletal muscle EVs increased similarly in men and women following exercise (+20%; $p = 0.027$) and from day 1 to day 3 (+37%; $p = 0.001$) and were higher in women than men (+27%; $p = 0.049$). VAMP3+ EVs and THSD1+ EVs were stable across stress conditions and were no different between sexes. Total EV concentration and SGCA+ EVs displayed robust variation according to sex, whereas total EV size and CD63+ and SGCA+ EV subpopulations varied in response to single versus multi-stressor conditions.

5.2 Introduction

Multicellular organisms coordinate an array of physiological functions through a complex network of communication systems that promote near and distant tissue crosstalk (182). Circulating hormones have been the focus of endocrine research and biomarker discovery for decades, but, more recently, extracellular vesicles (EV) have emerged as a novel mechanism through which cells communicate in an autocrine, paracrine, and endocrine manner (134). Mostly studied in the context of pathological states such as cancer (120, 183), cardiovascular disease (184, 185), and neurodegenerative disease (186, 187), EVs have provided novel insights into disease diagnosis, progression, and prognosis, with high sensitivity and specificity, earning the nomenclature as a “liquid biopsy” (188). However, comparatively less is known about changes in EV characteristics in the context of stress physiology. Investigation of EVs under physiologically relevant stress related conditions has the potential to provide important advancements in our ability to objectively monitor and quantify physiological stress and identify targets for future therapeutic applications (5, 189).

For decades, military research has provided important insights into stress physiology with far-reaching implications dating back to the early work of Ancel Keys' studies of human starvation and prolonged physical work in men demonstrating the negative impact on physical fitness (71, 190). Since then, researchers have followed the trail blazed by Dr. Keys by detailing the impact of stress during military operations on soldier readiness and performance (7, 75, 79, 84, 190). Military operational stress, which consists of combined multi-stressor environments including rigorous physical and cognitive demands, sleep restriction/disruption, undernutrition, and/or environmental extremes, serves as a useful model for studying human stress physiology and provides good ecological validity with applications that extend beyond the military to other populations such as emergency personnel, shift workers, and athletes to name a few.

Circulating hormones (e.g., cortisol, insulin-like growth factor-I, testosterone) have conventionally been used to objectively monitor and quantify stress in military research (9, 74, 191). More recently, EVs have gained notoriety as biomarkers of pathology and health. EVs are a heterogeneous population of nano-sized particles that can be classified by their biogenesis into exosomes (endosomal origin), microvesicles (ectosomal origin), and apoptotic bodies (ectosomal origin). EVs can be further differentiated based on biophysical characteristics, such as size, density, and morphology (134). EVs are characterized by a lipid bilayer membrane, which makes them stable in the extracellular environment. One of the more interesting attributes of EVs are their robust cargo. EVs are enriched with lipids, proteins, and nucleic acids according to the state of their parent cell during EV biogenesis. This makes them information-rich packages that are analogous to a "biological thumbprint". Once released into the extracellular space, EVs can be collected and isolated from virtually any biological fluid and analyzed to decipher both cell-specific and systemic messages from and across a range of tissues. Their ubiquitous presence in

biological fluids, stability in the extracellular environment, and robust cargo make them appealing targets for biomarker research.

Insights into the physiological functions of EVs may be found in their features, including size, shape, morphology, and their cargo (134). For example, size and shape may suggest a particular class of EV, with larger and more irregular particles being indicative of apoptotic bodies and smaller and more circular vesicles associated with exosomes. Similarly, EV surface markers may be related to their origin or biogenesis pathway with markers such as CD63, vesicle-associated membrane-3 (VAMP3), and thrombospondin-1 (THSD1) being present in exosomes, microvesicles, and apoptotic bodies, respectively, though not exclusively (122). Alpha-sarcoglycan (SGCA) has also been used as a marker for skeletal muscle-derived EVs, which is useful for studying EVs in the context of exercise and muscle activation (138, 142, 143). Common approaches for examining EV features include crude or low throughput methods such as Western blotting and electron microscopy to single-particle markerless instrument such as nanoparticle tracking analysis or tunable resistive pulse sensing to high-throughput methods like flow cytometry (192). More recently, imaging flow cytometry has been demonstrated as an method of EV analysis through its combination of microscopy and fluorescence detection combined into one in a high-throughput manner (193, 194). Despite substantial advancements, there remains a large degree of variability in EV methodologies and workflow, including isolation, enrichment, and analysis, resulting in some degree of inter-study variability.

Extracellular vesicles have primarily been studied under isolated stress conditions, and few data exist comparing men and women. Specifically, acute exercise (mostly aerobic) and, to a lesser extent, nutrient manipulation have been two of the more commonly tested physiological conditions (36, 37, 137–139, 141–143, 195–201). In studies comprised of men only or those that include both

sexes but lack a comparison of men and women, aerobic exercise has been shown to cause an acute rise in circulating EV concentration followed by return to baseline within minutes to hours post-exercise (137–139, 141, 142, 195–201). Studies that directly compare men and women have reported an effect of sex whereby EV concentration is unaltered in men but decreases in women following exercise (36, 143). Previous data also indicate an effect of exercise intensity on EV release (36, 201). Interestingly, EV mean size is unchanged with exercise (202). EV subpopulations, as defined by specific surface protein markers, are altered by exercise as well. Specifically, EVs positive for CD63, CD81, and CD9—tetraspanins commonly associated with exosomes—have been observed to increase with exercise (202). More specifically as they related to exercise, others have noted a significant increase in SGCA+ EVs (138, 143). A similar rise and fall of EV concentration has been noted following food intake (195, 197, 199, 200), and there is some data to suggest that exercise and nutrient intake may interact (199). However, the interaction of multi-stress environments common in real-world settings on EV characteristics remains largely unexamined.

The purpose of this study was to compare the effects of two stress conditions—exercise only versus exercise plus combined sleep and caloric restriction—on EV size, concentration, and surface proteins according to sex. We hypothesized that EV concentration would decrease following exercise in women only and that size would remain stable in men and women across stress conditions. We further hypothesized that EV surface markers would increase from pre- to post-exercise but would not be different from baseline to peak stress or between men and women. The results from this study will further establish the interactive effects of multi-stressor conditions common in real-world settings and delineate EV features that differ by sex for consideration in future studies using mixed-sex samples.

5.3 Methods

Data presented here are a subset of a larger prospective cohort study (US Department of Defense award # W81XWH-17-2-0070). The University of Pittsburgh Institutional Review Board approved all study procedures. Research activities were compliant with the U.S. Army Medical Research and Development Command Human Research Protection Office and in accordance with the Declaration of Helsinki.

Subjects were recruited via electronic correspondence, flyers, and in-person briefings. Volunteers supplied written, informed consent prior to participation. Participants were free to withdraw at any time. Male and female service members between 18-41 years old were eligible for the study. Volunteers were physically fit and had to meet their service-specific physical fitness standards to qualify for the study. Individuals were excluded if they were pregnant or had moderate to severe obstructive sleep apnea as determined by an apnea hypopnea index of ≥ 15, measured on the first night of the study.

5.3.1 Experimental Procedures

This study utilized a within-subject subjects, repeated measures, prospective cohort experimental design and was conducted in a controlled, laboratory setting. The protocol consisted of 5-days of simulated military operational stress characterized by daily physical and cognitive exertion and simulated marksmanship. The details of the parent study have been previously reported (34). In short, subjects arrived on day –1 to provide written, informed consent and complete additional screening procedures prior to participation. On day 0, subjects completed physiological testing and were familiarized to all tasks. Physiological testing included a $\dot{V}O2_{peak}$

test using the Bruce protocol (158) on a Woodway treadmill (Woodway; Waukesha, WI), vertical and broad jump measurements, and a body composition assessment measured via air displacement plethysmography (BOD POD®; Cosmed, Concord, CA, USA). Individual caloric needs were determined based on total daily energy expenditure estimated from air displacement plethysmography. On day 1, subjects were afforded 100% of their individual estimated caloric needs and 8 h of sleep opportunity on night 1 (2300-0700 h). On days 2 and 3, caloric intake was restricted to 50% of estimated caloric needs, and subjects were only allowed two 2-hour blocks of interrupted sleep from 0100-0300 and 0500-0700 h on nights 2 and 3. Meals were standardized and consumed each day at ~0830 h, 1530 h, and 2000 h, except on days 2 and 3 when the third meal was not provided during caloric restriction. Water was consumed *ad libitum*, and caffeine and alcohol were prohibited during the entire study period. Sleep was monitored using standard polysomnography. An overview of the 5-day protocol is presented in Figure 14.

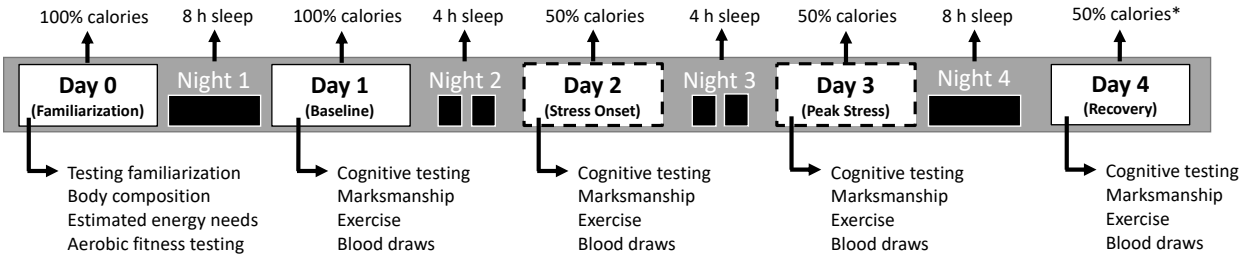


Figure 14. Overview of the 5-day simulated military operational stress protocol.

Dashed lines indicate days/nights with caloric and sleep restriction. A single black box indicates uninterrupted sleep from 2300-0700 h. Two small black boxes indicates interrupted sleep consisting of two 2 h blocks from 0100-0300 h and 0500-0700 h. *Participants were released before dinner on the final day.

Maximum strength of the lower limbs was assessed via bilateral isometric knee extensions using an S-type load cell sampling at 2000 Hz (SSM-AJ-500, Interface Inc., Scottsdale, AZ, USA). Participants were seated with ankle-, knee- and hip joint angle fixed at 90º and performed a series of four 3-5s maximal voluntary contractions (MVC) with at least one minute of rest between sets.

Verbal encouragement was provided and trials displaying excessive postural deviations were excluded from analysis. MVCs were averaged between legs, and only the best of the four trials was retained for analysis.

Each day, subjects completed a physical testing battery designed to mimic common military occupational tasks and was completed in an obstacle course format. The exercise protocol consisted of nine consecutive events, with minimal rest between each event. Events were conducted in the following order: unloaded and loaded (16-kg vest) vertical jumps, 2-min water can carry (20-kg in each hand), fire and movement course, 20-m casualty drag (91-kg), unloaded 300-m shuttle run, loaded (16-kg vest) 300-m shuttle run, unloaded and loaded (16-kg vest) vertical jumps, 4-mile loaded (16-kg) ruck march, and unloaded and loaded (16-kg vest) vertical jumps. Standardized rest periods were administered for three minutes prior to the unloaded and loaded shuttle runs and 10 minutes prior to the ruck march. The exercise protocol began at ~1200 h each day and took ~90 min to complete. Participant ratings of perceived exertion were recorded by a test administrator before and after each event using a 6-20 Borg scale (150), with a 6 indicating little to no effort and a 20 indicating maximal effort. The 6-20 Borg scale is a reliable indicator of overall effort and fatigue and was designed to correspond with heart rate (e.g., RPE of 15 corresponds to a heart rate of 150 beats per minute) (150, 151).

5.3.2 Blood Processing and Storage

Blood was drawn from an upper arm vein before and immediately after the physical exertion protocol using a 21-guage or 23-guage butterfly needle (Becton, Dickinson and Company Vacutainer, Franklin Lakes, NJ, USA). Ethylenediaminetetraacetic acid (EDTA)-lined tubes were immediately placed on ice, and serum tubes were allowed to clot for 30 min at ambient

temperature. Within 60 min of blood draw, samples were centrifuged at 1500 x *g* at 4°C for 15 min. Platelet-poor plasma and serum supernatants were aliquoted and stored at −80°C for later analysis.

5.3.3 Blood Assays

Serum myoglobin, creatine kinase, estrogen, and progesterone concentrations were measured using enzyme-linked immunoassays according to each manufacturer's protocol. Myoglobin (Alpco 25-MYOHU-E01; Salem, NH, USA) assays had a range of 25-1000 ng/mL and sensitivity of 5 ng/mL. Creatine Kinase MM (LSBio LS-F20706-1; Seattle, WA, USA) assays had a range of 1.563-100 ng/mL and sensitivity of 0.94 ng/mL. Estradiol (Alpco 11-ESTHU-E01; Salem, NH, USA) assays had a range of 20-3200 pg/mL and sensitivity of 10 pg/mL. Progesterone (Alpco 11-PROHU-E01; Salem, NH, USA) assays had a range of 0.3-60 ng/mL and sensitivity of 0.1 ng/mL. Each analyte was measured in duplicate with coefficients of variation of ≤ 10%. Myoglobin and creatine kinase were measured from blood drawn pre- and post-exercise and days 1 and 3. Estrogen and progesterone were measured from morning blood drawn in a fasted state on day 0.

5.3.4 Extracellular Vesicle Analysis

5.3.4.1 Size Exclusion Chromatography

Extracellular vesicles were isolated from plasma using size exclusion chromatography (SEC) (qEV 70 nm Original; IZON, Medford, ME, USA) according to the manufacturer's protocol. Thawed plasma samples were centrifuged at 1500 x *g* at 4°C for 10 min using a FiberLite

F21-48x1.5/2.0 fixed angle rotor (Thermo Scientific, Waltham, MA, USA) to remove any cells or large particles. Following stored conditions, SEC columns were flushed with 10 mL of 0.22 μm filtered phosphate buffer solution (PBS) before loading with 450 μl of plasma. The first 3 mL of eluate was discarded, and the subsequent 1.5 mL was collected as the EV fraction. This process was repeated for each sample. SEC columns were used no more than five times per column and were flushed with 15 mL of 0.22 μm filtered PBS between samples.

5.3.4.2 Nanoparticle Tracking Analysis

Extracellular vesicle size and concentration (particles/mL) were determined using nanoparticle tracking analysis (NanoSight NS300, Malvern Panalytical Ltd, Malvern, UK) equipped with a 532 nm (green) laser. Samples were analyzed by a single, trained user. Ten microliters from each EV sample were diluted 1:100 in type 1 EV-free water and infused into the flow cell using a syringe pump (Harvard Apparatus 98-4730). Three 45-second videos were recorded for each sample with the camera level set to 14. The flow cell was flushed with 1 mL of type 1 water between each sample. All samples were batch analyzed using computer software (NTA 3.4, build 3.4.003). Samples from each within-subject time point were assessed on the same day. The remaining isolated EV sample was divided into 150 μl aliquots and stored at −80°C for later analysis.

5.3.4.3 Staining

Isolated EVs (suspended in PBS) were fixed for 10 min using equal parts (140 μl each) of sample and 4% paraformaldehyde. Fixed samples were then centrifuged for 30 min at 16,000 x *g* at 4°C before removing 140 μl of supernatant and subsequently adding 140 μl of blocking buffer (3% bovine serum albumin, 0.1% Triton-X), then incubating on a rocker for 1 h at ambient

temperature. Following the 1 h incubation, samples were centrifuged for 30 min at 16,000 x *g* at 4°C and 140 µl of supernatant was removed. Samples were then stained with the following antibodies: 0.5 µL anti-human CD63 Alex Fluor® 700 (1:280, NBP2-42225AF700, Novus Biologicals, CO, USA), 0.5 µL anti-human vesicle-associated membrane-3 (VAMP3) Alex Fluor® 405 (1:280, NBP1-97948AF405, Novus Biologicals, CO, USA), 1.4 µL anti-human thrombospondin-1 (THSD1) Alex Fluor® 594 (1:100, FAB5178T-100UG, Novus Biologicals, CO, USA), and 0.35 µL anti-human alpha-sarcoglycan (SGCA) FITC (1:400 dilution, orb29665, Biorbyt, MO, USA). Stained samples incubated overnight at 4ºC. The following day, samples were centrifuged for 30 min at 16,000 x *g* at 4°C before removing 60 µL and adding 20 µL for a final volume of 100 µL.

Single-stained compensation controls (UltraComp eBeads™ Plus; Invitrogen) were used to correct for fluorescence carryover between channels. Fluorescence minus one (FMO) controls were used to establish gates for each spectral channel. Compensation and FMO controls followed the same staining protocol as outlined above with the exception that antibody volumes were half that of the volume used in samples.

5.3.4.4 Imaging Flow Cytometry

Imaging flow cytometry (ImageStream®X Mark II, EMD, Millipore Sigma, Seattle, WA, USA) combines conventional flow cytometry capabilities, including forward and side scatter detection, with up to 10 fluorescent channels and two channels of high-resolution microscopy of up to 60x magnification in a high-throughput manner (154). Data was collected using INSPIRE™ software with the following settings: normal gain mode, objective 60x, slow speed, high sensitivity, 7µm core size, auto-focus and auto-centering. Data was collected for 3 min for all EV samples, and 2,000 events were collected for compensation and FMO controls. SpeedBeads® were

gated out during data collection by plotting a histogram for channel 6 (side scatter) and collecting events less than the high side scatter peak indicative of SpeedBeads® (i.e., <1e+5 channel 6 intensity x-axis) (Appendix B Figure 1). The same gate was applied to all samples. Lasers were set to maximum voltage as follows: 405 nm 175 mW, 488 nm 200 mW, 561 nm 200 mW, 642 nm 150 mW, and SSC 70 mW. Brightfield images were collected using channels 1 and 9. Samples from the same subject point were stained and analyzed on imaging flow cytometry on the same day to control for potential day to day variability.

Following data collection in INSPIRE™, samples were analyzed using the IDEAS® 6.2 software. Positive events in each EV subpopulation were manually gated using the following procedures (Appendix B Figure 1). Scatterplots were made by plotting each stained fluorescence marker (y-axis) against its neighboring channel (x-axis). A blinded assessor gated events with a positive intensity for each fluorescence channel. The file was then saved as a template, and gates were adjusted using each FMO control. Samples were batch processed using the template containing the final adjusted gates so that all samples run on the same day were gated in the same manner. Features exported for analysis included objects per mL, percentage of total events, and intensity of channels 2 (SGCA), 4 (THSD1), 7 (VAMP3), and 11 (CD63). *Intensity* is the sum of fluorescence within the defined pixel region for each EV after correction for background pixel values. Intensity is used to measure protein expression (e.g., SGCA, THSD1, VAMP3, CD63) and estimated fluorescence activity.

5.3.5 Statistical Analysis

A sample size of 20 (10 men) was determined to be adequate to detect differences between men and women using a repeated measures design with one within-subjects factor based on

previously reported data (36). Data were tested for assumptions prior to inferential statistical analysis. Normality was assessed using Shapiro-Wilk, and Levene's test was used to determine homogeneity of variance. Data violating assumptions were transformed using natural log, square root, or reciprocal transformations for analysis; raw data is presented for reader interpretability. Independent variables included sex (men, women), time (pre-, post-exercise), and day (day 1, or baseline; day 3, or peak stress). Primary outcomes included: a) total EV mean size, b) total EV mean concentration, c) objects per mL for each subpopulation (i.e., CD63+, THSD1+, VAMP3+, SGCA+ EVs), d) percentage of each subpopulation as a proportion of total EVs, e) sum, mean, and median fluorescence intensity within each subpopulation of EVs. Secondary outcomes included sum, mean, and median fluorescence intensity of CD63, THSD1, and VAMP3 within the SGCA+ EV subpopulation as a measure changes in EV subtype tetraspanin expression within the skeletal-muscle-derived EVs. Changes in intensity were measured to assess changes in overall tetraspanin expression within each subpopulation, and as an estimate of tetraspanin surface density when normalized to particle count. A separate three-way analysis of variance was used to examine interactions or main effects of sex, time, or day on each of the primary outcomes followed by Bonferroni adjusted pairwise comparisons when appropriate. Subject characteristics were analyzed using independent samples *t-tests*. Partial eta squared (η_p^2) and Cohen's *d* effect sizes were calculated for analysis of variance and *t*-tests, respectively. Significance was considered $p < 0.05$ (two-tailed). Statistical analyses were performed using SPSS, version 27 (IBM, Armonk, NY).

5.4 Results

Twenty participants (10 men) were included in this study. Participant characteristics are presented in Table 4. Women were shorter, had lower body mass, higher body fat percentage, and lower cardiorespiratory fitness compared with men ($p < 0.05$ each); ages were similar across groups ($p > 0.05$). Mean relative rate of oxygen consumption is considered "excellent" for men and women according to American College of Sports Medicine criteria (203).

Table 4. Participant characteristics.

	Men (***n*** **= 10)**	**Women** (***n*** **= 10)**
Age (y)	25.6 ± 5.8 (19-35)	27.1 ± 5.9 (20-37)
Height (cm)	178.3 ± 7.2 (166-188)	168.0 ± 7.7 (152-178)*
Body mass (kg)	81.4 ± 7.8 (64-95)	70.8 ± 8.1 (59-87)*
Body fat (%)	18.8 ± 4.2 (11-24)	28.2 ± 6.7 (18-41)*
$\dot{V}O2_{peak}$ (ml·kg·min^{-1})	50.9 ± 5.3 (43-59)	39.0 ± 5.2 (32-47)*
Average KE MVC (N)	1141.1 ± 373.0 (704-1738)	900.1 ± 234.5 (530-1247)

Data are presented as mean ± standard deviation (range). *Indicates a significant difference from men at $p < 0.05$, according to independent samples *t*-tests. [#]Sample size for men (*n* = 9) and women (*n* = 8). KE = knee extension. MVC = maximum voluntary contraction.

Menstrual cycle and contraception use was self-reported; one female participant had missing data. Five women reported using contraceptive therapy and four women were not currently using or had not used any form of hormonal contraception within the past six months. Of those not using any contraceptive therapy, three experienced regular menstrual cycles and one reported averaging five weeks between periods. Mean ± standard deviation for estrogen and progesterone were 298.1 ± 264.9 ng/mL and 5.9 ± 6.0 pg/mL, respectively. Individual menstrual cycle and

contraception use information as well as corresponding sex hormone data for female participants are presented in Appendix B Table 1 and Appendix B Figure 2.

Mean caloric intake was 42% lower on restricted days (average of days 2 and 3) compared with baseline ($p < 0.001$; 95%*CI*: –1,085.4, –817.4 kcal). This was less than the targeted 50% restriction due to *ad libitum* underconsumption at baseline. Total sleep was reduced by 49% on restricted versus unrestricted nights ($p < 0.001$; 95%*CI*: –228.2, –215.2 min). The extent of caloric and sleep restriction did not differ between men and women (sex*day: $p = 0.481$ and $p = 0.618$, respectively), and total sleep and caloric intake was similar among sexes (main effect of sex: $p = 0.338$ and $p = 0.055$, respectively).

5.4.1 Qualitative and Quantitative Measures for Physical Effort

In order to demonstrate adequate physical stress during exercise, participants were asked to report their rating of perceived exertion before and after each event during the exercise protocol using a Borg scale (6-20). We also measured conventional markers of acute (pre- to post-exercise) and delayed (day 1 to day 3) muscle damage, including myoglobin and creatine kinase, respectively. Participants reported a significant increase in physical effort before and after each event during the ~90 min exercise protocol, with no differences according to sex (main effect of time: $p < 0.05$ each event) (Appendix B Figure 3). In agreement with these subjective data, reciprocal transformed serum myoglobin, increased by 169% from pre- to post-exercise (main effect of time: $p < 0.001$, $\eta_p^2 = 0.830$) (Appendix B Figure 4). Men also had a significantly higher myoglobin concentration than women when averaged by time or day (main effect of sex: $p = 0.006$, $\eta_p^2 = 0.351$). Reciprocal transformed serum creatine kinase increased 12% from day 1 to

day 3, nearing statistical significance and demonstrating the cumulative stress on skeletal muscle from baseline to peak stress (main effect of day: $p = 0.115$, $\eta_p^2 = 0.132$) (Appendix B Figure 4).

5.4.2 Total Extracellular Vesicle Size and Concentration

We began our analysis of EVs by examining their features using nanoparticle tracking analysis. We observed that exercise alone and exercise plus 48 h of 50% sleep and caloric restriction had a significant impact on EV concentration and size (Figure 15). There was a significant interaction of sex and time on EV natural log transformed EV concentration (sex*time: $p = 0.010$, $\eta_p^2 = 0.316$). Pairwise comparisons of pre- and post-exercise at each level of sex revealed a significant decline in women on day 1 (-37%; $p = 0.014$; Cohen's $d = 0.965$) and day 3 (-38%; $p = 0.003$, Cohen's $d = 1.287$), whereas there was no change in men (Figure 15A). Natural log transformed EV concentration also declined 29% in response to sleep and caloric restriction with no sex-specific differences (main effect of day: $p = 0.001$, $\eta_p^2 = 0.447$) (Figure 15A). Regardless of sex, EV mean size increased 13% from pre- to post-exercise and 8% from day 1 to day 3 (main effect of time: $p < 0.001$, $\eta_p^2 = 0.466$; main effect of day: $p = 0.002$, $\eta_p^2 = 0.433$) (Figure 15B).

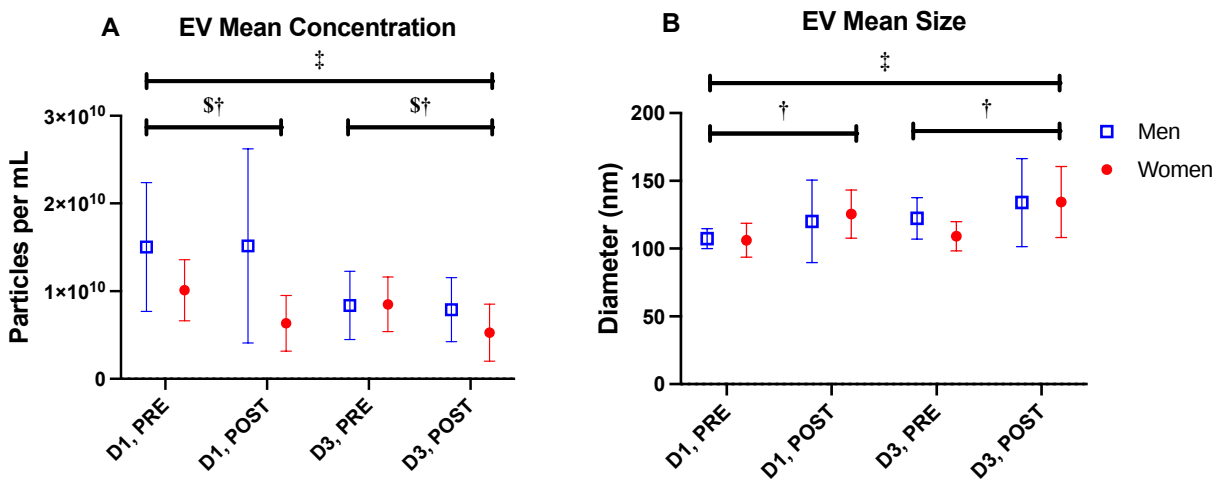


Figure 15. Extracellular vesicle (EV) mean concentration (A) and size (B) before and after exercise (time) on day 1 and day 3 (day) in men and women (sex).

EV concentration data was natural log transformed for analysis; raw data was used for EV size analysis. All data are presented as raw mean ± standard deviation. nm = nanometer. †Main effect of time. ‡Main effect of day. $†Sex by time interaction. Significance was set at $p < 0.05$.

5.4.3 Extracellular Vesicle Subpopulation Profiles

Next, we set out to broadly characterize various EV subpopulations by probing EVs with surface protein markers associated with exosomes (CD63), microvesicles (VAMP3), and apoptotic bodies (THSD1), as well as a marker of skeletal muscle-derived EVs (SGCA). Intensity features were exported for each subpopulation and analyzed to determine the effect of each condition on 1) objects per mL, 2) percentage of total EVs, and 3) intensity of each subpopulation. As a secondary aim, we also examined changes in CD63+, VAMP3+, and THSD1+ intensities within the skeletal muscle-derived EV population. Baseline comparisons revealed no differences between men and women in any EV subpopulation outcome ($p > 0.05$ each). Means ± standard deviation for all subpopulation outcomes are presented in Appendix B Tables 2-7.

5.4.3.1 CD63+ Extracellular Vesicles

A significant two-way interaction of time*day was present in CD63+ EV objects per mL (p = 0.033, η_p^2 = 0.228). Subsequent pairwise comparisons of time at each level of day revealed a 43% increase in CD63+ EVs from pre- to post-exercise following 48 h of sleep and caloric restriction (day 3), but there were no differences between pre- and post-exercise measures on day 1 (day 3: p = 0.017, Cohen's d = 0.583 vs. day 1: p = 0.773, Cohen's d = 0.065) (Figure 16A). A similar pattern emerged for the percentage of CD63+ EVs, whereby the proportion of total EVs that were CD63+ increased 76% from pre- to post-exercise on day 3 but did not differ on day 1 (time*day: p = 0.039, η_p^2 = 0.216; day 3: p = 0.018, Cohen's d = 0.579 vs. day 1: p = 0.727, Cohen's d = 0.079) (Figure 16B). While objects per mL and percentage of CD63+ EVs increased pre- to post exercise on day 3, reciprocal transformed CD63 median fluorescence intensity normalized to count decreased 55% from pre- to post-exercise on day 3, with no difference on day 1, suggesting a possible decline in CD63 surface density per particle (time*day: p = 0.028, η_p^2 = 0.241; day 3: p = 0.010, Cohen's d = 0.643 vs. day 1: p = 0.646, Cohen's d = 0.104) (Figure 16C). There were no differences according to sex (p > 0.05 each).

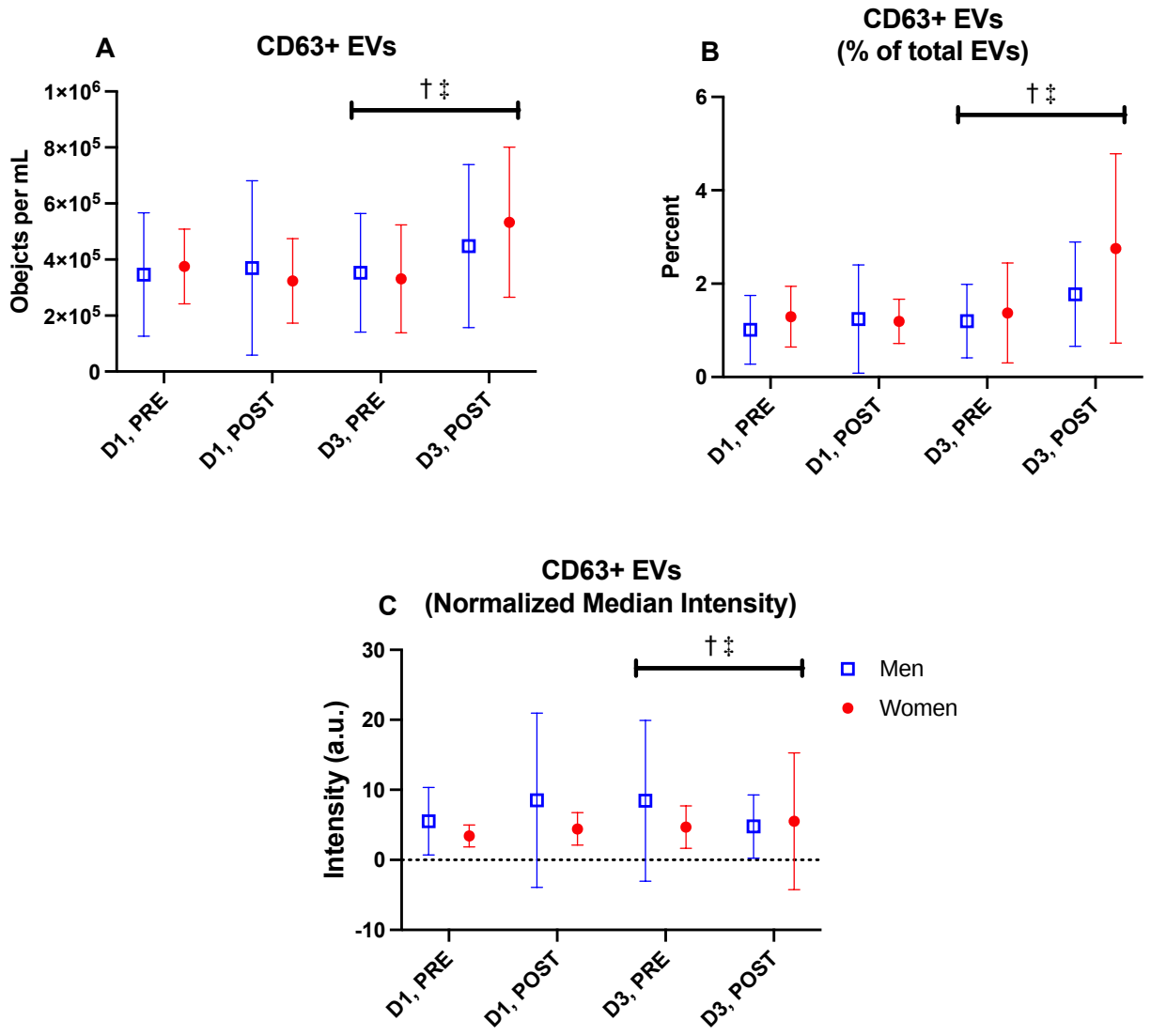


Figure 16. CD63+ extracellular vesicle (EV) objects per mL (A), percentage of total EVs (B), and median fluorescence intensity (C) before and after exercise (time) on day 1 and day 3 (day) in men and women (sex). Raw data was used for analysis of CD63+ EV objects per mL and percentage of total EVs; reciprocal transformed data was used for median fluorescence intensity. All data are presented as raw mean ± standard deviation for reader interpretation. †‡Time by day interaction. Significance was set at $p < 0.05$.

5.4.3.2 SGCA+ Extracellular Vesicles

While the objects per mL of SGCA+ EVs did not differ across time or day or between sex, there were significant main effects for each of these factors on the percentage of SGCA+ EVs.

Specifically, from pre- to post-exercise, there was a 20% increase in the percentage of SGCA+ EVs, and a 37% increase from day 1 to day 3 (main effect of time: p = 0.027, η_p^2 = 0.244; main effect of day: p = 0.001, η_p^2 = 0.438) (Figure 17A). When averaged across time and day, the percentage of SGCA+ EVs was 27% higher in women compared to men (main effect of sex: p = 0.049, η_p^2 = 0.198) (Figure 17A). Despite no difference in SGCA+ EV objects per mL from pre- to post-exercise, SGCA mean fluorescence intensity increased significantly following exercise, suggesting a possible increase in SGCA proteins per EV (main effect of time: p = 0.005, η_p^2 = 0.361) (Figure 17B).

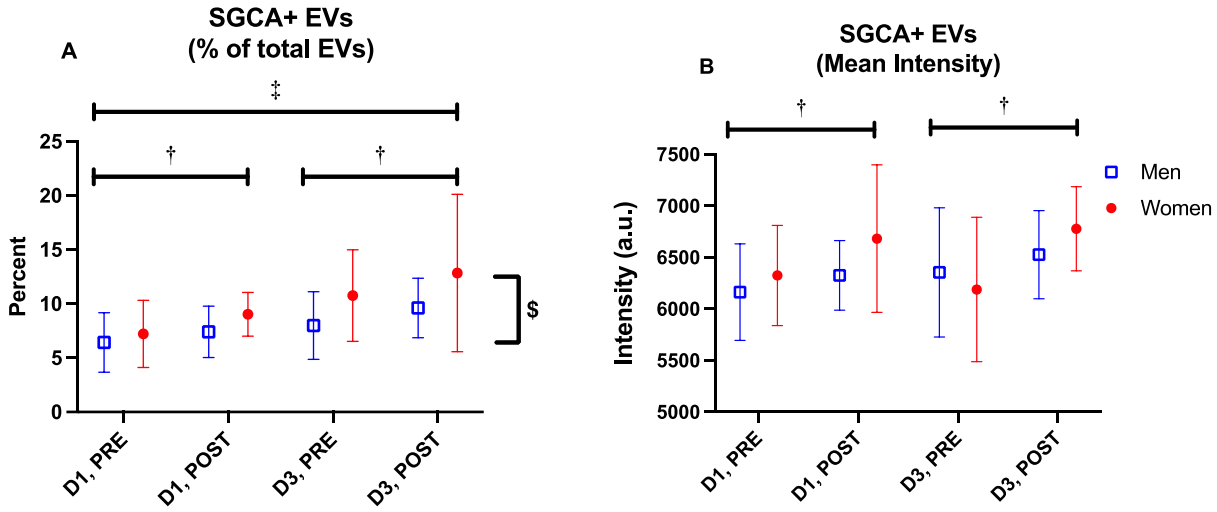


Figure 17. SGCA+ extracellular vesicle (EV) percentage of total EVs (A and mean fluorescence intensity (B) before and after exercise (time) on day 1 and day 3 (day) in men and women (sex).

Raw data were used for analyses of each outcome and are presented as mean ± standard deviation. $Main effect of sex. †Main effect of time. ‡Main effect of day. Significance was set at p < 0.05.

5.4.3.3 THSD1+ Extracellular Vesicles

There were no significant interaction or main effects of sex, time, or day on THSD1+ EV objects per mL, percentage of total EVs, or measure of fluorescence intensity (p > 0.05 each).

5.4.3.4 VAMP3+ Extracellular Vesicles

Although there was a modest increase in percentage of VAMP3+ EVs from pre- to post-exercise, it failed to meet statistical significance (main effect of time: $p = 0.086$, $\eta_p^2 = 0.155$). There were no significant differences in VAMP3+ EV objects per mL or measure of fluorescence intensity across time or day or between sex ($p > 0.05$ each).

5.4.3.5 CD63, THSD1, VAMP3 Intensities Relative to SGCA+ Extracellular Vesicles

We next examined the fluorescence intensities of exosomes (CD63), apoptotic bodies (THSD1), and microvesicles (VAMP3) within the skeletal-muscle derived EVs. CD63 sum intensity increased significantly from day 1 to day 3 in SGCA+ EVs (main effect of day: $p = 0.038$, $\eta_p^2 = 0.218$). CD63 mean intensity was greater in men than women, although this difference did not reach statistical significance (main effect of sex: $p = 0.086$, $\eta_p^2 = 0.680$). There were no differences in THSD1 or VAMP3 intensities within the skeletal muscle-derived EV subpopulation ($p > 0.05$ each).

5.5 Discussion

The purpose of this study was to characterize changes in EV profile characteristics (i.e., size, concentration, and surface proteins associated with EV subpopulations) across two stress conditions—exercise alone (single stress) versus exercise plus 48 h of sleep and caloric restriction (multi-stress)—in men and women. General EV characteristics such as total concentration demonstrated sex-specific changes, whereas EV size increased similarly in men and women following exercise and combined exercise plus sleep/caloric restriction. When examining EV

subpopulation characteristics, there was substantial variation according to sex and stress condition in SGCA+ and CD63+ EVs, respectively. Previous studies have primarily focused on single stress conditions, such as exercise (137, 138, 141–143) or nutrient intake alone (142, 197, 199), and few have included men and women (36, 37, 143, 204). To our knowledge, this is the first study to examine the combined effects of sleep and caloric restriction in addition to exercise on EV profiles in men and women.

Exercise resulted in a 37-38% decline in total EV concentration in women, whereas there was no change in men. Others have noted similar changes in EV concentration according to sex following an acute bout of exercise. In a mixed-sex group, Shill et al. (36) reported a 19% decline in CD62E+ (endothelial-derived) EV concentration in women only after a single bout of treadmill exercise, and Rigamonti et al. (143) observed a post-exercise decline in EVs in the 30-130 nm size range in women, but not men. Despite these intriguing observations, what appears to be a sex-specific response in EV concentration to exercise should be interpreted with caution. Although size exclusion chromatography is able to isolate EVs from most plasma proteins and small lipoproteins (205), other molecules with a similar size as EVs, such as low-density lipoproteins (LDL), very low-density lipoproteins (VLDL), and chylomicrons, may still be present as co-isolates (206). Bellou et al. (207) reported a 30% decline of VLDL in women following exercise, whereas, in a separate study, Brahmer et al. (142) reported no change in high density lipoproteins (HDL) and LDL in men. Taken together, changes in similarly sized particles that are co-isolated with EVs could account for at least some of the sex-specific response patterns noted in EV concentration following exercise as measured by nanoparticle tracking analysis.

Following nanoparticle tracking analysis, we next examined alterations in concentration and proportion of EV subpopulations and their corresponding fluorescence intensities. While there

no significant differences in the THSD1+ and VAMP3+ EVs—markers associated with apoptotic bodies and microvesicles, respectively—CD63+ EVs (exosomes) changed substantially. CD63+ EV concentration, proportion of total EVs, and fluorescence intensity were differentially impacted by stress condition; specifically, there was no change from pre- to post-exercise at baseline, but on day 3, CD63+ concentration increased by 43%, proportion of CD63+ EVs increased by 76%, and median fluorescence intensity declined by 55%. The inverse directional change in quantity of CD63+ EVs and median fluorescence intensity suggests a possible decline in the average number of CD63 proteins per particle (i.e., surface density of CD63 protein). CD63 is commonly used as a marker for exosomes due to its role in the endosomal pathway (208), though it is not exclusive to exosomes as it has been detected to a lesser extent in larger EVs, such as microvesicles (134, 209). CD63 expression may also vary according to cell type, adding to the complexity of analyzing its presence in circulation (209). In addition to its use as a marker for exosomes, CD63 is thought to play a role in target cell adhesion (208), allowing EVs to deliver their cargo and induce physiological changes such as immunomodulation and angiogenesis (210–212). CD63 is also involved in cell proliferation and survival (211, 213, 214). Previous studies have reported similar increases in circulating CD63+ EVs (137, 215, 216) and total CD63 protein content (142, 215) following exercise, though direct quantifiable comparisons cannot be made relating to the magnitude of CD63 expression due to variation among detection methods and lack of fluorescence normalization between studies. Nonetheless, we demonstrate significant shifts in the CD63+ EV subpopulation and CD63 surface protein cargo which vary according to single versus multi-stress conditions.

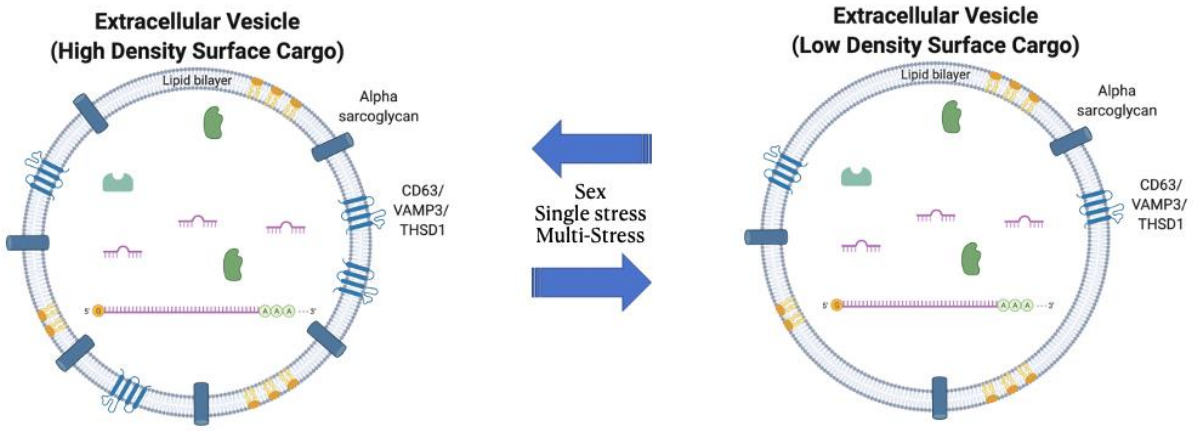


Figure 18. Illustration representing changes in surface protein density across the conditions of time (pre- to post-exercise) and day (baseline to peak stress) and between sexes.

Differences in median fluorescent intensity when normalized to count of SGCA, THSD1, VAMP3, or CD63 proteins, respsectively, suggests a increase or decrease in the number of these surface proteins per extracellular vesicle. Created with BioRender.com.

Given the exercise-based stress conditions in this study, we were particularly interested in EVs from skeletal muscle origin. Therefore, we probed EVs for alpha-sarcoglycan, which is highly enriched in skeletal muscle, and has been previously used as a marker to analyze skeletal muscle-derived EVs in circulation (138, 143). The proportion of SGCA+ EVs increased from 8.1% to 9.7% of total EVs from pre- to post-exercise (main effect of time) and ranged from 6.4% to 9.6% in men and 7.2% to 12.8% in women from pre-exercise on day 1 to post-exercise on day 3, respectively. The increase in proportion of SGCA+ EVs suggests augmented signaling originating from skeletal muscles, relative to other cell types, during exercise. These data are substantiated by others who have reported a similar increase in SGCA+ EVs following exercise (138, 143). The increase in proportion of skeletal muscle-derived EVs was complemented by an increase in SGCA mean fluorescence intensity despite no change in SGCA+ EV objects per mL, suggesting greater SGCA protein surface density (Figure 18). When looking at baseline (i.e., exercise only condition),

our data substantiate the directional changes reported by others (138, 143) and is similar, but slightly higher, than the 1-5% SGCA+ of total EVs peri-exercise observed by Guescini et al. (138). The difference between our results and those of Guescini et al. (138) may be accounted for by variations in exercise intensity and duration as well as methodological differences. Specifically, Guescini et al. (138) used anti-SGCA immunoaffinity capture beads, which may have lower recovery compared to our approach of directly staining the samples of isolated EVs.

A novel observation from this study is that the addition of sleep and caloric restriction (main effect of day) resulted in an increase in the proportion of SGCA+ EVs from 7.5% on day 1 to 10.3% on day 3. The increased presence of SGCA+ EVs in the circulation suggests augmented cell-cell communication originating from skeletal muscle tissue during combined sleep and caloric restriction. It is well established that skeletal muscle undergoes proteolysis to liberate amino acids for energy production via gluconeogenesis during periods of underfeeding (217). Additionally, enzymes related to energy metabolism are among some of the most commonly identified proteins in proteomic studies of EVs (218). One explanation for the increase in proportion of SGCA+ EVs during energy restriction is that skeletal-muscle derived EVs have a greater contribution to energy metabolism during hypocaloric compared to eucaloric energy states. If true, this hypothesis would establish a new mechanism through which cells coordinate and share limited energy resources when nutrient intake is suboptimal. This increase in proportion of SGCA+ EVs may also be due to the cumulative effects of daily physical activity. Creatine kinase is a conventional marker of muscle damage that peaks ~48 h after onset of intense exercise (219). Interestingly, the increase in creatine kinase concentration did not reach statistical significance 48 h after baseline exercise in this study (p = 0.115), but the proportion of SGCA+ EVs steadily increased from pre- to post exercise on day 1 through post-exercise on day 3, suggesting skeletal muscle-derived EVs may be

a more sensitive marker of endocrine signaling following exercise. Finally, we observed a significantly higher proportion of SGCA+ EVs in women compared with men (main effect of sex: 10.0% in women vs. 7.9% in men). Prior literature demonstrates that women operate at a higher percentage of heart rate reserve than men when performing the same work and with no difference in ratings of perceived exertion (220–222). This higher relative demands of performing a given task in women compared with men may have accounted for some of the difference in proportion of SGCA+ EVs noted here. Additional research is needed to determine the implications of this sex-based effect.

SGCA is primarily used as a marker for subpopulation analysis in EV research. However, it has important physiological functions. SGCA is part of a family of glycoproteins that associate with dystrophin to make up a critical complex necessary for normal skeletal muscle development (223). Sarcoglycan abnormalities are a hallmark in numerous muscular dystrophies (223). *In vitro* (224) and *in vivo* (225) studies have shown rescued muscle cell development in dystrophic models via pharmacological intervention. SGCA+ EVs have also been shown to carry miRNA that target pathways involved in skeletal muscle differentiation (138, 226–229) and are taken up by a range of tissues (230). We isolated EVs in the size range of both exosomes and microvesicles and were also CD63+ and VAMP3+, respectively. Data from Le Bihan et al. (231) demonstrated that exosomes and microvesicles released from myotubes have distinct proteomic profiles (65% overlap), which have differing roles in myogenesis. Collectively, data from this study and others (138, 143) suggest a possible endocrine function of EVs from skeletal muscle which should be further investigated as a novel mechanism of tissue crosstalk during exercise.

This descriptive study establishes an important starting point to lay the foundation for future investigations aimed at distinguishing the effects of stress type(s) and sex on EV dynamics

and the role of EVs in systemic cell-cell communication. Though single stress studies provide good internal validity, generalizability to an applied setting is often compromised. Our use of two different stress conditions demonstrates the interactive effects that occur by combining multiple stresses as they occur in real-world settings. These data provide greater ecological validity and are more applicable to populations that may experience any combination of sleep disruption/restriction, undernutrition, and/or physical work such as military service members, shift workers, emergency services personnel, and individuals attempting to lose weight. We used contemporary methodologies to measure multiple features of EVs in a high throughput manner, including nanoparticle tracking analysis and imaging flow cytometry. Our inclusion of men and women adds to the scant pool of data examining EVs in both sexes and helps distinguish aspects of EV research that should consider sex for analysis and interpretation of results.

We used a combination of size exclusion chromatography and nanoparticle tracking analysis to isolate and broadly characterize EVs. Each of these are commonly used methods but are not without limitations. Size exclusion chromatography is a reliable and effective method for obtaining highly enriched EVs relative to other methods such as precipitation or filtration, specifically for particles within the 70-1000 nm diameter range (153, 232). No method exists, however, for achieving entirely pure samples, and co-isolation of similarly size molecules is likely. Though nanoparticle tracking analysis is considered a gold standard method for size and concentration characterization, it is unable to distinguish between EVs and similarly sized particles. In light of these facts regarding the isolation and size/concentration characterization methods used here, we cannot discount the fact that at least some of the variation in EV size and/or concentration can be accounted for by changes in co-isolates across conditions. Although our goal was to broadly characterize different EV subpopulations, including exosomes, microvesicles, and

apoptotic bodies, we used a limited panel of fluorescent markers for identifying these populations; therefore, EVs that were present but were any $CD63^{-}$, $THSD1^{-}$, or $VAMP3^{-}$ EVs went undetected. As more data is published and technologies aimed at studying nanoparticles evolve, our capability to more holistically assess EV subpopulation dynamics will also evolve. Finally, we examined the effects of two different exercise conditions—one with exercise alone and the other following 48 h of combined sleep and caloric restriction. While we noted interactive effects on EV characteristics between the two stress conditions, we are unable to distinguish between the effect of sleep restriction and energy deficit. Though sleep and caloric restriction often go hand in hand, future studies should tease out the effects of each.

5.6 Conclusion

Data from this study extends previous findings and adds to an exciting and burgeoning field of EV research. We demonstrated sex- and stress-specific changes in EV profile dynamics which lays a foundation for future studies to examine changes in EV cargo and determine their implication on physiology with the long-term end goal of EV therapeutics. Basic science research is showing exceptional advancements using EVs in this manner (233). One can conceptualize similar translational benefits applied in humans whereby EVs may be isolated from healthy/high performing individuals, or synthetic EVs may be bioengineered, and administered to individuals with the goal of promoting health and optimizing performance. For now, characterization of EVs in humans is a necessary first step for identifying physiological targets of interest.

6.0 Manuscript 3: Resistance Exercise Alters Extracellular Vesicle Size and Subpopulation Characteristics Differently in Men and Women

6.1 Abstract

Extracellular vesicles (EV) released during exercise have been shown to carry cargo targeting pathways associated with myogenesis and are established mediators of adaptation to exercise. Sexually dimorphic responses to exercise have been documented in literature, but there are no published data comparing changes in EV profiles in men and women after resistance exercise. We tested the hypothesis that EV profiles would demonstrate a sex-specific signature following resistance exercise. We compared 10 men and 10 women undergoing a bout of acute heavy resistance exercise test (AHRET) using 75% of their one-repetition maximum on back squats. Blood was drawn before and immediately after AHRET. EVs were isolated from plasma using size exclusion chromatography and probed for surface proteins associated with exosomes (CD63), microvesicles (VAMP3), and apoptotic bodies (THSD1) as well as a marker for skeletal muscle EVs (SGCA). Despite no differences in total EV concentration following AHRET, analysis of EV subpopulations using imaging flow cytometry revealed significant increases in exosome and microvesicle subpopulations. Exosome concentration and proportion of total EVs increased 23% ($p = 0.006$) and 113% ($p = 0.005$) in both sexes, but the change was primarily driven by men. In concordance with these observations, total EV mean size declined in men ($p = 0.020$), suggesting a relative increase in small EVs, whereas total EV mean size did not change in women ($p = 0.275$). Microvesicle (VAMP3+) concentration and proportion of total EVs increased by 93% ($p = 0.025$) and 61% ($p = 0.030$), respectively, in men and women. Skeletal muscle-derived EVs

(SGCA+) trended upward following AHRET, regardless of sex ($p = 0.084$). Interestingly, SGCA+ EV concentration was 69% higher in women compared to men (main effect of sex: $p = 0.007$). Differences were also observed for CD63 median fluorescence intensity (MFI) (sex*time: $p = 0.045$), VAMP3 MFI (main effect of time: $p = 0.028$), and SGCA MFI (main effect of sex: $p = 0.014$; main effect of time: $p = 0.012$), suggesting altered surface protein density according to interactive and main effects of sex and time. There were no effects of time or sex on THSD1+ EVs or fluorescence intensity. Results from this study lay a foundation for future investigations to identify molecular mechanisms underpinning sexually dimorphic responses to resistance exercise training which may inform optimized training for men and women.

6.2 Introduction

Resistance exercise confers a wide range of benefits, including health promotion, physical performance improvements, and anti-aging (234). Some of the adaptive responses to resistance training have been shown to differ according to sex. For example, a recently published meta-analysis by Roberts et al. (93) concluded that women experienced a greater response in upper body strength compared to men, whereas there were no differences in lower body strength or total body hypertrophy between sexes. Mounting evidence suggests part of the benefits derived from exercise are mediated through the release of myokines, or functional molecules released into circulation during muscle contraction (235). Skeletal muscle is one of the largest organs in the body and can function in an endocrine manner by secreting factors involved in organ crosstalk (4). Extracellular vesicles (EV) are now recognized as a novel class of myokines with roles in myogenesis (231, 236), immunomodulation (237), and angiogenesis (201, 238).

Most of the EV research to date has focused on aerobic exercise with considerably less data published using resistance exercise training (139, 144, 145, 239, 240) and no studies comparing EV characteristics in men and women after weight training. Elucidation of sex-dependent EV responses may help identify biomarkers to better inform optimized training for men and women.

Extracellular vesicles are a heterogeneous population of lipid membrane bound vesicles secreted by nearly all cells (134). There are three EV subclasses: exosomes, microvesicles, apoptotic bodies. Each of these are defined by their biogenesis. Exosomes are formed though an endosomal pathway with endpoints of either lysosomal degradation or extracellular release (241). Microvesicles bud directly from the plasma membrane, and apoptotic bodies bud or bleb from the plasma membrane during programmed cell death (241). EVs can be further characterized by additional descriptive features such as shape or size. Though the latter is commonly used to classify EV subpopulations, there are no definitive size cutoffs, and overlap exists between EV subtypes which range from 30-150 nm for exosomes, 100-1000 nm for microvesicles, and 50-5000+ nm for apoptotic bodies (117). EVs are enriched with cargo including lipids, proteins, and RNA, which are transferred to target cells to induce physiological changes (18, 136). Apoptotic bodies may also contain organelles or other cellular components derived during the fragmentation period of apoptosis. EV cargo is reflective of the parent cell state during formation (134) and is enriched with proteins that function in their formation, such as CD9, CD81, or CD63 for exosomes; vesicle associated membrane-3 (VAMP3) for microvesicles; and Annexin-V or thrombospondin-1 (THSD1) for apoptotic bodies. Although no protein has yet been identified as an exclusive marker of any EV subclass (122), the aforementioned proteins are commonly associated with each of the respective subclasses (132, 242). Others have also used alpha-sarcoglycan (SGCA) as a marker of skeletal muscle-derived

EVs during exercise (138, 142, 143). Muscle cells release EVs and are taken up by other myocytes/myotubes to increase muscle differentiation and growth (231, 236, 243).

There is a growing body of evidence characterizing changes in EVs during aerobic training (for comprehensive reviews, see 198, 240), but far fewer data reported using resistance training (139, 144, 145, 239). Men completing an acute bout of flywheel-based resistance exercise (squats) experienced a 2-fold increase in EV mean concentration, accompanied by an increase in EV-associated miR-206 and miR-146a, two hours after exercise compared to baseline (144). Just et al. (139) reported no change in EV size or concentration 1 h after blood flow restriction exercise compared to baseline. However, EV surface proteins and miRNA were altered in a hypertrophic manner. Similar results were reported by Lovett et al. (198) in men completing muscle damaging exercises consisting of plyometrics and downhill running. Following 8-weeks of resistance training in elderly men, EV CD63 surface protein expression declined in the training group compared to controls (239). Finally, Garner et al. (145) observed an increase in proteins and miRNAs associated with exosome biogenesis following aerobic plus resistance training exercise, though circulating EVs were not measured in that study. Taken together, initial evidence in men demonstrates that resistance exercise increases EV concentration with no change in size and alters molecular machineries that promote EV biogenesis. Currently, there are no comparisons of EV profiles in men and women undergoing acute resistance exercise.

The purpose of this study was to examine total EV size and concentration, EV subpopulation concentration, and EV surface proteins in men and women after AHRET. We hypothesized that total EV concentration would increase, and size would not change in men and women after resistance exercise. We further hypothesized that EV subpopulation concentrations

and surface proteins would differ according to sex. Results from the study will be the first of its kind to detail changes in EV profiles according to sex following resistance exercise. This experiment is important for identifying molecular targets for mechanisms underlying sexual dimorphisms in strength training. Ultimately, we anticipate that data from this study may inform future investigations aimed at optimizing training programs for men and women.

6.3 Methods

Data presented here are a subset of a lager prospective cohort study (United Kingdom Ministry of Defense, Award # WGCC 5.5.6 - Task 0107) aimed at optimizing physical readiness and training for military occupational performance in men and women. This study was approved by the University of Pittsburgh Institutional Review Board (IRB # 19030387) and United Kingdom Ministry of Defense Research Ethics Committee. All study procedures were performed in accordance with the Declaration of Helsinki.

6.3.1 Participants

Participants were recruited via email, listservs, electronic mailing list, flyers, social media, new stations, online advertisement (Pitt+Me), and in-person communications. Following a telephonic pre-screen, eligible participants provided written, informed consent and were free to withdraw from the study any time.

Recreationally active, health men and women were included in the study if they were between the ages of 18-36 years, participating in a minimum of 30 min of physical activity three

times per week, free of any upper or lower body injuries or impairments, and comfortable with multiple blood draws. Individuals were excluded from the study if they were training for a competitive sporting event, had a ≥ 10 lbs weight fluctuation within the previous two months (self-report), had a medical condition precluding them from participating in intense physical activity, a heart condition or high blood pressure, experienced chest pain at rest or during exercise, experienced dizziness or loss of consciousness in the previous 12 months, diagnosed with a chronic medical condition, had been advised by a physician to only participate in physical activity under supervision, had an injury that prevented physical activity for more than one month in the previous two years, had any psychological disorders, were pregnant or planning to become pregnant during the study, or were unwilling to perform any of the testing procedures.

6.3.2 Experimental Design

Using a within-subjects, repeated measures design, we assessed circulating EV characteristics before and immediately after acute heavy resistance exercise test (AHRET) in men and women. AHRET is a well-established resistance exercise protocol for inducing hormonal stress changes (33, 245, 246). Testing was performed at the Neuromuscular Research Laboratory / Warrior Human Performance Center, Pittsburgh, PA and occurred over multiple testing sessions separated by at least seven days (Figure 19). Familiarization to testing procedures and baseline measurements of aerobic fitness, strength, and body composition were completed prior to the AHRET. Beginning with testing Day A, body composition was determined using dual-energy x-ray absorptiometry (Lunar iDXA, GE, Wauwatosa, WI, USA), and aerobic fitness was assessed on a treadmill (Woodway; Waukesha, WI) using the Bruce protocol (158). One-repetition maximum (1-RM) assessments of the back squat, bench press, and deadlift were performed

according to National Strength and Conditioning Association testing guidelines (162) on testing Day B. A 1-RM was considered the highest weight an individual could lift with each respective exercise using proper technique. Testing Day C consisted of measurements of performance on a military-style obstacle course (data not used in this study). The AHRET was performed on test Day D. Participants arrived at the testing facility between 0500-1000 h having withheld from food for 10 h, caffeine for 8 h, non-steroidal anti-inflammatory drugs for 72 h, and exercise for 72 h. Following a dynamic warmup, subjects performed six sets of 10 repetitions of back squats using 75% of their 1-RM as determined at a prior visit (Day B). Squats were performed on a smith machine rack (Star Trac, Max Rack) which allowed for movement along the x- and y-axes. An adjustable box was positioned to standardize squat depth so that the subject's thighs were parallel to the ground at the end range of flexion. Subjects were instructed not to bounce off the box. If, despite his/her best effort, a subject was unable to perform 10 repetitions, the weight was adjusted to allow for completion of all repetitions. Participants were allotted two minutes of rest between each set. Rating of perceived exertion was recorded before and after each set using the 1-10 Borg ratio scale (150), where 0 is an effort of "nothing at all" and 10 is "very, very strong". Trained staff provided spotting and monitored completion of all sets and repetitions during the AHRET. All sessions were supervised by a Certified Strength and Conditioning Specialist®.

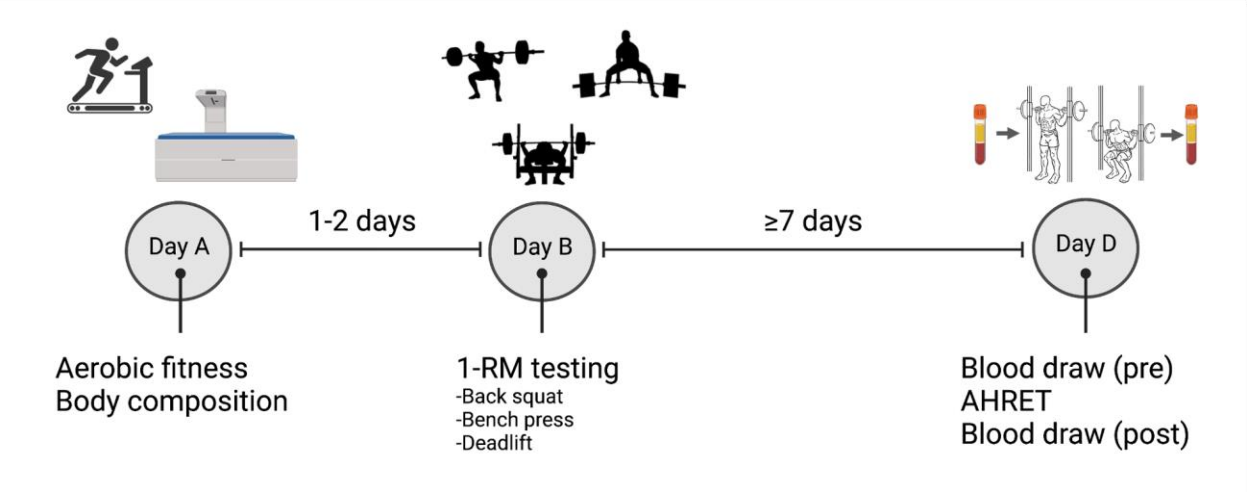


Figure 19. Overview of Soldier Performance and Readiness as Tactical Athletes (SPARTA) testing procedures. On testing Day A, aerobic fitness ($V\dot{O}2_{peak}$) was assessed using the treadmill Bruce protocol, and body composition was determined by dual energy x-ray absorptiometry scan. On Day B, participants completed one repetition maximum (1-RM) testing on the back squat, bench press, and deadlift. On Day D, participants completed the acute heavy resistance exercise test (AHRET) bout consisting of six sets of 10 repetitions of back squats at 75% 1-RM; each set was separated by two minutes. Blood was drawn before and immediately after the AHRET. Testing Days A and B were separated by 1-2 days and Days B and D were separated by at least seven days. Figure was created using BioRender.com.

6.3.3 Blood Collection and Analysis

Venous blood was drawn shortly before exercise in a rested and seated or supine position and again immediately after exercise using a 21-guage or 23-guage needle (Becton, Dickinson and Company Vacutainer, Franklin Lakes, NJ, USA). Blood was collected into 10 mL serum and 6 mL EDTA plasma tubes. Serum was allowed to clot for 30 min at ambient temperature and subsequently centrifuged at 1500 x *g* for 15 min at 4ºC. Plasma was centrifuged immediately after blood draw using the same settings as serum. Serum and plasma were aliquoted and stored at –80ºC until analysis.

Estradiol (baseline) and myoglobin (pre-/post-AHRET) were measured from serum using standard enzyme-linked immunoassay procedures. The range and sensitivity for estradiol assays (Alpco 11-ESTHU-E01; Salem, NH, USA) were 20-3200 pg/mL and 10 pg/mL, respectively. Myoglobin assays (Alpco 25-MYOHU-E01; Salem, NH, USA) had a range of 25-1000 ng/mL and sensitivity of 5 ng/mL. Intra-assay coefficients of variation were ≤9.3% and ≤6.6% for estradiol and myoglobin, respectively. All samples were measured in duplicate.

6.3.4 Extracellular Vesicle Analysis

6.3.4.1 Size Exclusion Chromatography

Extracellular vesicles were isolated from plasma using size exclusion chromatography (SEC) (qEV 70 nm Original; IZON, Medford, ME, USA) according to manufacturer's guidelines. Stored SEC columns were flushed with 10 mL of 0.22 μm filtered phosphate buffer solution (PBS) before adding 450 μl of plasma. The first 3 mL of eluate was discarded, and the next 1.5 mL (EV fraction) was collected for analysis. Isolated EV samples were aliquoted and stored at –80ºC until analysis.

6.3.4.2 Vesicle Flow Cytometry

Vesicle size and concentration were measured using the Vesicle Flow Cytometry™ assay (Cellarcus Biosciences, San Diego, CA, USA) on a CytoFLEX™ flow cytometer (Beckman Coulter, Brea, CA, USA) according to manufacturer's recommendations. Preliminary experiments were run to determine the optimal dilution for subsequent staining and analysis (Appendix C Figure 1). Samples were stained using a membrane dye (1:100; vFRed™, Cellarcus Biosciences) and incubated for one hour in the dark at ambient temperature. Data was acquired on the

CytoFLEX™ using the following settings: forward scatter (488 nm), side scatter (488 nm), and violet side scatter (405 nm) gains at 100; fluorescence gains at 1000 (all channels); primary trigger threshold using violet side scatter (405 nm); manual threshold of fluorescence above background at 1500; and width channel to 690/50. Data was collected for 120 seconds for each sample using the fast setting (60 μL/min) and analyzed using FCS Express 7 software (De Novo, Pasadena, CA, USA).

6.3.4.3 Imaging Flow Cytometry

We used the ImageStream®X Mark II imaging flow cytometer (EMD, Millipore Sigma, Seattle, WA, USA) for EV analysis. Imaging flow cytometry combines conventional flow cytometry and brightfield microscopy capabilities into one instrument, enabling multiparameter detection of sample characteristics in a high-throughput manner. The use of time delayed integrated (TDI) charged coupled device (CCD) camera technology in the ImageStream®X Mark II makes it more sensitive for measuring small particles than most conventional flow ctyometers (247). Channels 1 and 9 were used for brightfield imaging (60X) in this study. Channels 2, 4, 7, and 11 were used for fluorescence detection of muscle-derived EVs (SGCA), apoptotic bodies (THSD1), microvesicles (VAMP3), and exosomes (CD63), respectively (132, 138, 242).

Prior to sample analysis in imaging flow cytometry, samples were prepared using the following procedures. For each sample, 140 μL of 4% paraformaldehyde was added to 140 μL of isolated EVs in PBS and allowed to fix at ambient temperate for 10 min. Fixed samples were then centrifuged for 30 min at 16,000 x *g* at 4°C. After centrifugation, 140 μL of supernatant was then carefully removed, making sure not to disturb the EV pellet, and 140 μL of blocking buffer (3% bovine serum albumin in PBS) was then added. Samples incubated on a rocker for one hour at ambient temperature. Following incubation, the samples were again centrifuged for 30 min at

16,000 x *g* at 4°C and 140 µL was carefully removed. Samples were then stained with the following antibodies: 0.5 µL anti-human CD63 Alex Fluor® 700 (1:280, NBP2-42225AF700, Novus Biologicals, CO, USA), 0.5 µL anti-human vesicle-associated membrane-3 (VAMP3) Alex Fluor® 405 (1:280, NBP1-97948AF405, Novus Biologicals, CO, USA), 1.4 µL anti-human thrombospondin-1 (THSD1) Alex Fluor® 594 (1:100, FAB5178T-100UG, Novus Biologicals, CO, USA), and 0.35 µL anti-human alpha-sarcoglycan (SGCA) FITC (1:400 dilution, orb29665, Biorbyt, MO, USA). Stained samples incubated overnight at 4ºC. The following day, samples were centrifuged for 30 min at 16,000 x *g* at 4°C before removing 60 µL of supernatant and adding 20 µL of PBS for a final volume of 100 µL.

Single-stained compensation controls (UltraComp eBeads™ Plus; Invitrogen) were used to correct for fluorescence carryover between channels. Fluorescence minus one (FMO) controls were used to establish gates for each spectral channel. Compensation and FMO controls followed the same staining protocol as outlined above with the exception that antibody volumes were half that of the volume used in samples.

Data were collected using an ImageStream®X Mark II imaging flow cytometer (EMD, Millipore Sigma, Seattle, WA, USA) and INSPIRE™ software using the following settings: objective 60x, slow speed, high sensitivity, 7µm core size, auto-focus, auto-centering, and high gain mode. High gain mode enables better detection of small, dim particles on the ImageStream®X Mark II instrument compared to normal gain mode (155). Each sample was run for 3 min, and 2,000 events were collected for compensation and FMO controls. SpeedBeads® were gated out during data collection by plotting a histogram for channel 6 (side scatter) and collecting events less than the high side scatter peak indicative of SpeedBeads® (i.e., <1e+5 channel 6 intensity x-axis) (Appendix C Figure 2). The same gate was applied to all samples. Laser voltage settings

were as follows: 405 nm 175 mW, 488 nm 200 mW, 561 nm 200 mW, 642 nm 150 mW, and SSC 70 mW. All timepoint samples for a given subject were stained and analyzed on imaging flow cytometry on the same day to control for potential day-to-day variability.

Following data collection in INSPIRE™, samples were analyzed using the IDEAS® 6.2 software. Positive events in each EV subpopulation were manually gated using the following procedures (Appendix C Figure 2). Scatterplots were made by plotting each stained fluorescence marker (y-axis) against its neighboring channel (x-axis). Events with a positive intensity were gated for each fluorescence channel. The file was then saved as a template, and gates were adjusted using each FMO control. Samples were batch processed using the template containing the final adjusted gates so that all samples run on the same day were gated in the same manner. Features exported for analysis included objects per mL, percentage gated of total events, and intensity of channels 2 (SGCA), 4 (THSD1), 7 (VAMP3), and 11 (CD63). *Intensity* is the sum of fluorescence within the defined pixel region for each EV after correction for background pixel values. Intensity is used to measure protein expression (e.g., SGCA, THSD1, VAMP3, CD63) and estimated fluorescence activity. When normalized to EV count, median fluorescence intensity gives an estimate of the surface protein density per particle. An increase in median fluorescence intensity per particle is indicative of a greater number of surface proteins per particle whereas a decrease suggests less proteins per particle.

6.3.5 Statistical Analysis

A sample size of 20 (10 men) was determined *a priori* to be adequate to detect differences between men and women using a repeated measures design with one within-subjects factor (time) based on previously reported data (36, 37). Subject characteristics were analyzed using

independent samples *t-tests*. The interaction and main effects of sex and time were analyzed using a two-way mixed analysis of variance with Bonferroni *post hoc* adjustments, as appropriate. Data were natural log, square root, or reciprocal transformed and used for analysis when assumptions of normality were not met. If assumptions were still not met following data transformations, Wilcoxon signed-rank exact tests were used to compare pre- and post-exercise, split by sex. Dependent variables included: total EV size and concentration, concentration (objects per mL) of each EV subpopulation (CD63+, VAMP3+, THSD1+, SGCA+ EVs), proportion of each subpopulation as a percentage of total EVs, and median fluorescence intensity (normalized to count) (MFI) for each subpopulation. Total EV size and concentration measurements consisted of all vesicles with a positive intensity above background using fluorescence triggering (VSSC) as assessed using vesicle flow cytometry. Subpopulation assessments were only particles within each respective subpopulation gate (i.e., CD63+, VAMP3+, THSD1+, SGCA+ EVs) as measured using imaging flow cytometry. The percentage of each subpopulation as a proportion of total EVs was defined as the number of gated particles within a subpopulation divided by the total number of particles collected during the 3-min run time on imaging flow cytometry. Raw data are presented as mean ± standard deviation or estimated marginal means ± *95%CI* for pairwise comparisons following a significant omnibus test for all outcomes. Effect sizes were calculated using partial eta squared (η_p^2) for analysis of variance, Cohen's *d* for paired samples *t*-tests, and Cohen's *r* for Wilcoxon signed-rank tests (163). Statistical significance was set at $p < 0.05$ (two-tailed). Analysis was performed using SPSS, version 27.1 (SPSS, IBM Corp., Armonk, NY, USA).

6.4 Results

Twenty participants (10 men) completed this study to characterize the effects of resistance training on EV profiles in men and women. Baseline demographic, anthropometric, and physiological characteristics were measured and recorded at least seven days prior to completing the AHRET and are outlined in Table 1.

Table 5. Baseline subject characteristics.

	Men (***n*** **= 10)**	**Women** (***n*** **= 10)**
Age (y)	24.8 ± 5.2 (18 − 32)	27.7 ± 5.0 (20 − 35)
Height (cm)*	178.9 ± 8.1 (161.0 − 193.0)	162.6 ± 5.7 (154.1 − 172.1)
Body mass (kg)*	83.9 ± 20.4 (60.9 − 124.4)	64.8 ± 12.1 (51.9 − 85.0)
Body fat (%)*	20.5 ± 8.4 (13.7 − 38.0)	34.0 ± 6.4 (23.2 − 47.0)
Fat-free mass (kg)*	62.5 ± 9.3 (47.8 − 72.7)	40.0 ± 4.4 (33.9 − 49.2)
Fat mass (kg)	18.5 ± 12.7 (9.1 − 46.6)	22.5 ± 8.3 (12.1 − 39.7)
$\dot{V}O2_{peak}$ **(ml·kg·min^{-1})***	50.9 ± 9.4 (32.1 − 65.3)	40.5 ± 4.9 (34.7 − 48.4)
1-RM Squat (kg)*	121.1 ± 29.2 (84.1 − 170.5)	56.6 ± 15.3 (25.0 − 75.0)
1-RM Bench Press (kg)*	92.7 ± 21.0 (70.5 − 131.8)	35.2 ± 4.0 (27.3 − 40.9)
1-RM Deadlift (kg)*	137.3 ± 25.8 (102.3 − 165.9)	73.4 ± 8.0 (61.4 − 86.4)

***Indicates a significant difference between men and women. Data are presented as mean ± standard deviation (range). Significance was set at $p < 0.05$.**

Menstrual cycle and contraception use was self-reported. Four women reported using contraceptive therapy, and six women were not currently using or had not used any form of hormonal contraception within the past year. Of those not using any contraceptive therapy, five experienced regular menstrual cycles, and one was amenorrheic. Mean ± standard deviation for estradiol was 37.2 ± 42.0 ng/mL. Individual menstrual cycle and contraception use information as well as corresponding sex hormone data for female participants are presented in Appendix C, Table 1 and Appendix C, Figure 3.

Myoglobin concentration was measured before and after exercise as a conventional marker of acute muscle damage. Data transformations were unsuccessful in achieving homogeneity of variance, but were able to correct normality; therefore, within-subjects comparisons were made using paired *t*-tests, split by sex. Natural log transformed myoglobin increased 132% from pre- to post-exercise in men but there were no statistical differences in women (men: $p < 0.001$, Cohen's $d = 2.536$; women: $p = 0.291$, Cohen's $d = 0.355$) (Figure 20).

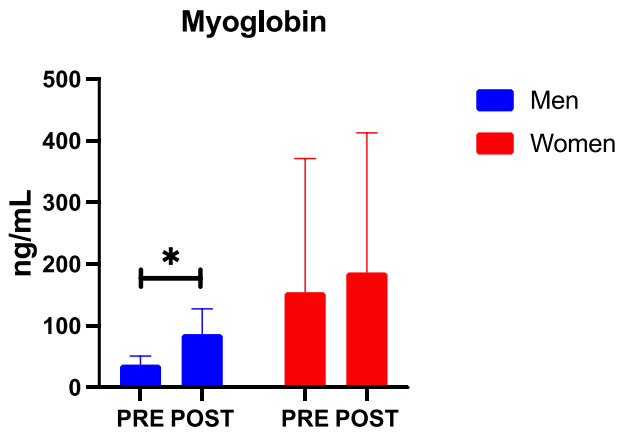


Figure 20. Serum myoglobin concentration measured before and immediately after a bout of acute resistance exercise training in men and women.

Data were natural log transformed and analyzed using paired t-tests, split by sex. Data are presented as raw mean ± standard deviation. †Main effect of time. PRE = pre-exercise. POST = post-workout. Significance was set at p < 0.05.

6.4.1 Total Extracellular Vesicle Concentration and Size

Total EV mean concentration and size were measured using vesicle flow cytometry. Parametric testing assumptions were unmet following data transformation; therefore, pre- and post-exercise comparisons were made using Wilcoxon signed-rank exact tests, split by sex. There were no significant differences in EV mean concentration between pre- and post-exercise in men or women (men: $p = 0.193$, Cohen's $r = 0.435$; women: $p = 0.770$, Cohen's r = 0.113) (Figure 21A). EV mean size decreased 3% in men following AHRET but there was no difference in women (men: $p = 0.020$, Cohen's $r = 0.725$; women: $p = 0.275$, Cohen's $r = 0.371$) (Figure 21B).

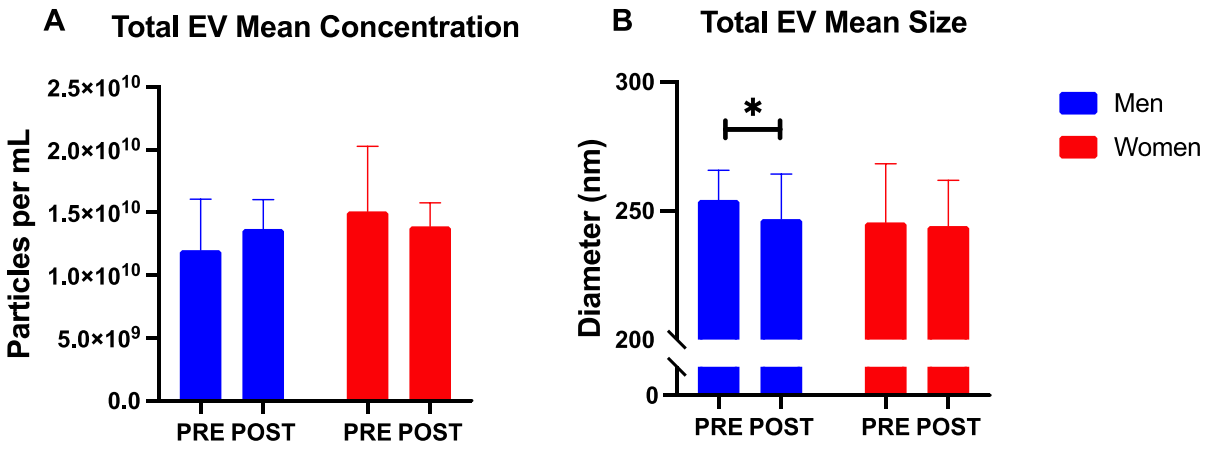


Figure 21. Total EV mean concentration (A) and size (B) measured before and immediately after a bout of acute heavy resistance exercise in men and women.

Data was analyzed using Wilcoxon signed-rank exact test, split by sex. Data are presented as raw mean ± standard deviation. *Significantly different. PRE = pre-exercise. POST = post-workout. Significance was set at $p < 0.05$.

6.4.2 CD63+ Extracellular Vesicles

Natural log transformed CD63+ EV concentration (objects per mL) increased 23% following AHRET (sex*time: $p = 0.082$, $\eta_p^2 = 0.159$; main effect of time: $p = 0.006$, $\eta_p^2 = 0.356$) (Figure 22A and 22D). A similar pattern was observed for the proportion of CD63+ EVs which increased 113% after exercise (sex*time: $p = 0.091$, $\eta_p^2 = 0.150$; main effect of time: $p = 0.005$, $\eta_p^2 = 0.359$) (Figure 22B and 22E). Although the sex by time interactions did not reach statistical significance, visual inspection of the mean plots shows an increase in CD63+ EV concentration and proportion to total EVs in men but a slight decrease or no change in women following AHRET, suggesting that the main effects of time were primarily driven by men. There was, however, a significant interaction of sex and time on natural log transformed CD63 MFI (sex*time: $p = 0.045$, $\eta_p^2 = 0.205$) (Figure 22C). Pairwise comparisons of pre- and post-exercise at each level of sex revealed a significant decrease in men but not women (men: $p = 0.001$, Cohen's $d = 1.508$; women: $p = 0.564$, Cohen's $d = 0.189$) (Figure 22F).

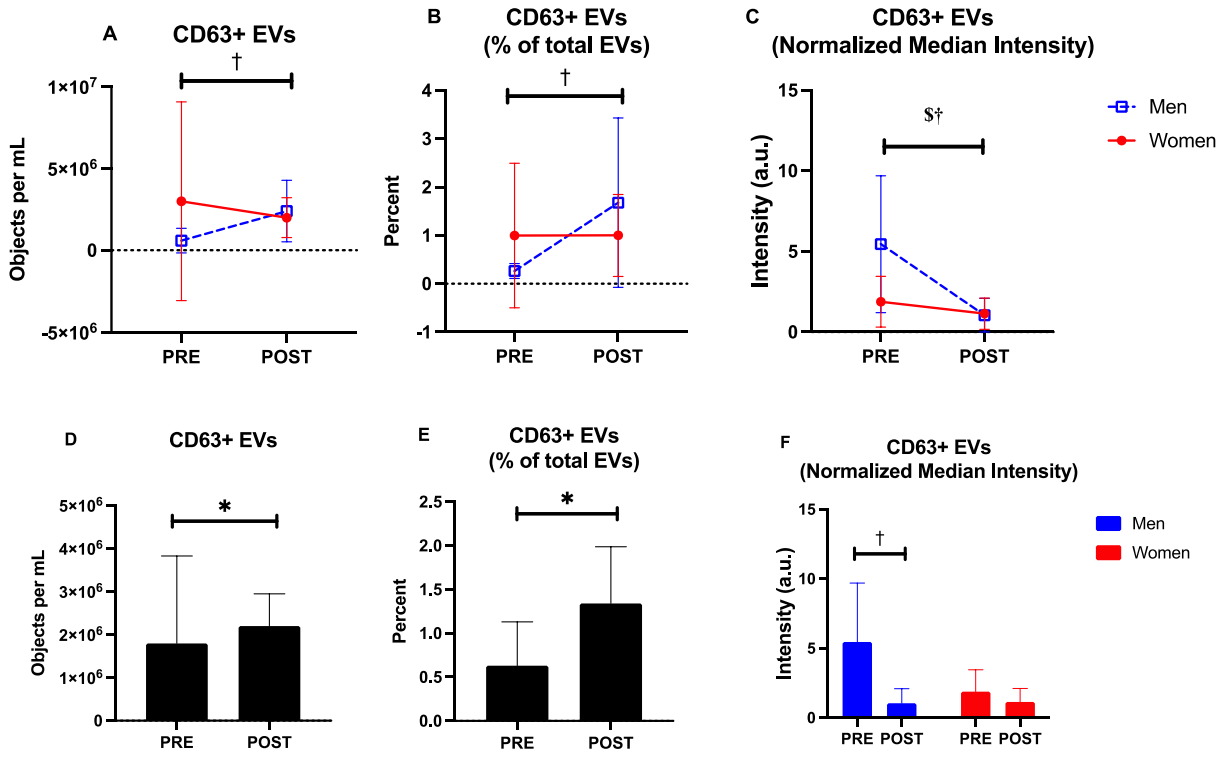


Figure 22. CD63+ EVs concentration (objects per mL), proportion of CD63+ EVs as a percentage of total EVs, and CD63 median fluorescence intensity (normalized to count) before and after exercise in men and women. Data were natural log transformed for analysis using two-way mixed analysis of variance. Outcomes are presented as raw mean ± standard deviation or mean ± 95%*CI* (main effect of time only; D, E). The rows are aligned vertically according to outcome. The top row shows sex and time on one graph (A-C). The bottom row shows the corresponding main effect of time (D, E) or pairwise comparisons for pre- and post-exercise split by sex following a significant interaction effect (F). $†Interaction of sex and time. †Main effect of time. *Significantly different. PRE = pre-exercise. POST = post-workout. Significance was set at $p < 0.05$.

6.4.3 SGCA+ Extracellular Vesicles

There were no differences in natural log transformed SGCA+ EV concentration (objects per mL) from pre- to post-exercise according to sex or when averaged by sex (sex*time: $p = 0.280$,

η_p^2 = 0.064; main effect of time: p = 0.084, η_p^2 = 0.157) (Figure 23A and 23C). However, SGCA+ EV concentration was 69% higher in women compared to men (main effect of sex: p = 0.007, η_p^2 = 0.344) (Figure 23B). Square root transformed SGCA MFI was 38% lower in women compared with men and declined by 34% from pre- to post-exercise when averaged by sex (main effect of sex: p = 0.014, η_p^2 = 0.293; main effect of time: p = 0.012, η_p^2 = 0.304) (Figure 23E and 23F). There were no interactions or main effects of sex and time on the proportion of SGCA+ EVs as a percentage of total EVs (p > 0.05).

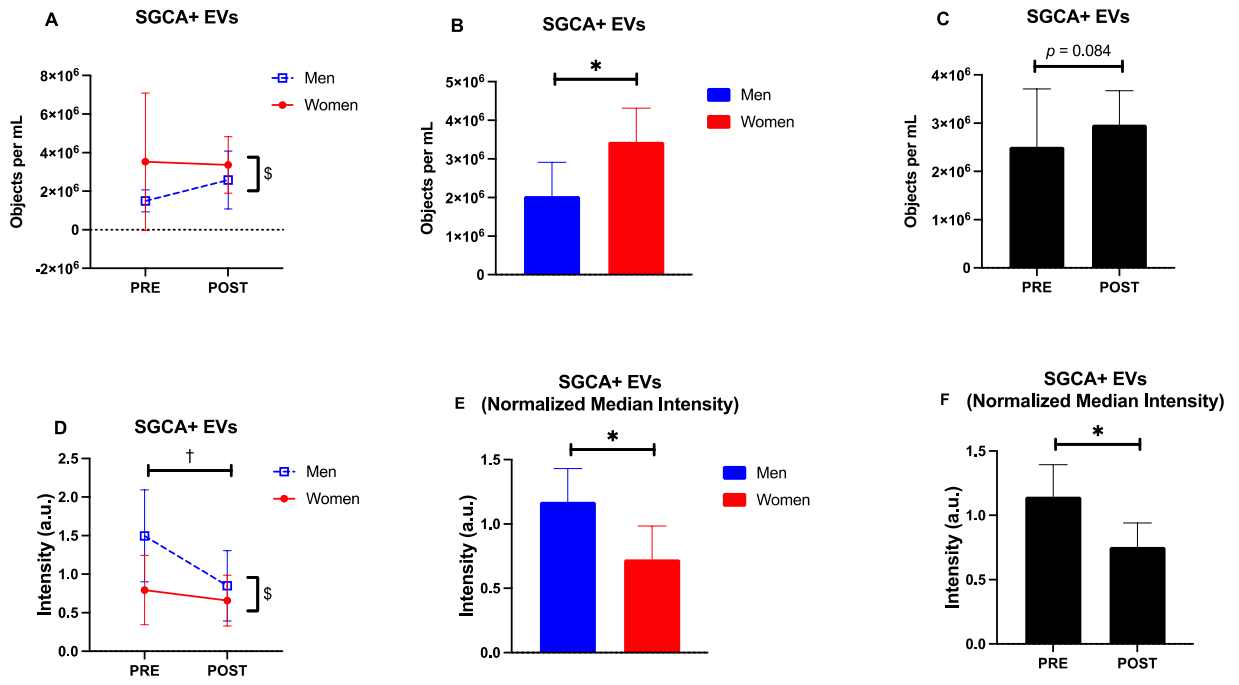


Figure 23. SGCA+ EVs concentration (objects per mL) and SGCA median fluorescence intensity (normalized to count) before and after exercise in men and women.

Data were natural log (concentration) or square root (intensity) transformed for analysis using two-way mixed analysis of variance. Outcomes are presented as raw mean ± standard deviation or mean ± 95%*CI* (main effects of time or sex only; B, C, E, F). $Main effect of sex. †Main effect of time. *Significantly different. PRE = pre-exercise. POST = post-workout. Significance was set at p < 0.05.

6.4.4 VAMP3+ Extracellular Vesicles

Natural log transformed VAMP3+ EV concentration (objects per mL) response to exercise and overall concentration did not differ according to sex (sex*time: p = 0.173, η_p^2 = 0.101; main effect of sex: p = 0.212, η_p^2 = 0.085) (Figure 24A). However, there was an effect of exercise; VAMP3+ EV concentration increased 93% following the AHRET (main effect of time: p = 0.025, η_p^2 = 0.250) (Figure 24D). The proportion of VAMP3+ EVs as a percentage of total EVs changed similarly with a 61% increase from pre- to post-exercise but no differences according to sex (sex*time: p = 0.140, η_p^2 = 0.117; main effect of sex: p = 0.882, η_p^2 = 0.001; main effect of time: p = 0.030, η_p^2 = 0.237) (Figure 24B and 24E). Similar to CD63+ and SGCA+ EVs, square root transformed VAMP3 MFI decreased by 36% following resistance exercise (main effect of time: p = 0.028, η_p^2 = 0.241) (Figure 24F). There were no sex-specific differences (sex*time: p = 0.233, η_p^2 = 0.078; main effect of sex: p = 0.137, η_p^2 = 0.119) (Figure 24C).

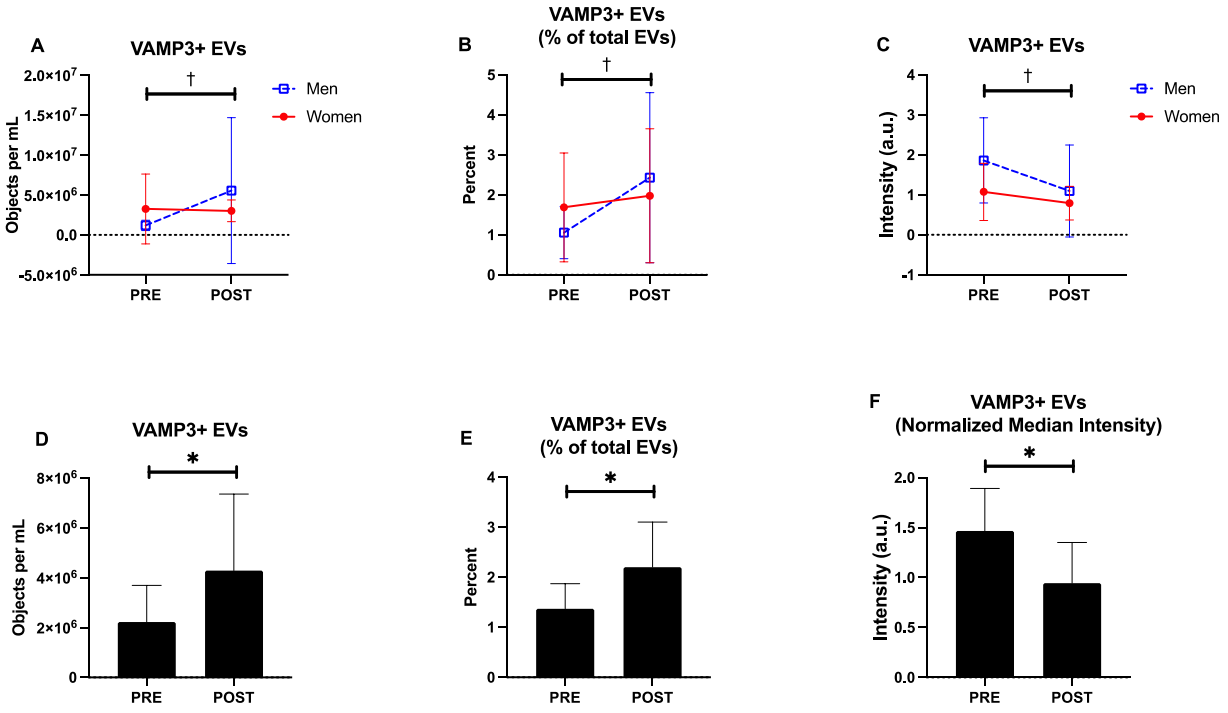


Figure 24. VAMP3+ EVs concentration (objects per mL), proportion of VAMP3+ EVs as a percentage of total EVs, and VAMP3 median fluorescence intensity (normalized to count) before and after exercise in men and women.

Data were natural log (concentration and proportion) or square root (intensity) transformed for analysis using two-way mixed analysis of variance. Outcomes are presented as raw mean ± standard deviation or mean ± 95%*CI* (main effect of time only; D-F). †Main effect of time. *Significantly different. PRE = pre-exercise. POST = post-workout. Significance was set at $p < 0.05$.

6.4.5 THSD1+ Extracellular Vesicles

There were no significant interactions or main effects of sex and time on any THSD1+ EV outcome ($p > 0.05$) (Figure 25).

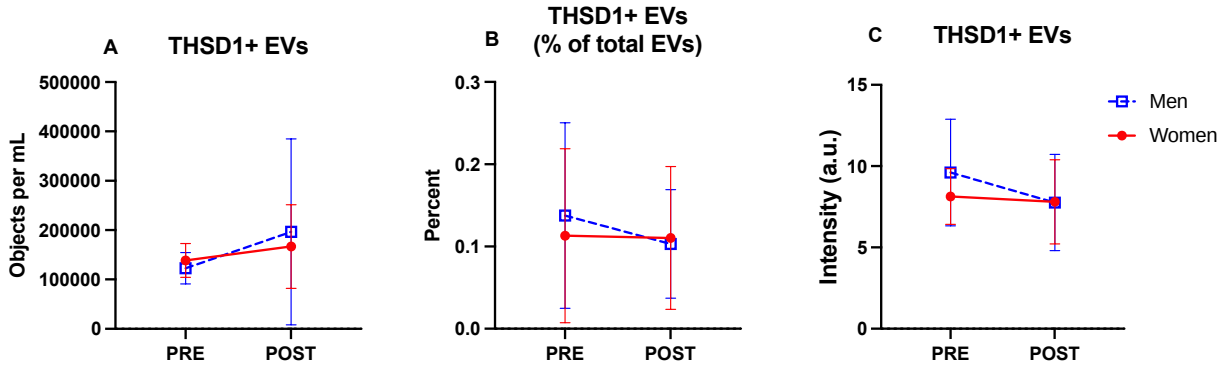


Figure 25. THSD1+ EVs concentration (objects per mL), proportion of THSD1+ EVs as a percentage of total EVs, and THSD1 median fluorescence intensity (normalized to count) before and after exercise in men and women.

Data were reciprocal transformed for THSD1+ EV concentration, and raw data was used for intensity for analysis using two-way mixed analysis of variance. Assumptions were not met for proportion of THSD1+ EVs; therefore, data were analyzed using Wilcoxon signed-rank exact test, split by sex. Outcomes are presented as raw mean ± standard deviation. PRE = pre-exercise. POST = post-workout. Significance was set at $p < 0.05$.

6.4.6 CD63, VAMP3, THSD1 Intensities Relative to SGCA+ Extracellular Vesicles

Given the nature of the protocol, we were particularly interested in changes in fluorescent intensities of markers associated with exosomes (CD63), microvesicles (VAMP3), and apoptotic bodies (THSD1) within the skeletal muscle-derived EV (SGCA+) subpopulation. To do this, we calculated the MFIs of CD63, VAMP3, and THSD1 within the SGCA+ EV subpopulation and normalized to SGCA+ EV count. Data transformations were unable to meet assumptions for statistical analysis using a two-way mixed analysis of variance; therefore, we used Wilcoxon signed-rank exact tests, split by sex, for each of the outcomes. CD63 MFI significantly increased in men but did not change in women from pre- to post- exercise (men: $p = 0.002$, Cohen's r =

0.886; women: $p = 0.131$, Cohen's $r = 0.500$). Similarly, VAMP3 MFI increased in men only following exercise (men: $p = 0.020$, Cohen's $r = 0.725$; women: $p = 0.193$, Cohen's $r = 0.435$). There were no differences in THSD1 MFI following exercise in either sex (men: $p = 0.432$, Cohen's $r = 0.274$; women: $p = 0.846$, Cohen's $r = 0.081$) (Figure 26). Means ± standard deviations for all EV subpopulation outcomes are presented in Appendix C Tables 2-5.

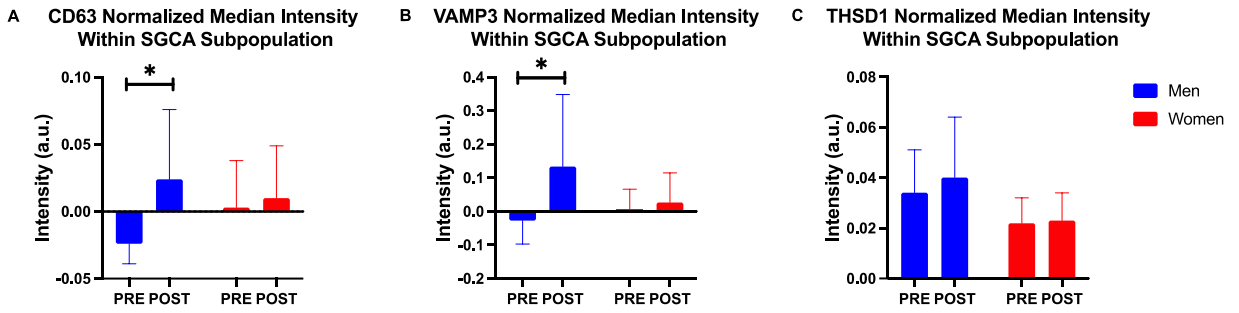


Figure 26. Median fluorescent intensities of CD63, VAMP3, THSD1 within the skeletal muscle-derived EV subpopulation (SGCA+) and normalized to SGCA count pre- and post-exercise in men and women. Assumptions were not met for any outcome; therefore, data were analyzed using Wilcoxon signed-rank exact test, split by sex. Outcomes are presented as raw mean ± standard deviation for reader interpretation. *Significnatly different. PRE = pre-exercise. POST = post-workout. Significance was set at $p < 0.05$.

6.5 Discussion

Despite a growing body of evidence characterizing the effects of acute and chronic *aerobic* exercise on EV features (reviewed in Estébanez et al., 2020), relatively little is known about the impact of *resistance* training on EV characteristics (139, 144, 145, 239, 240), and there is no published data comparing EV responses in men and women undergoing acute resistance training. In this study, we examined size, concentration, and surface proteins of circulating EVs to

determine if there were differences in men and women before and after AHRET. Through a combination of vesicle flow cytometry and imaging flow cytometry, we identified numerous distinctions in EV features according to sex, specifically related to EV size and within EV subpopulations associated with exosomes (CD63+ EVs) and of skeletal muscle origin (SGCA+ EVs). Acute resistance exercise also altered VAMP3+ EVs, though there were no differences between sexes.

We observed an increase in CD63+ EV concentration and proportion of total EVs from pre- to post-exercise, which was primarily driven by men, juxtaposed by a decline in CD63 median fluorescence intensity in men only. The latter suggests a decrease in CD63 surface protein density. This finding is in accordance with a report by Estébanez et al. (239) that demonstrated diminished CD63 protein expression following 8 weeks (2 sessions per week) of resistance training in elderly men. We also observed an increase in VAMP3+ EV concentration and proportion of total EVs with a concurrent decline in VAMP3 MFI that was similar in men and women.

The functional distinction between exosomes and microvesicles remains unclear, but both are believed to mediate many of the physiological processes associated with exercise (235). Beginning with evidence from *in vitro* studies, early work by Guescini et al. (228) demonstrated that C2C12 myoblast cells release EVs enriched with cargo that participate in signal transduction in a paracrine and endocrine manner. Later work by the same group (236) showed that myotubes exposed to oxidatively stressed muscle EVs displayed increased myoblast proliferation. In an elegant series of experiments using *in vitro* and *in vivo* models, Vechetti et al. (248) demonstrated that mechanical loading causes an increase in EVs enriched with miR-1, which is preferentially taken up by epididymal white adipose tissue to promote lipolysis via enhanced beta 3 adrenergic receptor signaling. Using a murine model, Castano et al. demonstrated that high intensity interval

training results in upregulation of EV-associated miR-133a and miR-133b which subsequently targets forkhead box 01 to improve insulin sensitivity. Finally, in a study of men, Annibalini et al. (144) reported increases in EV-associated miR-206 and miR-146a two hours after resistance exercise. miR-206 and miR-146a target pathways involved in myogenesis (249) and inflammation (250), respectively. Collectively, these data highlight the role of EVs in mediating physiological processes that promote a multitude of systemic benefits gained from exercise.

Prior literature supports the notion that exercise-induced circulating EVs predominately originate from platelets, endothelial cells, and leukocytes (142). Given the nature of the protocol, we were also interested in EVs originating from skeletal muscle (i.e., SGCA+ EV). Contrary to our hypothesis, we did not observe a significant increase in SGCA+ EVs following exercise in men and women, though an upward trend was identified (main effect of time: $p = 0.084$). Previous studies have noted an increase in SGCA+ EVs following acute aerobic exercise which accounted for 1-5% of total circulating EVs (138, 143). Others have reported similar increases in EVs enriched with skeletal muscle specific miR-206 after aerobic and resistance exercise (138, 144). The discordance between this study and others may be explained by differences in exercise modality (aerobic vs. resistance) (138, 143) or a lack of statistical power to detect change in SGCA+ EVs in this study (main effect of time, observed power = 41%). Nonetheless, this study builds from previous aerobic exercise paradigms measuring skeletal muscle-derived EVs by comparing both sexes, in which we observed significantly higher SGCA+ EV concentration in women compared with men. Additionally, SGCA MFI was higher in men compared with women, suggesting higher SGCA surface protein density in EVs in men. Although the significance of skeletal muscle-derived EV signaling relative to other EV sources is not yet known, it is important

to begin unveiling the nuances behind tissue-specific EV origins to determine the contributions of each cell source in multisystemic tissue crosstalk.

In the present study, there were no observable differences in total EV concentration after exercise. This observation is supported by Lovett et al. (198) who observed no change in EV concentration following muscle damaging exercises consisting of plyometric jumps and downhill running, but is in disagreement with Annibalini et al. (144) who reported a 2-fold rise in EV concentration after flywheel-based resistance exercise. The discrepancy in total EV concentration between our study and Annibalini et al. (144) is likely explained by methodological differences, including differences in timing of blood draw (immediate post vs. 2 h post), training modality (free weights vs. flywheel), intensity (75% 1-RM vs. peak power output), and/or method of measuring EV concentration (vesicle flow cytometry vs. nanoparticle tracking analysis). A review by Nederveen et al. (244) highlighted differences in experimental design that lead to mixed results, with some studies reporting an increase, decrease, or no change in EV concentration following aerobic exercise.

Mean EV size, on the other hand, decreased significantly in men following AHRET, but there was no change in size for women. The majority of previous reports have noted no change in size following aerobic exercise (reviewed in Estébanez et al., 2020; Nederveen et al., 2020), but, to our knowledge, this is the first study to report EV size following resistance exercise. Size is commonly used to describe EV subpopulations with exosomes being small EVs (<200 nm) and microvesicles and apoptotic bodies corresponding to medium and large sized vesicles (>200 nm). Though the functional differences between various EV sizes remain to be elucidated, exosomes undergo endosomal pathway biogenesis, whereas microvesicles and apoptotic bodies bud or bleb from the cell surface membrane. The distinction between each EV subpopulation biogenesis most

likely affects the cargo enrichment process and, ultimately, their downstream function (241). Taken together, the decrease in EV mean size in response to resistance exercise in men, but not women, may indicate greater production of exosomes relative to larger vesicles, which agrees with the point made earlier regarding CD63+ EV concentration increasing after exercise primarily due to men.

It is well-established that individuals respond differently to the same training program. Previous attempts at stratifying high versus low responders to exercise training have produced mixed results. Physiological characteristics that seem to influence response to training include certain genetic, physical, etc. characteristics as well as molecular factors. Specific to molecular factors, ribosomal RNA cellular concentration, muscle stem cells, skeletal muscle androgen receptor density, and certain miRNA have established positive relationships with muscle several hypertrophy indices (251, 252). Only one study to date has attempted to connect EV features and training responses. Secondary analysis of EV data from the EXIT trial (253) established a relationship between EV features and high- versus low-responders to a six-week resistance exercise training program in obese youth (240). Specifically, individuals demonstrating greater improvements in metabolic health in response to chronic resistance exercise training (i.e., high-responders) had significantly larger EVs (150-250 nm) compared to low-responders (50-100 nm) following a bout of acute aerobic exercise at baseline. High responders also had 25% lower expression of the tumor susceptibility gene 101 (TSG101) and 85% higher matrix metallopeptidase 2 (MMP2) protein content in EVs than low responders. Other groups have also demonstrated substantial changes in EV cargo with acute and chronic exercise training (reviewed in (202, 244). Collectively, these data are some of the first to suggest that both acute and chronic exercise potentiate changes in EV features that may be useful as biological indicators for tailoring exercise

training to the individual. Ongoing (Molecular Transducers of Physical Activity Consortium, MoTrPAC) and future studies will likely expand our understanding molecular factors related to optimized physical training programs.

This study was the first to compare EV features in men and women undergoing resistance exercise. A key strength of this study was the use of sophisticated methods and instruments for assessing EVs. Conventional flow cytometers have a limit of detection of ~150 nm (254). In this study, we used ImageStream®X Mark II in high gain mode for EV analysis. High gain mode was specifically developed for the detection of smaller particles in the size range of EVs. Previously published data have demonstrated up to 5-fold greater detection of small particles compared with conventional flow cytometry (155). Another strength of the study was our method of measuring EV concentration and size. Most EV studies have used tunable resistive pulse sensing or nanoparticle tracking analysis to measure EV size and concentration (192). While tunable resistive pulse sensing and nanoparticle tracking analysis can measure particle attributes on a particle-by-particle basis, they both have limitations. Nanoparticle tracking analysis measures light scatter from individual objects under Brownian motion to estimate size (192). This method is non-specific and unable to differentiate between EVs and other similarly sized particles. Nanoparticle tracking analysis is also susceptible to swarm detection, when particles cluster together, or missed detection of smaller particles that are aligned behind larger particles. Tunable resistive pulse sensing overcomes the shortcomings of light scatter-based methods by detecting objects through voltage changes when particles suspended in an electrolyte fluid pass through and disrupt a voltage field (192). However, the range of detection for tunable resistive pulse sensing is narrow and based on the size of nanopore filter being used. Vesicle flow cytometry overcomes some of these shortfalls by using an intercalating lipid membrane dye (vFRed™) to trigger detection of particles based on

fluorescence, which ensures the particles being detected have a lipid bilayer membrane and are not protein aggregates. Size can then be calculated from fluorescence intensity which corresponds to vesicle surface area.

This study had several strengths but is not without limitations. Although we attempted to select women by keeping hormonal status as homogenous as possible, there was some variation in oral contraception use and menstrual cycle phase. As reported by Toth et al. (255), women in the luteal phase of their menstrual cycle demonstrated higher concentrations of platelet- and endothelial-derived microparticles, including those expressing CD63, compared with women in the follicular phase. The luteal phase is characterized by elevated progesterone concentration which is also a feature of the type of oral contraception used in four out of ten of the women in this study. As such, this could have explained some of the variation within women and between sexes. Additionally, sample size selection was based on previous literature able to detect sex differences in EV concentration following exercise (36). Our sample size may have been underpowered to detect changes in some of our outcomes as evidenced by the *post hoc* power analysis of SGCA+ EV concentration. Nonetheless, results from this study lay a foundation for future research questions investigating sex-based differences in EV profiles.

6.6 Conclusion

Extracellular vesicles are becoming increasingly present in the literature with respect to their role in physiological adaptations and homeostasis regulation. Specific to exercise, previous reports demonstrate the role of muscle-derived EVs in the process of muscle growth and differentiation through participation in the inflammatory cascade and myogenic responses that

occur following exercise (236, 256). In this study, we observed several sex differences in EV characteristics in context of resistance training, including: 1) an increase in CD63+ EVs after resistance exercise that was primarily driven by men, 2) greater SGCA+ EVs in women compared to men, and 3) a decline in EV mean size in only men after resistance exercise. Still unknown are whether these differences will manifest in terms of adaptive responses to exercise, which is an area for future exploration. Recent efforts to draw relationships between EV features and high versus low responders to resistance exercise raise new and interesting questions as to how the study of EVs may inform optimized training for men and women (240). Future investigations using more holistic assessments of EVs and their cargo following resistance training exercise may provide new information regarding the use of EVs as biomarkers to inform human performance optimization through precision training programs.

7.0 Conclusions and Future Directions

The purpose of this series of studies was to examine sex-specific response patterns of circulating blood biomarkers and determine their relationship with physical performance in settings relevant to two distinct types of military training—simulated operational stress and resistance exercise training. Previous studies have characterized the effects of military training on endocrine signaling and physical performance in men (9, 10, 164, 257), but few comparisons have been made between men and women undergoing the same types of training stress (108, 168, 258). Results from the first study (Chapter 4) demonstrated that, over the 5-day simulated operational stress period, there were no differences in physical performance changes based on sex. This is an encouraging result given that women have been recently integrated into direct ground combat positions, which are some of the most physically challenging military occupations. The response patterns of physical performance in men and women suggests overall unit performance is impacted by operational stress to a similar extent regardless of the proportions of men and women in a mixed-sex unit. However, as hypothesized, men demonstrated greater performance than women in most tactically-relevant physical events. The gap in physical fitness between sexes may be reduced with targeted training programs (93, 95–97). In contrast to physical performance, conventional markers of anabolic status (GH, IGF-I) and metabolism (BDNF) were differentially impacted in men and women by simulated operational stress. Specifically, GH and BDNF increased after exercise in men only, and IGF-I declined to a greater extent in women than men following 48 h of sleep and caloric restriction. There were also significant relationships between these biomarkers and changes in physical performance, highlighting the utility of biomarkers for monitoring physical readiness.

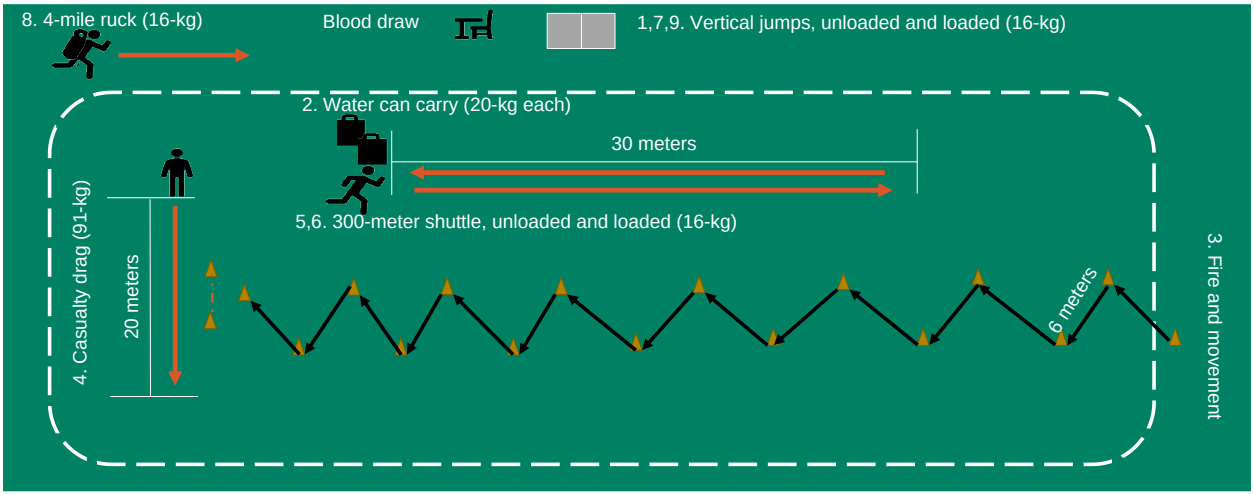
As an extension of the conventional biomarker analysis in Chapter 4, EV features were analyzed in a subsample of 10 men and 10 women in Chapter 5. This was the first study to use a model of military operational stress to examine EV profiles. Several sex-specific response patterns were noted, with the most prominent difference being that EV concentration declined by almost half in women in response to physical exertion but did not change in men. Unlike the conventionally measured biomarkers measured in Chapter 4, an interaction of time and day emerged for CD63+ EVs from the analysis in Chapter 5. CD63+ EVs, which are commonly associated with small EVs known as exosomes, have been implicated in immune regulation, angiogenesis, and metabolism. This intriguing observation may serve as a basis for future study to investigate the role of exosomes in physiological adaptations that occur during exercise versus exercise plus sleep and caloric restriction. Another interesting observation was the increase in the proportion of skeletal muscle-derived EVs from pre- to post-exercise, which aligns with data reported by other groups (138, 143). A novel finding from these studies was that skeletal muscle-derived EVs also increased relative to total EVs in response to sleep and caloric restriction, which suggests an enhanced role of skeletal muscle in tissue crosstalk during augmented stress.

In the final study, EV profiles in men and women were compared in response to an acute heavy resistance exercise test. Strength is a fundamental element for military occupational performance and is developed through resistance exercise. In contrast to the results from Chapter 5 using a mostly aerobic-based exercise protocol, there was a decrease in EV mean size following resistance exercise in men but not women. This observation corresponded with an increase in CD63+ EV concentration which was primarily driven by men. Comparisons of the changes in CD63+ EV in Chapters 5 and 6 suggest that exosome release into circulation may be dependent on exercise modality and sex (in resistance training only). Interestingly, there was a main effect of

sex on circulating skeletal muscle-derived EVs in both Chapter 5 and 6 studies, whereby women had a significantly greater proportion of SGCA+ EVs and higher SGCA+ EV concentration than men, respectively.

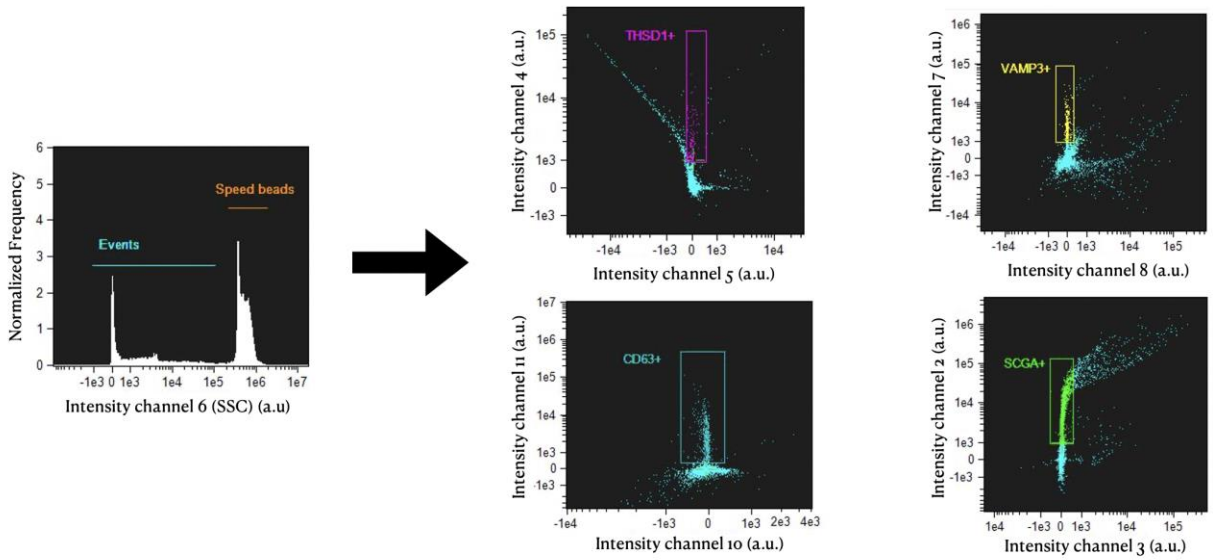
Given that data comparing men and women during military training are lacking, these studies add important results to the existing body of literature in military stress physiology in men. The novelty of the study design in Specific Aims 1 and 2 also provides new insights into how additional stress, including sleep and caloric restriction, have an interactive effect with exercise on circulating EVs. Future studies should examine changes in EV cargoes, such as lipids, mRNA, miRNA, and a wider variety of proteins, following operational stress and exercise to determine their impact on physiological adaptations related to military readiness. Moreover, examination of the effects of individual operational stressors will enable researchers to tease out the impacts sleep versus caloric restriction. Acute resistance exercise revealed significant effects on EV subpopulations in Chapter 6. Future investigations should examine the impact of chronic resistance training according to sex to determine potential sex-based predictors of training response and inform optimized training practices. Collectively, results from the studies presented here demonstrate sex differences in physiology that may serve as useful targets to enhance military readiness.

Appendix A Supplementary Materials for Specific Aim 1



Appendix A Figure 1. Tactial Mobility Test (TMT) layout.

Appendix B Supplementary Materials for Specific Aim 2

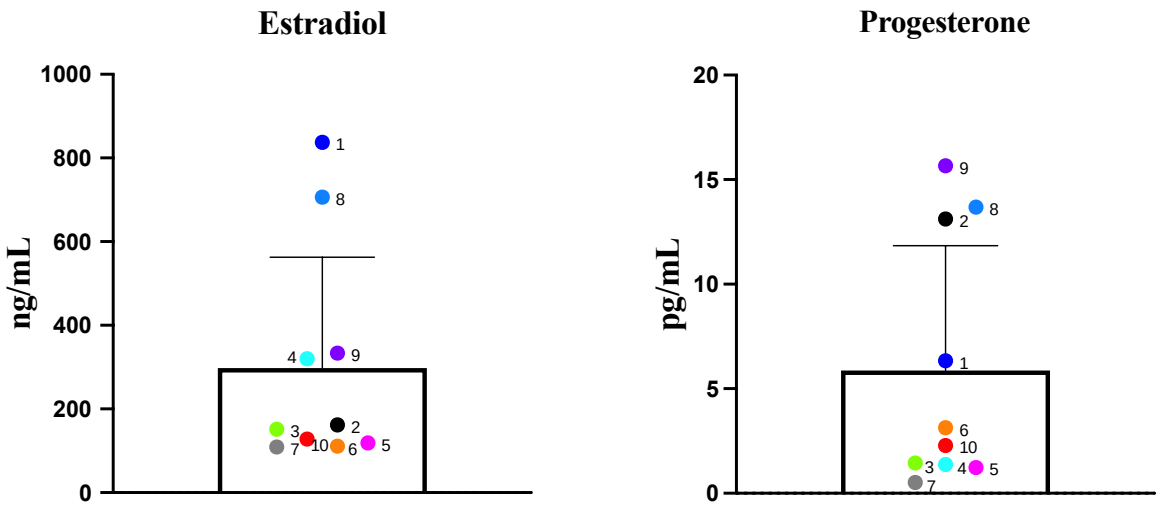


Appendix B Figure 1. Imaging flow cytometry gating.

Appendix B Table 1. Menstrual cycle and contraception data for female participants.

Subject	Contraception Use	Contraception Name	Contraception Type	Regular Menstrual Cycles	Days Since Last Period
1	Yes	Medroxyprogesterone acetate	Injection	No	17
2	Yes	Levonorgestrel	IUD	Yes	18
3	Yes	Etonogestrel	Implant	No	>365
4	Yes	Etonogestrel	Implant	No	15
5	Yes	Etonogestrel	Implant	No	11
6	No	N/A	N/A	Yes	33
7	No	N/A	N/A	Yes	6
8	No	N/A	N/A	Yes	0
9	No	N/A	N/A	No	34
10*					

Notes: Colored circles correspond to the same subject estradiol and progesterone data in Appendix B Figure 2. An asterisk (*) indicates missing menstrual cycle/contraception data. IUD = intrauterine device.



Appendix B Figure 2. Estradiol and progesterone concentrations for female participants. Numbers and colored circles in the figure correspond to the subject numbers and row information in Supplemental Table S1. Data are plotted as individual points and summarized as mean ± standard deviation.

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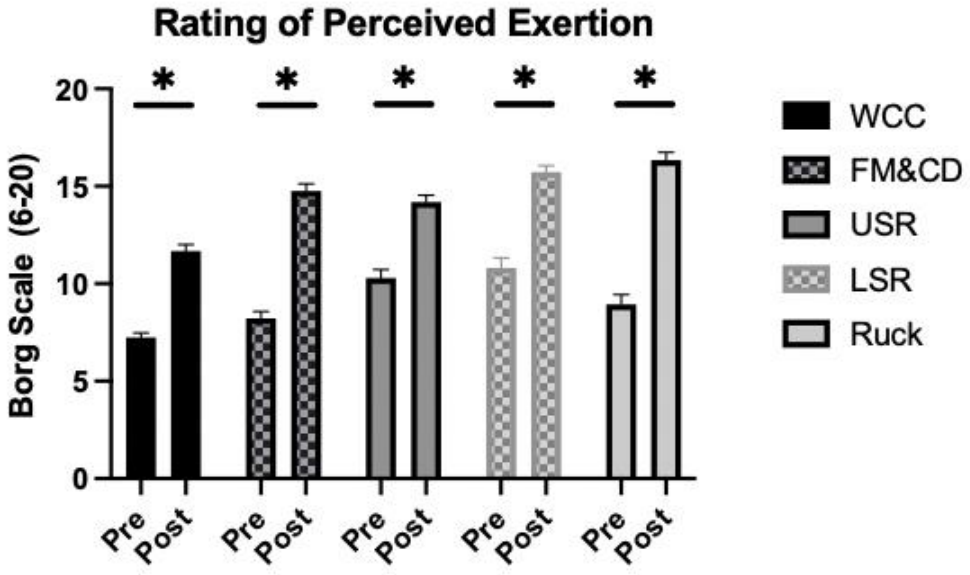
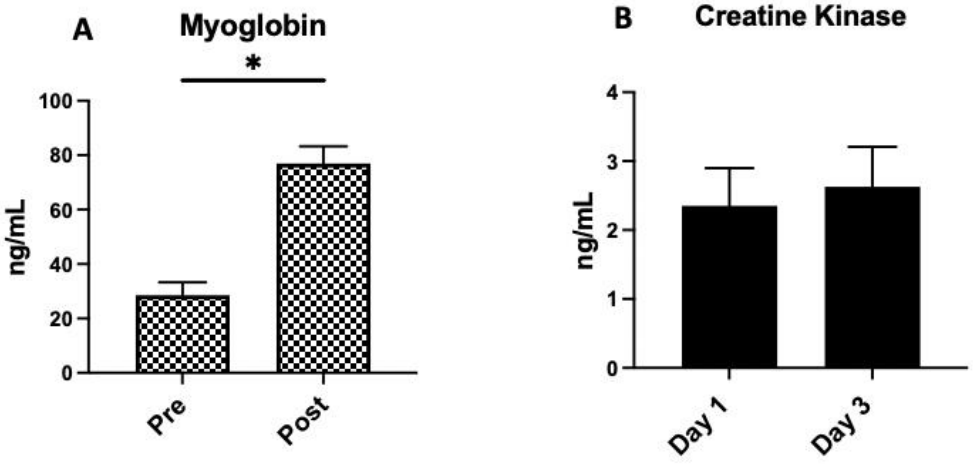


Appendix B Figure 3. Rating of perceived exertion (Borg Scale 6-20).

WCC = water can carry. FM&CD = fire and movement plus casualty drag. USR = 300 m unloaded shuttle run. LSR = 300 m loaded shuttle run. Ruck = loaded 4-mile ruck march. Data are presented as estimated marginal means ± standard error. An asterisk (*) indicates a significant difference in rating of perceived exertion before and after an event. Significance was set at $p < 0.05$.



Appendix B Figure 4. Serum myoglobin and creatine kinase concentrations.

Myoglobin (A) and creatine kinase (B) were measured as markers of acute and delayed muscle damage, respectively. Data for both variables were reciprocal transformed analysis to meet statistical assumptions. Raw data are presented as mean ± 95%*CI* for reader interpretation. An asterisk (*) signifies a significant difference between time points. Significance was set at $p < 0.05$.

Appendix B Table 2. Total extracellular vesicle mean concentration and size before and after exercise on day 1 (baseline) and day 3 (peak stress) in men and women as measured by nanoparticle tracking analysis.

Variable	Sex	Time	Day 1 (Baseline)	Day 3 (Peak Stress)
Concentration (particles per mL)	Men	Pre	1.50e+10 ± 7.36e+09	8.37e+09 ± 3.88e+09
		Post	1.52e+10 ± 1.11e+10	7.90e+09 ± 3.65e+09
	Women	Pre	1.01e+10 ± 3.40e+09	8.49e+09 ± 3.11e+09
		Post	6.34e+09 ± 3.17e+09	5.26e+09 ± 3.25e+09
Diameter (nm)	Men	Pre	107.4 ± 7.4	122.3 ± 15.3
		Post	120.1 ± 30.5	134.0 ± 32.5
	Women	Pre	106.2 ± 12.5	109.1 ± 10.8
		Post	125.4 ± 17.7	134.4 ± 26.2

***Raw data are presented as mean ± standard deviation. nm = nanometers. Pre = pre-exercise. Post = post-exercise.**

Appendix B Table 3. Concentration (objects per mL) of extracellular vesicle subpopulations for men (n =10) and women (n = 10) before and after exercise on day 1 and day 3.

EV Subpopulation	Sex	Time	Day 1 (Baseline)	Day 3 (Peak Stress)
CD63+ EVs (particles per mL)	Men	Pre	346626 ± 220115	352994 ± 211529
		Post	369815 ± 311242	448072 ± 291056
	Women	Pre	375640 ± 133450	331214 ± 192242
		Post	323673 ± 150575	533230 ± 268051
VAMP3+ EVs (particles per mL)	Men	Pre	1167270 ± 576824	1248020 ± 655750
		Post	1270030 ± 804149	1341591 ± 1098900
	Women	Pre	1155221 ± 819362	1233172 ± 699708
		Post	1518882 ± 1097383	1282550 ± 826610
THSD1+ EVs (particles per mL)	Men	Pre	52052 ± 37290	65340 ± 35772
		Post	61621 ± 26531	62156 ± 28076
	Women	Pre	128833 ± 247520	58892 ± 36039
		Post	145491 ± 278516	78307 ± 52772
SGCA+ EVs (particles per mL)	Men	Pre	2286593 ± 1070940	2521541 ± 1214122
		Post	2329741 ± 709428	2610159 ± 938381
	Women	Pre	2284692 ± 1062076	2898534 ± 1510189
		Post	2387346 ± 645470	2728045 ± 1266908

***Raw data are presented as mean ± standard deviation. EVs = extracellular vesicles. Pre = pre-exercise. Post = post-exercise.**

Appendix B Table 4. Proportion of extracellular vesicle subpopulations gated as a percentage of total extracellular vesicles as assessed by imaging flow cytometry.

EV Subpopulation	Sex	Time	Day 1 (Baseline)	Day 3 (Peak Stress)
CD63+ EVs (% of total EVs)	Men	Pre	1.01 ± 0.74	1.20 ± 0.79
		Post	1.24 ± 1.16	1.77 ± 1.12
	Women	Pre	1.29 ± 0.65	1.37 ± 1.07
		Post	1.19 ± 0.47	2.75 ± 2.03
VAMP3+ EVs (% of total EVs)	Men	Pre	3.20 ± 1.63	3.88 ± 1.75
		Post	4.06 ± 2.61	5.13 ± 4.24
	Women	Pre	3.81 ± 3.08	5.17 ± 3.20
		Post	5.86 ± 4.31	6.08 ± 5.19
THSD1+ EVs (% of total EVs)	Men	Pre	0.16 ± 0.15	0.22 ± 0.15
		Post	0.20 ± 0.10	0.23 ± 0.10
	Women	Pre	0.41 ± 0.78	0.22 ± 0.11
		Post	0.48 ± 0.84	0.42 ± 0.43
SGCA+ EVs (% of total EVs)	Men	Pre	6.43 ± 2.75	7.98 ± 3.13
		Post	7.40 ± 2.37	9.62 ± 2.75
	Women	Pre	7.22 ± 3.10	10.76 ± 4.23
		Post	9.03 ± 2.02	12.85 ± 7.27

***Raw data are presented as mean ± standard deviation. EVs = extracellular vesicles.**

Pre = pre-exercise. Post = post-exercise.

Appendix B Table 5. Median fluorescence intensity (normalized to count) of extracellular vesicle subpopulations for men (n =10) and women (n = 10) before and after exercise on day 1 and day 3.

EV Subpopulation	Sex	Time	Day 1 (Baseline)	Day 3 (Peak Stress)
CD63+ EVs (intensity per particle, a.u.)	Men	Pre	5.53 ± 4.83	8.44 ± 11.48
		Post	8.52 ± 12.43	4.78 ± 4.51
	Women	Pre	3.40 ± 1.57	4.68 ± 3.05
		Post	4.43 ± 2.32	5.51 ± 9.76
VAMP3+ EVs (intensity per particle, a.u.)	Men	Pre	1.97 ± 1.19	1.69 ± 0.69
		Post	2.40 ± 2.18	1.91 ± 0.95
	Women	Pre	2.60 ± 2.25	2.03 ± 1.48
		Post	2.29 ± 2.16	2.09 ± 1.38
THSD1+ EVs (intensity per particle, a.u.)	Men	Pre	41.78 ± 29.06	30.77 ± 17.71
		Post	27.97 ± 12.21	28.13 ± 17.60
	Women	Pre	33.72 ± 19.31	28.45 ± 10.60
		Post	31.27 ± 19.26	27.25 ± 16.67
SGCA+ EVs (intensity per particle, a.u.)	Men	Pre	1.36 ± 0.83	1.17 ± 0.47
		Post	1.14 ± 0.35	1.12 ± 0.49
	Women	Pre	1.20 ± 0.32	1.11 ± 0.60
		Post	1.16 ± 0.28	1.10 ± 0.38

***Raw data are presented as mean ± standard deviation. EVs = extracellular vesicles. Pre = pre-exercise. Post = post-exercise.**

Appendix B Table 6. Mean fluorescence intensity of extracellular vesicle subpopulations for men (n =10) and women (n = 10) before and after exercise on day 1 and day 3.

EV Subpopulation	Sex	Time	Day 1 (Baseline)	Day 3 (Peak Stress)
CD63+ EVs	Men	Pre	2887.76 ± 1542.53	6240.56 ± 8826.66
		Post	3013.98 ± 1585.87	3550.22 ± 1981.05
	Women	Pre	2774.43 ± 947.06	2735.11 ± 1211.69
		Post	3125.48 ± 1351.09	3264.81 ± 1016.64
VAMP3+ EVs	Men	Pre	3028.38 ± 300.12	3071.33 ± 493.92
		Post	3286.87 ± 570.13	3096.41 ± 434.41
	Women	Pre	3269.91 ± 710.69	3111.85 ± 406.29
		Post	3154.89 ± 558.10	3091.86 ± 509.91
THSD1+ EVs	Men	Pre	2453.31 ± 679.31	2657.97 ± 490.55
		Post	2545.97 ± 341.22	2776.71 ± 598.65
	Women	Pre	2470.68 ± 590.40	2624.21 ± 553.48
		Post	2509.46 ± 385.29	2659.53 ± 382.61
SGCA+ EVs	Men	Pre	6163.51 ± 468.49	6355.35 ± 627.55
		Post	6326.40 ± 337.09	6527.50 ± 427.67
	Women	Pre	6325.20 ± 487.25	6188.97 ± 701.12
		Post	6683.03 ± 715.66	6778.71 ± 408.61

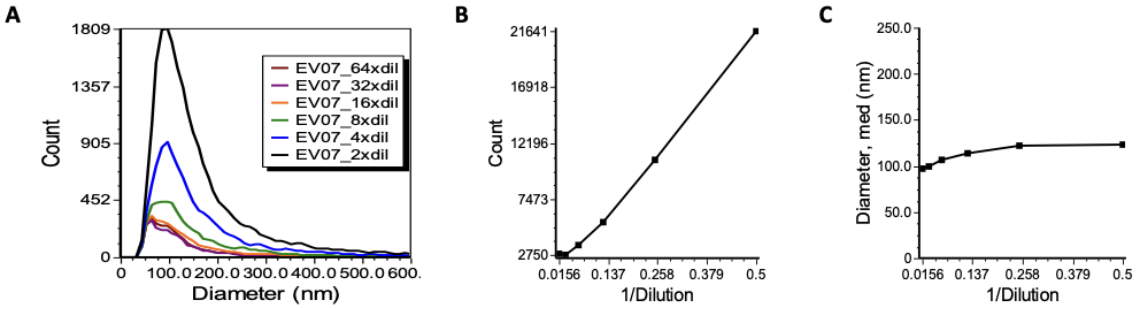
***Raw data are presented as mean ± standard deviation. EVs = extracellular vesicles. Pre = pre-exercise. Post = post-exercise.**

Appendix B Table 7. Sum fluorescence intensity of extracellular vesicle subpopulations for men (n =10) and women (n = 10) before and after exercise on day 1 and day 3.

EV Subpopulation	Sex	Time	Day 1 (Baseline)	Day 3 (Peak Stress)
CD63+ EVs	Men	Pre	1431570 ± 1430369	2431252.21 ± 2848194.08
		Post	1672491 ± 1767732	2274878 ± 2087224
	Women	Pre	1210924 ± 666395	1131269 ± 1045876
		Post	1287313 ± 1003309	2164574 ± 1471872
VAMP3+ EVs	Men	Pre	3985012 ± 2083656	4373963 ± 2649364
		Post	4495522 ± 2684911	4981635 ± 4812748
	Women	Pre	4352692 ± 3524995	4370466 ± 2609583
		Post	5494645.08 ± 4238160	4578297 ± 3291923
THSD1+ EVs	Men	Pre	158189 ± 140000	203173 ± 126350
		Post	180410 ± 90914	203908 ± 117870
	Women	Pre	401511 ± 810462	180891 ± 124948
		Post	444020 ± 888605	237188 ± 165925
SGCA+ EVs	Men	Pre	15960516 ± 7983817	17662881 ± 7454812
		Post	16603592 ± 5731050	19099389 ± 6905108
	Women	Pre	16243691 ± 8163940	19568624 ± 8809468
		Post	18126879 ± 6352290	20659154 ± 9777275

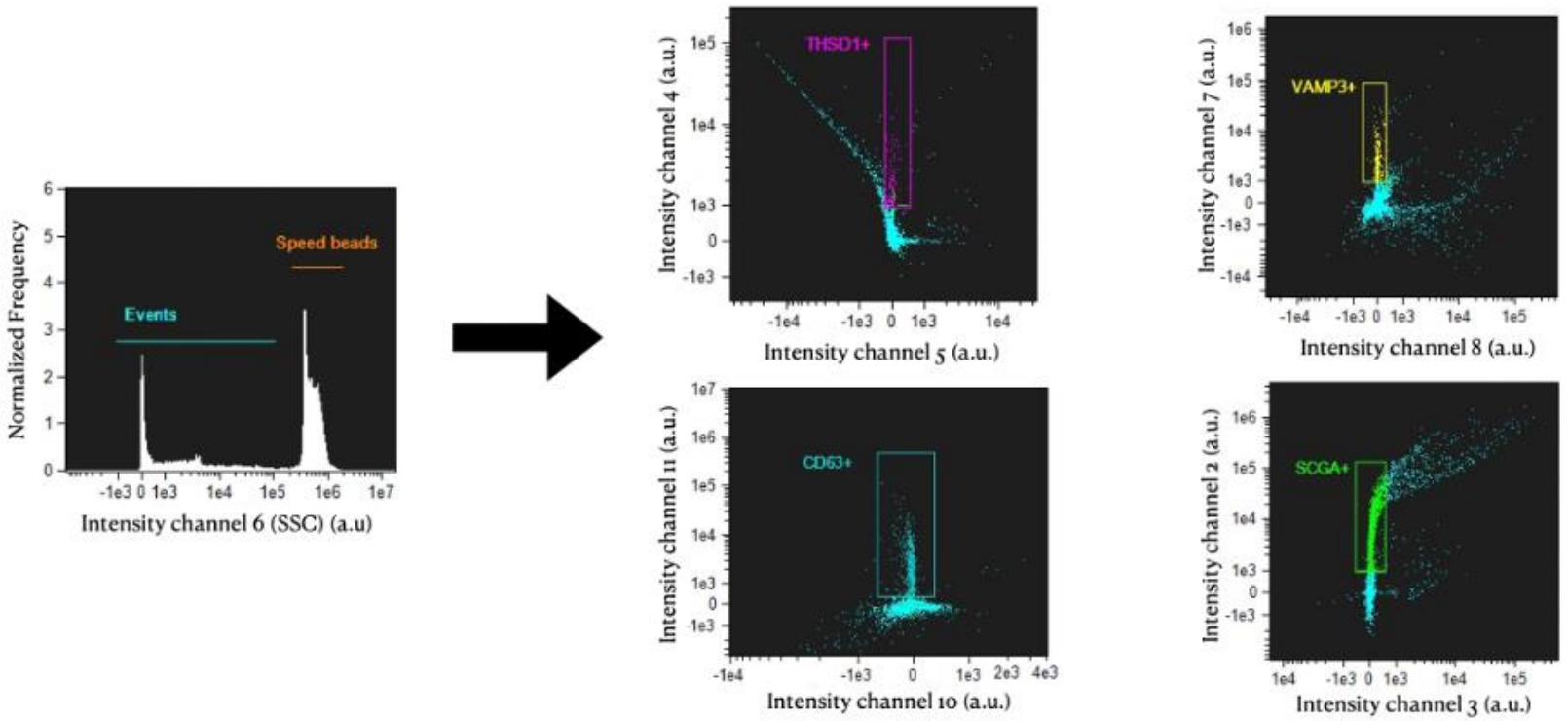
***Raw data are presented as mean ± standard deviation. EVs = extracellular vesicles. Pre = pre-exercise. Post = post-exercise.**

Appendix C Supplementary Materials for Specific Aim 3



Appendix C Figure 1. Vesicle Flow Cytometry dilution series experiment.

Samples were serially diluted to determine the optimal concentration for sample measurement. (A) Histogram of sample concentration at dilutions ranging from 2x to 64x. (B) Concentration decreased in a linear manner with each subsequent dilution. (C) Vesicle diameter was stable across dilution series demonstrating absence of swarm detection

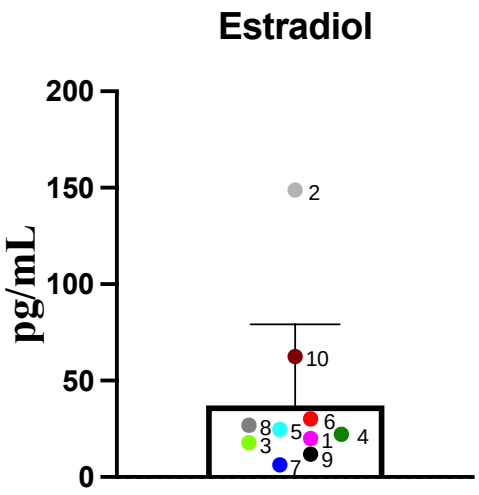


Appendix C Figure 2. Imaging flow cytometry gating.

Appendix C Table 1. Menstrual cycle and contraception data for female participants.

Subject	Contraception Use	Contraception Name	Contraception Type	Regular Menstrual Cycles	Number of Cycles per Year	Days Since Last Period
1	No	N/A	N/A	Yes	12	22
2	No	N/A	N/A	Yes	12	29
3	No	N/A	N/A	Yes	13	8
4	Yes	Levonorgestrel	IUD	No	4	21
5	No	N/A	N/A	Yes	12	15
6	No	N/A	N/A	Yes	12	7
7	No	N/A	N/A	No	0	>365
8	Yes	Levonorgestrel	IUD	No	0	>365
9	Yes	Levonorgestrel	IUD	No	1	32
10	Yes	Levonorgestrel	IUD	No	3	165

Note: Colored circles correspond to the same subject estradiol data in Appendix C Figure 3. IUD = intrauterine device.



Appendix C Figure 3. Estradiol concentration for female participants.

Numbers and colored circles in the figure correspond to the subject numbers and row information in Appendix C Table 1. Data are plotted as individual points and summarized as mean ± standard deviation.

Appendix C Table 2. EV mean concentration and size (diameter) before and after acute heavy resistance exercise test in men (n =10) and women (n =10).

Variable	Sex	Pre-AHRET	Post-AHRET
Concentration (particles per mL)	Men	1.20e+10 ± 4.07e+09	1.37e+10 ± 2.33e+09
	Women	1.51e+10 ±5.19e+09	1.39e+10 ± 1.88e+09
Diameter (nm)	Men	254.2 ± 11.5	246.8 ± 17.5
	Women	245.5 ± 22.8	244.1 ± 17.7

***Raw data are presented as mean ± standard deviation. AHRET = acute heavy resistance exercise test. Nm = nanometers. Pre = pre-exercise. Post = post-exercise.**

Appendix C Table 3. Concentration (objects per mL) of extracellular vesicle subpopulations for men (n =10) and women (n = 10) before and after a bout of acute heavy resistance exercise.

EV Subpopulation	Sex	Pre-AHRET	Post-AHRET
CD63+ EVs	Men	587991.25 ± 749680.98	2397354.25 ± 1879233.15
	Women	2997158.10 ± 6070404.73	1999628.39 ± 1214627.76
VAMP3+ EVs	Men	1198623.40 ± 636521.40	5547462.63 ± 9125076.45
	Women	3250507.73 ± 4356597.74	3019347.44 ± 1363283.10
THSD1+ EVs	Men	122701.59 ± 31574.68	196500.59 ± 188495.77
	Women	138539.37 ± 34043.64	166701.67 ± 84826.88
SGCA+ EVs	Men	1497067.18 ± 571241.52	2581667.25 ± 1500878.79
	Women	3526152.89 ± 3559876.62	3363207.50 ± 1469255.63

***Raw data are presented as mean ± standard deviation. AHRET = acute heavy resistance exercise test. EVs = extracellular vesicles. Pre = pre-exercise. Post = post-exercise.**

Appendix C Table 4. Proportion of extracellular vesicle subpopulations as a percentage of total extracellular vesicles for men (n =10) and women (n = 10) before and after a bout of acute heavy resistance exercise.

EV Subpopulation	Sex	Pre-AHRET	Post-AHRET
CD63+ EVs	Men	0.26 ± 0.15	1.68 ± 1.75
	Women	1.00 ± 1.50	1.00 ± 0.85
VAMP3+ EVs	Men	1.06 ± 0.65	2.43 ± 2.13
	Women	1.69 ± 1.36	1.98 ± 1.68
THSD1+ EVs	Men	0.14 ± 0.11	0.10 ± 0.07
	Women	0.11 ± 0.11	0.11 ± 0.09
SGCA+ EVs	Men	1.52 ± 1.15	2.03 ± 1.91
	Women	2.02 ± 1.54	1.94 ± 1.39

***Raw data are presented as mean ± standard deviation. AHRET = acute heavy resistance exercise test. EVs = extracellular vesicles. Pre = pre-exercise. Post = post-exercise.**

Appendix C Table 5. Median fluorescence intensity (normalized to count) of extracellular vesicle subpopulations for men (n =10) and women (n = 10) before and after a bout of acute heavy resistance exercise.

EV Subpopulation	Sex	Pre-AHRET	Post-AHRET
CD63+ EVs	Men	5.45 ± 4.24	1.05 ± 1.04
	Women	1.89 ± 1.58	1.14 ± 0.97
VAMP3+ EVs	Men	1.86 ± 1.06	1.10 ± 1.15
	Women	1.08 ± 0.71	0.79 ± 0.42
THSD1+ EVs	Men	9.61 ± 3.27	7.76 ± 2.96
	Women	8.14 ± 1.71	7.81 ± 2.58
SGCA+ EVs	Men	1.50 ± 0.60	0.85 ± 0.45
	Women	0.79 ± 0.45	0.66 ± 0.33

***Raw data are presented as mean ± standard deviation. AHRET = acute heavy resistance exercise test. EVs = extracellular vesicles. Pre = pre-exercise. Post = post-exercise.**

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