

# ASSESSMENT OF PERIPHERAL BDNF LEVELS OVER 30 DAYS

By

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## **Abstract**

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Brain health, and the benefits of exercise have been linked to the biological signaling molecule called brain-derived neurotrophic factor (BDNF). Animal and human studies have provided some support for physical exercise as a mechanism for increasing BDNF levels. However, results have been inconsistent, which may be attributed in part to incomplete information about normal variation in circulating peripheral BDNF levels. This investigation examined capillary-drawn whole blood samples from nine healthy adult participants over 30 days with the goal of documenting variability in resting BDNF levels and changes that may be attributed to physical exercise. It was hypothesized that BDNF concentrations would stay relatively consistent (overall coefficient of variance not exceeding 15%) and that physical exercise within 12 hours of blood sampling would increase BDNF levels. In contrast to these expectations, the current study showed high within-subject variability in resting BDNF levels across 30 days, and no association between recent physical exercise and BDNF levels. However, having a variability quantification is equally important for future methodology designs. While it remains unclear if there are valid cognitive benefits link to BDNF, understanding human BDNF

variability can be of general utility as a benchmark for designing and interpreting future BDNF-related studies.

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## Introduction

Neurotrophic factors are critical in brain development but also continue to function in the mature nervous system to promote growth of new neurons (adult neurogenesis; Gage, 2000) and maintain neuronal health and plasticity (as reviewed in Binder & Scharfman, 2004). There are likely to be multiple biological mechanisms in the periphery and the brain that mediate these beneficial effects, but one of the most researched protein associated with brain health and aging is brain derived neurotropic factor (BDNF). Many lines of evidence link BDNF concentration levels to brain and cognitive health. Aging and neurological disease are both associated with reductions of BDNF, while physical exercise and other methods have been used to increase BDNF levels and provide some protection against those stresses (O'Leary, Berchtold, Isackson, & Cotman, 1998).

Disruptions in BDNF signaling have been linked to deadly effects on neurons, including cell deterioration, impaired cellular metabolism and apoptosis (Miller & Kaplan, 1998). Genetic variations such as the BDNF polymorphism gene referred to as the Val66Met results in lower BDNF concentration levels and is associated with smaller brain size and decreased activity-induced neurogenesis (Herting, Keenan, & Nagel, 2016). A study done by Hopkins, Davis, Vantighem, Whalen, and Brucci (2012) showed that having the homozygous polymorphism can impede the benefits of acute exercise sessions. The studies cited above, and many other published studies, indicate the

importance of BDNF proteins for maintaining brain and behavioral health and suggest that physical exercise may promote health through a BDNF-dependent mechanism.

The effects of BDNF homeostasis on brain and behavior have also been studied broadly in humans but with more variable results, which is not surprising considering the many sources of uncontrolled variability in human subject samples. A major obstacle to researching BDNF in humans is that there is no way to precisely measure BDNF levels in brain tissue noninvasively. Most human studies measure peripheral BDNF levels using saliva, serum, plasma, platelets and whole blood (as reviewed in Fernandes et al., 2015; Ferris, Williams, & Shen, 2007; Hötting, Schickert, Kaiser, Röder, & Schmidt-Kassow, 2016; Kim et al., 2005; Klein et al., 2011; Komulainen et al., 2008; Lommatzsch et al., 2005). Research supports peripheral blood BDNF appears to be a good indicator of brain BDNF levels (Mandel, Ozdener, & Utermohlen, 2009; Marais, Stein, & Willie, 2009). In psychopharmacology, there is an understanding that suggests that if it is present in the platelets, then it's almost certainly concurring in the brain, too. Studies by Thompson, Weickert, Wyatt, and Webster (2011) found evidence of similar BDNF concentration levels in brain and periphery of depressed patients. Further evidence suggests peripheral BDNF modulates subpopulations of neuronal function (Lommatzsch, Bruan, & Renz, 2003). These studies suggest that we could use the peripheral as a "window to the brain" to study BDNF concentration levels.

However, there is still inconsistency when assessing BDNF levels through peripheral blood. Average BDNF found in serum levels are 100-fold higher when compared to plasma levels. A possible explanation is that platelet degranulation occurs

the clotting process (Fujimura et al., 2002). Other studies such as one done by Fujimura et al. found evidence that suggests BDNF levels in serum is proportional to BDNF levels in lysed platelets. This may be because a primary source of peripheral BDNF comes from platelets (Karege, Bondolfi, Gervasoni, Schwald, Aubry, & Bertschy, 2005), studies support BDNF levels in whole blood and plasma correlate with BDNF levels in hippocampus; therefore, this shows the potential of peripheral measures of BDNF as a biomarker tool (Klein et. al., 2011).

Several animal BDNF studies with different types of peripheral blood do corroborate higher BDNF levels post recent physical exercise (Avdoshina et al., 2013; Koo et al., 2013; Lee et al., 2009), but few speak of normal resting BDNF concentration levels. In human participants, it is recommended to study BDNF through serum or whole blood as plasma BDNF requires a more stringent sample handling (Elfving, Ploughmann, & Wegener, 2010). This study aimed to evaluate the variability of BDNF levels under normal life conditions over 30 days through capillary blood. This longer study design generated new information about the variability of BDNF concentrations, which is a longer span of time than has been measured previously, and also shed some light on how reliable capillary-whole blood BDNF measurements are.

## Literature Review

BDNF is one of the most abundant neurotrophic proteins produced in the mammalian and animal central nervous system (CNS) that is highly implicated in the neural plasticity, maintaining cell structure and promoting new neuronal cell growth. They are found largely in the hippocampus followed by the cerebrum and other brain areas. Like other neurotrophins, BDNF is firstly synthesized as a precursor, proBDNF, which splits to generate the mature BDNF. Reichardt (2006) provided an overview for the two ways BDNF exerts its effects: the tyrosine kinase receptor (TrKB) and pan-neurotrophin receptor p75 (p75NTR). The role of Trk receptors regulate axonal strength and integrity as well as plasticity. TrK activation has different outcomes with downstream cascades of ion channel activations, cell signaling, and mechanism specific signaling with p75NTR being one of them (Huang & Reichardt, 2003).

The discovery of the TrK receptors led to an upsurge of neurotrophic research and its various signaling pathways (Huang et al., 2003). While it is still being debated if BDNF crosses the blood brain barrier (BBB) and circulates in the peripheral nervous system (PNS), a study done by Pan, Banks, Fasold, Bluth, and Kastin (1999) concluded that the peripheral BDNF in adult mice crosses the BBB via an efficient and saturable transport system. Additionally, there is evidence of peripherally secreted BDNF from non-neuronal peripheral cells, vascular smooth muscle cells, endothelial cells, lymphocytes, and monocytes (Donovan et al, 1995). Whether BDNF crosses the BBB or

is secreted peripherally, it supports that BDNF levels in the PNS correlates with the BDNF levels in the CNS and brain diseases.

Maintaining BDNF homeostasis is imperative to maximal cell functioning. For example, an imbalance in BDNF signaling can lead to abnormal cell development patterns during critical visual cortex development and dendritic morphology (McAllister et al., 1999). Low levels of BDNF signaling in non-dementia ageing women have been correlated to cognitive impairments (Komulainen et al., 2008). Such correlations were also seen in lower BDNF levels in depressed women when compared to depressed men (Karege et al., 2002), Alzheimer's disease, (Yasutake, Kuroda, Yanagawa, Okamura, & Yoneda, 2006), and multiple sclerosis (Castellano, Patel, & White, 2008). Low BDNF levels have also correlated with bipolar disorders on the mania spectrum but are much lower during episodes on the depression spectrum (as reviewed in Fernandes et al., 2015). These studies support the importance of maintaining BDNF homeostasis, but unfortunately, direct connections between BDNF and cognitive function still need more research.

There are external factors that could also influence BDNF signaling such as environmental, seasonal changes and the amount of sunlight. A longitudinal, between-subjects study assessed serum BDNF levels of Netherland residents from August 2004 to March 2007. They found that on average, serum BDNF levels were lower from winter to spring and highest from summer to winter (Molendijk et al., 2012). BDNF levels were highest during the season with the most sunlight in summer and were lowest in winter when there was the least amount of sunlight; sunlight within any given week varied from

2 hours to 131 hours per week. They did a Post-hoc correlation of BDNF levels and found lower levels associated with higher body mass index (BMI) with the higher BMIs during the winter months (Molendijk et al., 2012). A possible explanation may be that since there is less sunlight during the winter, that may be associated with less physical exercise due to more time spent indoors due to winter conditions.

Daily circadian routines may have an impact on BDNF levels. Diurnal BDNF secretion patterns have been linked to circadian rhythm of cortisol and hormones. In a study with 34 healthy, young male participants, there were higher BDNF and cortisol levels correlated with the morning samples and the BDNF concentrations decrease throughout the day, showing lowest trends at night (Begliuomini et al., 2008) but other studies showed BDNF level differences between genders with higher BDNF levels in females (Choi, Bhang, & Ahn, 2011). In respect to hormones, oral contraception can pose as a mediator of BDNF signaling. A study conducted to assess how a woman's menstrual cycle can affect BDNF signaling found that BDNF levels were highest during the follicular phase and had a significant increase in BDNF during the luteal phase (Pluchino et al., 2009). However, further research is necessary to address hormones related to BDNF fluctuations.

### **BDNF and Physical Exercise**

A growing body of literature is investigating mechanisms, such as physical exercise, in order to maintain a wholesome and healthy lifestyle. Physical exercise is essential for survival and has been engrained by evolution into many aspects of animal

and mammalian biology. On a popular account, humans for example, were “born to run” (Mattson, 2012). However, there is no consensus on the effect of physical exercise intensity and duration i.e., differences between acute and regular physical exercise and its effects on BDNF levels. Because of this, we still rely heavily on animal models to explain this relationship (Cotman & Berchtold, 2002; van Praag, Shubert, Zhao, & Gage, 2005). For example, running has been shown to immediately impact rodent neurogenesis and this trend is consistent with *regular* running-type activities (Kerr & Swain, 2011). Regular exercise can increase baseline BDNF levels within the hippocampus, striatum, and other cortical regions in laboratory animals (Ding et al., 2004; Neeper, Gómez-Pinilla, Choi, & Cotman, 1996; Rasmussen et al., 2009). This speaks to the biological need of maintaining a physically active lifestyle which may indirectly benefit other factors such as mental or physiological health.

**Physical exercise duration.** Literature has increasingly supported the positive effects of physical exercise on physical and mental health and longevity (Campos et al., 2016). However, activity induced-BDNF signaling has been shown to be dependent on duration of exercise and intensity (Ferris et al., 2007). Low intensity and shorter durations may not significantly increase BDNF levels and extremely intense or long periods of exercising can cause detrimental effects. Aerobic, resistance, endurance and high intensity interval training (HiIT) all affect BDNF concentration levels in different ways. Most studies report seeing effects after 30-minutes (reviewed in Roig, Norbrandt, Geertseen, & Nielsen, 2013; Ferris, et al., 2007). Single sessions of acute 30-minute exercises (short-term) of cycling at 60% VO<sub>2</sub> max increased serum BDNF concentrations



in healthy participants and multiple sclerosis patients (Gold et al., 2003). Long-term, regular exercise has positive effects as well. Regular exercise facilitates long term potentiation (LTP) which is repeated strengthening of synapses from its repeated usage (van Praag, Christie, Sejnowski, & Gage 1999). Other benefits include physiological changes such as hippocampal volume increases. For example, after one year of physical exercise, hippocampal volume increased by 2% in aerobic exercising group when compared to the stretching control group (Erickson et al., 2011). Typically, hippocampal volume decreases by 1-2 % per year. This increase can equate to 1-2 years' worth of hippocampal volume. Therefore, we speculate that acute bouts of exercise could be involved in jumpstarting or keeping this process going. Hopkins et al. (2012) demonstrated that even short bouts of exercise can result in increased cognitive benefits for individuals who are regular exercisers i.e., the cognitive benefits are dependent on physical exercise history. This suggests the need for some type of physical exercise routine.

**Voluntary versus forced physical exercise.** Self-selected routines have been shown to be more beneficial in increasing BDNF signaling. Rodent models show that voluntary, aerobic exercise (running wheel) increases hippocampal activity, growth and BDNF circulation when compared to other forms of exercise and the control (Cotman & Berchtold, 2002). But, having genetic predispositions or gene polymorphisms can also influence BDNF signaling, brain structure and morphology even with participation in physical exercise regimens. The BDNF gene polymorphism, Val66met, (Martinez-Levy & Cruz-Fuentes, 2014) and the high affinity BDNF receptor, TrkB, are a correlate with

deviations in synaptic physiology and disease risk (Avdoshina et al., 2013). This provides evidence for BDNF signaling in brain homeostasis.

Other mediators include selective-phenotypic breeding. Nokia et al. (2016) conducted a study using a contrasting rat model system. Adult male rats were selectively bred up to the 17<sup>th</sup> generation to either have an innate high response trainer (HRT) or low response trainer (LRT) to aerobic exercise. They were subjected to various exercise activities: voluntary running wheel, forced endurance, forced resistance and a sedentary group. The rats who were bred to have a HRT showed a greater adaptive ability to run farther distances, carry more weight, and exhibited higher BDNF levels when compared to controls and other forms of physical exercise. They also had more doublecortin-positive cell growth and more adult hippocampal neurogenesis (AHN). What's more intriguing is that the HRT rats in the voluntary running wheel group displayed the maximum running speed increase, increase in doublecortin-positive cells, and most increase in AHN (Nokia et al., 2016). Conversely, in another study rats who were subjected to low-intensity, forced aerobic exercise had increased BDNF expression while moderate-intensity, forced aerobic exercise suppressed it (Soya et al., 2007). Further investigation is needed to support how genetic predispositions and physical exercise routines can affect BDNF concentration levels.

Some additional factors to consider are inadequate training or overtraining, which can show negative effects to the health, too. Inactivity has been related to increase health mortality rates. While physical exercise has been linked to higher BDNF concentration levels and better memory, short durations of 20 minutes or less did not show any effect

on cognitive performance (Brisswalter, Collardeau, & Rene, 2002; Lambourne & Tomporowski, 2010). In particular, when assessing memory, it is recommended that physical exercise is performed at low intensities for 20 minutes or longer (reviewed in Roig et al., 2013). However, it is important to consider that after 60 minutes of exercise may cause exhaustion and dehydration (Tomporowski, 2003).

**Intensity preference-based approaches.** Preference-based approaches have been gaining attention due to its beneficial practices. As seen in rodents, voluntary, self-paced exercises with minimal outside stress induces positive results (Nokia et al., 2016). Self-selected exercise intensities increase the runner's willingness to adhere to an exercise program (Ekkekakis, 2009; Parfitt, Blisset, Rose, & Eston, 2009). Low to moderate intensity programs also can increase working memory (Martins, Kavussanu, Willoughby, & Ring, 2012). This may be attributed to how stress or continually elevated cortisol levels can hinder one's physical activity performance (Keinan, Friedland, Kahneman, & Roth, 1999). Increases in stress and cortisol release could in turn lead to more erroneous or unwanted responses. In a study with active females who were subjected to exercise with the intention to keep the exercise intensity to *feel* "fairly good" or "good", the "fairly good" group did significantly better in the "good" group.; this supports the notion of using affect to integrate into exercise regimens (Keinan et al., 1999; Williams et al, 2016). When conducted with sedentary participants' willingness to participant in exercise 6 to 12 months, participants were more likely to do so after having a positive affect to a small bout (30-minutes) of moderate-intensity exercise (Williams et al., 2008). This

provides evidence in the willingness to apply physical activity research in sensible, preference-based exercise routines.

### **BDNF, Genetics and Mental Health**

Lower BDNF concentration levels have been correlated in neurodegenerative disorders and diseases. It has been supported that lower BDNF mRNA expression has been linked to depression (Altar, 1999). A meta-analysis found a significant association between the BDNF Val66Met polymorphism and panic disorder (as reviewed in Chen, et al., 2015). Although there are limitations in the research, this supports that having this polymorphism gene poses the predisposition to panic disorder. Other studies have investigated the neurotrophic hypothesis of depression (Dunman & Monteggia, 2006) and found consistent evidence that having decreased levels of BDNF can lead to neuronal atrophy and the use of antidepressants may help to reverse this. However, typical short-term antidepressant treatment doesn't increase BDNF levels. It's important to note that in animal models, the deletion of the BDNF gene don't enable depressive-like states. This may be the cause of precise, targeted location deletion of BDNF in certain brain regions.

In inflammatory disease research with rodents, there is an increase in BDNF concentration levels. A possible theory is that the upsurge in circulating BDNF is due to BDNF's function in protecting neuronal structures from further damage (Ha, Kim, Hong, Kim, & Cho, 2001; Mannion et al., 1999). The extent of the relationship between peripheral BDNF and diseases and mood disorders is not fully understood. Therefore, the

need for a better understanding of BDNF variability and the possible therapeutic effects underscores its role for further research.

### **Statement of the Problem**

The primary goal of this study was to evaluate BDNF concentration levels over the course of 30 days using capillary whole blood samples obtained through finger pricks. This was important for two reasons: First, resting BDNF levels under normal life conditions has never been measured over a period as long as 30 days (to my best knowledge of the published research), so this study provided new information about normal variability in BDNF levels. A better estimate of normal variability would be useful in designing studies aimed at examining methods to alter BDNF levels, such as physical exercise, as variability is a factor in determining statistical power and adequate sample sizes. The longest duration of BDNF measurement currently reported in the literature is three days (Dinoff et al., 2016; D'Sa et al., 2012; Szuhany, Bugatti, & Otto, 2015). Second, the most commonly used method for measuring peripheral BDNF is to use blood from veins (venipuncture). This current study's method of sample retrieval was from finger-pricks, which would be a more efficient and versatile method for future research (Dinoff et al., 2016; Szuhany et al., 2015). Capillary sampling has become increasingly common in medicine and research due to the process being less-invasive, quicker and less painful. Obtaining blood samples by finger-pricks rather than by venipuncture can be especially helpful to stay within blood sample volume limits (Howie, 2011) while still measuring variability.

While it has been long supported that BDNF levels acquired through serum is 100-folds higher when compared to BDNF concentrations in plasma, this difference may

be due to BDNF being stored in circulating platelets; the platelets act as storage centers for BDNF (Serra-Millás, 2016). Possibly, tissue trauma and nerve injury activates BDNF release from these circulating platelets (Radka, Holst, Fritsche, & Alter, 1996). These sources of BDNF may be a contributing factor to differences in BDNF quantification. Elfving et al. (2010) conducted a study with recommendations to study BDNF concentration levels through serum or whole blood since whole blood is more stable for longer sample storage studies. Different research designs with different methods could be used to determine if capillary-drawn whole blood BDNF levels are comparable to venous blood (plasma or serum) BDNF levels, such as obtaining venous and capillary blood samples at the same time over this longer study duration, but obtaining venous blood was considered not feasible for the current study. Therefore, I assessed for the variability of BDNF levels in the capillary whole blood samples under regular life circumstances over a course of 30 days while also logging their recent physical exercise activity.

## **Research Hypothesis and Rationale**

**Hypothesis 1.** BDNF concentrations will stay relatively consistent throughout the course of 30 days, with the overall coefficient of variance (CV) not exceeding 15%.

***Rationale for Hypothesis 1.*** Meta-analyses reveal high variability in measurements of resting peripheral BDNF levels and of exercise-induced BDNF increase, due both to methodological heterogeneity and to unknown inconsistencies (Dinoff et al., 2016). What this study hoped to elucidate is typical BDNF concentrations under normal life conditions over the course of 30 days. Statistically, having a lower

standard deviation doesn't always mean there is less variance. But a CV gives us a point of reference to assess how spread out the data is. A CV not exceeding 15% of the mean is proposed as an a priori criteria of stability over time. This criterion matches a level of variability considered typical at the population level for some quantitative traits such as intelligence.

**Hypothesis 2.** We expect to see that recent exercise will significantly increase BDNF concentration. Average BDNF concentration in samples taken more than 12 hours after exercise will be significantly lower than average BDNF concentration in samples taken within 12 hours of exercise.

*Rationale for Hypothesis 2.* Many previous studies, and meta-analyses, cited in the literature review above, have shown that voluntary aerobic exercise increases peripheral BDNF levels in humans and animals within minutes or hours, therefore, I expect to replicate this finding. Methods vary greatly across previous studies and the exercise and BDNF sampling methods proposed in this study are within the general parameters expected to produce effects based on previous studies in humans (Dinoff et al., 2016). This recent meta-analysis confirmed the association between extended aerobic (but not resistance) training and increased BDNF levels in blood in humans, but found no moderating effects of exercise intensity or duration, participant age or sex, or of the use of blood serum versus plasma, suggesting that the effect is robust and generalizes across various types of aerobic exercise, participant characteristics, and BDNF measurement methods. Berchtold, Castello, and Cotman (2010) did a study with mice that revealed that after an exercise session, BDNF levels remained elevated and returned to baseline 3-4



weeks after exercise ended. This finding was consistent with their previous finding using a rodent model (Berchtold, Chinn, Chou, Kesslak, & Cotman, 2005). Though there hasn't been a translatable study to assess human BDNF variability and how long BDNF levels may stay elevated post-exercise. We made the decision to cut off "recent exercise" at 12 hours based on our timeframe of collecting samples. Earliest sample collection began at 12:00 p.m.; therefore, by making the cut-off at 12 hours ago, we were able to operationalize and categorize the physical exercise to that *day* of sample collection.

## Method

### Participants

Participants were recruited through flyers posted around campus and class announcements at Humboldt State University (HSU). Due to limited resources prior to the start of data collection, we were only able to accept, at most, 10 participants. Participants were not offered monetary or school credit to participate. However, they were offered the option to participate as a co-investigator on the project. As a critical confidentiality precaution to those who took on co-investigator positions, they were only allowed to participate in certain aspects of data collection. All participant identifying information was numerically re-coded and the key was kept confidential with the principal investigator which permitted anonymous data entry and certain aspects of data analysis.

As a critical health safety precaution, participants were asked to affirm their self-rated willingness to participate and consent to understanding that the study is asking for repeated-sampling of up to 300  $\mu$ l of blood three times a week over a course of 30 days (refer to Appendix A). Participants were also separately required to reaffirm their health during each blood draw session, specifically, that they were fit to contribute blood samples, knew of no reason their blood may be dangerous to others, and knew of no reasons their own health may be harmed by the blood draw procedures (refer to Appendix B). Demographic questions included their age, weight, height and most recent sleep quality (refer to Appendix C). Other selection criteria included non-smoking status, as

cigarette smoking is known to influence BDNF levels (Collie et al., 2016; Zhang, et al., 2016). Women were asked about the first date of their most recent menstrual cycle since hormone cycles are known to influence BDNF levels. Since BDNF levels have been supported to be highest in the morning with lowest levels at night, these meetings were always in the afternoon between 12 p.m. and 5 p.m. to minimize confounding effects of high BDNF levels correlated to circadian rhythm (Begliuomini et al., 2008). Three participants were excluded due to meeting one or more of the exclusion criteria.

## **Procedures**

This study was conducted at Humboldt State University in Arcata, California from April 2017 to May 2017. This study and methods were approved by the Institutional Review Board of Humboldt State University (IRB # 16-164). Following the first meeting, the investigator(s) met with each participant three times a week up to four weeks total for repeated blood sampling.

Participants followed their normal routine as the focus of this study was on natural variability in BDNF levels. Although the participants were not instructed to participate in a specific exercise regimen, an exercise activity and intensity log was asked at each session. Specific times of blood collection was chosen by the participant within the constraints of the protocol and these sessions remained relatively consistent during each blood-sampling meeting. Meetings took place in BSS 122, where facilities for blood draws and sample processing were set up (see Appendix D for BDNF collection protocol).

**Blood draw.** All investigators took and received a certificate for an HSU Bloodborne Pathogen workshop prior to being trained to administer fingertip punctures. Co-investigators were trained by the PI to use the safety lancets (MediPurpose SLB250) for ‘finger-prick’ blood collection. Participants were instructed to wash their hands before each sample collection with lukewarm water and soap to decrease likelihood of infection and to increase blood flow to the injection site. Single-use hand heating pads were available afterwards to further promote blood flow to the finger. Participants always sat down with arm relaxed on an armrest or table at a slight  $-10^{\circ}$  to  $-30^{\circ}$  angle to assist with a comfortable blood flow direction. Investigators provided a short hand massage to promote blood flow to selected fingertip (which was always the middle or ring finger). The selected fingertip collection site was wiped with a single use 70% isopropyl alcohol pad prior to puncture and after sample collection. The spring-loaded safety lancet was positioned and activated to puncture skin just off center of the finger pad, following manufacturer’s instructions. The first drop was always wiped with a clean cotton pad to reduce contamination of initial tissue fluid or environmental debris. A minimum of 50  $\mu$ l up to 300  $\mu$ l of blood was collected into a single-use plastic ethylenediaminetetraacetic acid (EDTA) tube (EDTA, which maintains blood in a fluid state), and the tube was inverted gently five to 10 times before being placed on dry ice in an insulated ice chest. Band-Aids were applied to the puncture site after sample collection. Once participants were dismissed, samples were transported to the Humboldt State University Biology Core and stored at  $-80^{\circ}\text{C}$  prior to being analyzed.

**Sample preparation.** Within two weeks of freezing, samples were homogenized using a homogenization buffer made following manufacturer's instructions (refer to Appendix E for protocol) and returned to the -80 °C freezer. The manufacturer confirmed that homogenized blood samples could be re-stored in the -80 °C freezer until ready for plate analysis.

**BDNF sandwich ELISA.** This study started with two BDNF assay kits (two plates per kit, four plates total; CYT306, EMD Millipore) which were funded by a program for interdisciplinary research collaboration run by the Humboldt State University Dean of Research. Kits were stored in a 2-8°C until usage. This assay is a sandwich enzyme-linked immunosorbent assay (ELISA) where BDNF in the blood samples reacts with molecules in the wells to alter the fluid's optical density (light absorbance) in a BDNF concentration-dependent manner. The BDNF plates were read using the Humboldt State University Biology Core's microplate reader (SpectraMax i3 Plate Reader, Molecular Devices).

All plates were assayed per manufacturer's instructions. It's important to note that appropriate adjustments were made to the standard serial dilution series in order to conserve the remaining standard diluent solution for the remaining samples (refer to Table 1 for standards solution series). One hundred µl of Standards and samples were pipetted into each well, sealed and incubated at 2-8°C overnight. Using a multi-channel pipet, 250 µl of diluted Wash Buffer was pipetted into each well. The fluid was removed by inverting the plate over a sink and gently flicking the fluid out of the wells and followed by blotting the plate on clean paper towels. This process was repeated five to six

times in order to thoroughly wash the plate to reduce background noise. One hundred  $\mu\text{l}$  of diluted biotinylated mouse-anti-BDNF monoclonal antibody was added into each well. The plate was sealed and incubated at room temperature for 3 hours on a shaker and then the wash step was repeated. This was followed by 100  $\mu\text{l}$  of diluted streptavidin-horseradish peroxidase conjugate solution. The plate was sealed and incubated at room temperature for one hour on a shaker. The plate was washed again following wash instructions. TMB/E was warmed to room temperature and 100  $\mu\text{l}$  of the TMB/E was added into each well and the plate was incubated at room temperature for 15 minutes. The solution achieved a range of blue colors. To stop the reaction, 100  $\mu\text{l}$  of Stop solution provided in the kit was added into each well which caused the blue color to turn yellow. The plate was immediately read at 450 nm.

Table 1

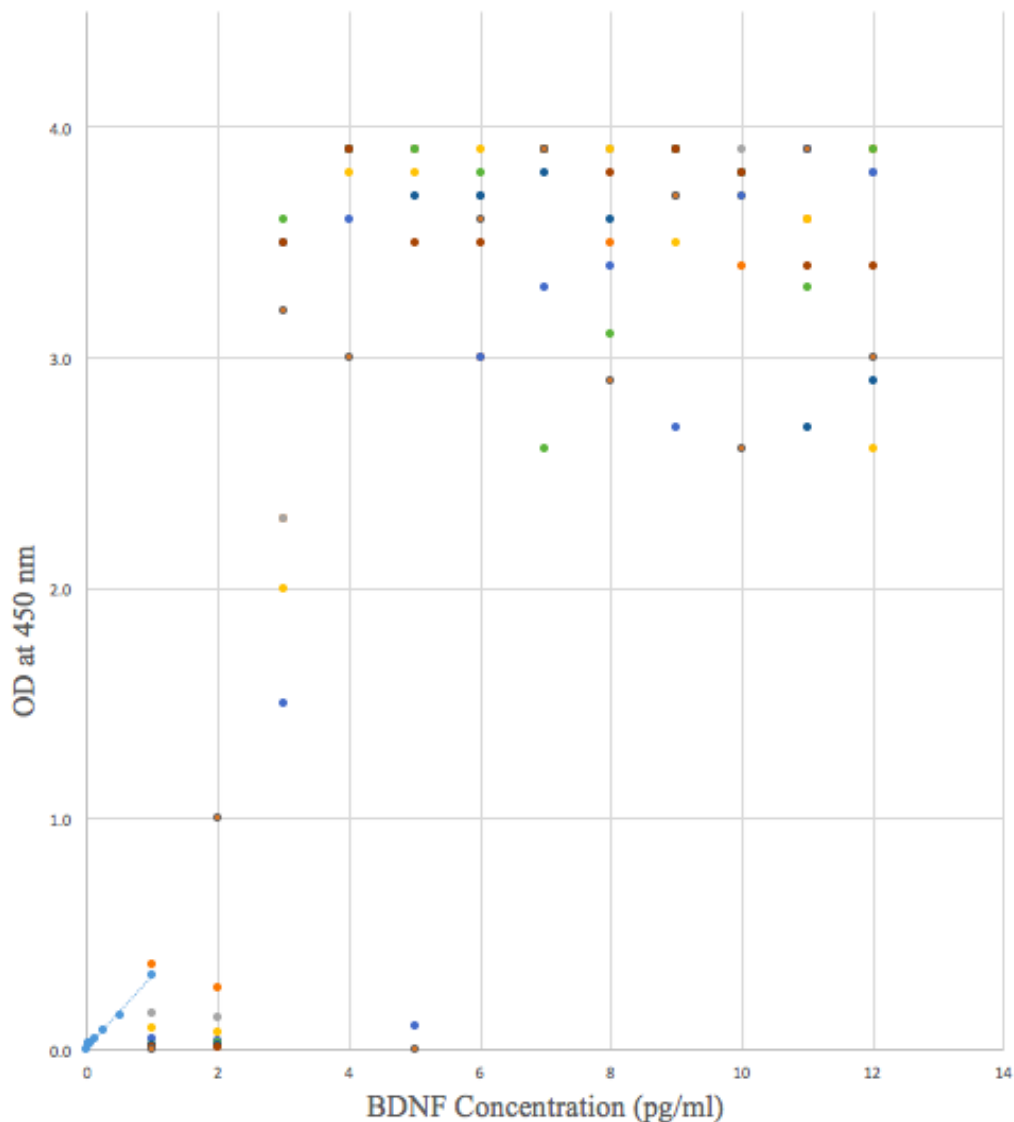
*Standards Dilution Series*

Standard Number	#1	#2	#3	#4	#5	#6	#7	0 Dose
Initial Volume ( $\mu\text{l}$ )	475	250	250	250	250	250	250	250
Concentration (pg/ml)	1000	500	250	125	62.5	31.25	15.63	0.0

*Note.* This study adjusted the Standard Curve volumes to allow for maximum preservation of the kit's standard diluent to dilute blood samples. Standard curve volumes were decreased in half from the default instructions and appropriate changes were made with the stock standard solution (25  $\mu\text{L}$ ) to provide the same strength (1000 pg/ml).

Due to insufficient literature on dilution procedures for capillary-drawn whole blood, we ran a preliminary plate analysis with the first plate to find a proper dilution series. Samples with larger volumes were chosen for the dilution series to provide preliminary data and still have enough volume left for subsequent analysis. Previous BDNF ELISA studies have diluted whole blood samples at 1:50, 1:200, and 1:500 (Elfving, Plougmann, and Wegener, 2009). But, for the purpose of this study, in order to find the lowest possible dilution and conserve the maximum amount of the kit's sample diluent solution, we utilized the first plate to obtain preliminary data on possible dilution ratios. Samples were analyzed at full concentrations to observe undiluted optical readings; other samples were diluted with the sample diluent at 1:1 and 1:3 dilutions (refer to Figure 1 for Plate One's data).



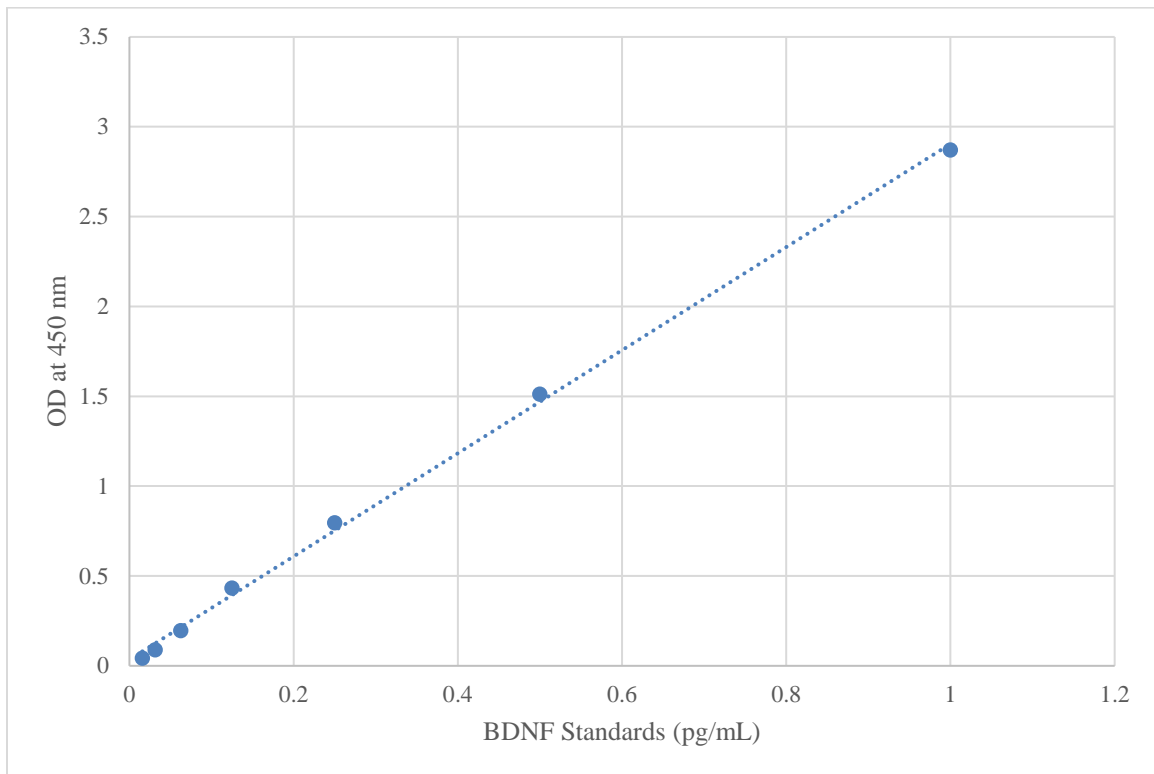


*Figure 1.* Plate One Assay. A representative chart displaying Plate One's standard curve (shown with a blue trend line on bottom left). The standard curve is an average from duplicated standards with a  $r^2 = 0.996$ . Optical density (OD) is the amount of light that is absorbed by the sample. All dots represent a data point. All data points above the 1.5 OD were samples at full concentration. All points below the 1.5 OD were samples either at 1:1 or 1:3 dilution.

Plate One's results produced absorbance readings outside the limit of detection (too high). We contacted Millipore, and Millipore agreed to send us a new replacement kit. While waiting for the new kit to arrive, we used the remaining plate from the first kit (Plate Two) to do a second dilution series including a 1:25 and 1:50 dilution (e.g., one individual sample was plated in duplicates at the 1:25 and the 1:50 dilutions in order to compare dilution readings.) As a manipulation check, we also analyzed possible background noise by plating sample diluent from four clean EDTA tubes. Absorbance readings confirmed that 1:50 dilutions fit within the standard curves the best.

For Plate Three, homogenized samples were plated as follows: samples were removed from  $-80^{\circ}\text{C}$ , thawed on ice, then centrifuged at 3000 x grpm for 1 minute. Two microliters of the sample were pipetted into an Eppendorf tube with 147  $\mu\text{l}$  of sample diluent. Diluted sample was vortexed for a few seconds then returned to ice. One hundred  $\mu\text{l}$  of freshly diluted sample was added per well on a 96 well plate, with 16 wells reserved for the plate standards. It's worth mentioning that while all samples were tested in duplicate, the duplicates were tested on different plates. This decision served a dual purpose: 1) to maximize preliminary analysis output data and 2) to determine if certain samples needed to be analyzed for a triplicate run since the dilution series in this protocol experimented with a 1:50 sample dilution series. This aided in careful preliminary data review and provided invaluable insight for the next sample plating. For the purpose of this study, sample concentrations that fell below or above the limit of detection (highest and lowest point on each plate's standard curve) were not included.

After preliminary analysis of the data from Plate Three, we realized that an error was made, specifically, an old solution from the first kit was used during the plate-prepping phase. This may have resulted in the standards having very low BDNF concentration values. So, we called and received a second replacement kit from the manufacturer. In order to streamline the plate processing time, we diluted samples ahead of time, refroze them and thawed them to be used on Plate Four. But, with this protocol change we experienced yet again another poor plate reading with the concentrations being outside the limit of detection. After contacting the manufacture for a third time, they agreed to send out a new replacement kit. For the purposes of organization, this study ended up with eight total plates which were referred to as Plates One through Eight, labeled according to the order processed. This study went through four preliminary plate readings which produced unusable but insightful data for protocol adjustments on the remaining plates. Out of eight total plates, the final dataset came from samples that were assayed on plates Five, Six and Seven (refer to Appendix F for final BDNF assay protocol). Generally, each of those plate's standard curve included seven points with a correlation coefficient of  $r > .99$  (as shown in Figure 2).



*Figure 2.* Representative Standard Curve. A representative seven-point standard curve for Plate Seven's whole blood BDNF ELISA. This curve was obtained using duplicate BDNF standards in the Millipore Chemikine BDNF ELISA and produced a correlation coefficient of  $r = .999$ . Only data within each plate's standard curve can be quantified and used.

**Data analysis.** Statistical analyses were performed using R 3.4.3 software (R Core Team, 2017; see Appendix G for R script). A coefficient of variance analysis assessed the overall within-subject variability (standard deviation) in BDNF levels against the overall mean. In addition, a linear mixed-effects model analysis was performed to assess BDNF concentration levels between those who recently exercised within 12 hours and those who didn't. This analysis is robust to both skewness and kurtosis and handles multiple sources of variance, especially those from repeated-measures (which is not possible with a traditional ANOVA) which allows for the use of both continuous and categorical data as predictors (Baayen, Davidson & Bates, 2008). This analysis doesn't give a probability value to determine the statistical significance of the model in order to reject or fail to reject the null hypothesis. But, in order to obtain a significance value, we performed a likelihood ratio test that compared the fixed effects and random effects against a model with only the random effects to understand what variance that can be attributed to the random effect variable. Three data points were missing due to the troubleshooting phases with Plates One through Four, which depleted the samples entirely; therefore, the final data set from plates Five, Six and Seven were missing those three data points. Therefore, data imputation using predictive mean matching (PMM) was performed through the R package "mice" (van Buren, 2011). PMM helps to estimate a linear regression and uses data from a multivariate normal distribution. Values were generated for both missing and non-missing data and used to fill in the three missing values. In particular, for each of the missing values a predicted value is generated and is as close to a prediction of what the missing data may have been

based on the other data points. Typical cases have five imputation values with five iterations each. Three observations were missing from the initial analysis and a series of 10 iterations and 50 imputations were utilized to fill in the missing data points. The final analysis utilized the dataset with three imputed data points out of 108 total data points.

## Results

### Stability of BDNF Over Time

The final cohort of participants were healthy volunteers ( $N = 9$ ; 5 female) from Humboldt State University between the ages 18 and 39 years with a mean age of  $27.88 \pm 7.04$  years and a mean BMI of  $23.29 \text{ kg/m}^2$ . Samples were duplicated and assayed on separate plates (each of the sample duplicate was assayed on a separate plate and the average of those two concentration values were used). All samples were duplicated and assayed on Plate Five through Seven fell within each plate's standard curve; therefore, their sample averages were used in the final dataset. The overall coefficient of variance ( $CV = .19$ ) failed to meet our a priori stability over-time criterion (refer to Table 2).

Table 2

*Participant Average BDNF Measurements over 4 weeks, Mean, Standard Deviations and Coefficient of Variances*

ID	1	2	3	4	5	6	7	8	9	10	11	12	<i>M</i>	<i>SD</i>	<i>CV</i>
1	35.32	48.03	33.03	34.40	33.15	35.28	38.38	43.60	29.63	30.26	44.86	36.23	36.84	5.82	0.16
2	47.43	55.25	25.61	21.41	40.03	43.25	33.38	49.20	33.03	26.83	40.64	39.64	38.42	10.23	0.27
3	36.88	36.64	33.47	21.72	26.56	28.39	25.26	24.22	21.30	24.37	28.75	28.72	28.02	5.27	0.19
4	22.33	25.33	26.11	19.22	18.87	13.06	21.66	28.42	20.83	21.41	22.65	23.68	22.00	3.95	0.18
5	19.24	13.56	11.14	15.59	15.07	15.35	14.60	16.39	10.86	12.21	16.02	14.67	14.60	2.36	0.16
6	29.99	34.31	29.84	28.30	35.045	46.36	19.17	26.35	29.47	28.59	31.75	22.73	30.20	6.75	0.22
7	37.53	56.51	48.45	35.24	27.09	29.535	26.87	34.58	30.23	46.36	19.59	30.23	36.00	10.50	0.29
8	41.76	27.03	31.54	33.37	33.5	37.75	31.66	33.04	37.09	38.42	27.7	31.80	33.72	4.35	0.13
9	35.76	27.98	29.45	28.7	29.97	21.69	32.55	34.64	28.54	28.09	32.53	36	30.50	4.06	0.13

*Note.* *M* = Mean; *SD* = Standard Deviation; *CV* = Coefficient of Variance. All values including *M*, *SD* and *CV* are

measurements in pg/ml. The a priori *CV* was set at 15% as a method to assess stability over time. The *CV* is calculated as

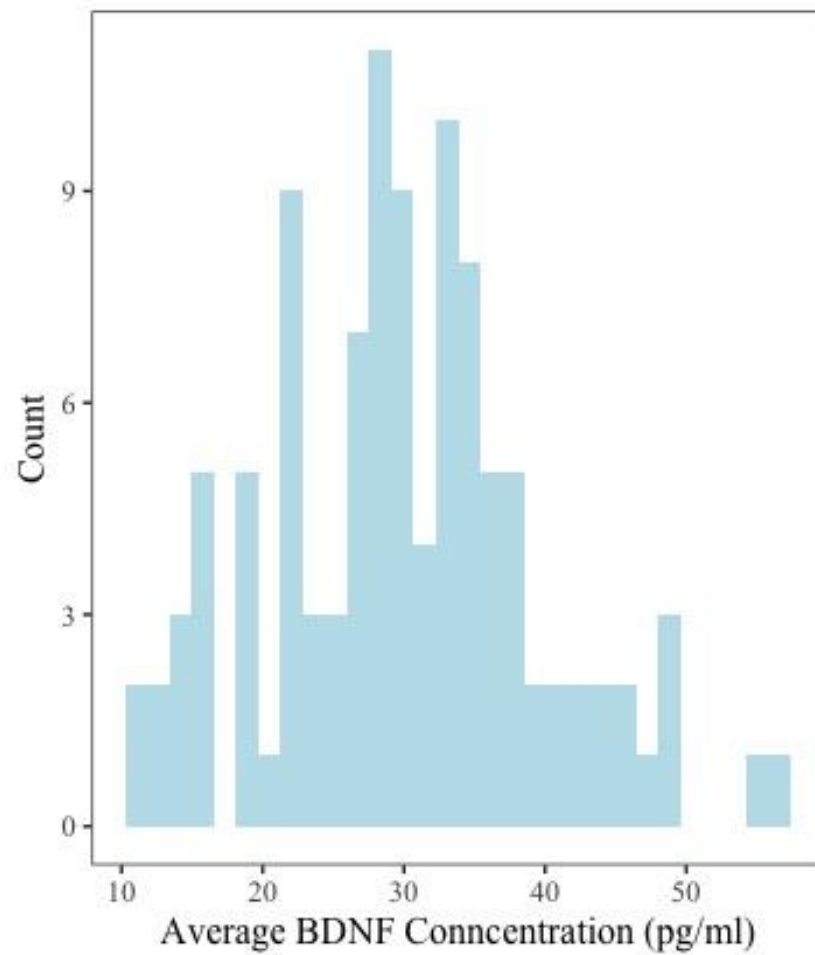
follows:  $SD/M$ . While some individuals did fall within this a priori *CV*, the current data indicates an overall  $CV = 19\%$ . The

overall was  $M = 29.80$  ( $SD = 5.92$ ).



### **Relationship of Individual Characteristics to BDNF Levels**

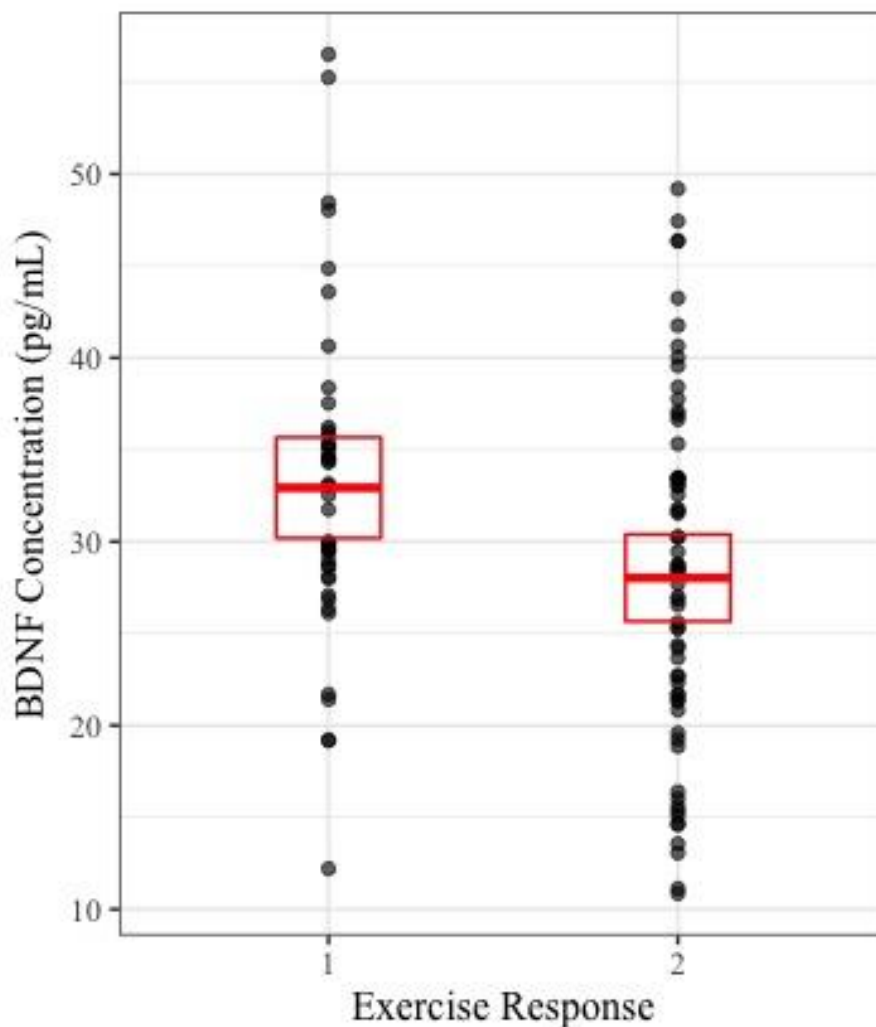
Initial data inspection of histogram plots did not reveal any obvious deviations from normality (as seen in Figure 3). But, further statistical analysis used to examine the relationship between BDNF levels and recent physical exercise utilized a linear mixed-model analysis.



*Figure 3.* Data Distribution. Duplicated BDNF blood samples ( $N = 105$ ) were assayed.

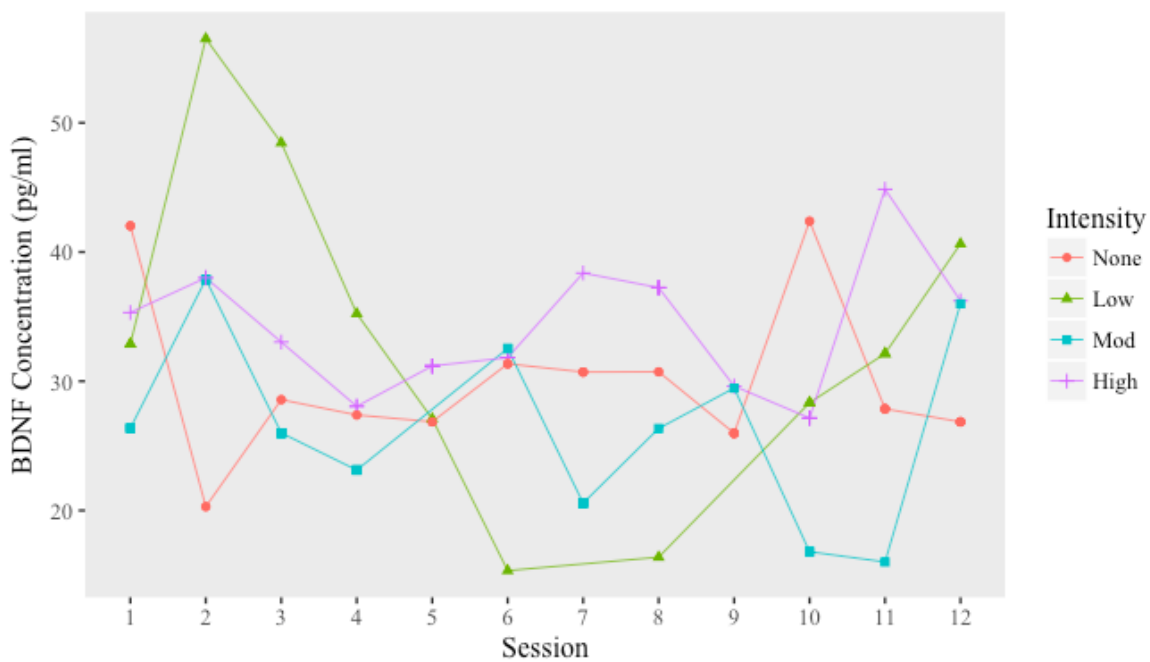
Data did not present any obvious deviations from normality. Averages of assayed duplicate samples were used in the final statistical analyses.

The R package *lme4* (Bates, Maechler & Bolker, 2012) was used to perform a linear mixed effects analysis of the relationship between repeated-measures BDNF levels and recent physical exercise. Recent exercise history was entered into the model as a fixed effect, and participants were entered as random effects. Fixed effects are factors that are constant and defined. Random effects are factors that are random samples of possible levels. In this case, the participants are random selections from the larger population. Probability values were obtained by likelihood ratio tests of the full model with ‘Exercise Response (Yes/No)’ against a model with just the participants to assess the variance with and without the fixed effect (refer to Appendix H for linear mixed-effect output). While samples taken after recent exercise had a higher median ( $Mdn = 32.14$ ) and wider range overall, the likelihood ratio test no significant differences in BDNF levels between the group of post-exercise samples and the samples not associated with recent exercise  $\chi^2(1, N = 9) = 2.28, p = .13$ . This analysis had an effect size of  $R^2 = 0.57$  (refer to Figure 4 for exercise response distribution).



*Figure 4.* Exercise Response and BDNF Levels. Each dot is a data point with darker dots represent overlapping data points. Exercise responses to recently exercising within 12 hours of sampling session: 1 = “yes” and 2 = “no.” Visual inspection displays a larger range of BDNF levels for those who reported “yes” to recently exercising ( $M = 32.75$ ,  $SD = 8.93$ ) when compared to those who reported “no” to not having recently exercised within 12 hours of the sampling session ( $M = 27.66$ ,  $SD = 9.40$ ). However, these results are not statistically significant.

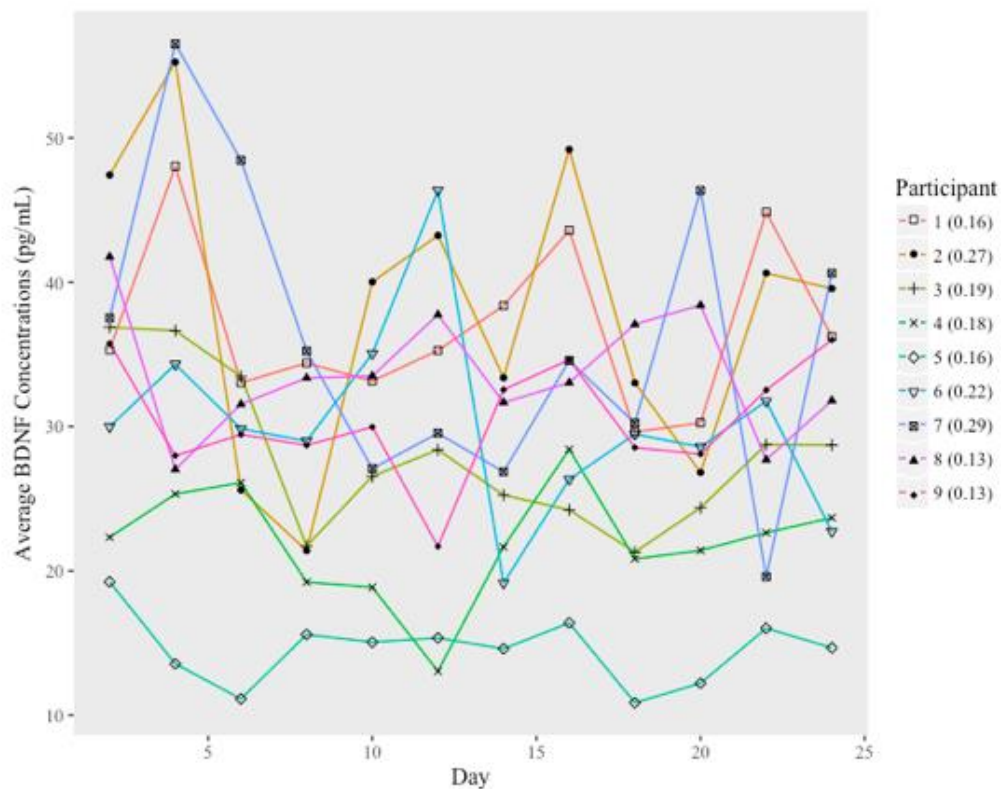
Targeted post-hoc analyses were performed to obtain a better understanding of BDNF concentration variability: self-reported exercise intensity, BMI, gender, female menstrual cycling. Participants reported exercise intensity level on a 4-point ordinal scale (none, low, moderate, high) at each sampling time. We grouped exercise intensity into these categories based on the literature that suggests that self-selected intensity preferences have been associated to higher BDNF levels (Keinan et al., 1999; Williams et al, 2016). Because we didn't measure exercise intensity levels, this was our way to assess the relationship between them. I analyzed the relationship of exercise intensity to BDNF concentration using a linear mixed effects analysis with exercise intensity as a fixed effect and participant as a random effect (refer to Appendix I for linear mixed-effects output). Probability values were obtained by likelihood ratio tests of the full model with exercise intensity in question against the model without exercise intensity. There was no significant difference between intensity types and BDNF levels  $\chi^2(3, N = 9) = 5.22, p = .15$ . This model had an effect size of  $R^2 = .57$  (refer to Figure 5 for a plot illustrating the fluctuating mean BDNF levels of each intensity response).



*Figure 5.* Exercise Intensity and BDNF Levels. Plot showing mean BDNF levels of each self-reported exercise intensity at each blood draw session over the 12 blood-draw sessions. Responses were based off a 4-point ordinal scale (none, low, moderate, high).

Correlation analysis showed that BMI was not correlated with BDNF concentration levels  $r(7) = .06, p = .50$ . BDNF levels were not significantly correlated with gender  $r(7) = 0.27, p = .18, d = 0.27$ . An ANOVA was done to analyze female BDNF concentration levels during Follicular phase (days 1-13) and Luteal phases (first stage: days 13-21 and second stage: days 22-29) of the menstrual cycles. BDNF levels were not significantly different during the Follicular and Luteal phases  $F(1, 4) = 0.16, p = .68, \text{partial } \eta^2 = .004$ .

Lastly, a post-hoc time-series analysis was done to obtain a better assessment of BDNF variability between participants (refer to Figure 4). Time-series analysis are used in exploratory analysis for evaluating and predicting future trends within a time frame.



*Figure 6.* Time Series Analysis. Graph depicts a time-series plot of BDNF concentration levels for each participant taken three times a week over 4 weeks. Each line represents a different individual with varying ranges of BDNF concentration levels (*Min* = 10.86 pg/ml, *Max* = 56.51pg/ml). Individual coefficient of variance displayed next to each participant key.



## Discussion

### BDNF Variability Over Time

In order to understand BDNF variability under naturalistic conditions, this study's primary objective was to track those levels over the course of 30 days. An a priori criterion was set for a stability baseline prior to investigating. This criterion was practical way to frame and set up a stability measure in order to evaluate BDNF variability. Our results show moderate BDNF variability within participants ( $CV = .19$ ) which did not meet our a priori criterion ( $CV = .15$ ) and there were high within-subject variability (refer to Appendix J for each participant's individual variability scores). However, having the mere variability quantification is equally important. Because variability influences the statistical power, understanding the naturalistic variability underscores its importance for future methodology designs. Having an estimate of variability is a useful benchmark for assessing the statistical power of future studies on BDNF health effects and is fundamental to interpreting findings of other BDNF related studies.

Two crucial components in this study was a longer time span and studying BDNF concentration levels through whole blood. Although D'Sa et al. (2012) studied BDNF through *serum* over three days and found no significant day-to-day differences between the serum BDNF levels. The type of blood sampling and time span may have been a reason for why our results had such a high variability. The data from these assay kits did produce BDNF concentrations that are within range to other BDNF levels measured in other studies (Dinoff et al., 2016; Elfving et al., 2010; Klein et al., 2011; Trajkovska,

Marcussen, Vinberg, Hartvig, Aznar & Knudson, 2007) ultimately, underscoring how important it is to understand normal BDNF variability and the potential of studying it through whole blood. Timing of sample collection is important as well. We tracked BDNF levels over 30 days from April to May and this may have introduced new variables that may have affected the concentration variability such as changing exercise routines (gearing up training for nationals/championships), increasing mental stressors leading up to finals week and environmental factors in preparation for life post-graduation.

### **Exercise and Individual Differences Related to BDNF Levels**

A secondary objective of this study was to investigate how recent physical exercise can affect BDNF concentration levels. New research still show evidence that short bouts of physical exercise increases BDNF levels (Tsai, Ukropec, Ukropcová, & Pai, 2018). The duration of these increases have been supported in a research which showed that platelets circulate in the peripheral blood for up to eleven days but BDNF proteins in plasma only circulates for up to an hour (Kishino et al., 2011; Poduslo & Curran, 1996). In animal models, BDNF has a half-life of 10 minutes in the blood and then removed from the circulation by the liver (Sakane & Pardridge, 1997). In order to better understand BDNF levels in human whole blood, assessment of BDNF levels after delayed physical exercise (within the past 12 hours) is a novel approach to operationalize and understand how BDNF levels may fluctuate on a daily basis. Because circadian rhythms can influence BDNF levels, we used this criteria in order to stay within the

standard 24-hour day since our earliest sample collection began at 12:00 noon each day. With this cut off, we found no statistical differences between samples post-exercise and samples from those who didn't recently exercise.

The environmental condition of this study was to investigate BDNF concentration levels under normal life circumstances. We wanted to minimize the amount of control we had on exercise regimens. While categorized exercise intensity levels based on a four-point ordinal scale based on the literature that self-selected intensity levels may be associated to higher BDNF levels (Keinan et al., 1999; Williams et al, 2016). However, we didn't provide a guideline as to what may be considered low, moderate or high intensity. A study performed by Pareja-Galeano et al. (2015) had controlled variables and exercise protocols, but these conditions don't typically translate to normal life circumstances. Perhaps future studies should consider operationalizing exercise regimens/intensity levels and investigating carry over effects to understand normal life BDNF variability post physical exercise activity. In addition, sampling multiple fluids simultaneously (plasma, serum, whole blood and saliva) at different time points post-exercise -- e.g., BDNF measured from capillary blood, venous blood, and saliva, at multiple points throughout the day -- would provide a more relevant measurement of BDNF variability.

Moreover, there are numerous factors mentioned in this study that may also affect BDNF levels, including circadian rhythms, hormonal cycling, sunlight exposure, genetic make-up, BMI, physical exercise history and mental health. In this current study, it is not likely that diurnal variation contributed to the observed variance since all samples were

collected at a common time for each participant at each session. Some literature suggests BDNF concentration levels in normally cycling women vary depending on the phase of the menstrual cycle (Pluchino et al., 2009). While there is evidence supporting different BDNF levels between gender (Lommatzsch et al., 2005; Pluchino et al., 2009; Trajkovska et al., 2007), we found no statistically significant differences between BDNF concentration levels of sex and the different phases (Luteal and Follicular) of the menstrual cycle.

Factors such as genetic background and mental health history also may have contributed to individual variances between BDNF levels. These effects need further examination. For example, healthy populations at risk for mood disorders and depressed patients have lower serum BDNF levels that correlate with the severity of their disorder (Karege et al., 2002). Genetic variants are a crucial factor when assessing BDNF levels. Future studies involving capillary-drawn whole blood should consider obtaining thorough genetic BDNF information, as well. Developing a protocol to assess natural BDNF concentration levels in participants with the Val66Met polymorphism may aid in the investigation if of BDNF variability between populations with and without this polymorphism.

### **Methodological Limitations**

The biggest limitation was sample size. Due to timing, resources and funding, this study was only able to evaluate BDNF concentrations from 9 participants. These participants were between the ages of 18 and 39 years with a mean age of  $27.88 \pm 7.04$

years. If our results were generalized in anyway, it's important to consider that our participants were within a limited age range. Not to mention, they averaged a mean BMI of  $23.29 \pm \text{kg/m}^2$ . This participant pool consisted of one HSU athlete and physically active participants. While they were healthy adults, this sample may not have been a representative sample of the general population.

Moreover, our initial resources included two assay kits, each with two 96 well plates (192 wells/kit) for roughly \$420 each/kit. Sixteen wells are reserved on each plate for running standard curve solutions. Recruiting 10 participants to contribute up to 12 samples each yields 120 samples. Samples are recommended to be assayed in duplicates, at minimum, which would require three plates. Because this protocol required at least one plate to be used to test a dilution series, this meant that obtaining more participants was not feasible for this study. A post-hoc power analysis was conducted in order to understand how many participants would be necessary to have 80% power for detecting an effect size of 0.8, the study would require 222 participants in each group (exercise vs. no exercise). Thus, sample size was a huge limiting factor and contributed to an underpowered study.

In order to properly account for the post-hoc power analysis' minimum sample size in turn to achieve the desired effect size, it would require at least 122 plates (60 kits, estimated to cost ~\$25,200) to be sufficient to analyze 12 duplicate samples from each participant. This is in addition to having additional resources for making homogenization buffers and sample diluent solutions. The minimum recommendation is to assay samples in duplications; but, it is highly recommended to run samples at triplicates or by serial

dilutions to allow for a more thorough sample assessment. More samples would require more personnel in general, in order to hold more blood-draw sessions. All co-investigators would need proper Bloodborne safety and finger-prick training. Scheduling and re-scheduling blood draw sessions were a recurrent theme in this study. Therefore, to increase the volume in sample size brings other variables that this study just simply could not handle.

Another potential source of laboratory error related to the sampling method used is the possibility of hemolysis from excessive squeezing or massaging of the fingers, which may have been done to encourage blood flow. While co-investigators were trained and cognizant that these procedures are not recommended (Lenicek Krleza, Dorotic, Grzunov & Maradin, 2015), it could have contributed to the large variance between duplicate samples because BDNF within broken cells degrade quicker (Trajkovska et al., 2007). BDNF concentration levels were highly variable between participants, which is consistent with this possibility.

There were numerous technical limitations that had to be taken into consideration. Timing of sample homogenization is extremely crucial to avoid samples from further degrading. For example, the samples went through numerous freeze-thaw cycles after homogenization. Trajkovska et al. (2007) found that whole blood can be thawed up to three times without compromising its BDNF concentrations. They also found that repeated freezing cycles at a minimum storage of  $-20^{\circ}\text{C}$  decreased serum BDNF concentrations but not whole blood BDNF concentration. This finding suggests that

platelets protect BDNF from further degradation. This current study homogenized whole blood samples within two weeks of obtainment and stored the homogenized samples at  $-80^{\circ}\text{C}$  for six months. Therefore, our samples are not likely to be affected by physical storage or freeze/thaw conditions.

In addition, changing protocol steps may have contributed to the inconsistency in BDNF concentration levels and increased inter-plate variance. For example, this study's protocol assayed duplicates samples on separate plates. This decision was made due to the initial resources (four BDNF plates) and time constraint in order to provide preliminary dilution series data. While we understood that duplicates are typically assayed on the same plate, due to our resources, this decision was intended for us to dilute samples and rerun samples at a larger dilution, if necessary, for a triplicate run should they fall outside the standard curve during the first two dilutions. In addition, when we processed Plate Six, the manufacturer's sample diluent was running low. We obtained the sample diluent recipe from Millipore and made a new solution in-house. A manipulation check was performed on randomly selected samples ( $n = 9$ ) that were diluted with the manufacturer's sample diluent and the new solution. This number was chosen due to the amount of wells we could spare to perform the manipulation check. This change in solution can be expected to add to measurement noise. When using the kit solutions from the same batch and pipetting techniques are consistent, there would likely be less variance between sample values.

Since technical factors such as method, preparation, analysis and storage of blood samples are crucial in BDNF concentration quantification, it could be difficult to produce

and replicate data. Perhaps a protocol modification to consider is to aliquot undiluted samples into separate vials intended for specific plates in order to reduce freeze/thaw cycles, contamination and degradation. This may help to obtain more within- subject reproducible BDNF concentration values with less variance. However, it's worth noting that most of the above-mentioned concerns are sources of error that would be expected to increase BDNF variability, so our measure of an overall CV of 19% is likely to be over-estimate within-subject BDNF variability. On the other hand, factors such as cell damage during collection that could degrade BDNF in the sample before the detection assay is processed could decrease variability by causing a floor effect on measurements. But since all readings were above the detection threshold shown by the assay's standard curve, this such a floor effect seems unlikely.

### **Relationship Between Brain and Peripheral BDNF Levels**

The current study analyzed peripheral BDNF as a proxy for brain BDNF levels, but the relationship of brain and peripheral BDNF concentrations is not entirely clear, so caution is required for interpreting the relevance of these findings to the construct of main interest, which is BDNF's positive effects on brain and cognitive health. Findings from an animal study conducted by Klein et al. (2011) suggests that BDNF concentrations in blood and plasma are comparable to BDNF concentrations in the hippocampus, which warrants using peripheral BDNF as a biomarker. However, other animal studies indicate different levels of BDNF in different regions of the brain (Krishnan et al., 2007). Radka et al. (1996) did a study that involved rodents and humans and showed that there was



higher plasma BDNF concentrations in rodents when compared to humans. A possible explanation includes the locale of sampling from the rats' trunk after decapitation compared to humans' peripheral blood sampling. There is support that BDNF crosses the BBB in both directions (Pan et al., 1999; Poduslo et al., 1996), but there are other explanations and sources of peripheral BDNF release such as liver, smooth muscle cells, and endothelial cells (Ernfors, Wetmore, Olson, & Persson, 2000; Nakahasi et al., 2000;). It is not entirely clear that peripheral BDNF concentrations reflects levels of BDNF released from the CNS.

### **Future Directions for BDNF Research**

Existing evidence supports physical exercise as a mechanism to up-regulate BDNF concentration levels, in turn, improving mood and cognitive function (Zoladz & Pilc, 2010). Aerobic exercise training has been supported over and over with improvements in attention, memory and executive functioning, although not working memory (as reviewed by Smith et al., 2010), whereas, Wang and Wang (2016) showed that low intensity treadmill exercises could improve short-term working memory. Van Dongen, Kersten, Wagner, Morris, & Fernandez (2016) showed that participating in physical exercise after a period of learning can actually increase memory recall which is consistent with hippocampal activation during memory retrieval. But it is still unclear how these mechanisms directly affect BDNF secretion and concentration (Campos et al., 2016). Thus, there is demand for a standardized and systematic approach across studies to measure BDNF levels and how physical exercise can affect them (Knaepen et al., 2010).

The prevalence of BDNF-related physiology research is to understand the molecular underpinnings of BDNF as it can be a crucial component for insight into the genetic disparities and possible therapeutic avenues. A breakthrough study by Egan et al. (2003) found that the BDNF polymorphism gene Val66Met was linked to poorer memory recall and atypical hippocampal activation. Such studies help to provide understanding and justification for animal research since we currently rely on animal models to support the value of conducting clinical trials for humans. Subsequently, molecular studies in humans help to provide a more genetically personalized treatment for brain or behavioral changes due to the high sources of variability. Research studying how physical exercise affects BDNF concentration levels help to provide an empirical basis for therapeutic interventions.

While animal research has shown that neural stem cells within the subgranular zone continue onto adult mouse neurogenesis (van Praag, 2002), there has been some recent push-back on a central premise of BDNF's beneficial effects, namely, that increasing BDNF can increase the rate of neurogenesis in adult brains. Sorrells et al. (2018) found evidence that young neurons begin to decline in the dentate gyrus during infancy and continues to decline onto early childhood. There was no evidence of young neurons or dividing cells in adults with epilepsy and the control group. If any was seen in the control participants, it wasn't notably significant. While it was found that neurogenesis happens postnatally in macaques, in adult macaques (17 and 24 years old), there was little to no evidence of young neurons. These evidences propose that the presumed BDNF-linked neurogenesis may not be real at all. This suggests that most of

the literature on BDNF from animal models don't fully translate to the human species. Feasibly, this is a motivation for more continued research to understand the function of BDNF in humans. For example, while BDNF may not control the birth of new, young neurons in adult brains, it may still influence cognitive health by promoting healthy functioning of existing neurons.

Problems related to inconsistent research methods and underpowered studies make conclusions difficult. BDNF research is easier in animal models and are more difficult with human participants. Since we currently have to assess BDNF through peripheral methods, a standardized protocol is needed for measuring human blood BDNF levels since there is such inconsistency between serum, plasma, platelet, whole blood, coagulated or anticoagulated blood forms (Knaepen et al., 2010; Trajkovska et al., 2007; Sanchis-Gomar & Lippi, 2014). Not to mention, different components (i.e., serum, plasma, whole blood, and platelet count) that have been studied from the same participant still end up with varying results depending on sample type (Pareja-Galeano et al., 2015; Trajkovska et al., 2007). An important distinction is that whole blood must be lysed before being plated so this adds an extra step where confounding variables could be introduced. For example, Karege et al. (2005) found a non-significant difference between whole blood BDNF levels in depressed participants when compared to the controls. However, their serum BDNF concentrations in depressed patients were lower when compared to their controls, perhaps, due to BDNF's mechanism of release. Thus, more research is needed to understand the locale and timing of peripheral BDNF retrieval.

While this study worked within the constraints of its limitations in attempt to provide a fuller picture of BDNF variability over time, it still remains unclear if there are valid cognitive benefits link to BDNF. Perhaps, understanding human BDNF variability can be a start and be used as a benchmark for designing and interpreting future BDNF-related studies.

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## Appendices

### Appendix A: Consent to Participate

#### **Purpose of the study:**

The aim of this study is to assess how levels of a protein, called brain-derived neurotrophic factor (BDNF), vary over 30 days in blood samples drawn from ‘finger prick’ safety lancets. BDNF is a protein that is found in your body and nervous system and is important for brain health. We hope to learn more about BDNF by examining levels over a longer time period than has been examined before.

**What we are asking our participants to do.** Your involvement in this study to completely voluntary and you can quit at any time. However, to get the most reliable results possible, we hope our participants will meet with us to contribute a ‘finger-prick’ blood sample 3x a week for up to 30 days, for a total of 12 samples. In addition, since BDNF levels are linked to exercise, we will ask you about your most recent physical exercise routine at each session. But, we are not asking you change your physical exercise habits in any way, just to report the time and intensity of your last exercise. We are asking your permission to contact you periodically by email or cell phone during the 30-day study to arrange the timing of meetings.

#### **Procedure and time line of the study.**

At the first meeting, you will be asked to fill out a brief health screening form and to sign this consent form. Then, for three times a week, you will be asked to meet the investigator (Sally Hang), at your convenience within the constraints of the study, for about 10 minutes to do three things: (1) answer a blood collection readiness question (2) contribute a blood sample (3) answer questions about the time and intensity of recent exercise.

#### **Information about the blood collection methods.**

A spring-loaded ‘safety lancet’ will be used to puncture skin at the fingertip and produce a few drops (50 - 300  $\mu$ l) of blood that will be collected into a plastic tube. For most people, this results in mild discomfort lasting a few second to a minute. The following steps will be taken for safety and comfort

- The principle investigator, Sally Hang and responsible co-investigators, will have completed a blood-borne pathogen safety training class
- Investigators will always wear new, nitrile gloves with each participant
- Participant will wash hands prior to each session with Luke warm water and soap for at least 20 seconds
- Investigator will perform a gentle palm massage to promote blood flow



- Your skin will be wiped with an alcohol pad before and after collection
- Your fingertip will be pricked using a safety lancet
- You may prick your own finger if you prefer
- A heating pad will be available to promote blood flow in your finger
- Band-Aids will always be applied after blood collection and a first aid kit will be available onsite
- Contact information for the HSU Health Clinic, local hospitals, and Counseling and Psychological Services (CAPS) will be readily available for participant's use should an emergency occur.

**Risks, Benefits, and safety measures:****Confidentiality.**

Your blood samples and questionnaires will be labeled with a numerical code and will never be directly connected to your name. Only the principle investigator will have a key linking your name to your code number and she will keep it secure and confidential.

**Blood sample handling.**

Your blood samples will be stored securely at HSU. Your blood will be tested only for BDNF; no other tests will be done. Your sample will be safely disposed of after testing, which will be no more than 1 year after collection.

**Risks and safety measures.**

Possible risks from blood collection include dizziness and the rare possibility of light headedness or injury from bleeding. Although the risks are small, we will have a first-aid kit on hand if needed. You will be asked to affirm your readiness for the blood collection before each collection. As noted, alcohol disinfectant wipes and gloves will be used to reduce the risk from pathogens.

**Benefits.**

You may directly benefit from your involvement in this study by choosing to become a co-investigator on the project. Becoming a co-investigator is an option open to all participants and includes the opportunity to participate in analyzing and interpreting data and, potentially, presenting results. Co-investigators will have to complete a human subjects training course and receive IRB approval before beginning work as an investigator. Indicate below if you would like to be a co-investigator in this study and Sally will contact you with more information.

**Voluntary participation.**

Your participation is voluntary and you may also withdraw from the study at any time without jeopardizing your position as a co-investigator if you choose to be one.

**Contacts:**

If you have any questions about this study you may contact the investigator, Sally Hang at [sh1738@humboldt.edu](mailto:sh1738@humboldt.edu), or the faculty supervisor, Ethan Gahtan, at [eg51@humboldt.edu](mailto:eg51@humboldt.edu) or 707-826-4545. If you have any concerns with this study or questions about your rights as a participant, contact the Institutional Review Board for the Protection of Human Subjects at [irb@humboldt.edu](mailto:irb@humboldt.edu) or (707) 826-5165.

By signing below, you consent to participate in this study.

Signature \_\_\_\_\_ Date \_\_\_\_\_

(Check and initial if applicable) I would like to be a co-investigator on the project \_\_\_\_\_

**Appendix B: Health Reaffirmation at Each Session****Date:** \_\_\_\_\_

1. Do you feel well and healthy enough today to contribute a blood sample? Yes/ No
2. Did you recently exercise? Yes/ No  
If yes, please answer the following?

- a. Date: \_\_\_\_\_ Time: \_\_\_\_\_ A.M. / P.M.

- b. Type of Physical Activity: \_\_\_\_\_

- c. Intensity of Physical Activity: Low / Moderate / High

### Appendix C: Demographics

Name: \_\_\_\_\_

Sex: M F Age: \_\_\_\_\_

1. Do you use Tobacco? Yes. No.

If yes, how many packs a week? \_\_\_\_\_

2. Are you generally in good health? Yes / No

3. Do you exercise regularly? Yes/ No. If yes, for how long? \_\_\_\_\_  
month/years

4. What is your main form of exercise (e.g., biking to school, walking, running, cycling, weight lifting, high intensity interval training (Hiit), walking across campus...etc):  
\_\_\_\_\_

5. Do you know of any reason why your health might be harmed by the procedures described for this study? Yes / No

6. Do you know of any reason why your blood might be dangerous to others? Yes/ No

7. Typical hormone cycles in females are known to influence BDNF levels. To control for that in the study, if you are a female with *typical hormone cycling*, please estimate the first day of your current cycle: \_\_\_\_\_ days ago / Not Applicable

8. Please estimate your height \_\_\_\_\_ and weight \_\_\_\_\_, as these factors may also be related to BDNF levels.

9. Please rate your general sleep quality over the previous month from 1 (poor sleep over the past month) to 10 (excellent sleep over the past month): 1 2 3 4 5 6 7  
8 9 10

## **Appendix D: Blood Draw Protocol**

Note: this protocol was adapted from a similar protocol shared with the investigators by Chris Benassi, Trevor Caudle, Thomas Williams (all HSU Alumni) and Tina Manos (HSU faculty). Some of the wording is unchanged from the original protocol.

### **Testing Supplies:**

- Single Use Safety Lancets (referred to as ‘finger prick devices’ in the protocol application)
- Disinfecting 70% isopropyl wipes (alcohol prep pads)
- Cotton Squares
- Band Aids & First-Aid kit
- Biohazard disposal container with hard shell, lined with biohazard bag and non-penetrable lid.
- Disposable, sterile, absorbent pads for covering table surfaces during blood draws and blood handling.
- Separate biohazard bag for table covering pads
- Nitrile exam gloves
- 300 µl SAFE-T-FILL® Capillary Blood Collection Tubes with EDTA for blood collection and storage

### **General Safety:**

- All persons (Sally Hang and responsible co-investigators) administering blood collection must have current Hepatitis B vaccine.
- All researchers participating in blood collection must have proper Blood Borne Pathogen (BBP) training.
- All tests are to be treated as potentially infectious.
- Hands should be washed with soap and warm water. If a glove is ripped, hands should be washed and gloves changed before continuing to collect blood. Hands should be washed thoroughly after removal of gloves.
- Dump used lancet, cotton square, capillary tube, into biohazard container immediately following use.
- The blood collection table will be set up within 50-meters of a hand-washing facility.
- Eating or drinking is prohibited in testing area.
- In case of an Emergency Call 911 then Administer First Aid.
- Sterile absorbent pad will cover the blood collection table in order to absorb any blood spills on the table. A new pad will be used for each participant.
- Protective clothing must be worn by persons administering test including:
  - Nitrile Gloves

- Laboratory Coat
- Safety Glasses
- Closed Toe Shoes

### **Management of Accidents and Injuries:**

*First*, attend to any injured personnel. Cell phone must be present. Call 911 for emergency assistance and inform responders of biohazards that may be a threat. Notify the faculty supervisor and complete an incident report form at the soonest possible opportunity.

Accidents that may occur include the following:

- **Fainting-** A subject may become dizzy or faint from the sight of blood. If this occurs, move the subject into the supine position with their legs slightly elevated. Make sure to keep up communication with subject in order to assure proper recovery.
- **Hematoma-** May occur either when the needle has penetrated through the vein or when insufficient pressure is applied on the puncture site immediately following test. The puncture area will swell and become discolored. If this occurs, apply firm pressure directly on the site for 5 minutes.
- **Needle-Stick Injuries-** May happen to either the subject or the tester and is classified by any puncture injury caused by exposed lancets or any other sharp object. Extreme caution is advised. If injury does occur, wash wound immediately with water and treat with 70% isopropyl alcohol.

### **Exposures and Needle-Stick Injury:**

- Although fewer than 0.3% of HIV-infected needle sticks lead to cross infection, this possibility should be known to blood collection staff (Sally Hang) prior to involvement in blood handling.
- Report exposures and/or needle-stick injuries verbally to the UPD responders. Suspected exposures should also be reported.
- Report the exposure and/or needle-stick to the EH&S biosafety officer at (707) [826-5711](tel:826-5711) for a review of laboratory protocols and procedures.
- Report adverse event incident to the IRB.

### **Procedure for Collection:**

Blood

- Outside tips of ring or middle fingers will only be used as sample sites. Clean and sterilize the area with an alcohol prep pad and allow moisture to evaporate before testing or dry with sterile cotton square.

- Using single-use safety lancet, hold cotton pad in one hand underneath the site and apply pressure with lancet on ideal site. Prick finger of test subject and allow a drop of blood to form on the surface of the skin. The first drop of blood may contain perspiration and other contaminants so wipe clean with cotton square and obtain a second drop of blood. Give the cotton square to the subject in the non-sampled hand so they can clot the sampling site after the sample has been collected. Avoid squeezing the sampled finger because it introduces contamination.
- The subject will place finger over the top of the blood collection tube and allow the blood to fill the tube until 300 $\mu$ L is collected (about 2-4 drops).
- A band-aid will be applied after sample collection.
- Used cotton squares with blood can be disposed of in a regular trash bin per OSHA standards.

### **Clean Up:**

- Using a 70% isopropyl alcohol wipe, wipe down all equipment
- Properly dispose of lancets and contaminated collection supplies in designated biohazard container after each use.

Procedures adapted from: Australian Sports Commission, Physiological Tests for Elite Athletes (2000), ch 6.

**Appendix E: Whole Blood Homogenization Protocol**

---

Step	Procedure
1	Prepare Homogenization Buffer w/ Protease (HB w/P) per manufacturer's instructions
2	Prep micro-centrifuge tubes with appropriate recoded ID numbers
2	Thaw blood samples on ice (keep on ice until plated)
3	Estimate and record total sample volume by comparing it against pre-measured, water-filled micro-centrifuge tubes set at 50 $\mu$ l intervals
4	Add HB w/ P at 1:10 ratio
5	Vortex for 2 – 5 second pulses
6	Visually check viscosity
	6a. If solution is too runny, add 5 $\mu$ l HB w/ P as needed and recheck viscosity
7	Record total sample volume + HB w/ P
8	Refreeze or plate sample
9	Repeat with next sample

---



**Appendix F: Final Whole Blood Assay Protocol**

Step	Procedure
1	Obtain blood samples and store in -80 °C
2	Homogenize samples in 1:10 ratio to homogenization buffer within 2 weeks of storage
3	Dilute samples 1:50 to Sample Diluent solution
4	Assay samples and obtain response
5	Calculate plate's Standard Curve
6	Complete statistics for converting plate responses to BDNF concentration values
7	Average duplicate sample BDNF concentration values

---

*Note:* Final Protocol for Assaying Whole Blood BDNF samples

## Appendix G: R Script

```
library(dplyr)
library(tidyr)
library(mice)
library(Amelia)
library(readr)
library(janitor)
library(lubridate)
library(ggplot2)
library(car)
library(multcomp)
library(ez)
library(tidyverse)
library(readr)

bdnfdata <- read_csv("Desktop/bdnfdata.csv")
View(bdnfdata)

# replace with your own path
path <- ("Desktop/bdnfdata.csv")

# import and clean
bdnf <- readr::read_csv("Desktop/bdnfdata.csv") %>%
  janitor::clean_names()

#turn into factors
bdnf <- bdnf %>%
  separate(id, into = c('id', 'nothing'), sep = 1) %>%
  mutate(x12hoursago = factor(x12hoursago),
         id = factor(id),
         sex = factor(sex),
         sessions = factor(session),
         re = factor(re))

View(bdnf)
#impute data
bdnf.t <- bdnf %>%
  mice(m = 10, maxit = 10, method = 'pmm', seed = 1619) %>%
  mice::complete()
View(bdnf.t)

missmap(bdnf)
```

```

missmap(bdnf.t)

glimpse(bdnf.t)

####-----
#### Exploratory Graphs
####-----

install.packages("extrafont")
library(extrafont)
font_import()
loadfonts(device="win") #Register fonts for Windows bitmap output
fonts()

library(Hmisc)
library(ggplot2)
#avg, those who did exercise had a higher median of BDNF levels vs. those who didn't
exercise
ggplot(bdnf.t, aes(x12hoursago, avgbdnf)) +
  geom_point(alpha = 0.6) +
  stat_summary(fun.data = 'mean_cl_normal', geom = 'crossbar', width = .3, col= 'red') +
  theme_bw() +
  theme(text=element_text(family="Times New Roman", face="plain", size=12)) +
  labs(x="Exercise Response",y="BDNF Concentration (pg/mL)")

bdnf.t %>%
  filter(re %in% c("1", "2")) %>%
  group_by(re) %>%
  summarize(the_medians = median(as.numeric(re), na.rm = TRUE))

#diff version
ggplot(bdnf.t, aes(x=x12hoursago, y=avgbdnf,fill=x12hoursago)) +
  geom_point(alpha = 0.6) +
  stat_summary(fun.data = 'mean_cl_normal', geom = 'crossbar', width = .3, col= 'red') +
  theme_bw() +
  scale_fill_discrete(name="Exercise Response",
                      breaks=c("1", "2"),
                      labels=c("Within 12 hours", "More than 12 hours ago")) +
  theme(text=element_text(family="Times New Roman", face="plain", size=12)) +
  xlab("Exercise Response") + ylab("BDNF Concentration (pg/mL)")

#overall bell curve of samples - looks pretty normal
ggplot(bdnf.t, aes(avgbdnf)) +

```

```

geom_density(fill = 'lightblue', alpha = 0.8) +
theme_bw() +
labs(x="Average BDNF Concentration (pg/ml)",y="Count")

#same but just in histogram bell curve form
ggplot(bdnf.t, aes(avgbdnf)) +
  geom_histogram(fill = 'lightblue', alpha = 0.8) +
  theme_bw() +
  labs(x="Average BDNF Concentration (pg/ml)",y="Count") +
  theme(text=element_text(family="Times New Roman", face="plain", size=12)) +
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank())

#dichotomize M and F and those who exercise vs. not exercise
ggplot(bdnf.t, aes(avgbdnf)) +
  geom_histogram(fill = 'lightblue', alpha = 0.8) +
  facet_wrap(sex ~ x12hoursago) +
  theme_bw()

####-----
#### HYPOTHESIS 1: COEFFICIENT OF VARIANCE: OVERALL SD/OVERALL
MEAN
#### CALCULATED IN EXCEL ALREADY
####-----

####-----
#### HYPOTHESIS 2: Mixed effect model (repeated measures)
####-----
library(lme4)

#### This model utilized mean imputation - leaning towards nonsig side b/c xhoursago =
-1.489

model1 <- lmer(avgbdnf ~ x12hoursago + (1|id), data = bdnf.t)
summary(model1)
model1

model2 <-lmer(avgbdnf ~ 1 + (1|id), data = bdnf.t)
model2
anova(model2, model1)

tapply(bdnf.t$avgbdnf, bdnf.t$x12hoursago, mean)
tapply(bdnf.t$avgbdnf, bdnf.t$x12hoursago, sd)
tapply(bdnf.t$avgbdnf, bdnf.t$x12hoursago, median)

```

#There was no significant difference between recent exercise activity within 12 hours and BDNF levels ( $\chi^2(1)=2.28$ ,  $p=0.13$ )

```
r2.corr.mer <- function(m) {
  lmfit <- lm(model.response(model.frame(m)) ~ fitted(m))
  summary(lmfit)$r.squared}
```

```
r2.corr.mer(model1)
r2.corr.mer(model2)
```

```
#BDNF lvls and intensity
bdnf.t <- bdnf.t %>%
  mutate (intensity.factor = as.factor(intensity))
```

```
View(bdnf.t)
```

```
str(bdnf.t$intensity.factor)
model1.int <- lmer(avgbdnf ~ intensity.factor + (1|id), data = bdnf.t)
summary(model1.int)
plot(model1.int)
```

```
model2.int <- lmer(avgbdnf ~ 1 + (1|id), data = bdnf.t)
model2.int
anova(model2.int, model1.int)
```

```
r2.corr.mer(model1.int)
r2.corr.mer(model2.int)
```

```
#descriptive statistics
tapply(bdnf.t$avgbdnf, bdnf.t$intensity.factor, mean)
tapply(bdnf.t$avgbdnf, bdnf.t$intensity.factor, sd)
tapply(bdnf.t$avgbdnf, bdnf.t$intensity.factor, median)
```

```
tapply(bdnf.t$avgbdnf, list(bdnf.t$intensity.factor, bdnf.t$sessions), mean)
tapply(bdnf.t$avgbdnf, list(bdnf.t$intensity.factor, bdnf.t$sessions), sd)
tapply(bdnf.t$avgbdnf, list(bdnf.t$intensity.factor, bdnf.t$sessions), median)
```

```
#####-----
```

```
##### POST HOC - Mixed effect model (repeated measures) FOR INTENSITY & BDNF LEVELS
```

```
#####-----
```

```
bdnf.t <- bdnf.t %>%
```

```

group_by(sessions,intensity.factor) %>%
mutate(mean.avgbdnf = mean(avgbdnf))

View(bdnf.t)

bdnf.t$intensity.factor <- factor(bdnf.t$intensity.factor, levels=c("none", "low", "mod",
"high"), labels=c("None", "Low", "Mod", "High"))

ggplot(data=bdnf.t, aes(x=sessions, y=mean.avgbdnf, group = intensity.factor,color =
intensity.factor, shape = intensity.factor)) +
  geom_line(size = .25) +
  geom_point() +
  labs(x="Session",y="BDNF Concentration (pg/ml)") +
  scale_linetype_discrete("Intensity") +
  scale_shape_discrete("Intensity") +
  scale_colour_discrete("Intensity") +
  theme(text=element_text(family="Times New Roman", face="plain", size=12)) +
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank())

#do not run#
#There was no significant difference between intensity types within 12 hours and BDNF
levels Chi square (3,1) = 5.22, p = 0.15)
#intensity <- ordered(c("none", "low", "mod", "high"))
#intensity.factor<- ordered(intensity.factor, levels = c("none", "low", "mod", "high"))
#intensity

#looks at imputed model and the dots are pretty scattered
plot(model1)

# They lie kinda heavily on the linear line
library(car)
qqp(resid(model1))

library(dotwhisker)
dwplot(model1)

#EXPLORATORY: non-imputed data show no decision for participant 2, 6 and 7
bdnf%>%
  group_by(id) %>%
  dplyr::summarize(mean = mean(avgbdnf), sd = sd(avgbdnf), upper = mean + sd, lower
= mean - sd) %>%
  mutate(within = ifelse((mean + sd) <= upper & (mean - sd) >= lower,
"TRUE", 'FALSE') )

```

```
##### TO-DO
```

```
bdnf.t$avgbdnf
```

```
####-----
```

```
#### ADDITIONAL POST HOC ANALYSES
```

```
####-----
```

```
# 1 - Time Series Analysis
```

```
#new plot w/ better legend and also CV next to each participant
```

```
bdnf.time <- bdnf.t %>%
  separate(id, into = c('id', 'nothing'), sep = 1) %>%
  mutate(time = case_when(session == 1 ~ ymd(20170403),
    session == 2 ~ ymd(20170405),
    session == 3 ~ ymd(20170407),
    session == 4 ~ ymd(20170410),
    session == 5 ~ ymd(20170412),
    session == 6 ~ ymd(20170414),
    session == 7 ~ ymd(20170417),
    session == 8 ~ ymd(20170419),
    session == 9 ~ ymd(20170421),
    session == 10 ~ ymd(20170424),
    session == 11 ~ ymd(20170426),
    session == 12 ~ ymd(20170428)),
    recent_exercise = case_when(x12hoursago == 1 ~ "yes",
    x12hoursago == 2 ~ "no"),
    time_factor = as.factor(session),
    time_test = ts(time),
    avg_bdnf = mean(avgbdnf),
    upper = avg_bdnf + (avgbdnf * .15),
    lower = avg_bdnf - (avgbdnf * .15),
    intensity = as.factor(intensity),
    sex = as.factor(sex),
    avgbdnf = round(avgbdnf,2),
    id = as.factor(id),
    session.2 = session*2) %>%
  mutate(id = as.factor(id),
    Participant = case_when(id == 1 ~ "1 (0.16)",
    id == 2 ~ "2 (0.27)",
    id == 3 ~ "3 (0.19)",
    id == 4 ~ "4 (0.18)",
```

```

id == 5 ~ "5 (0.16)",
id == 6 ~ "6 (0.22)",
id == 7 ~ "7 (0.29)",
id == 8 ~ "8 (0.13)",
id == 9 ~ "9 (0.13)")

```

View(bdnf.time)

```

ggplot(bdnf.time, aes(x = session.2, y = avgbdnf)) + geom_line(aes(color = Participant))
#rename axis titles
dat <- ggplot(data=bdnf.time, aes(x=session.2, y=avgbdnf, shape = id)) +
  geom_line(aes(color = Participant)) +
  geom_point() +
  labs(x="Day", y="Average BDNF Concentrations (pg/mL)") +
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank()) +
  theme(text=element_text(family="Times New Roman", face="plain", size=12))

print(dat + scale_shape_manual(values = c(0, 16, 3, 4, 5, 6, 7, 17, 18)))

dat + scale_shape_manual(values = c(0, 16, 3, 4, 5, 6, 7, 8, 9)) +
  geom_line(aes(color = Participant)) +
  guides(shape = FALSE) # you can switch this to color = FALSE instead if you want
#
print(dat + scale_shape_manual(values = c(0, 16, 3, 4, 5, 6, 7, 17, 18)) +
  geom_line(aes(color = Participant)))
#
ggplot(data=dat, aes(x=session.2, y=avgbdnf, shape = id)) +
  geom_line(aes(color = Participant)) +
  geom_point(shape=1) +
  labs(x="Day", y="Average BDNF Concentrations (pg/mL)") +
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank()) +
  theme(text=element_text(family="Times New Roman", face="plain", size=12)) +

print(dat + scale_shape_manual(values = c(1, 2, 3, 4, 5, 6, 7, 8, 9)))
dat <- ggplot(bdnf.time, aes(session, avgbdnf, shape = id, colour = id)) + geom_point()

print(dat + scale_shape_manual(values = c(0, 16, 3, 4, 5, 6, 7, 8, 9)))

#### Post Hoc # 2: does BMI and BDNF correlate? No.
cor.test(bdnf.time$bmi, bdnf.time$avgbdnf)
#need effect size

#notes: power analysis for a correlation?

```



```

pwr::pwr.r.test(n = 2200, r = .06, sig.level = .05)

#### Post Hoc # 3: Sex and bdnf averages over time correlation? Nope.
t.test(avgbdnf~sex, bdnf.time)
library(lsr)
lsr::cohensD(bdnf.time$avgbdnf~bdnf.time$sex)

#power analysis for gender correlation (maybe could even be repeated measures amt in
bdnf exercise group)
pwr::pwr.t.test(n = 222, d= .27, sig.level = .05)

### Post Hoc # 4 : Menstrual Phases (3) and BDNF levels

#Is there a difference between the Avgbdnf for females against their different menstrual
time periods.
#Follicular = Day 1-13, Luteal = Day 14-28. Nope.

females <- bdnf.time %>% filter(sex == "F") %>%
mutate(menstrul_1 = case_when(menstrul < 13 ~ "Follicular",
menstrul >= 13 & menstrul < 22 ~ "Luteal 1st half",
menstrul >= 29 ~ "Luteal 2nd half"),
menstrul_1 = as.factor(menstrul_1))

aov_menst <- aov(avgbdnf~menstrul_1, females)
Anova(aov_menst, type = 3) #type 3 b/c ~ spss output typical in APA
lsr::etaSquared(Anova(aov_menst, type = 3))

Testers <- glht(aov_menst, linfct=mcp(menstrul_1 = "Tukey"))
summary(Testers)

tapply(females$avgbdnf, females$menstrul_1, mean)
tapply(females$avgbdnf, females$menstrul_1, sd)
library(lsr)
lsr::cohensD(females$avgbdnf~females$menstrul_1)

```

## Appendix H: Linear Mixed Model Analysis for Exercise Response

### *Linear Mixed-Effects Analysis of Exercise Response and BDNF Levels*

#### ***Exercise Intensity – Fixed Effects***

	Estimate	SE	t
(Intercept)	31.60	2.69	11.744
Recent Exercise	-2.69	1.81	-1.49

*SE* = Standard Error

#### ***Participant - Random Effects***

	Response	
	<i>SD</i>	No Exercise
ID	7.4	
Recent Exercise	8.9	-0.40
No Exercise	9.5	

Note: Estimates of variance/covariance are reported as Standard deviation (*SD*) with correlations. *SD* of model with only random effects residual= 6.49, *N*=9.

## Appendix I: Linear Mixed Model Analysis for Exercise Intensity

### *Exercise Intensity – Fixed Effects*

	Estimate	SE	t
(Intercept)	28.37	2.84	9.97
Low	5.39	2.40	2.25
Mod	2.62	1.82	1.44
High	1.17	2.27	0.52

SE = Standard Error

### *Participant - Random Effects*

	SD	Intensity		
		None	Low	Mod
ID	7.42			
None	9.27			
Low	11.32	-0.23		
Mod	9.91	-0.28	0.46	
High	33.56	-0.27	0.29	0.34

Note: Estimates of variance/covariance are reported as standard deviation (SD) with correlations. SD of model with only random effects residual= 6.49, N=9.

**Appendix J: Duplicate Sample Variability for all Participants**

Participant 1

Session	ID	Run 1	Run 2	Run 1-Run 2
1	1	34.35	36.28	1.93
2	1	49.78	46.28	3.5
3	1	30.4	35.66	5.26
4	1	34.95	33.84	1.11
5	1	33.31	32.99	0.32
6	1	31.62	38.93	7.31
7	1	43.95	32.8	11.15
8	1	50	37.17	12.83
9	1	37.5	21.75	15.75
10	1	25.51	35.01	9.5
11	1	54.48	35.24	19.24
12	1	38.07	34.38	3.69

*Note:* Raw data for sample duplicate runs and the variability between each sample. Participant 1's overall duplicate sample variability averaged 7.6 pg/ml.

## Participant 2

Session	ID	Run 1	Run 2	Run 1-Run 2
1	2	57.51	37.35	20.16
2	2	58.3	52.2	6.1
3	2	18.79	32.42	13.63
4	2	32.63	10.19	22.44
5	2	33.56	46.49	12.93
6	2	39.46	47.03	7.57
7	2	35.03	31.74	3.29
8	2	61.6	36.79	24.81
9	2			
10	2	39.74	13.91	25.83
11	2	44.59	36.68	7.91
12	2	35.65	43.48	7.83

*Note:* Raw data for sample duplicate runs and the variability between each sample. The participant had a missing value for the ninth session due to the sample running out during the troubleshooting phases. Participant 2's overall duplicate samples variability averaged 14.2 pg/ml.

## Participant 3

Session	ID	Run 1	Run 2	Run 1-Run 2
1	3	28.06	45.7	17.64
2	3	42.24	31.04	11.2
3	3	25.12	41.81	16.69
4	3	21.49	21.95	0.46
5	3	27.27	25.84	1.43
6	3	25.74	31.03	5.29
7	3	24.02	26.49	2.47
8	3	18.82	29.62	10.8
9	3	19.15	23.44	4.29
10	3	25.43	23.31	2.12
11	3	32.02	25.48	6.54
12	3	33.99	23.44	10.55

*Note:* Raw data for sample duplicate runs and the variability between each sample. Participant 3's overall duplicate samples variability averaged 7.5 pg/ml.

Participant 4

Session	ID	Run 1	Run 2	Run 1-Run 2
1	4	19.2	25.46	6.26
2	4	15.2	35.45	20.25
3	4	22.84	29.38	6.54
4	4	18.17	20.27	2.1
5	4	18.94	18.79	0.15
6	4	11.52	14.59	3.07
7	4	20.27	23.04	2.77
8	4	37.88	18.96	18.92
9	4	26.67	14.99	11.68
10	4	23.4	19.42	3.98
11	4	25.5	19.79	5.71
12	4	29.05	18.31	10.74

*Note:* Raw data for sample duplicate runs and the variability between each sample. Participant 4's overall duplicate samples variability averaged 7.7 pg/ml.

**Participant 5**

Session	ID	Run 1	Run 2	Run 1-Run 2
1	5	14.87	23.61	8.74
2	5	13.05	14.07	1.02
3	5	9.91	12.36	2.45
4	5	13.89	17.28	3.39
5	5	12.84	17.29	4.45
6	5	11.6	19.09	7.49
7	5	12.56	16.63	4.07
8	5	13.64	19.13	5.49
9	5	8.05	13.66	5.61
10	5	12.64	11.78	0.86
11	5	16	16.03	0.03
12	5	15.4	13.94	1.46

*Note:* Raw data for sample duplicate runs and the variability between each sample. Participant 5's overall duplicate samples variability averaged 3.8 pg/ml.



## Participant 6

Session	ID	Run 1	Run 2	Run 1-Run 2
1	6	36.94	23.04	13.9
2	6	39.91	28.71	11.2
3	6	28.27	31.41	3.14
4	6	26.85	31.14	4.29
5	6	36.56	33.53	3.03
6	6			
7	6	17.58	20.75	3.17
8	6	21.61	31.08	9.47
9	6	37.56	21.38	16.18
10	6	27.34	29.84	2.5
11	6	35.87	27.62	8.25
12	6	25.19	20.26	4.93

*Note:* Raw data for sample duplicate runs and the variability between each sample. The participant had a missing value for the sixth session due to the sample running out during the troubleshooting phases. Participant 6's overall duplicate samples variability averaged 7.3 pg/ml.

## Participant 7

Session	ID	Run 1	Run 2	Run 1-Run 2
1	7	29.64	45.42	15.78
2	7	81.72	31.3	50.42
3	7	42.18	54.72	12.54
4	7	30.49	39.98	9.49
5	7	30.11	24.07	6.04
6	7	34.83	24.24	10.59
7	7	24.08	29.65	5.57
8	7	39.84	29.31	10.53
9	7	27.74	32.72	4.98
10	7	20.89	71.83	50.94
11	7	23.93	15.24	8.69
12	7			

*Note:* Raw data for sample duplicate runs and the variability between each sample. The participant had a missing value for the ninth session due to the sample running out during the troubleshooting phases. Participant 7's overall duplicate samples variability averaged 16.9 pg/ml.

**Participant 8**

Session	ID	Run 1	Run 2	Run 1-Run 2
1	8	41.13	42.39	1.26
2	8	33.27	20.79	12.48
3	8	34.87	28.21	6.66
4	8	37.33	29.41	7.92
5	8	24.11	42.89	18.78
6	8	34.14	41.36	7.22
7	8	32.13	31.19	0.94
8	8	37.76	28.32	9.44
9	8	35.67	38.5	2.83
10	8	48.9	27.93	20.97
11	8	30.79	24.61	6.18
12	8	32.87	30.72	2.15

*Note:* Raw data for sample duplicate runs and the variability between each sample. Participant 8's overall duplicate samples variability averaged 8.1 pg/ml.

## Participant 9

Session	ID	Run 1	Run 2	Run 1-Run 2
1	9	44.23	27.29	16.94
2	9	28.71	27.25	1.46
3	9	34.31	24.58	9.73
4	9	25.22	32.18	6.96
5	9	37.27	22.67	14.6
6	9	24.87	18.51	6.36
7	9	27.87	37.23	9.36
8	9	31.71	37.56	5.85
9	9	26.63	30.45	3.82
10	9	32.31	23.87	8.44
11	9	32.79	32.27	0.52
12	9	41.47	30.53	10.94

*Note:* Raw data for sample duplicate runs and the variability between each sample. Participant 9's overall duplicate samples variability averaged 7.9 pg/ml.