Stress Levels Measured through Salivary Cortisol in Nationally Ranked Fencers

by
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ABSTRACT

The sport of fencing is not only physically challenging, but mentally strenuous as well. The sport of fencing consists of three disciplines, for this study the discipline of epee was the focus. Epee has longer competitions, longer bout period, and utilizes the game of strategy at every moment. This study followed United States nationally ranked fencers to three domestic tournaments during the 2017-2018 fencing season. New research suggests that there is an optimal level of glucocorticoids in the system for positive effect on cognition and vigilance. This study aims to show that there is also an optimal level of stress measured through salivary cortisol for athletes during competition.

DEDICATION

Dedicated to my mother, Susan Vie, and my siblings. For they have continuously supported me throughout both my personal growth and academic journey. My family is my village.

ACKNOWLEDGMENTS

The work described in this thesis is possible because of the attention, time, and energy given by my advisor Dr. Debra Page Baluch. Thank you to Dr. Beckett Sterner and Dr. Donna Cataldo for serving on my committee and always giving valuable suggestions and advice. Thank you all for helping make this work come to fruition.

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ABBREVIATIONS

USFA	United States Fencing Association
USOC	United States Olympic Committee
DHEA	Dehydroepiandrosterone
NAC	North American Cup
HPA	Hypothalamic-Pituitary-Adrenal
ACTH	Adrenocorticotropic Hormone
ELISA	Enzyme-Linked Immunosorbent Assay
OD	Optical Density

Chapter 1. The Sport of Fencing and Cortisol

Introduction - topic

Fencing is becoming increasingly popular in the United States, in the last 15 years the participation at summer nationals, the largest event of the season, has tripled. Membership to the United States Fencing Association (USFA) has grown 20% in the last year. Worldwide fencing has been and continues to be one of the more popular sports, it is one of the eight original Olympic sports dating back the 1896 Games in Athens. In recent years the athletes that represent the United States in this sport have been breaking barriers and setting new records. In the 2017-2018 season the women's epee team won a world cup gold medal for the first time in U.S. women's epee team history. The same women's epee team also has the first Latina to represent the United States at the Pan American Championships. In the 2016 Olympics, U.S. women's saber team USA member Ibtihaj Mohammad broke barriers and became the first athlete in the history of the United States to wear a hijab while competing at the Olympics. There are numerous other records that the current team USA athletes have set in recent year making fencing and influential sport and important to the fabric of our country. Fencing has been called the "physical chess", not only is it physically challenging but mentally as well. The director of Sports Medicine for US fencing, Jeremy Summers, has set as the goal for the department stating, "Our Sports Medicine Program's ultimate goal is to help change a culture of fencing through education by providing our athletes, coaches, and parents with the tools of optimal performance" (Jomantes 2015). While this study is independent from U.S. Fencing, it can still provide insight into the stress the athletes face for this sport. Past

research on fencing is very limited. This research is an expansion to a previous pilot study researching salivary cortisol in fencers.

Many countries that compete against team USA have their countries government backing them financially, paying for athlete training, travel, competition costs, and paying them a bonus for wins. The majority of fencers in the United States are not funded through the U.S. Olympic Committee (USOC). The balance between paying bills, schooling and or having a full time job along with training to maintain being an elite athlete add the stressors these athletes face.

The USOC is "the only 501(c) non-profit sanctioned by Congress to represent the United States at the Olympic Games" (Crumpton 2013). The organization *Charity Navigator* states under the mission of the USOC that "The United States Olympic Committee (USOC) was incorporated by Congress for the purpose of establishing national goals for amateur athletic activities, and to aid and encourage the attainment of those goals." (Charity Navigator). In 2013, *Charity Navigator* reported that only 10.3% of the $795,917,076 budget for grants/expense in the 2009-2012 quad went directly to athletes grants (Crumpton 2013). When the funding does not come from the government or the USOC the athletes look to their sanctioned governing body for help, in this case the USFA. However, the USFA only funds international tournament and travel costs for the top 4 athletes in the country, and once one falls from the rankings the funding is automatically pulled. When finances are limited, and one is dedicating their life to a sport, making it their career, it is integral that each training session and performance at tournaments results in a medal. This is why the opportunity for athletes to have their

physiological response of stress analyzed would be to their benefit to make their training more effective for optimal performance during stressful situations at tournaments.

Currently there are no studies on how stress effects fencers during tournaments. According to Nixon P: Practitioner 1979, Yerkes RM, Dodson JD. there is an optimal range for stress, however it is not clear if this is individual or an optimal range for all. Additionally, there was one study that tested serum cortisol in saber fencers during practice. While that was a novel study, stress response associated with practice is generally lower than during a tournament (Haneishi et al. 2007). Filair and colleagues back this statement by stating "true competition induces greater hormonal response compared with laboratory exercise" (Filaire et al. 2001)

Cortisol is a glucocorticoid that is associated with both acute and chronic stress after intense exercise. Previous studies on cortisol and performance have shown a number of things. One study shows that Cortisol peaks after a loss (Casto & Edwards 2016) Another study shows that if one embraces the stress and knows how to manage it that the body will begin to produce Dehydroepiandrosterone (DHEA) which will counter act the effects of the stress hormone cortisol (Crum, Akinola, Martin, & Fath 2017). Some have suggested to study androgens instead of cortisol, however cortisol is a "more appropriate hormonal parameter of stress in women because it is less influenced by the menstrual cycle" (Haneishi et al. 2007). In this study cortisol was measured by way of salivary cortisol which numerous studies "consistently report high correlations between serum and salivary cortisol, indicating that salivary cortisol levels reliably estimate serum cortisol levels" (Salimetrics 2016).

The primary purpose of this study was to (a) compare how cortisol levels fluctuate during a tournament and (b) analyze cortisol levels to see if there is an optimal rage for performance. It was hypothesized that (a) cortisol levels would peak after a loss or stressful bout and (b) there would be an optimal range of cortisol for peak performance.

Fencing Around the World and the 3 weapons

While armed combat was common in the bronze age throughout the world the sport of fencing developed as a way to practice in combat situations. The modern sport of fencing has three disciplines: epee, foil, and saber. Saber has its roots in the cavalry form of fencing. The target area includes the entire upper body from the midsection until the head including the arms. Saber is only one of the three disciplines where fencers can score a touch with both the tip of the weapon and the side of the weapon. The rules and target area developed from the maneuvers necessary on horseback. It was considered unsportsmanlike to hit the opponents horse for example so that is why the target are is only from the belly up. The slashing motions were also necessary because the fencer would pass by each other very fast on horseback and so hitting with the precision of the point was not necessary to be lethal. A slashing motion with lots of velocity could also be lethal. Saber is also the fastest of the three weapons. Saber fencers must develop their fast twitch muscles for quick explosive movements and there is very little preparation and wait time between touches.

The disciple of foil developed as a practice for duals to the death. It was common for disputes to be resolved in duals to first blood. Meaning that if any part of the body bled, the fencer loses. It was not uncommon for duals to last for hours. If a fencer bled

from a blister that also counted as first blood, so fencers started wearing a single glove on their armed hand and continue to do so today. The target area of foil also excludes the arms and the legs because they are not considered lethal targets. It was also considered unsportsmanlike to hit an opponent's head. In the case of duals to the death where the family might need to identify a body or perhaps they would want to have an open casket funeral the face needed to be identifiable and intact. In modern foil, a fencer can only score by touching the body and the neck. Unlike saber where a fencer can score using both the side and the tip of the blade, in foil a fencer can only score by compressing the tip of the blade with at least 500grams of force. In terms of speed, foil is not as fast as saber but a match does not take as long as in epee. Foil requires both explosion and preparation so in terms of speed and endurance it lies somewhere in between saber and epee.

Epee is considered to be the true dueling weapon as it is the disciple that derived from actual duals. In epee, the entire body is a valid target area. The toe, the chest, a finger, the head is all valid for 1 point. In epee, similar to foil, the fencer must hit with the tip of the weapon with at least 750 grams of force. Epee is the only of the three weapons that does not require the rule of right of way, which essentially means that in right of way a fencer must be on offense to score a touch. Due to the fact that fencers can hit anywhere on the body and a touch can be scored at anytime, epee is extremely strategic. The dynamic is such that there is lots of preparation time between each point and so a single fencing match in the direct elimination rounds can last up to 20 minutes in total. An epee fencer must also possess a lot of endurance as the matches last a long time and the tournaments can also last up to 12 hours and span over two days.

Why the discipline of epee

Epee is the discipline from which this study was focused around. All of the participants were epee fencers. This decision was made for a few reasons. The first is that this is the disciple that is most familiar to the researcher. Second it is the weapon where there was the most access to the national and Olympic teams. The third reason behind the choice of testing only epee fencers is because the travel schedule for the three weapons, men and women, is completely different, so attempting to cover all dispinplines is too wide of a scope and virtually impossible to be able to research all 3 weapons simultaneously. There are 3 disciplines and male and female genders in each so 6 weapons in total. Additionally, because of the nature of each weapon the stress levels may differ. In a short burst sport like saber tournaments only last a few hours. In epee, tournaments can last 12 hours. Additionally, the type of skeletal muscle used for the disciplines differ, saber uses more fast twitch muscles for the bursts of movement while epee uses more slow twitch muscles for endurance.

How fencing developed into a sport

The sport of fencing is one of the original 8 Olympic sports and the modern sport of fencing has been greatly influenced by its status as an Olympic sport. From the very first modern Olympic Games in 1896, the three disciplines of fencing continued to derive away from the practice of dueling. Electronic scoring was introduced into fencing in the 1930's which further separated it from the practice of dueling. As fencing developed away from combat and as it became a sport there were a lot of crossover that developed between fencing and the traditional martial arts that also eventually became martial arts like judo and ju-jitsu.

Format of North American Cups (NAC)

North American Cups are an integral part of the qualification process for fencers to become nationally ranked on the United States Fencing Association's points system. This ranking process is how athletes qualify for the Olympic Games and the World Teams. There are 4 domestic tournaments a season which include 3 North American Cups and United States Nationals. The accumulated 2 best results plus the accumulated results from five of the eight international tournaments is what puts athletes in high standing on the United States points list.

2-day format

North American Cups have a 2-day format however athletes in the top 32 only compete on day 2. On day 2 the winners from day 1 are seeded into pools with the top 32. The pools are usually composed of 7 fencers who then fence a round robin after which all win percentages and indicators from all of the pools are combined to form a final seeding chart. The bottom 20% of the seeding chart are cut and a direct elimination tableau is formed. The top fencer is matched up with the bottom fencers, the second highest ranked fencers is matched with the second lowest ranked fencer, etc. From there fencers continue fencing until they are either eliminated or until they win.

The Endocrine System

In order to holistically understand this study one key element that is of great significance is the endocrinology in the human body and how it responds to stress. Glands throughout the body compromise the endocrine system producing and secreting hormones. The hormone specific to this study, also dubbed as the "stress" hormone, is cortisol. The three components of this major neuroendocrine system that will be the area

of focus are the hypothalamus, the pituitary gland, and the adrenal glands. One of the responsibilities of the hypothalamus is to keep in check various homeostatic systems such as hunger, thirst, and core body temperature. The pituitary gland is rather small in size and is located below the thalamus resting in the sella turcica of the cranium, while the adrenal glands are located on top of the kidneys. The pituitary gland is made up of the anterior and posterior lobes. The conical shaped organs, adrenal glands, have a core termed the medulla with the surrounding layer named the cortex. These three organs work together to form the hypothalamic-pituitary-adrenal axis, or more commonly known as the "HPA axis" (Malenka, Nestler, & Hyman 2009). This HPA axis is one of the 4 major neuroendocrine systems found within the human body.

Stress, digestion, and the immune system are a few of the various body processes the HPA axis controls. The acute stress response of the HPA axis begins with a neural response from the hypothalamus. This causes the pituitary gland to secrete adrenocorticotropic hormone $(ACTH)^2$ which then enters the circulatory system stimulating the zona fasciculata of the adrenal cortex. The zona fasciculata releases cortisol glucocorticoids. The stimulation is done by way of the sympathetic nervous system; the adrenal glands then secrete the neurotransmitter epinephrine, colloquially referred to as adrenaline. This directly heightens the fight-or-flight response which many times is characterized by rushing of blood to the muscles, increased breathing rate, and increased blood pressure. The high levels of glucocorticoid cortisol in the system causes a negative feedback loop to the hypothalamus ordering the immediate reduction in production of ACTH.

Glucocorticoids

Cortisol is arguably the most critical glucocorticoid in the human system serving several purposes. This glucocorticoid is fundamental for the vitality and regulation of many immunological and homeostatic roles as well as is "involved in metabolic, inflammatory, cardiovascular and behavioral processes" (Wang 2005). Though recent studies have shown the positive effect glucocorticoids can have on vigilance and cognition (Lupien, Buss, Schramek, Maheu, Pruessner 2005), others support that long-term stress leads to prolonged heighted levels of glucocorticoids making it detrimental to the neurons in the hippocampal formation of the brain causing poorer memory performance (Belanoff, Gross, Yager, & Schatzberg 2001).

For years research perpetuated that glucocorticoids caused memory impairment, when in actuality the study titled "*Stress and cognition: are corticosteroids good or bad guys?*" reviewed these studies that stated these notions of memory impairment tied to high levels of glucocorticoids and acted. The authors "re-interpreted the effects of glucocorticoids on cognitive performance in line with the Type I/Type II ratio hypothesis, suggesting that cognitive function can be enhanced when most of the Type I receptors and only part of the Type II receptors are activated" (de Kloet, Oitzl, & Joëls 1999). The authors flipped the interpretation suggesting that the negative view of glucocorticoid "on human cognitive function could be partly explained by limitations in previous human experimental designs, which did not allow for the differential manipulation of Type I and Type II levels" (de Kloet, Oitzl, & Joëls 1999). Inspired by the *Kloet et al. (1999)* study, the article titled "*Hormetic Influence of Glucocorticoids on Human Memory*" decided to test what the previous researchers suggested but could not do. In following this path to

finding the true answer *Lupien et al. (2007)* conducted a study in which they removed and replaced a hormone (Lupien, Buss, Schramek, Maheu, Pruessner 2005). In this study the hormone that was removed and replaced was the glucocorticoid cortisol. The main point of this study was to test if glucocorticoids were a menace to memory as others had previously suggested. The protocol for the experiment was to "first pharmacologically lowered glucocorticoids levels by administrating metyrapone, a potent inhibitor of glucocorticoid synthesis, we then restored baseline circulating glucocorticoid levels by infusing hydrocortisone, a synthetic glucocorticoid. Memory performance of participants under each of these conditions was compared to that measured on a placebo day" (Lupien, Buss, Schramek, Maheu, Pruessner 2005). The results showed a significantly lower memory performance with the pharmacologically lowered glucocorticoids when compared to the placebo. Furthermore, the researchers were able to reverse the memory impairment with the administration of hydrocortisone. The results were able to show that a lack of circulating glucocorticoids caused memory impairment just as much as a significant increase in circulating glucocorticoids (Lupien, Buss, Schramek, Maheu, Pruessner 2005). The authors were able to show "the effects of stress hormones on human cognition are best understood in line with the inverted-U shape function between glucocorticoid and cognitive performance" (Lupien, Buss, Schramek, Maheu, Pruessner 2005). This is to say that there is a range in which the levels of glucocorticoids can improve cognition and there is also a range in which they cause impairment.

Stress

There are different types of stress, and many times the different types of stress are not mutually exclusive. Numerous studies have researched the various types of stress and

how they affect the Hypothalamic-Pituitary-Adrenal (HPA) axis in different contexts. One of the distinctions that is made is between "social stress" and "physical stress". These two distinctions both stimulate the HPA axis through different pathways. Helping regulate the HPA axis are monoamine neurotransmitters (Douglas 2009). These monoamine neurotransmitters include oxytocin, dopamine, and serotonin (Douglas 2009). Furthermore, studies have suggested that a rise in oxytocin from positive social interactions can suppress the stimulation of the HPA axis and counteract the cortisol and stress (Detillion, Craft, Glasper, Prendergast, & Devries 2004). This particular study even found that would healing began as a positive health effect from the stress being counteracted (Detillion, Craft, Glasper, Prendergast, & Devries 2004).

Early Life Stress

Animal models have been key in developing and studying how early life stress can program and effect the HPA axis (Macrí & Würbel 2006). HPA axis regulation has shown to support resilience to stress when one has been exposed to "mild or moderate stressors early in life" (Macrí & Würbel 2006). By contrast, the HPA axis can become hyper sensitive when one is exposed to extreme stress early in life causing one to be extremely vulnerable to stress (Macrí & Würbel 2006). Rat models have been studied in ordered to find an explanation of these findings on exposure to early-life stress and the mechanisms behind it. It is thought that perhaps during development there is a critical time in which "the level of stress hormones in the bloodstream contribute to the permanent calibration of the HPA axis" (Macrí & Würbel 2006). There has been evidence suggesting that when there are no environmental stressors, exposure of moderate levels of corticosterone in the early stages is linked with stress resilience in

adult rats, in contrast when exposed to higher levels it is linked with stress vulnerability (Macrí & Würbel 2006). Animal models allow for a more controlled environment as well as more control for manipulation. The effects of early life stress on the HPA axis have also been studied on humans. One population that we find is studied often for these effects are adult victims of childhood abuse. When psychosocial stress tasks were compared with healthy subjects and those with depression it was found that there is a rise in Adrenocorticotropic Hormone (ACTH) concentrations as a response (Macrí & Würbel 2006).

These early programming's are not fully determined by these early life events. The environment where the individual is developing can "match or mismatch" with how the individual's HPA axis is programmed for reactivity (Champagne, Frances, Mar, & Meaney 2003). The exact mechanism behind the programming of the HPA axis has yet to be fully explained. Among evolutionary biologist it is still disputed the exact adaptive significance of the programming of the HPA axis. This is to say that it is not clear whether having an increased HPA axis reactivity means greater evolutionary fitness. The HPA axis is critical in the production of corticosteroids, and as mentioned before is important in the brain development and response to environmental stress. If one is to think in terms of evolutionary biology, we would find that the more sensitive HPA axis contributes to evolutionary fitness and help keep us alive; keeping organisms safe from predators and sometime harsh environmental conditions by sparking migration.

Physiological Stress v Psychological Stress

While researching the different forms of stress I found that there were two simple categories, or so I thought. One being physiological stress; which by one account

describes it as stress being one of the most common ways a body reacts to a stressor such as a challenge, threat, or psychological and physical barrier (Ulrich & Herman 2009). These stimuli can alter the environment of an organism. It is also said that physiological stress can come from environmental factors such as the weather, whether it is hot or cold, the kind of physical stress there is on the body. For this study, physiological stress encompasses both the former and the latter. I have also come to the conclusion that most stressors we humans experience are psychological stressors, and our body reacts in a physiological way. Rather than defining stress by physiological or psychological I believe that the TYPE of stressor is what is important and the reaction that may follow.

Definitions of Stress and the Parameter for this Study

There are many different definitions of stress and they have been categorized in various ways. There is one method that suggests five types of stress; (Hardy)

1) acute time-limited stressors - Short Term Challenge

2) brief naturalistic stressors - Normal Event, but Challenging

3) Stressful event sequences – A Stressor that occurs and continues to yield stress into the immediate future

4) chronic stressors – long term stressor exposure

5) distant stressors – none immediate stressor

Another way stress has been categorized and defined is by Albrecht's four common types of stress (Albrecht 1979)

1) Time Stress – most common type of stress; worry about things one is tasked with and the lack of time there is to complete them

2) Anticipatory Stress – worried about the future; either the general future or a specific event

3) Situational Stress – more sudden stress that occurs in specific settings such as fencing bouts

4) Encounter Stress – worried about interactions with others

According to American Psychological Association (APA), there are 3 different types stress —

1) Acute stress - Brief; Most Common; can be negative thoughts or upcoming events

2) Episodic acute stress – Feelings of being in a constant rush or pressure for performance;

3) Chronic stress – Most harmful type of stress, causing damage to physical self and mental health; examples are long term poverty; repeated abuse

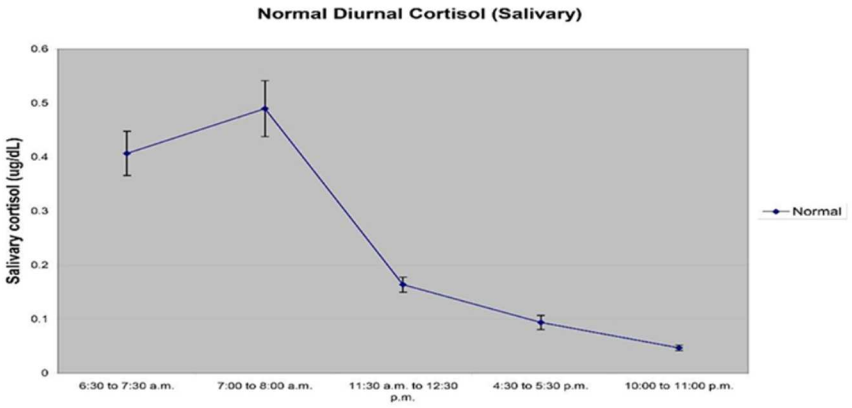
Stress and Performance

For this research the parameters around stress and stressors will be in accordance with how the American Psychological Association (APA) defines the three different types of stress above. For this study, it has been concluded that the type of stress that is experienced during competition falls under the category of *episodic acute stress.* When one experiences this type of stress the symptoms can include but are not limited to anxiety, conflict avoidance, muscle tension, low/excessive motivation, headaches, and tremors (Freshwater 2018). Many times, this can also be a sign of "heightened basal ganglia activity" in the brain (Freshwater 2018). These basal ganglia surround the limbic system. They are involved in the integrations of thoughts, movement, and feelings (Hikosaka, Takikawa, & Kawagoe 2000). Many times when the basal ganglia are overactive, like is seen with stress and anxiety, people show various physical responses such as immobility or muscles tensing. This is where stress can affect performance in a

negative way. However, stress can positively affect performance if one is able to embrace it become more cognitively aware.

Circadian Rhythm

The production of Cortisol is diurinal, this is to say that the levels peak in the morning and gradually decline through the day. Stress increases Cortisol levels independently from the circadian rhythm. This does not affect the samples taken from each participant because competition times throughout the three tournaments is comparable. The schedule does not change. Though, what could not be accounted for was athletes traveling to other times zones.



"Salivary Cortisol ELISA Kit – Salimetrics." *Salimetrics Cortisol Overview*, Salimetrics, www.salimetrics.com/assay-kit/salivary-cortisol-elisa-kit/.

Fig. A. Normal Diurnal Cortisol

Chapter 2. Cortisol and Performance

Abstract

Salivary cortisol is the least invasive way in measuring hormonal response during exercise without interruption. In nationally ranked fencers (n=21), changes in cortisol were monitored by measurement of salivary cortisol sampled throughout different rounds of three North American Cup tournaments during the 2017-2018 United States fencing season. The changes were also compared when looking at if a bout ended in a victory or defeat; the difference in rank between opponents; and the difference in score at the end of the bout. Immediately before the tournament cortisol levels were sampled, changes were in comparison to the initial sample as well as change from one bout to the next. The primary purpose of this study was to (a) compare how cortisol levels fluctuate during a tournament and (b) analyze cortisol levels to see if there is an optimal rage for performance. Eustress, "good stress" was considered optimal when the athletes were at peak performance. Here, peak performance means accomplishing the task, with the task being the bout ending in a victory. It was hypothesized that (a) cortisol levels would peak after a loss or stressful bout and (b) there would be an optimal range of cortisol for peak performance. This study supports the findings that cortisol peaks after a loss, and could point to optimal cortisol levels being more of an individualized range for each athlete. If these athletes can explicitly see just how their hormones rise and fall, then perhaps being more aware of these levels and being able to embrace them could lead to peak performance.

Study Overview

This research required approval from Arizona State University's Institutional Review Board (IRB). The submission for approval took place in January of 2017 and received approval in March of 2017, with approval through March of 2019. The protocol approval number from IRB is STUDY00005570. In the process of gaining IRB approval, this study was also approved by Christine Simons, Senior Director of Operations, along with Jeremy Summers, Director of Sports Medicine, of the United Stated Fencing Association. This research is independent of the United States Fencing Association. In order to collect and process the saliva samples all material was provided in the Expanded Range High Sensitivity SALIVARY CORTISOL ENZYME IMMUNOASSAY KIT through Salimetrics. The samples were taken from athletes at three integral North American Cup (NAC) competitions during the 2017-2018 fencing season. The athletes were competing in Division I women's epee and Division I men's epee. For the full protocol of the ELISA salivary cortisol testing can be found in Appendix A.

Participant Recruitment

This study used nationally ranked Division I fencers who are members of the United States Fencing Association. Potential participants were contacted via email in the months leading up to the first sampling, October 2017 NAC. The potential participants were identified under certain criteria. First, they must be ranked epee fencers in the United States, secondly, they must be athletes who regularly compete at NACs and have the training regimen of high performance athletes. These athletes must also be experienced fencers with a minimum of five years of training and competing. The ranking of athletes can easily be accessed through the updated 2017-2018 rolling points

list that US Fencing provides online. A copy of the recruitment email can be found in Appendix B, and the consent form can be found in Appendix C.

Sample Collection

The targeted tournaments where salivary samples were collected were the 3 major domestic tournaments for these Division I athletes during the 2017-2018 fencing season; October 2017 NAC, December 2017 NAC, and January 2018 NAC. All athletes were sampled during the same intervals of the tournament. Though, according to the performance of an athlete there is an unequal number of samples taken from each athlete. The intervals where saliva was collected was: morning of the tournament, directly after the first round (pools), and if applicable 13-15 minutes after a direct elimination bout. Each sample taken is after a victory except for the last sample which is after a defeat.

Stress was measured through concentration of cortisol in the saliva. Saliva samples were collected with salivettes in a cryotubes that were provided in the Salimetrics salivary cortisol kit. Gloves were used at all times. In order to ensure the sample matched with the participant the tube used was labeled and numbered to match the athlete's identifying code. Once the sample was collected [minimum of 75ul] it was placed in an ice chest until the samples were to be frozen and shipped to the lab at Arizona State University. During the weeks following the competition the samples were prepared with reagents provided in the Salimetrics kit, placed into a 96 well plate and then analyzed using a plate reader that is available in the School of Life Sciences DNA lab at 450 nm light absorbance. Samples were conducted in duplicate and with the proper titration controls. Refer to Appendix B for the full Salimetrics protocol for salivary cortisol assays.

Serologic testing – Processing the samples

"In blood, only about 5-10% of Cortisol is in its unbound or biologically active form. The remaining Cortisol is bound to serum proteins. Unbound serum Cortisol enters saliva via intracellular mechanisms; in saliva, the majority of Cortisol remains unbound to protein. Salivary Cortisol levels are unaffected by salivary flow rate and are relatively resistant to degradation from enzymes or freeze-thaw cycles. Studies consistently report high correlations between serum and salivary Cortisol, indicating that salivary Cortisol levels reliably estimate serum Cortisol levels." (Salimetrics protocol)

Enzyme-Linked Immunosorbent Assay (ELISA) was used for serologic testing of cortisol. A multi-welled (96) micro tittered plate was used so that the dilution of the serum could be easily prepared and tested. The wells of the plate come coated with the antigen of interest. The wells were then filled with the individual sample of saliva of the participant. If the antibodies of an antigen are present, then they will bind and be fixed to the bottom of the well. The wells are then washed out in order to remove all unbound antibodies. Next, a solution of an animal antibody against human antibodies is added, this second antibody is covalently conjugated to an enzyme. The wells are washed again this time to remove any unbound enzyme conjugated antibody. Finally, a solution of a colorogenic enzyme substrate is then added, this interaction of the substrate with the enzyme on the second antibody generates a visible color. The development of color can be seen and quantified with a plate reader.

Sample Population

In the October North American Cup (NAC) there were 164 participants for Division I women's epee. This study sampled 7 of those athletes. In Division I men's epee there were 286 participants. This study sampled 6 of those athletes.

In the December North American Cup (NAC) there were 129 participants for Division I women's epee. This study sampled 11 of those athletes. In Division I men's epee there were 207 participants. This study sampled 3 of those athletes.

In the January North American Cup (NAC) there were 179 participants for Division I women's epee. This study sampled 7 of those athletes. In Division I men's epee there were 285 participants. This study sampled 3 of those athletes.

On average for Division I women's epee there are 157 participants competing at North American Cups, this study sampled on average 9.5% of the population. On average for Division I men's epee there are 232 participants competing at North American Cups, this study sampled on average 5.17% of the population

Calculations

After processing the samples, the calculations performed were the first steps into viewing just how cortisol levels change during competition for athletes. The average optical density (OD) was computed for all duplicate wells. Then the average OD was subtracted from the OD of the zero, standards, controls, and saliva samples. Finally, in order to calculate the percent bound (B/Bo) for each the standard, high/low control, and

saliva sample one had to divide the OD of each well (B) by the average OD for the zero (Bo).

Statistical Analyses

Once the adjusted OD was calculated then the dataset was normalized before analysis. The concentrations of cortisol were normalized in order to account for individual differences. The three ways chosen to graph the results and analyze them were as follows.

Graphs

All of the data from the tournaments was compiled onto the same graph, originally these graphs were separated by gender, but after further research there is no difference between female and male cortisol concentrations (Thatcher, Thatcher, & Dorling 2004). Participants who did not make it to the Direct Elimination (DE) round only had two samples, so that data is left off of the graphs. The overall data, with normalized concentrations of cortisol was graphed in three separate distinct ways; Difference in Score v Change in Cortisol, Difference in Rank v Change in Cortisol, and Change in Cortisol v Victory (0) & Defeat (1).

Additionally, for better understanding of the data there are additional graphs which are more focused on the individual. These graphs move the scope of looking for an optimal range for every athlete, to an optimal range for each individual athlete. The concentration of cortisol on these graphs is not normalized and represents the difference in cortisol from one bout to the next.

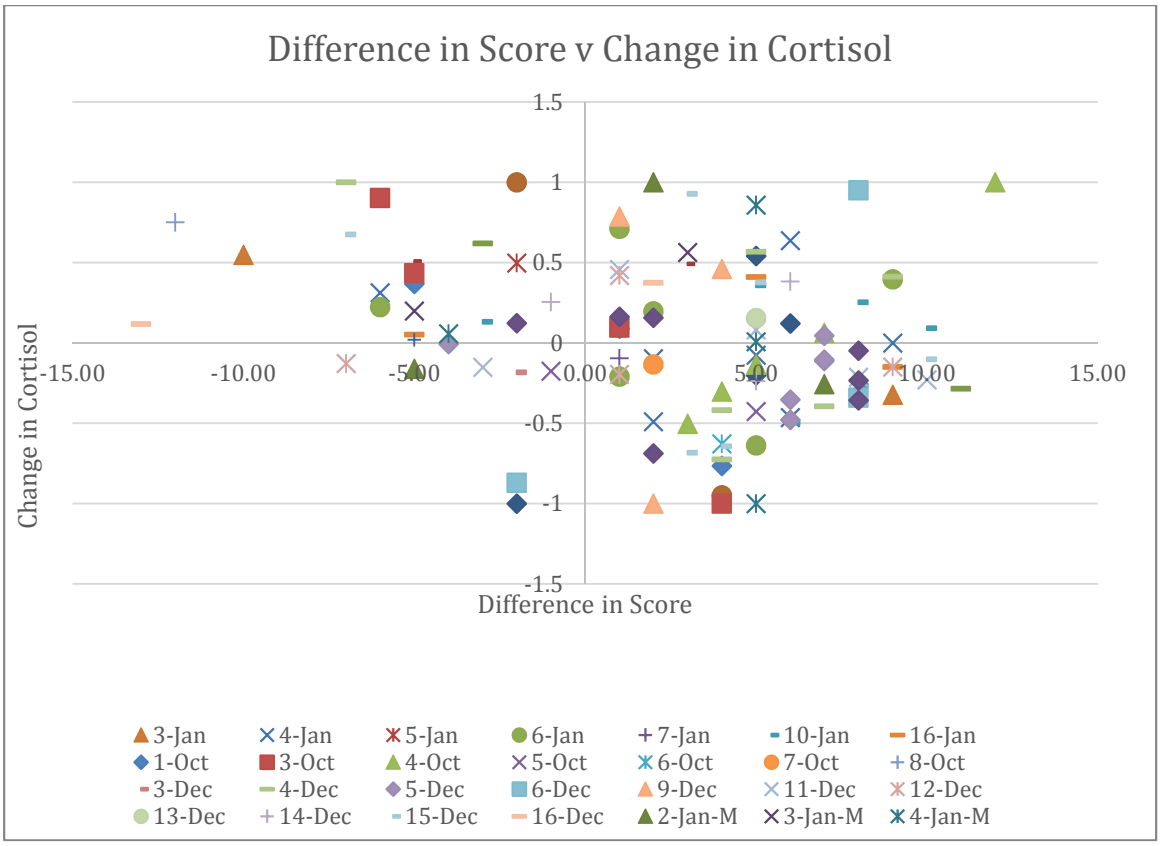


Fig. 1. Difference in Score v Change in Cortisol The difference in score during a single bout against the change in cortisol measured in µg/dL, Legend is participant number and the tournament sampled. M represents male sample.

The graph above indicates the difference in score during a single direct elimination bout against the change in cortisol measured in *µg/dL* from the previous direct elimination bout. A negative cortisol value represents a decrease in cortisol and a positive value indicates an increase in cortisol. A negative score difference value represents the participant experienced a defeat from their opponent by that margin, while a positive value indicates the participants came out with a victory with that difference. This graph has both men and women. Each series is named *participant number + tournament month + M (for men if applicable).*

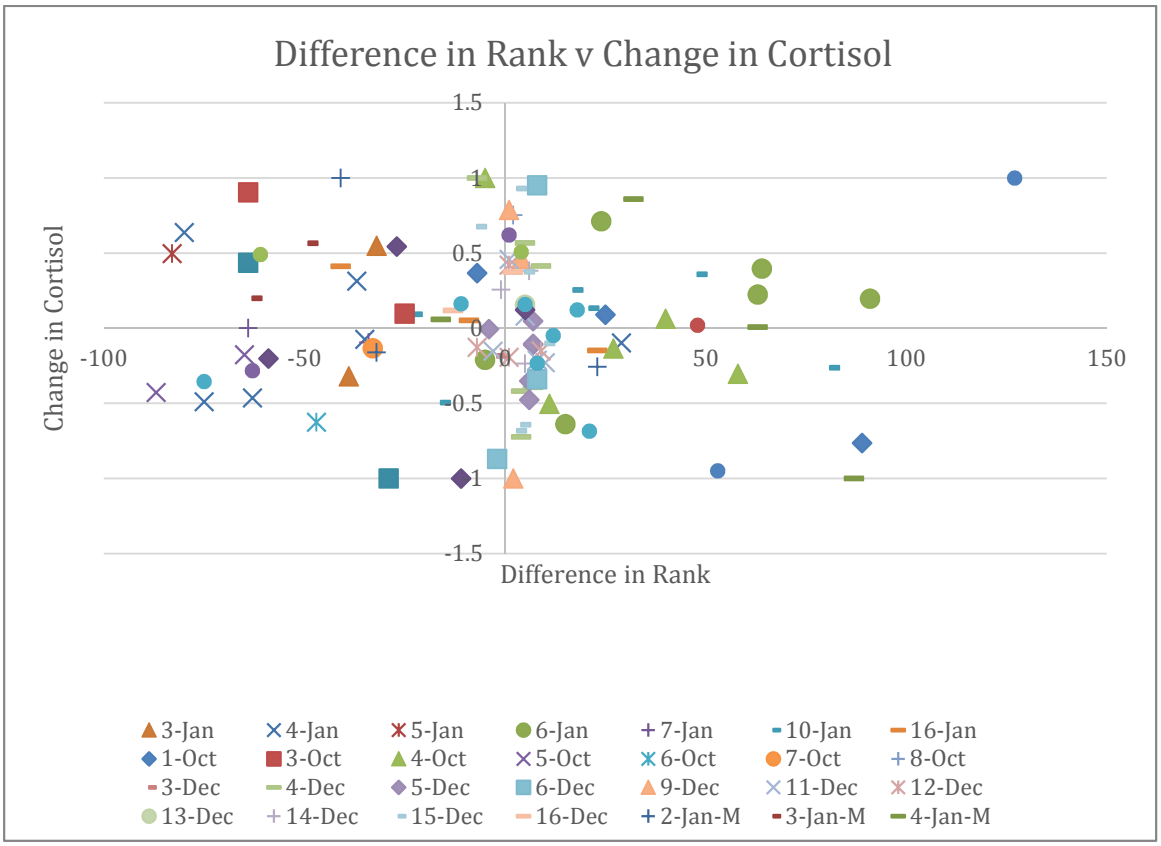


Fig. 2. Difference in Rank v Change in Cortisol The difference in rank in each individual direct elimination bout against the change in cortisol measured in µg/dL, Legend is participant number and the tournament sampled. M represents male sample.

The graph above shows the change in cortisol, measured in *µg/dL* from one bout to the next against the difference in rank between opponents. Again, a negative cortisol value represents a decrease in cortisol and a positive value indicates an increase in cortisol. The x-axis represents the rank difference between the opponents. A negative value represents the participant is ranked BELOW their opponent, while a positive value indicates the participants is ranked ABOVE their opponent. This graph has both men and women. Each series is named *participant number + tournament month + M (for men if applicable).*

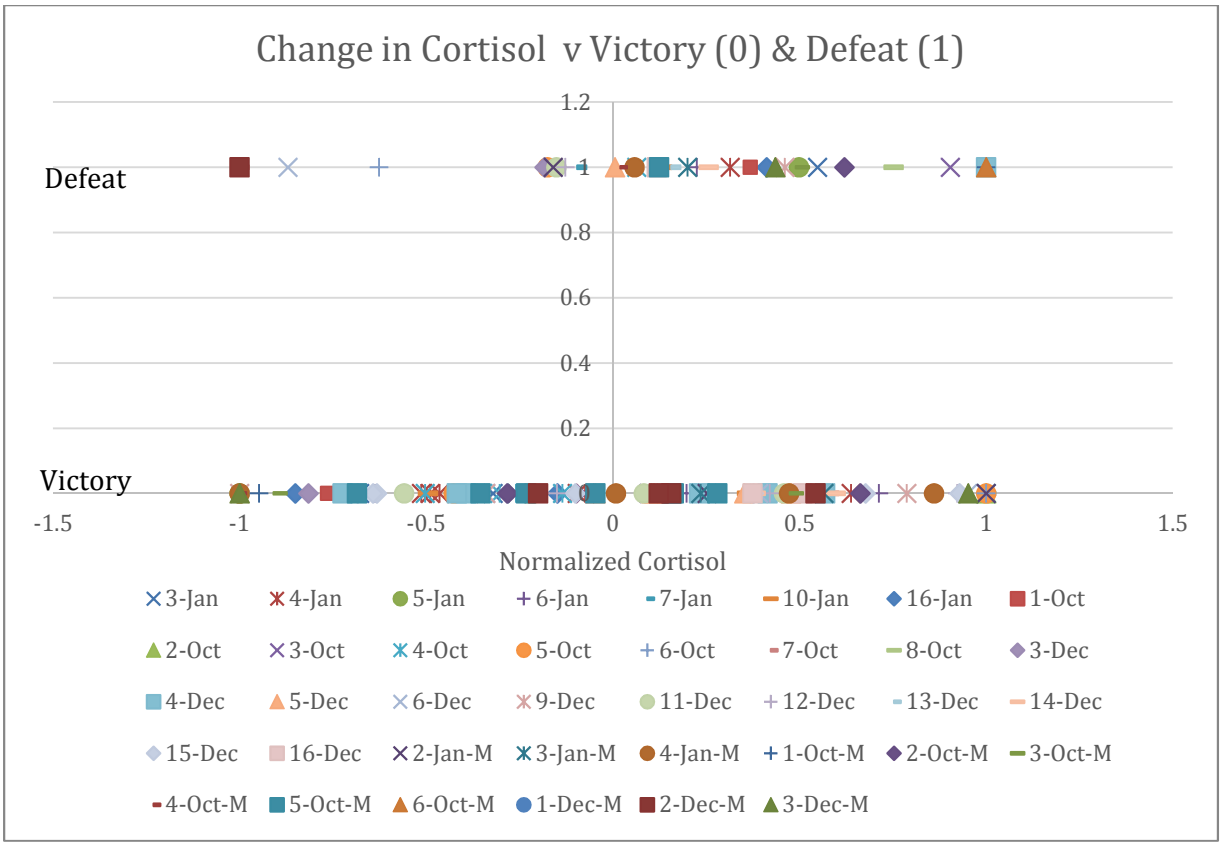


Fig. 3. Change in Cortisol v Victory (0) & Defeat (1) The change in cortisol measured in µg/dL against the outcome of a single direct elimination. Legend is participant number and the tournament sampled. M represent male sample.

Figure 3 shows the change in cortisol from one bout to the next against the outcome of that bout, either a victory or defeat. A negative cortisol value represents a decrease in cortisol and a positive value indicates an increase in cortisol. This graph has both men and women. Each series is named *participant number + tournament month + M (for men if applicable).*

	Oct. 2017 NAC	*Dec. 2017 NAC*	*Jan. 2018 NAC*
WOMEN			
Participant 1	X	--	X
Participant 3	X	X	--
Participant 4	X	X	X
Participant 5	X	X	X
Participant 6	X	X	X
Participant 7	X	--	X
Participant 8	X	--	--
Participant 9	--	X	--
Participant 10	--	--	X
Participant 11	--	X	--
Participant 12	--	X	--
Participant 13	--	X	--
Participant 14	--	X	--
Participant 15	--	X	--
Participant 16	--	X	X
MEN			
Participant 1	X	X	--
Participant 2	X	X	X
Participant 3	X	X	X
Participant 4	X		X
Participant 5	X	--	
Participant 6	X	--	--

"X" represents participant was

Fig. 4. Athletes who participated in the study and which tournaments their samples were collected

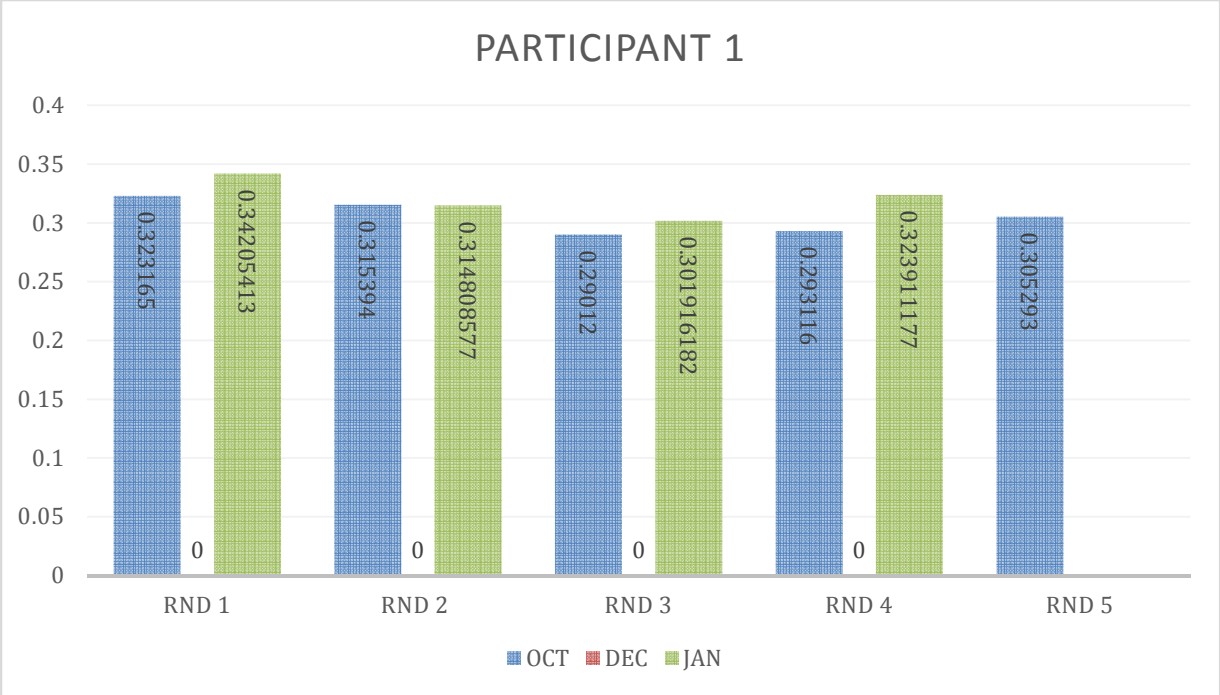


Fig. 5. Participant 1 Women's epee Direct Elimination round vs Salivary Cortisol concentration measured in µg/dL

	OCT	JAN
RND 3	(+89) +4	(+39) +9
RND 4	(+25) +1	(+32) -10
RND 5	(-7) -5	

*Number inside parenthesis indicates the rank difference between the participant and their opponent, and the number outside of the parenthesis is the margin of the victory/defeat.

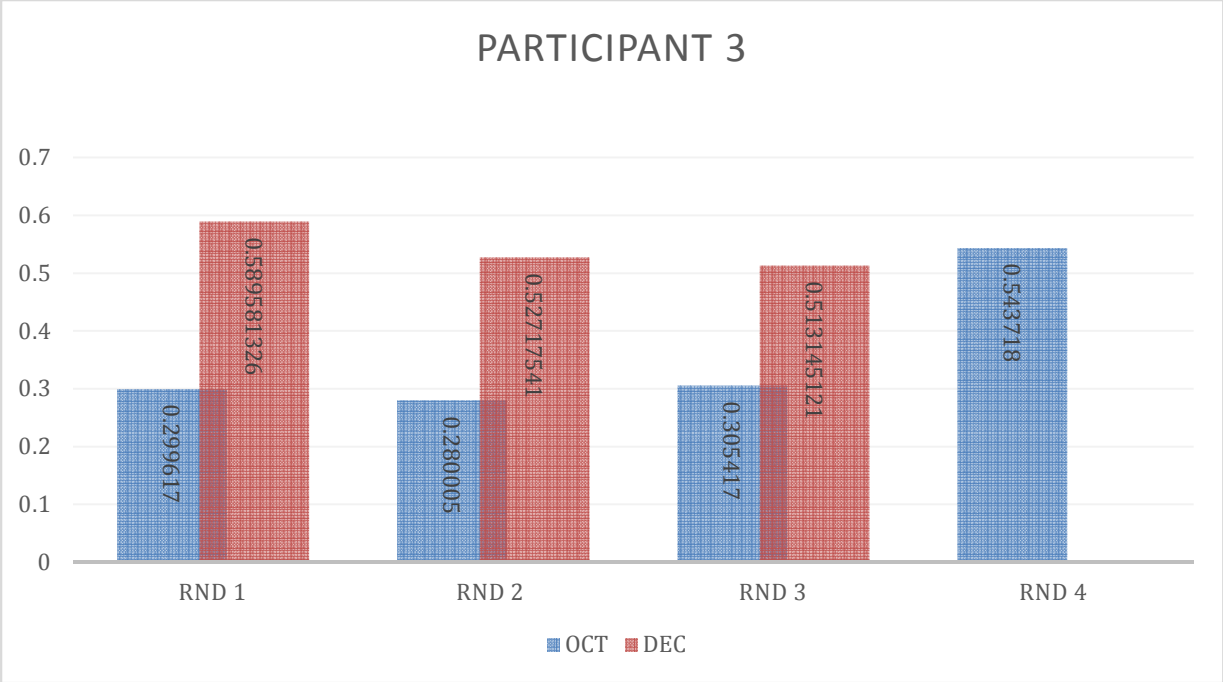


Fig. 6. Participant 3 Women's epee Direct Elimination round vs Salivary Cortisol concentration measured in µg/dL

	OCT	DEC
RND 3	(-25) +1	(+21) -2
RND 4	(-64) -6	

*Number inside parenthesis indicates the rank difference between the participant and their opponent, and the number outside of the parenthesis is the margin of the victory/defeat.

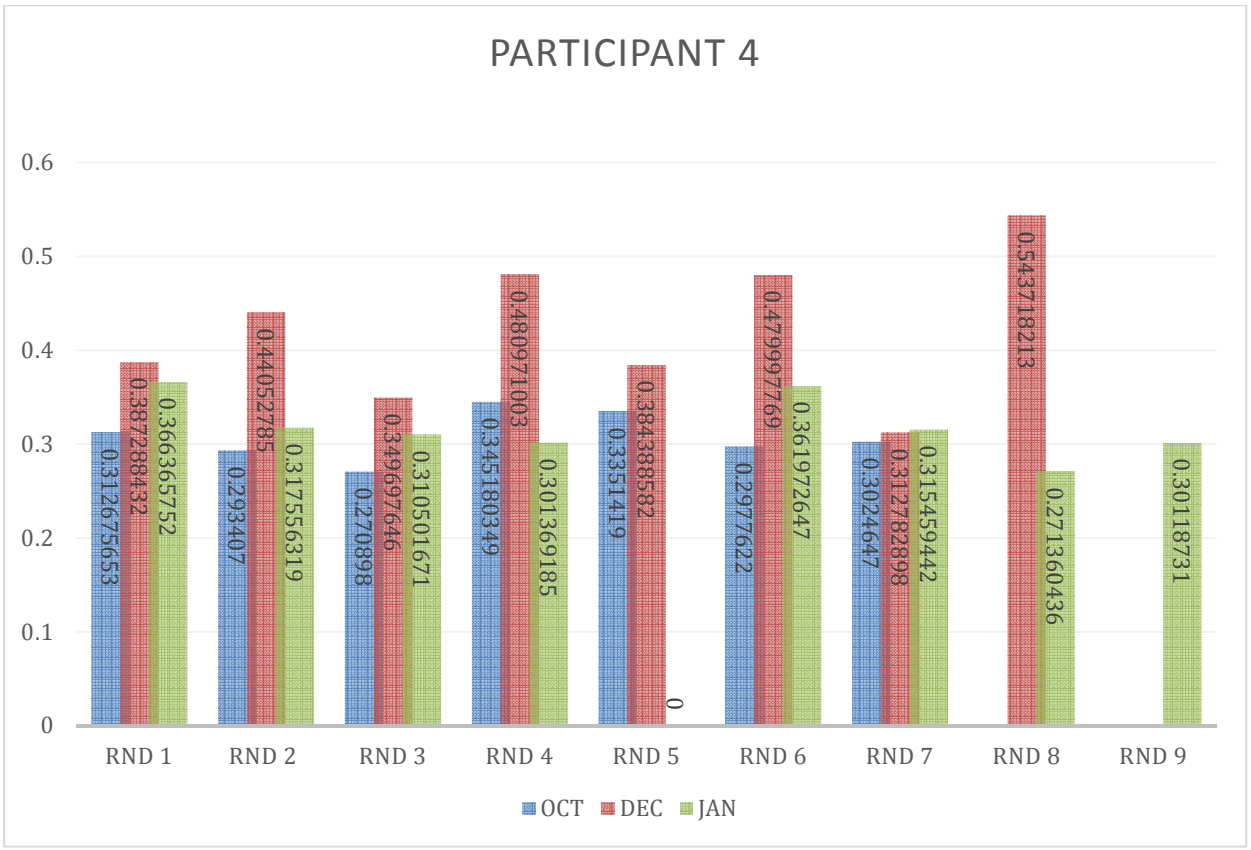


Fig. 7. Participant 4 Women's epee Direct Elimination round vs Salivary Cortisol concentration measured in µg/dL

	OCT	DEC	JAN
RND 3	(+58) +4	(+64) +7	(-35) +5
RND 4	(-5) +12	(+32) +5	(+29) +12
RND 5	(+27) +5	No Saliva (+48) +4	(-3) +9
RND 6	(+11) +3	(+8) +9	(-80) +6
RND 7	(+40) -6	(+40) +4	(-75) +2
RND 8		(+3) -7	(-63) +6
RND 9			(-37) -6

*Number inside parenthesis indicates the rank difference between the participant and their opponent, and the number outside of the parenthesis is the margin of the victory/defeat.

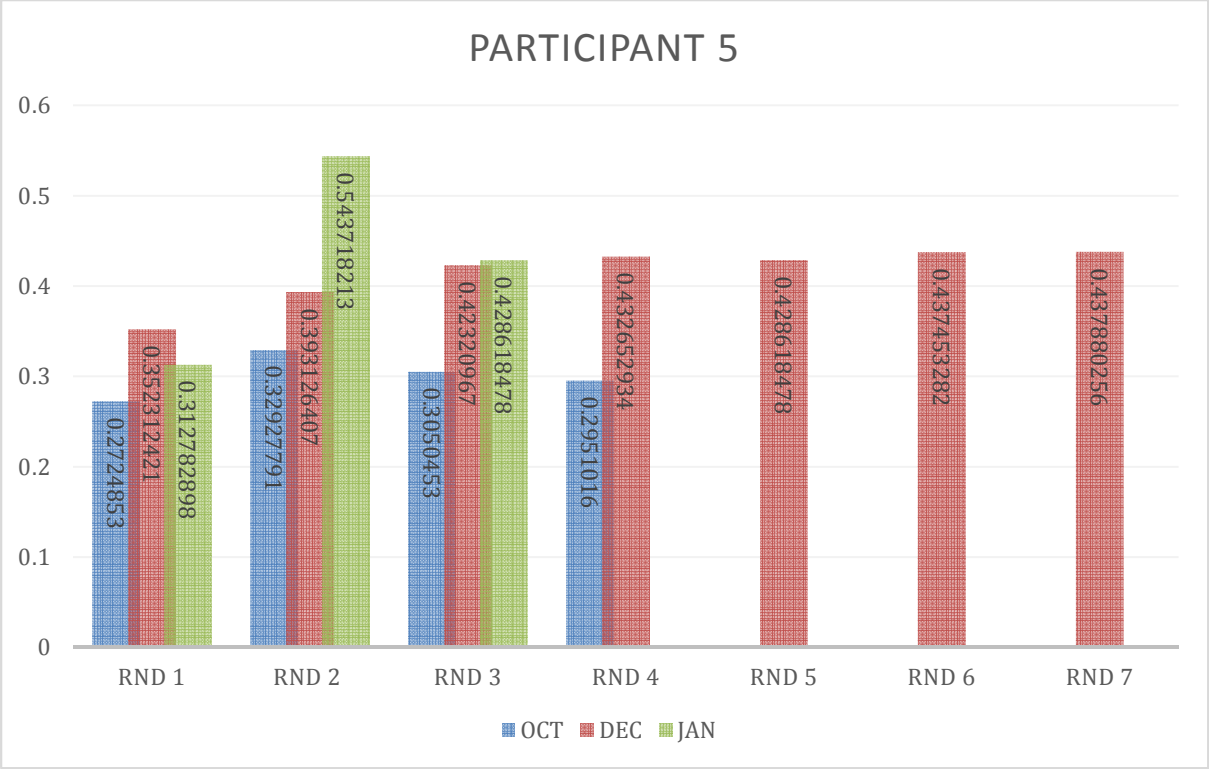


Fig. 8. Participant 5 Women's epee Direct Elimination round vs Salivary Cortisol concentration measured in µg/dL

	OCT	DEC	JAN
RND 3	(+87) +4	(+44) +6	(+83) -2
RND 4	(+65) -6	(-20) +6	
RND 5		(-4) +7	
RND 6		(-23) +7	
RND 7		(-40) -4	
RND 8			

*Number inside parenthesis indicates the rank difference between the participant and their opponent, and the number outside of the parenthesis is the margin of the victory/defeat.

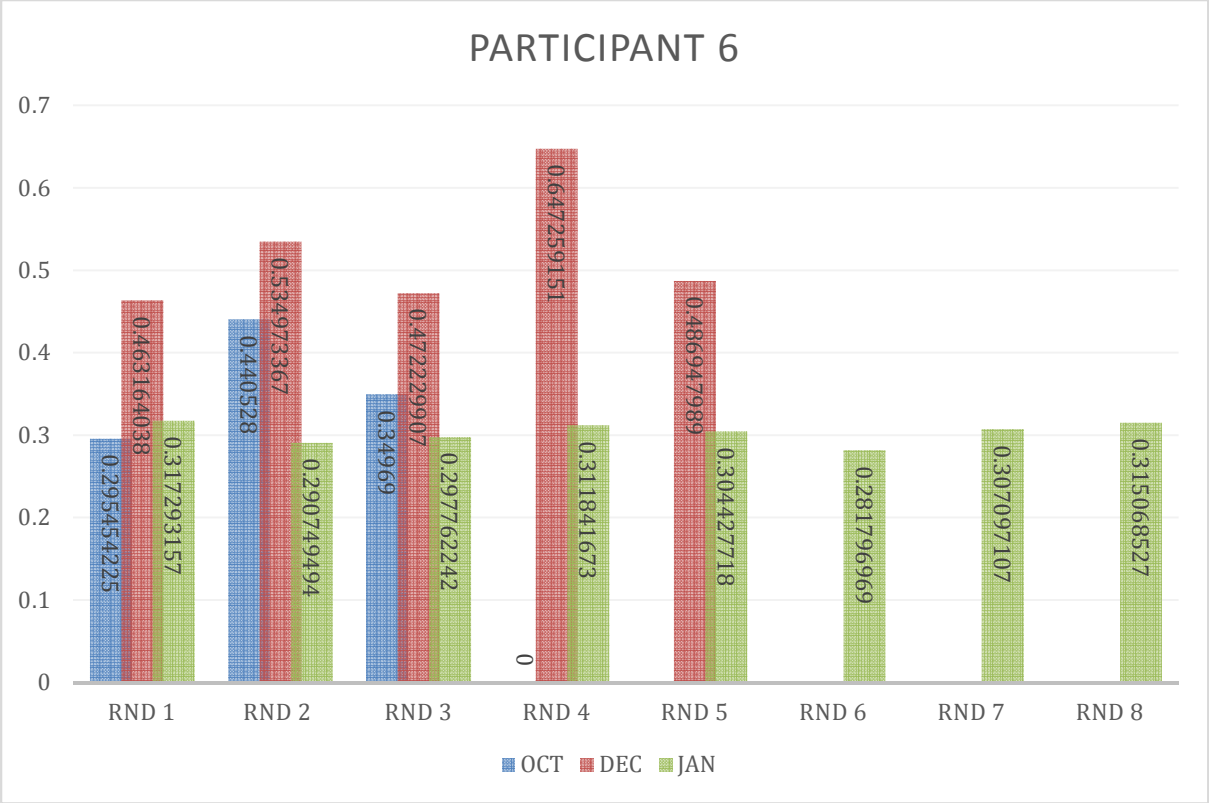


Fig. 9. Participant 6 Women's epee Direct Elimination round vs Salivary Cortisol concentration measured in µg/dL

	OCT	DEC	JAN
RND 3	(+47) +4	(+93) +8	(+91) +2
RND 4	(-16) -1 (sample not collected)	(+29) +8	(+64) +9
RND 5		(+32) -2	(-5) +1
RND 6			(+15) +5
RND 7			(+24) +1
RND 8			(+63) -6

*Number inside parenthesis indicates the rank difference between the participant and their opponent, and the number outside of the parenthesis is the margin of the victory/defeat.

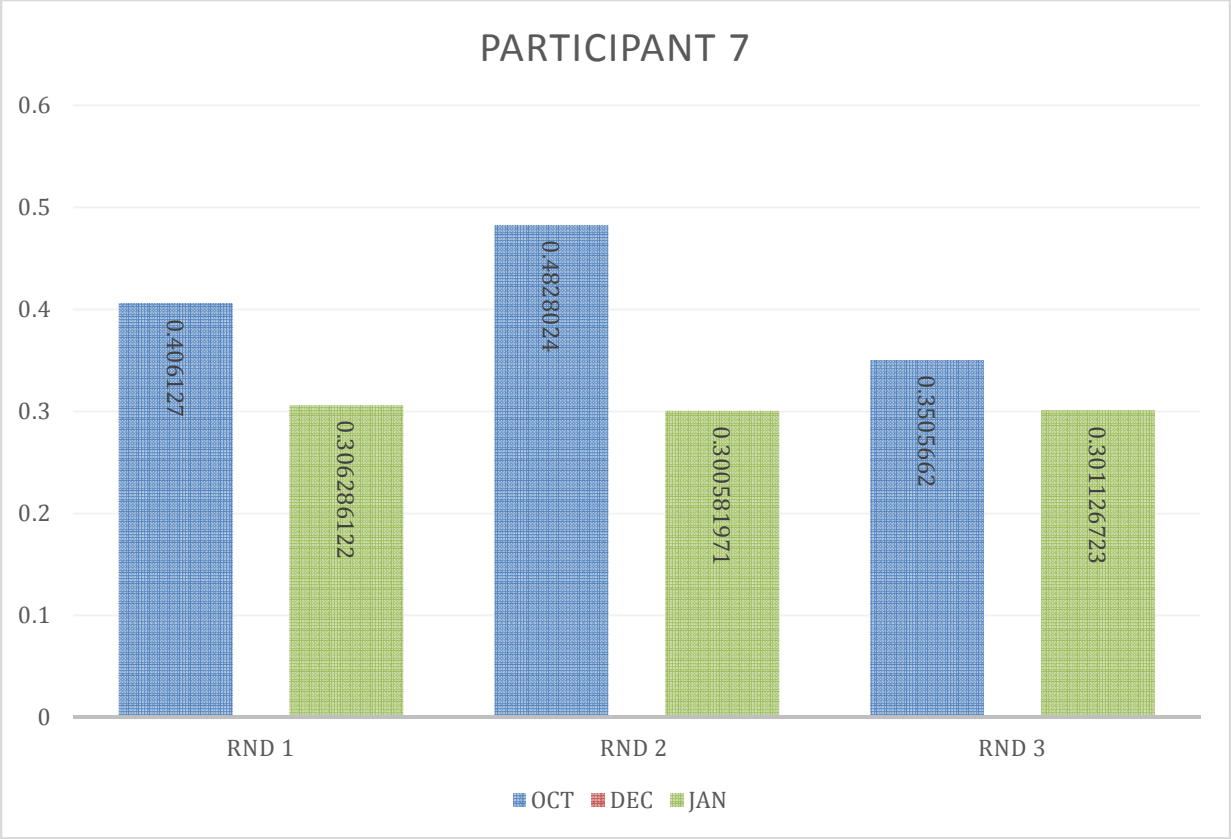


Fig. 10. Participant 7Women's epee Direct Elimination round vs Salivary Cortisol concentration measured in µg/dL

	OCT	JAN
RND 3	(+33) +2	(-34) +1
RND 4	(-31) -1 (SAMPLE NOT COLLECTED)	(-64) -1 (SAMPLE NOT COLLECTED)

*Number inside parenthesis indicates the rank difference between the participant and their opponent, and the number outside of the parenthesis is the margin of the victory/defeat.

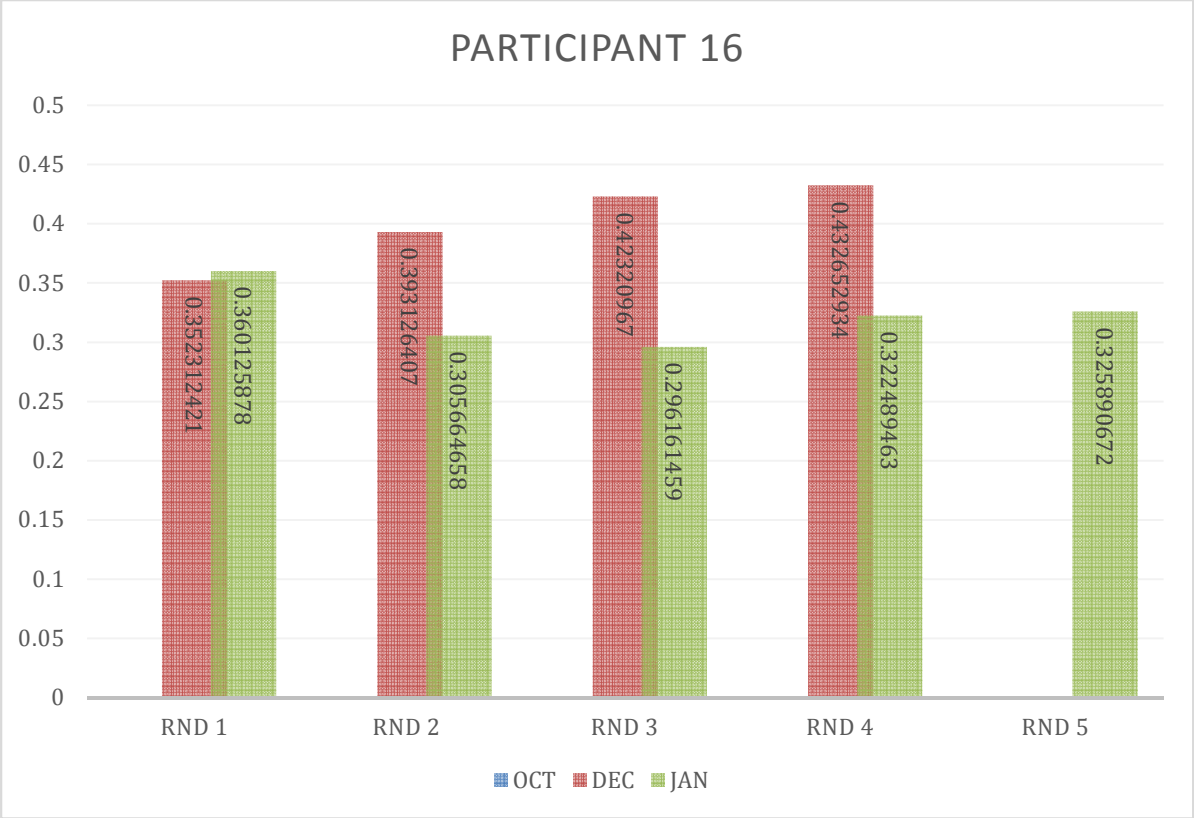


Fig. 11. Participant 16 Women's epee Direct Elimination round vs Salivary Cortisol concentration measured in µg/dL

	DEC	JAN
RND 3	(+64) +2	(+23) +9
RND 4	(+5) -13	(-41) +5
RND 5		(-9) -5

*Number inside parenthesis indicates the rank difference between the participant and their opponent, and the number outside of the parenthesis is the margin of the victory/defeat.

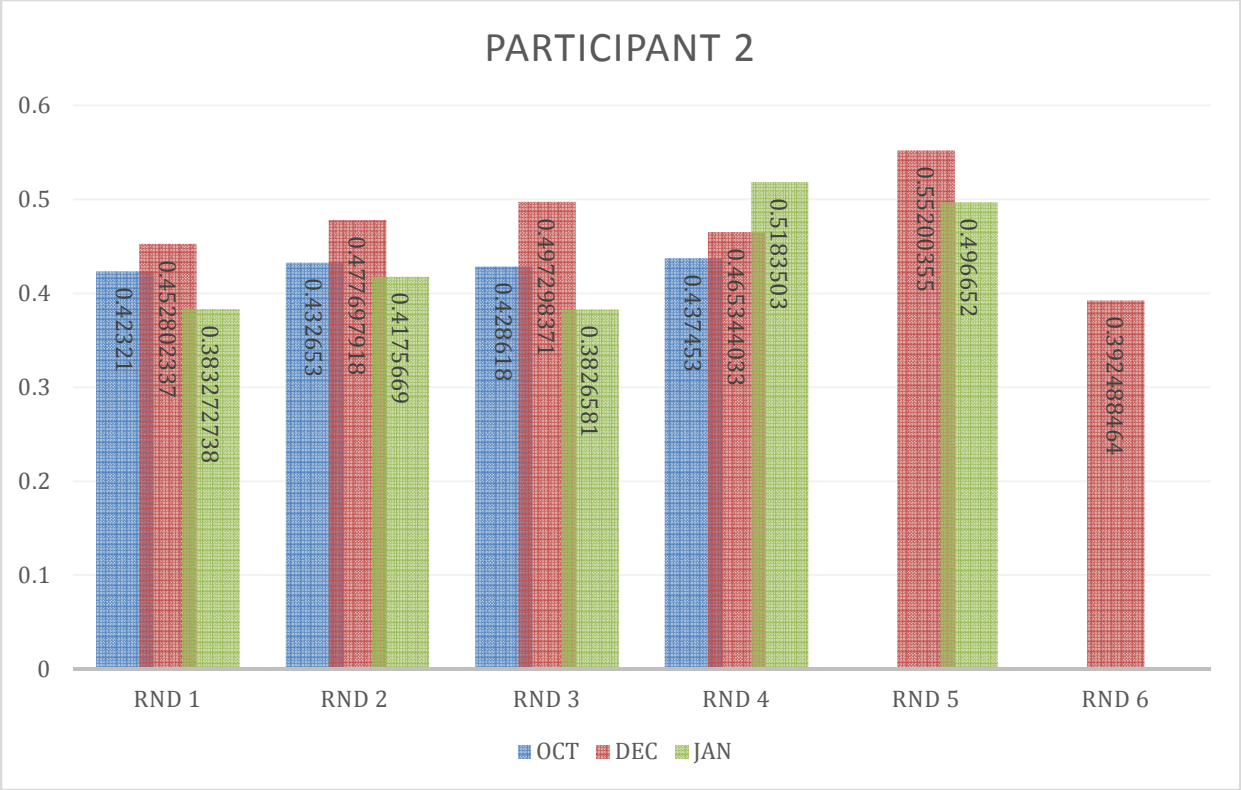


Fig. 12. Participant 2 Men's epee Direct Elimination round vs Salivary Cortisol concentration measured in µg/dL

	OCT	DEC	JAN
RND 3	(+63) +11	(+5) +6	(+23) +7
RND 4	(-1) -3	(-59) +5	(-41) +2
RND 5		(-27) +5	(-32) -5
RND 6		(-11) -2	

*Number inside parenthesis indicates the rank difference between the participant and their opponent, and the number outside of the parenthesis is the margin of the victory/defeat.

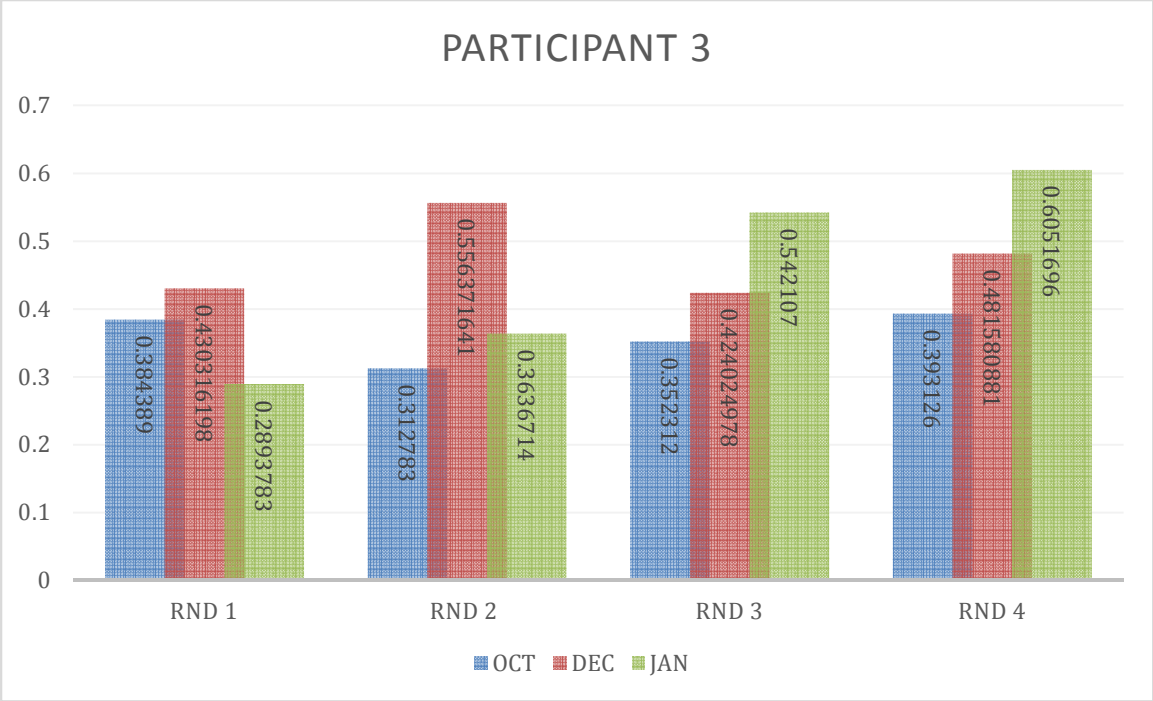


Fig. 13. Participant 3 Men's epee Direct Elimination round vs Salivary Cortisol concentration measured in µg/dL

	OCT	DEC	JAN
RND 3	(+61) +3	(-29) +4	(-40) +3
RND 4	(-4) -5	(-64) -5	(-63) -5

*Number inside parenthesis indicates the rank difference between the participant and their opponent, and the number outside of the parenthesis is the margin of the victory/defeat.

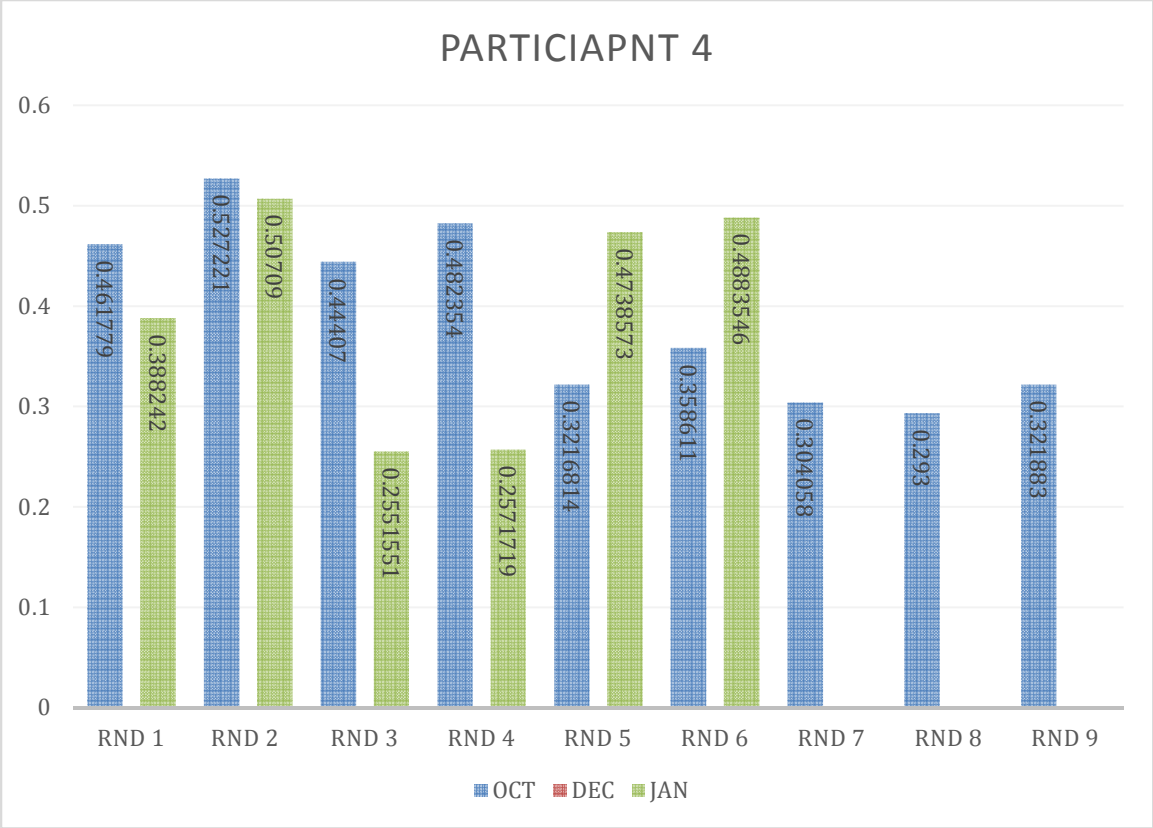


Fig. 14. Participant 4 Men's epee Direct Elimination round vs Salivary Cortisol concentration measured in µg/dL

	OCT	JAN
RND 3	(+75) +8	(+87) +5
RND 4	(+11) +1	(+63) +5
RND 5	(-21) +3	(+32) +5
RND 6	(-5) +2	(-16) -4
RND 7	(-8) +8	
RND 8	(-12) +8	
RND 9	(-18) -2	

*Number inside parenthesis indicates the rank difference between the participant and their opponent, and the number outside of the parenthesis is the margin of the victory/defeat.

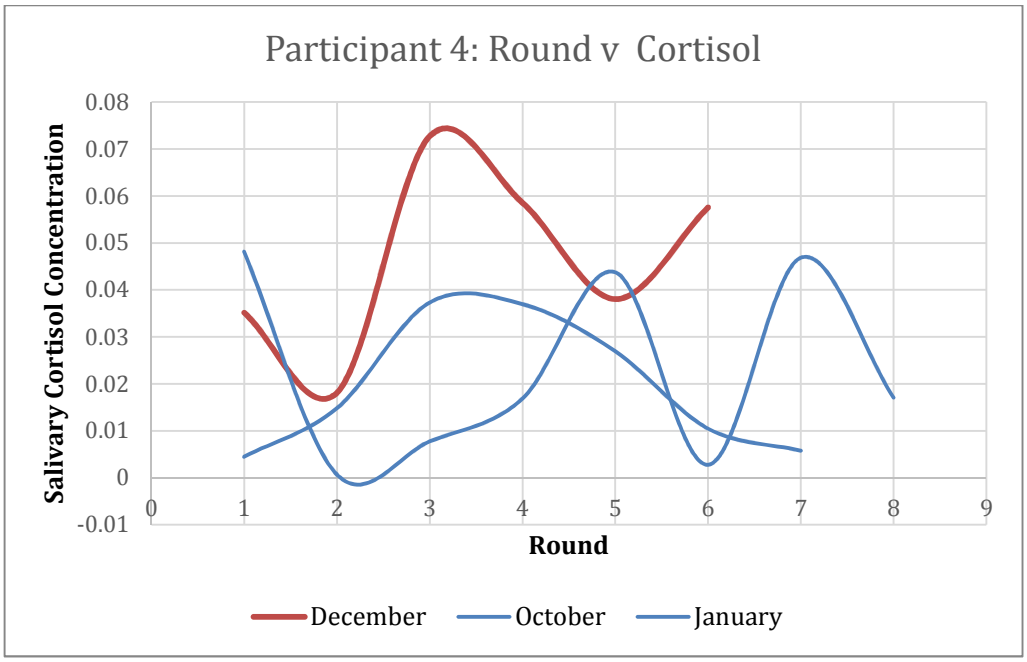


Fig. 15. Participant 4 Women's epee Direct Elimination round vs Salivary Cortisol concentration measured in µg/dL

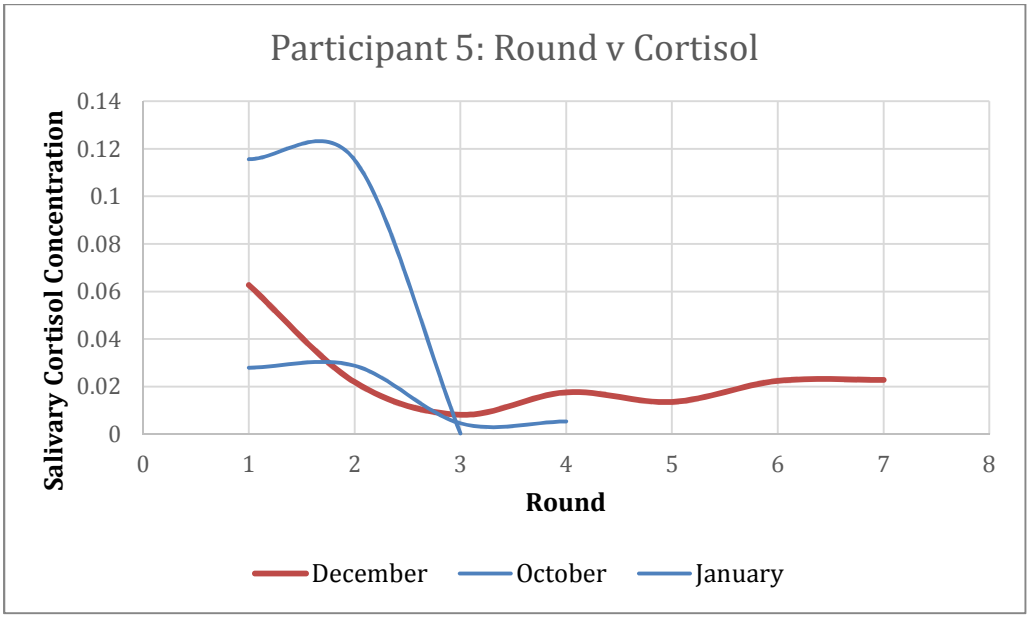


Fig. 16. Participant 5 Women's epee Direct Elimination round vs Salivary Cortisol concentration measured in µg/dL

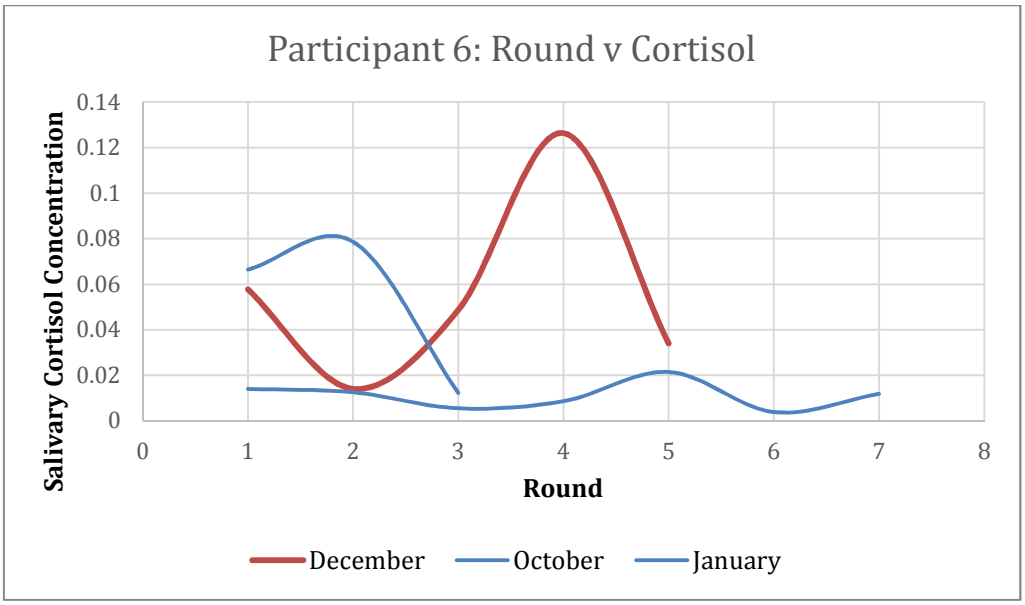


Fig. 17. Participant 6 Women's epee Direct Elimination round vs Salivary Cortisol concentration measured in µg/dL

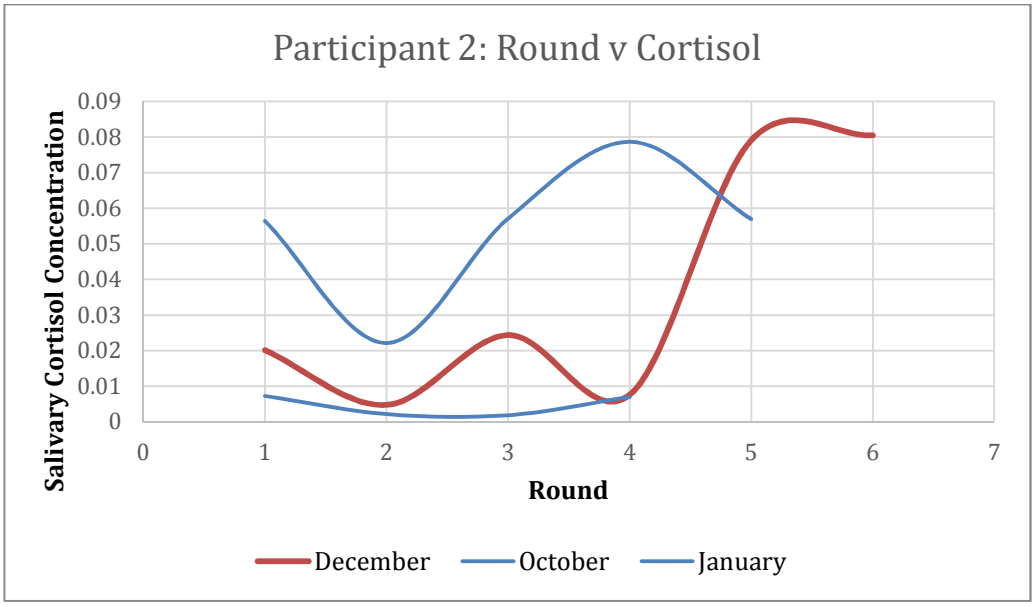


Fig. 18. Participant 2 Men's epee Direct Elimination round vs Salivary Cortisol concentration measured in µg/dL

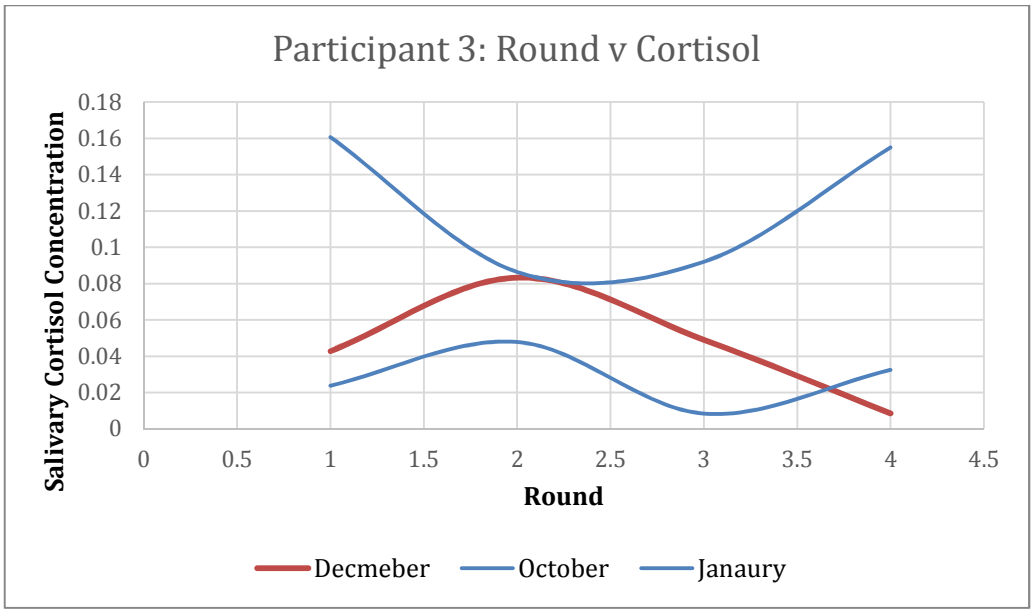


Fig. 19. Participant 3 Men's epee Direct Elimination round vs Salivary Cortisol concentration measured in µg/dL

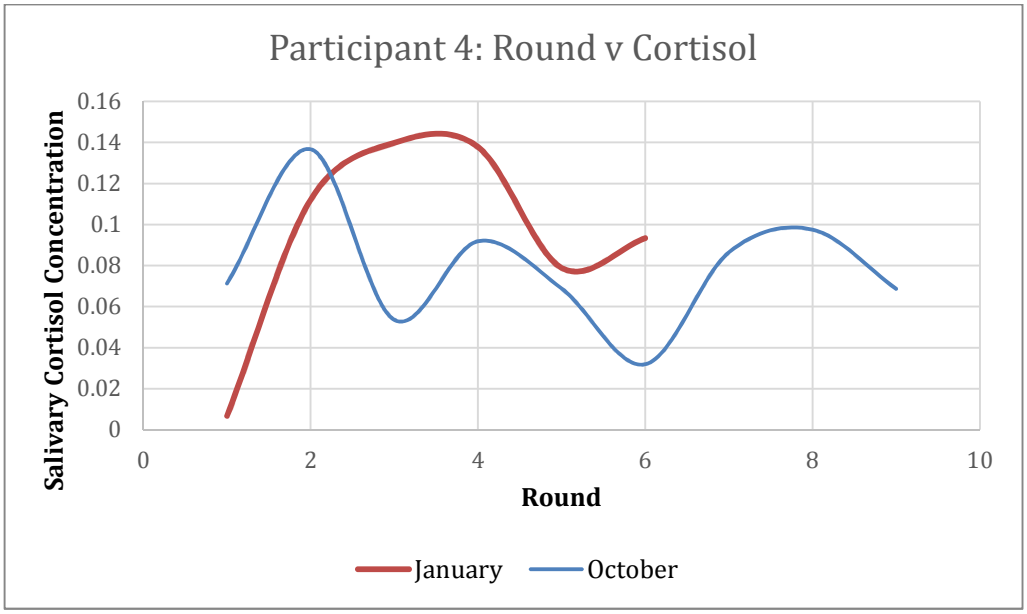


Fig. 20. Participant 4 Men's epee Direct Elimination round vs Salivary Cortisol concentration measured in µg/dL

Results

The numbers do show that many times there is a rise in cortisol after a defeat. We see with women's epee participant 1 a spike in cortisol during their losing bouts. In the October NAC the athlete's cortisol level in the penultimate round is 0.293116 µg/dL then it rises to 0.305293 µg/dL in the bout that the athlete was defeated. Again, with participant 1 in the January NAC the penultimate round the athlete's cortisol is measured at 0.301916182 µg/dL, and this rises to 0.32391177 µg/dL during the last eliminatory bout.

Participant 4 shows similar behavior to participant 1, in the December NAC the penultimate bout reading of cortisol is 0.312782898 µg/dL which then spikes to 0.543718213 µg/dL after a loss in the finals, perhaps the most critical bout of the tournament. Once again after defeat in a gold medal bout Participant 4 goes from 0.27130436 µg/dL to 0.30118731 µg/dL in the final round of the January NAC.

In the December tournament participant 6 had a victorious bout ending with a +8 margin, and their cortisol peaked during this bout at 0.647259151 µg/dL. It seems that although there was a spike in cortisol, this athlete knew how to embrace that stress and perhaps was more cognitively aware ending with a victory. Women's epeeist participant 16 showed rather steady levels of cortisol throughout both tournaments they participated in. The initial cortisol levels on both days were similar, and in both tournaments their levels rise slightly during each bout, with the final bout ending in defeat with their cortisol levels at their peak.

Men's epeeist, participant 3, lost the last bouts of all 3 tournaments by 5 touches. Again, we see that their cortisol rises after a defeat. During the October NAC men's epeeist participant 4 has an especially high peak during the 4th round. Looking at the bout score, the participant was victorious but only by 1 touch.

What Can and Cannot be Concluded

This study can tentatively answer the question of whether there is an optimal range of cortisol for peak performance among athletes. The hypothesis that there would be an optimal range of cortisol for peak performance among athletes was not supported. There was not a single trend among all participants showing the hypothesized inverted U where all have the same range of cortisol levels for peak performance. Previous studies have hypothesized an inverted "U" to describe the rise is eustress peaking for an optimal stress then decreasing in distress. In an attempt to recreate the inverted "U" figures 15-20 show the participants cortisol level at each round. The mean concentration of cortisol was subtracted from each individual cortisol level at each round. If the inverted "U" was suggested we would see a higher probability of losing when the cortisol levels are further from the mean.

This study further supports the claims in "Testosterone, cortisol, and human competition" by Kathleen V. Casto, &, David A. Edwards, saying that cortisol is the hormone that peaks after a loss. The study by Cast and Edwards also suggest that testosterone is the dominating hormone peaking after a victory.

Chapter 3. From Lab to the Everyday World – Biology to Society

Future Directions

For future studies, testosterone would be a way to further this study and dig deeper into the roles hormones play in athletes in relation to stress and performance. Fencing is a sport that has a potential to disproportionally have more victories than losses for elite athletes during a tournament. Being able to study both the "winning" hormone and the "losing" hormone would give better insight. Furthermore, being able to speak with each individual athlete before and after competing in order to understand how they perceive stress would add another layer to this study.

It does seem that the appraisal theory which states "the cognitive, emotional, and physiological effects of stress are determined not by the stress itself, but by ones perception of the stress as a threat, in which the demands of the situation exceed ones resources to cope, or as a challenge, in which resources exceed demands" (Lazarus & Folkman, 1984) could be playing a role as to why each individual has different levels of cortisol in relation to their performance.

In conclusion, this study supports the findings that cortisol peaks after a loss, and could point to optimal cortisol levels being more of an individualized range for each athlete. If these athletes can explicitly see just how their hormones rise and fall, then perhaps being more aware of these levels and being able to embrace them could lead to peak performance.

Practical Applications

The United States Olympic Committee has sport psychologists that travel with the athletes to most international tournaments. If these physicians are able to analyze how the athletes stress hormones vary then they would be better equipped in their mentoring sessions with the athletes. Recently, the U.S. women's epee team won a grant from the USOC. This grant awarded the team the opportunity to work with specialized sports phycologist to travel with the team to various tournaments and study the athlete's individual dynamic as well as the team dynamic. The information into the physiological response of the individual athlete has the potential to be successfully paired with how they approach stress, this could lead to not only a more successful athlete but a stronger Team USA as they prepare for the 2020 Tokyo Olympics.

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Appendix A

Salimetrics Protocol

Prepare the Reagents

- All reagents must be at room temperature before use.
- Microtitre Plate must be at room temperature before use.
- Prepare 1X buffer by diluting Wash Buffer Concentrate (10X) 10 fold with room temperature deionized water.

Procedure

Step 1 - Prepare the reagents before beginning assay.

Step 2 - Pipette 24mL of Assay Diluent into disposable tube.

Step 3

- Pipette 25 microliters of standards, controls, and saliva samples into appropriate wells
- Pipette 25 microliters of Assay Diluent into 2 wells to serve as the zero
- Pipette 25 microliters of Assay Diluent into each NSB well

Step 4 – Dilute the Enzyme Conjugate 1:1600 by adding 15 microliters of the conjugate to the 24 mL tube of Assay Diluent.

Step 5 – Mix plate on a plate rotator for 5 minutes at 500 rpm and incubate at room temperature for a total of 1 hour.

Step 6 – Wash the plate 4 times with 1X wash buffer.

Step 7 – Add 200 microliters of TMB Substrate Solution to each well with a multichannel pipette.

Step 8 – Mix on a plate rotator for 5 minutes at 500 rpm and incubate the plate in the dark (covered) at room temperature for an additional 25 minutes

Step 9 – Add 50 microliters of Stop Solution with a multichannel pipette

Step 10

- Mix on a plate reader for 3 minutes at 500 rpm. If green color remains, continue mixing until green color turns yellow.
- Wipe off bottom of plate with water-moistened, lint free cloth and wipe dry.
- Read in a plate reader at 450 nm. Read plate within 10 minutes of adding Stop Solution.

Quality Control

The Salimetrics' High and Low Cortisols Controls should be run with each assay. The control ranges established at Salimetrics are to be used as a guide.

Calculations

1. Compute the optical density (OD) for all duplicate wells.

2. Calculate the percent bound (B/Bo) for each standard, control, and saliva sample by dividing the OD of each well (B) by the average OD for the zero (Bo).

3. Determine the concentrations of the controls and saliva samples by interpolation using data reduction software.

4. Samples with Cortisol values greater than 3.0 micrograms/dL should be diluted with Assay Diluent and rerun for accurate results. If a dilution of the samples is used, Multiple the assay by the dilution factor.

Appendix B

Recruitment Email

Dear [Athlete's Name],

My name is Jerica Vie, fellow fencer and member of the USFA. I am conducting a study through Arizona State University, evaluating stress and how it relates to competition performance. The levels of stress can be measured through your cortisol hormone levels and this can be collected using a saliva sample. In my study, I would like to find volunteers who would be willing to provide saliva samples before, during and after a fencing competition. These samples will be processed directly by the lab I work in [Baluch Lab] at ASU and the results will be analyzed to determine the correlation between stress cortisol levels and competition performance. No names will be associated with the data through the analysis or published results and the samples are only analyzed for cortisol levels and then disposed. Once the results have been published you will be provided a copy of the story.

If you are interested in participating in this study I can send you additional information including a consent form, pre-event survey and there will be a post-event survey following the competition. Participation in this study is voluntary. Your participation will help us better understand how different levels of stress can either help or hurt athletic performance and may provide insight to improve training strategies.

I look forward to hearing from you.

Best Regards,

JericaVie
Arizona State University

Graduate Student School of Life Sciences/Biology and Society

Email: jnvie@asu.edu

Appendix C

Consent Form

CONSENT FORM: Phase II Study

Saliva Sample Collection and Cortisol Level Analysis

Arizona State University

You are being asked to participate in a research study. Participation in this study is voluntary. This form provides you with information about the study. The Principal Investigator (person who oversees this project) or her Graduate Student (person who will be collecting and analyzing the samples) is available to describe this study to you and answer all of your questions. Please read the information below and contact us with any questions you may have before deciding whether or not to take part of this study. Your participation is completely voluntary and you can refuse to participate without penalty or loss of any benefits to which may be entitled.

Title of Research Study: **Stress levels measured through salivary cortisol assays and its correlation with the tournament performance of United States nationally ranked fencers**

INTRODUCTION

In this study, participants will be recruited from the United States Fencing Association to measure stress levels at scheduled times during a tournament using salivary cortisol collection vials. Through this study, we aim to quantitatively determine the stress levels exhibited by professional fencing athletes as they prepare for and complete at qualifying Olympic competitions. From this data, the information obtained may demonstrate differences between male and female athletes and how they manage stress and may show time points of more extreme stress that could be modified such that it improves the performance of the athlete.

RESEARCHERS

Principal Investigator D. Page Baluch, PhD Research Scientist School of Life Sciences Arizona State University page.baluch@asu.edu

Graduate Student [Study coordinator]

JericaVie
Master's Student School of Life Sciences Arizona State University jnvie@asu.edu

Study Purpose

The purpose of this study is determine is cortisol levels, measured through salivary testing, is an indicator of increased stress during fencing competitions.

DESCRIPTION OF RESEARCH STUDY

Participants in this study can be male or female and are selected based on their ranking in the USA national rolling points list, if they compete in Epee style fencing, if they have been competing for a minimum of 5 years and are between the ages of 21-35 years old.

Selected participants will be asked to provide a saliva sample right after check-in, prior to the 1st match and then 10 min after a match. The participant will continue to provide a salivary sample 13-15 minutes after each consecutive match that he or she participates in until the end of the bout or elimination. During any breaks in which food must be consumed, a saliva sample cannot be collected until 60 minutes after consumption. If this conflicts with a sample collection, then that particular sample will be omitted. If a sports or energy drink is consumed, the participant must rinse their mouth with water 5 minutes prior to providing a sample.

Volunteers admitted into the study will be asked to complete a pre and post survey to be administered by the study coordinator.

The goal of this study is to identify if saliva based cortisol levels can be used as a stress indicator in the sport of professional fencing. The ability to successfully measure stress will enable trainers and athletes to improve or modify training to better prepare for competition. Data collected from this study will remain anonymous and no identifying information associated with the participants will be released. The results will be shared with the USFA and published in sports journals and popular science publications.

RISKS

There are no known risks from taking part in this study, but in any research, there is some possibility that you may be subject to risks that have not yet been identified.

BENEFITS

The benefits of your participation in this study are to improve the sport of fencing performance by improving stress management during training and competition. If requested, you may receive a copy of your saliva cortisol test results but acknowledge that these do not serve as a diagnosis of any kind for any medical condition and that only your physician can interpret these results.

CONFIDENTIALITY

All information obtained in this study is strictly confidential. The results of this study may be used in reports, presentations and publications, but all participants will remain anonymous. In order to maintain the confidentiality of your records, all documents associated with the study will remain with primary investigator and filed in a secure

location upon completion of the study. Only the participant ID number will appear on the samples and questionnaires.

VOLUNTARY CONSENT

Any questions you have concerning the research study or your participation in the study, before or after your consent, will be answered by Dr. Page Baluch/PI [page.baluch@asu.edu] or Ms. Jerica Vie [jnvie@asu.edu].

If you have questions about your rights as a subject/participant in this research, or if you feel you have been placed at risk; you can contact the Chair of the Human Subjects Institutional Review Board, through the ASU Office of Research Integrity and Assurance, at 480-965 6788.

This form explains the nature, demands, benefits and any risk of the project. By signing this form, you agree knowingly to assume any risks involved. Remember, your participation is voluntary. You may choose not to participate by using the enhanced materials without penalty or loss of benefit. In signing this consent form, you are not waiving any legal claims, rights, or remedies. If requested, a copy of this consent form can be provided. **Your signature below indicates that you consent to participate in this study.**

Subject's Signature Printed Name Date

INVESTIGATOR'S STATEMENT

"I certify that I have explained to the above individual the nature and purpose, the potential benefits and possible risks associated with participation in this research study, have answered any questions that have been raised, and have witnessed the above signature. These elements of Informed Consent conform to the Assurance given by Arizona State University to the Office for Human Research Protections to protect the rights of human subjects. I have provided (offered) the subject/participant a copy of this signed consent document."

Signature of Investigator_____
Date_____