CHRONIC OMEGA-3 SUPPLEMENTATION AND ERYTHROCYTE DEFORMABILITY, OXYGEN CONSUMPTION, AND PERFORMANCE DURING NORMOBARIC HYPOXIC EXERCISE

Shane A. Bielko

Submitted to the faculty of the University Graduate School in partial fulfillment of the requirements for the degree

Master of Science

in the Department of Kinesiology of School of Public Health,

Indiana University

Accepted by the Graduate Faculty, Indiana University, in partial fulfillment of the requirements for the degree of Master of Science.

Master's Thesis Committee

_____

Robert Chapman, Ph.D.

_____

Timothy Mickleborough, Ph.D.

_____

Joel Stager, Ph.D.

ABSTRACT

O_2 delivery is dependent upon the ability of erythrocytes (diameter of ~8μm) to deform and to pass through the smaller microvasculature (~3μm). Reduced erythrocyte deformability in hypoxia could ultimately compromise O_2 delivery to the microvasculature of skeletal muscles during exercise, thus impairing performance. Chronic supplementation with omega-3 fatty acids (PUFAs) has been shown to increase erythrocyte deformability, which may improve oxygenation and endurance exercise performance in acute hypoxia. **PURPOSE:** To determine if chronic ω-3 PUFA supplementation improves erythrocyte deformability, $\dot{V}O_2$, and cycling time to exhaustion during acute hypoxic exercise. **METHODS:** Thirteen young, healthy, endurance-trained subjects were divided into PUFA ($\dot{V}O_2$max=60.2 ± 4.5 $mL \cdot kg^{-1} \cdot min^{-1}$, n=6) and placebo (66.2 ± 4.1 $mL \cdot kg^{-1} \cdot min^{-1}$, n=7) groups. Subjects completed 6 weeks of supplementation with either ω-3 PUFAs (PUFA group; 3g EPA, 2g DHA/day) or placebo (placebo group; safflower oil). Subjects performed identical experimental sessions in acute normobaric hypoxia (F_IO_2=15%) pre- and post-supplementation that consisted of 3 min cycling / 10 min rest at 25% and 50% of normoxic peak power, as well as cycling to exhaustion at 75% of normoxic peak power. Erythrocyte elongation index (EI) via ektacytometry, $\dot{V}O_2$, and time to exhaustion were recorded for each trial. **RESULTS:** EI at 20 Pa of shear stress was significantly greater (e.g. higher deformability) within the PUFA group post-supplementation (pre: 0.574 ± 0.004; post: 0.580 ± 0.003; $p<0.05$). EI at 20 Pa was significantly greater post-supplementation in the PUFA group compared to the placebo group (PUFA: 0.580 ± 0.003; placebo: 0.574 ± 0.006; $p<0.05$). $\dot{V}O_2$ only during the 50% trial was significantly greater within the PUFA group post-supplementation compared to pre, while no significant differences in $\dot{V}O_2$ were seen between groups at 25%, 50%, or 75% of peak power, or at exhaustion. No significant improvements were seen in time to exhaustion within either group and no difference was seen between groups. **CONCLUSION:** In acute hypoxia, chronic PUFA supplementation significantly, but marginally (1%) improves erythrocyte deformability, but does not have a significant effect on oxygen uptake or performance.

SHANE A. BIELKO

CHRONIC OMEGA-3 SUPPLEMENTATION AND ERYTHROCYTE DEFORMABILITY, OXYGEN CONSUMPTION, AND PERFORMANCE DURING NORMOBARIC HYPOXIC EXERCISE

Table of Contents

Chapter 1: Introduction 1
Chapter 2: Review of Literature 6
Chapter 3: Methodology 24
Chapter 4: Results 31
Chapter 5: Discussion 40
Conclusion 46
References 47
Supplemental Materials 50
Appendix 1. Subject Characteristic data. 50
Appendix 2. Time to exhaustion, HR, and $\dot{V}O_2$ data. 51
Appendix 3. Elongation index data in normoxia. 53
Appendix 4. Elongation index data in hypoxia. 57
Appendix 5. Informed Consent Form. 61
Appendix 6. Modified Physical Activity Readiness Questionnaire (PAR-Q). 66
Appendix 7. General Study Questionnaire. 68
Appendix 8. Omega-3 Food Frequency Questionnaire. 69

Chapter 1: Introduction

The ability to perform endurance exercise is dependent upon both the ability to deliver oxygen to the working muscles and the ability to extract oxygen for oxidative metabolism. Alterations in either of these systems result in decreased endurance performance. Acute exposure to hypoxia has been shown to reduce endurance performance, but the mechanisms at the cellular level are still relatively unknown.

Of the many proposed mechanisms responsible for the decline in performance in hypoxia, a reduction in erythrocyte deformability has not been extensively investigated. In the capillary, erythrocytes of approximately 8.3μm diameter must deform to be able to pass through the rigid capillaries with diameters as small as 3μm (13). If the erythrocytes are unable to properly deform, they do not pass through the capillary to complete the transfer of oxygen, and are instead diverted to "accessory channels" (33) resulting in regional areas of hypoxia in the muscle tissue. Reduced erythrocyte deformability has been shown in diseased states, such as Type 2 Diabetes, Sickle cell anemia, and Malaria Tropica (10). Additionally, acute exposure to hypoxia, as well as prolonged exercise in hypoxia (18), has shown similar results of decreased erythrocyte deformability (31, 36). Ultimately, if erythrocyte deformability compromises oxygen delivery (coupled with an already reduced arterial content), a larger decrease than expected in maximal oxygen uptake ($\dot{V}O_2max$) and performance may be seen in hypoxia. Similarly, any intervention which could increase erythrocyte deformability could hold the potential to mitigate $\dot{V}O_2max$ and/or performance declines during exercise in hypoxia.

Chronic supplementation with omega-3 polyunsaturated fatty acids (ω-3 PUFAs) has been shown to increase erythrocyte deformability in both humans and animals (5,

41). Mechanistically, ω-3 PUFAs increase the number of unsaturated acyl chains in the phospholipid membrane (27) causing total blood viscosity to decrease and erythrocyte deformability to increase as a result (27, 41). Moderate ω-3 PUFA supplementation has also been shown to increase $\dot{V}O_2max$ in normoxia through the above mechanisms described (27). However, ω-3 PUFAs have yet to be tested to see if they have an effect on the decrease in maximal oxygen uptake and exercise performance in hypoxia in highly trained endurance athletes.

Although alterations in blood rheology with chronic ω-3 PUFA supplementation have been observed in normoxia, whether or not these changes have any effect in hypoxia remains unknown. If ω-3 PUFA supplementation alters erythrocyte deformability and increases the effectiveness of oxygen delivery to the skeletal muscle microvasculature, there should be measurable changes in maximal oxygen uptake and/or exercise performance in highly trained athletes in hypoxia. If no changes are seen, then perhaps erythrocyte deformability is not a factor limiting endurance exercise in hypoxia.

Therefore, in this study, we examined $\dot{V}O_2max$ and endurance exercise performance of highly trained athletes in hypoxia and normoxia pre and post 6-week supplementation of ω-3 PUFAs or placebo.

Hypotheses:

1. Erythrocyte deformability will be increased after chronic (6 week) ω-3 PUFA supplementation.
2. Performance, measured as TTE, in acute hypoxia will be improved in normoxia and hypoxia after chronic ω-3 PUFA supplementation.

3. Oxygen consumption at termination of exercise in normoxia and acute hypoxia would be increased after chronic ω-3 PUFA supplementation.
4. Microvascular oxygenation/extraction at termination of exercise in acute hypoxia will be increased after chronic ω-3 PUFA supplementation.

Delimitations:

This study was delimited to the following:

1. Fifteen highly trained cyclists with a $\dot{V}O_2max \geq 55$ $mL \cdot kg^{-1}min^{-1}$.
2. The experimental trials were randomized and blinded for each subject.
3. Subjects were blinded in regards to which treatment they receive (placebo/omega-3).
4. Performance was measured by time to exhaustion at a constant workload.
5. Subjects performed trials at the same time of day.
6. Subjects were required to make five visits to the lab: a preliminary $\dot{V}O_2max$ test, pre-tests in normoxia and hypoxia, and post-tests in normoxia and hypoxia.

Limitations:

The data generated from this investigation was interpreted with the following limitations:

1) Subjects were selectively chosen to be highly trained.
2) Performance measures could not account for subject motivation.
3) Subjects will be completing the trials while breathing gas from a facemask in a laboratory (normobaric hypoxia) which is markedly different from competition/training these athletes may undergo at terrestrial altitude (hypobaric hypoxia).

4) The findings from this study can only be attributed to a simulated altitude of approximately 3000m (9900ft)

Assumptions:

This study was based upon the following assumptions:

1) Subjects represent highly trained endurance athletes in general.
2) The apparatus used to measure exercise oxygen consumption and ventilation does not alter the normal breathing strategies of the athlete.
3) Simulated altitude correlates with actual altitude.
4) TTE at a fixed workload is a valid measure of exercise performance.
5) Motivation to exercise does not change between trials.
6) Subjects will follow instructions pertaining to pre-exercise behavior, and training will remain the same during the supplementation period as the pre-supplementation period.
7) Subjects will follow the omega-3 supplementation treatment by ingesting the required daily dosage for the entire 6 weeks.

Statement of the problem:

Purpose:

There is tangential evidence of the effects of chronic ω-3 PUFA supplementation on endurance exercise performance in hypoxia, but no direct data. Therefore, the purpose of this study was to determine if chronic ω-3 PUFA supplementation improves erythrocyte deformability and if any changes are seen, do they result in increased microvascular oxygenation, and performance during maximal exercise in acute hypoxia.

Definition of Terms

Endurance trained athletes: Individuals who train regularly for endurance competition and have a $VO_2max \geq 55$ mL/kg/min.

Erythrocyte deformability: The ability of erythrocytes to alter their shape to pass through smaller diameter microvasculature.

Hypoxia: Decreased partial pressure of oxygen in the ambient environment compared to sea level. When discussing the literature, P_IO_2 and/or altitude will be given for context.

Normoxia: Partial pressure of oxygen in the ambient environment at sea level.

ω-3 PUFA: Omega-3 polyunsaturated fatty acids (see chapter 2 for more detail).

Oxygen Consumption ($\dot{V}O_2$): The volume of oxygen consumed by the body per 1 minute of time (in mL/min).

POmax: Maximal power output achieved during a VO_2max test in normoxia.

Chapter 2: Review of Literature

Effects of Acute Hypoxia on Performance

The main characteristic of hypoxia is decreased barometric pressure, which concomitantly results in decreased ambient partial pressure of oxygen, leading to a reduced partial pressure of inspired oxygen in humans (P_IO_2) (32). During rest in hypoxia, the inability to compensate for the decrease in P_IO_2 results in reduced partial pressure of oxygen in the alveoli (P_AO_2). This reduces the gradient for diffusion between the alveoli and capillaries, resulting in a lower partial pressure of oxygen in the arterial blood (P_aO_2) (3, 32, 46). To prevent S_aO_2 from decreasing, ventilation needs to be increased to maintain P_AO_2.

It is widely accepted that $\dot{V}O_2$max begins to decline at altitudes greater than 1500m in untrained or moderately trained individuals (3, 32), although it has been shown to decrease by as much as 4% at 1250m (40). Linear relationships have been made describing a 10% decrease in $\dot{V}O_2$ per 1000m increase in altitude (32), but there is much variability between subjects (7, 26, 42). Additionally, trained subjects have shown significant decreases in $\dot{V}O_2$ at an altitude of 580m, whereas untrained subjects showed no decrease (16). Furthermore, Lawler et al (26) examined the $\dot{V}O_2$ of trained and untrained subjects in acute hypoxic exposure of 3000m and found that more aerobically fit subjects had a greater reduction in $\dot{V}O_2$max (20.8%) than untrained subjects (10.2%). This difference can be contributed to larger declines in S_aO_2 in the trained subjects. Chapman et al (8) examined this relationship and found that the extent of performance decline (3000m track time trials in highly trained runners) at altitude was significantly related to the drop in S_aO_2. Since significant correlations have already been shown

between decreased S_aO_2 and $\dot{V}O_2max$ impairment (6, 14, 15), it can be assumed that $\dot{V}O_2max$ decrements and performance impairments would also be strongly correlated.

Erythrocyte Deformability

During normal blood flow at rest, blood ejects from the left ventricle into the aorta, where it then travels through progressively smaller diameter vessels until it reaches the microcirculation. In the microcirculation, vital exchange of oxygen and carbon dioxide occurs between blood and tissues. In order for this exchange to occur, erythrocytes must pass through the smallest vessels of the microcirculation, the capillaries. This poses an issue as normal erythrocyte diameter exceeds 8 μm and it must pass through the rigid vessel wall of the capillary that can be as small as 3μm in diameter (44). In order for the erythrocyte to flow through the capillary, it must deform or flex in such a way where it is able to fit through the smaller diameter capillary. This phenomenon is referred to as erythrocyte deformability, and can be defined as all of the geometric and physical characteristics that allow erythrocytes the ability to pass through capillaries with diameters smaller than the erythrocyte itself (44). Therefore, an erythrocyte with high deformability has a greater capacity to pass through small capillaries.

Since capillary flow is bolus flow, that is single file flow of erythrocytes, the properties of the flow inside the capillary are rather unique (25). Canham and Burton (4) suggest that one critical factor that affects capillary flow is the minimum cylindrical diameter (MCD). MCD is described as the smallest cylindrical channel through which a flexible erythrocyte can pass through without increasing its membrane area. It has been

shown that for normal human erythrocytes, a MCD of approximately 2.8 µm is required for a channel 12µm in length (17, 45). If the channel length is greater than 12µm with a MCD of 2.8µm, the channel becomes prohibitive and the erythrocyte only enters partially into the channel. The remainder of the erythrocyte remains outside of the capillary incapable of deforming, and can become trapped (25).

Parthasarathi and Lipowsky (33) explored this mechanism by infusing rats with fluorescently labeled rat and human erythrocytes in hypoxia, which has been shown to increase regional blood flow and functional capillary density in the microcirculation. Erythrocytes were injected into the femoral artery contralateral to the cremaster muscle. They found that during the infusion of rat erythrocytes, cardiac output and vascular volume increased threefold in hypoxia. Normalized transit time and tissue hematocrit were not significantly different. During infusion of human erythrocytes, normalized transit time and tissue hematocrit decreased significantly (Figures 1 and 2, respectively). Cardiac output was increased but vascular volume did not exhibit an increase, suggesting that the erythrocytes were redistributed to larger-diameter pathways within the microcirculation. Therefore, if less deformable erythrocytes cannot fit through small diameter capillaries, they must be rerouted or they risk becoming trapped in the capillary.

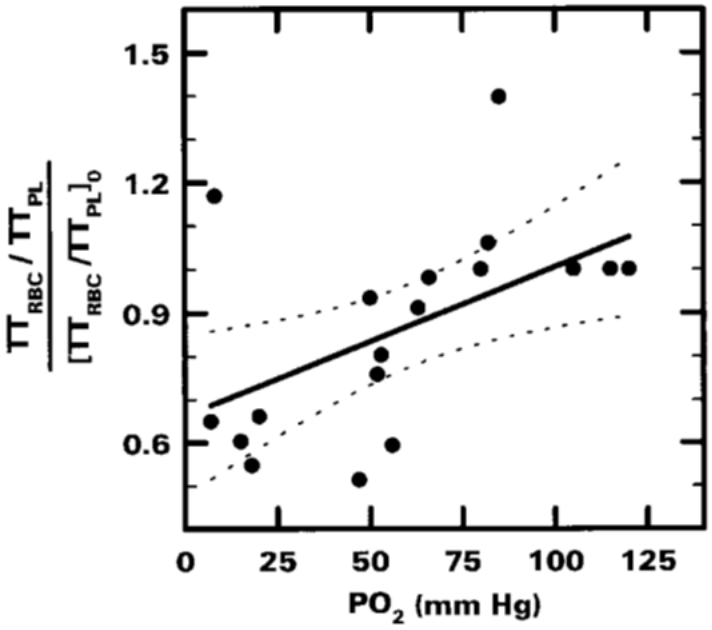


Figure 1. Normalized transit time (TT_{RBC}/TT_{PI}) during bolus infusions of human erythrocytes in 5 networks with normal vascular tone. Transit time decreased significantly ($P<0.01$) because of confinement of human erythrocytes to more centralized pathways of network, thus reflecting an attenuation of capillary recruitment (33).

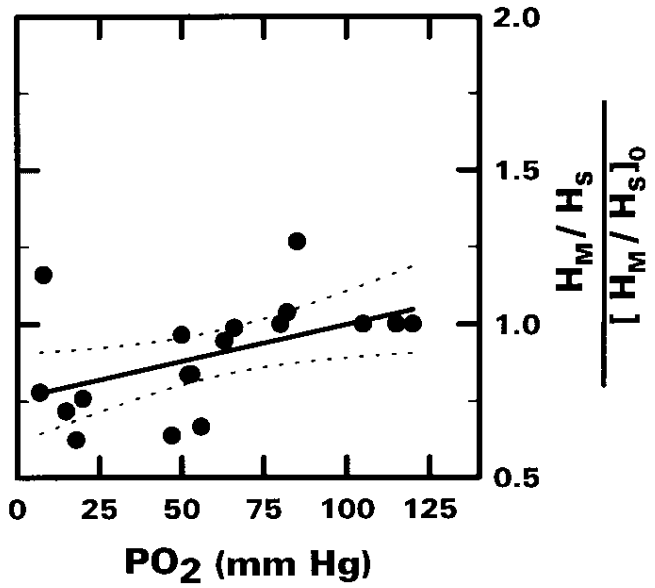


Figure 2. Microvascular hematocrit (H_M) with human erythrocytes perfusing normal tone vascular network plotted as a function of PO_2. H_M decreased with decreasing PO_2,

consistent with an attenuated increase in erythrocyte compartment volume. Hence, the larger and less deformable human erythrocytes were unable to take advantage of the increase in number of pathways generated by hypoxia (33).

Driessen et al (12) found similar results on rats by using the SH-oxidizing agent diamide to rigidify erythrocytes. Erythrocytes were incubated at two concentrations (0.5 $mmol \cdot l^{-1}$ and 1.5 $mmol \cdot l^{-1}$). After a control period of 30 min, 60-70% of the cells were exchanged for the rigidified cells. Then, they instilled a hypotension period of 30 min by bleeding the rats to a pressure of 4.7 kPa. They found that for the 0.5 $mmol \cdot l^{-1}$ group, erythrocyte velocity decreased from 0.91 $mm \cdot s^{-1}$ to 0.61 $mm \cdot s^{-1}$ (30% reduction from the control group). Additionally, during the hypotension period, erythrocyte velocity decreased to 0.04 $mm \cdot s^{-1}$ and flow stoppage was seen in many vessels. Surprisingly, further rigidification of erythrocytes led to a smaller decrease in erythrocyte velocity (14% reduction from the control group). These findings suggest that reduced erythrocyte deformability is the primary cause for disturbance of blood flow in the microcirculation, especially in the small capillaries.

Erythrocyte deformability is dependent upon three factors: the maintenance of the biconcave shape to maintain a high surface area ratio, normal internal fluidity of the cell, and intrinsic membrane deformability (44). The composition of the phospholipid cell membrane has shown to have a large impact on total blood viscosity and erythrocyte deformability (5, 41). When the acyl chains of the membrane phospholipid are unsaturated, the lipid fluidity of the erythrocyte membrane increases. Furthermore, this change causes total blood viscosity to decrease and erythrocyte deformability to increase

as a result (27, 41). Weed (44) states that a "decrease in the ratio of surface area to volume either by osmotic swelling or by loss of effective surface area will result in progressive loss of the biconcave shape and decrease in the ability of the cell to negotiate restricted passages within the microcirculation." Various chemical agents can alter the biconcave shape causing a decrease in surface area (34), including ATP depletion (29). Regeneration of ATP reverses this effect.

Maintenance of the internal fluidity of erythrocytes is regulated by two main factors. First, regulation of appropriate intracellular hemoglobin is needed. Abnormal hemoglobins, such as in sickle cell disease (20) and hemoglobin C disease (9, 28), are predisposed to intracellular crystallization resulting in cellular rigidity and reduced lifetime. Furthermore, Heinz body formations (denatured hemoglobin molecules within erythrocytes) also cause cellular rigidity and if in great enough numbers, lead to premature removal from blood circulation (37).

Lastly, intracellular ATP, calcium, and magnesium levels in erythrocytes have all been determined to be important in maintaining intrinsic membrane deformability (45). Weed (44) states that when intracellular calcium reacts with the erythrocyte membrane, the membrane becomes very rigid. This effect can be minimized by intracellular ATP binding calcium and magnesium, creating less calcium to react with the membrane. Therefore, high intracellular ATP levels are critical in maintaining erythrocyte deformability.

Decline of Erythrocyte Deformability at Altitude

It is apparent that the human erythrocyte has a great capacity to alter its deformability intrinsically. However, external factors can also play a significant role in altering erythrocyte deformability, such as altitude. La Celle and Weed (24) first demonstrated changes in erythrocyte deformability at low oxygen pressures. In this study, deformability was measured as the negative pressure sufficient enough to cause the erythrocyte to pass through a 2.9 μm diameter micropipette (P_t), which is similar to the MCD found by Canham and Burton (4). For normal erythrocytes, this is 6.8 mm H_2O. No reduction in P_t was found with a PO_2 as low as 30 mmHg. However, between 30 and 20 mmHg PO_2, P_t significantly increased to 45 mm H_2O, and as PO_2 approached zero, P_t rose to 500 mm H_2O. When PO_2 levels were restored to above 30 mmHg, normal deformability was restored. La Celle and Weed conclude that low levels of PO_2 significantly decrease erythrocyte deformability.

Continuation of La Celle and Weed's (24) research has examined the effect of hypoxia on erythrocyte deformability at a variety of conditions and in different species as well. Hakim and Macek (19) examined the effect of hypoxia (PO_2 = 40-70 mmHg) on erythrocyte deformability in various mammalian species. They concluded that changes in deformability are highly species dependent. A significant decrease in deformability in hypoxia was shown in rats, cats, rabbits, and hamsters, but no changes were seen in dogs or pigs (Figure 1). They contribute these findings to species component dependency of the hypoxic pressor response (HPR), which is an increase in arterial blood pressure in response to hypoxia. They propose that the HPR has two components: smooth muscle contraction (vasoconstriction) and capillary obstruction. The HPR for a species depends on the ratio of these factors. For example, the results suggest that the HPR in pigs is

independent of changes in erythrocyte deformability. Conversely, the HPR in rats is highly dependent upon change in erythrocyte deformability. Using a similar model, Kaniewski et al (23) found a decrease in erythrocyte deformability in rats, but no change in cat, dog, human, or rabbit in response to hypoxia (PO_2 = 47 ± 6 mmHg, Figure 2). Since these studies used in vitro models, we cannot say that changes do not occur in vivo. Thus, using animal models to correlate similar effects in humans might not be appropriate.

Figure 1. Mean relative filtration time (measure of erythrocyte deformability) during normoxic and hypoxic conditions (19).

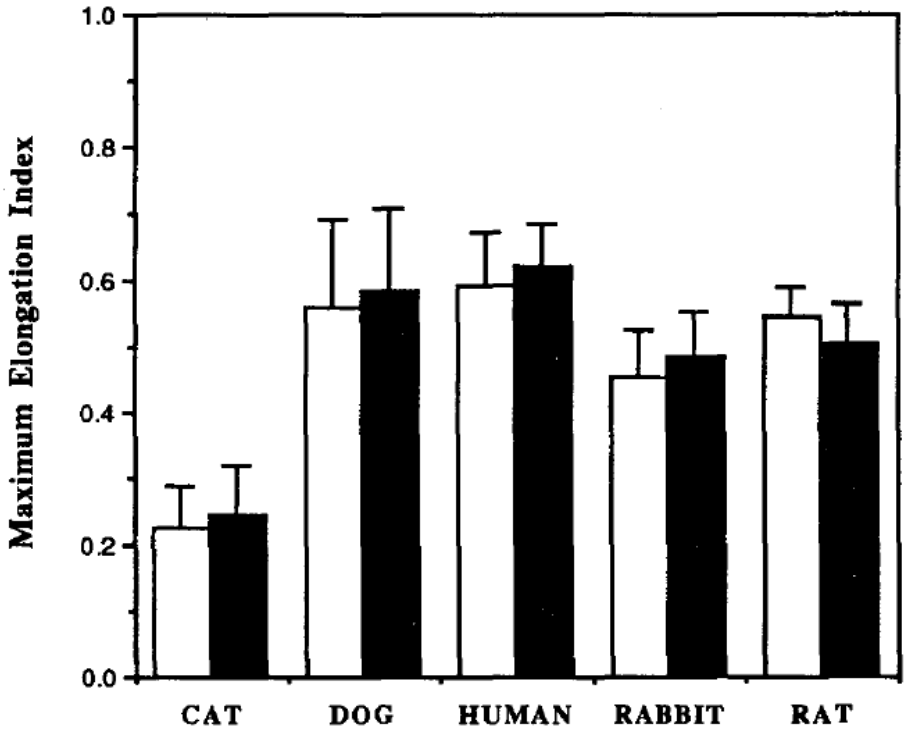


Figure 2. The maximum elongation index (EI_{max}) in normoxic (empty bars) and hypoxic (filled bars) conditions for erythrocytes from cats, dogs, humans, rabbits, and rats (23).

Additionally, variations in methodology have produced conflicting results. Hakim and Macek's (19) results were shown in animals in vivo. However, Doyle and Walker (11) performed similar a similar protocol on rats but instead examined isolated rat lungs in vitro. In line with Hakim and Macek's findings, they found that a reduction in erythrocyte deformability increased the HPR. Conversely, they found no difference in erythrocyte deformability between normoxic and hypoxic conditions. From this, Doyle and Walker concluded that acute hypoxic exposure does not affect erythrocyte deformability. However, it is not entirely known if either of these findings are translational to humans.

Although animal models have yielded conflicting results, Palareti et al (31) found that chronic high altitude exposure during a climbing expedition resulted in the deterioration of the rheologic properties of the blood. Fourteen professional male climbers were examined over the course of a 61 day climb to between 5250 and 7350m (P_IO_2 = 53-73 mmHg, Figure 3). Upon return to normoxia, significant decreases were shown in relative viscosity, fibrinogen, platelet count, and erythrocyte deformability. Treatment with Pentoxifylline, a pharmaceutical drug shown to decrease blood viscosity, was shown to attenuate these decreases, with only platelet count showing a significant decrease after the expedition. In regards to erythrocyte deformability, the authors state that when in hypoxia, deoxygenated hemoglobin is increased and thus, may exert a binding effect on intracellular ATP in the erythrocyte. As noted earlier, maintenance of intracellular ATP in the erythrocyte is one of the governing factors regulating erythrocyte deformability. Therefore, decreased erythrocyte deformability, due to decreased levels of intracellular ATP caused by higher concentration of deoxygenated hemoglobin, might be a limiting factor of exercise performance in hypoxia. To our knowledge, no studies have examined this relationship yet.

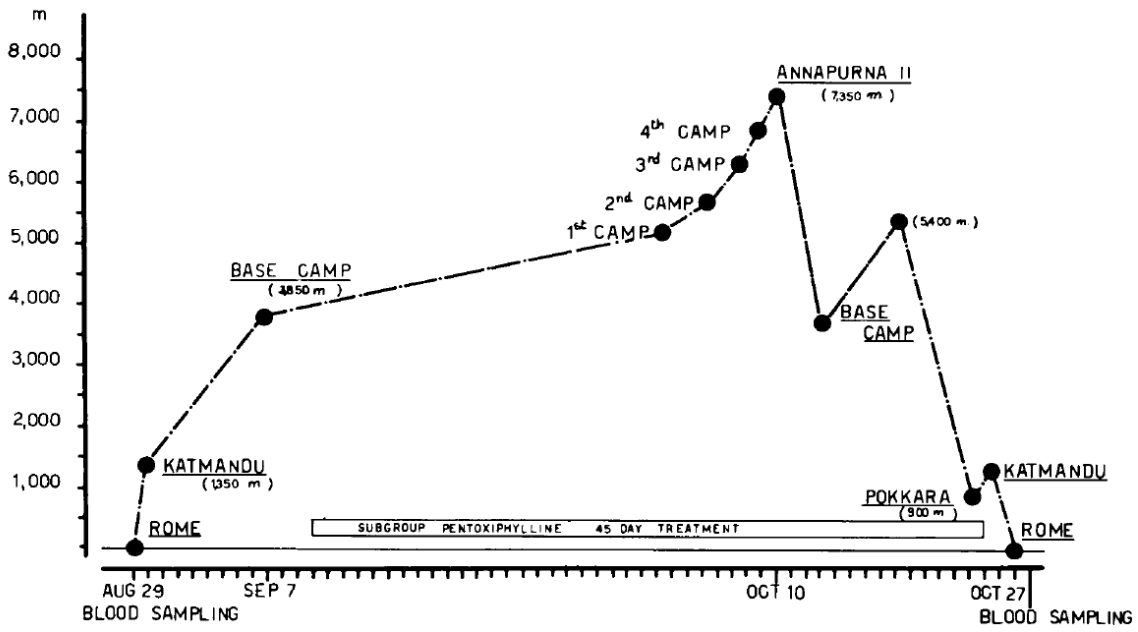


Figure 3. Altimetric graph of Palareti et al's climbing expedition (31).

Acute altitude exposure has also been shown to decrease erythrocyte deformability (18). Fourteen trained subjects were randomized into two groups: a control group that ate a "standard diet" consisting of fish and seafood no more than once a week, and a treatment group given 6g of ω-3 PUFA daily (18.1% EPA and 13.6% DHA by weight among other fatty acids; listed in Table 1). Subjects performed 1 hour of cycling at an intensity eliciting 70% of their $\dot{V}O_2max$ in normoxia and hypoxia (simulated altitude of 3000m, P_IO_2 = 101 mmHg). Tests were performed pre- and post-supplementation. They found that before supplementation in both groups, index of filtration I, which increases as erythrocyte deformability decreases, was significantly higher after exercise in hypoxia (Figure 4). Additionally, they found that after 6 weeks of ω-3 PUFA supplementation, there was no significant difference in erythrocyte deformability between normoxic and hypoxic exercise. Erythrocyte deformability was

significantly decreased in hypoxia in the control group. Therefore, the results show that not only does exercise in hypoxia decrease erythrocyte deformability, but that this decrease can be prevented with 6 weeks of ω-3 PUFA supplementation (Figure 5). The authors contribute the decrease in deformability to higher blood levels of ADP/AMP and an increase of 2-3 DPG blood concentration. However, it is not known exactly what fish oil affects to cause a change in deformability.

Fatty acid		Weight %
14:0		7.5
14:1		0.6
16:0		21.6
16:1		11.1
18:0		3.3
18:1		13.4
18:2	n-6	1.9
18:3	n-3	0.4
20:1		1.2
20:4	n-6	2.1
20:5	n-3	18.1
22:1		1.7
22:4	n-6	0.2
22:5	n-3	2.2
22:6	n-3	13.6

The fatty acid composition is described with the following notation C x:Y n-b.
x = the number of carbon atoms;
y = the number of double bonds;
b = the position of double bonds from the terminal carbon.

Table 1. Fatty acid composition of ω-3 PUFAs (18).

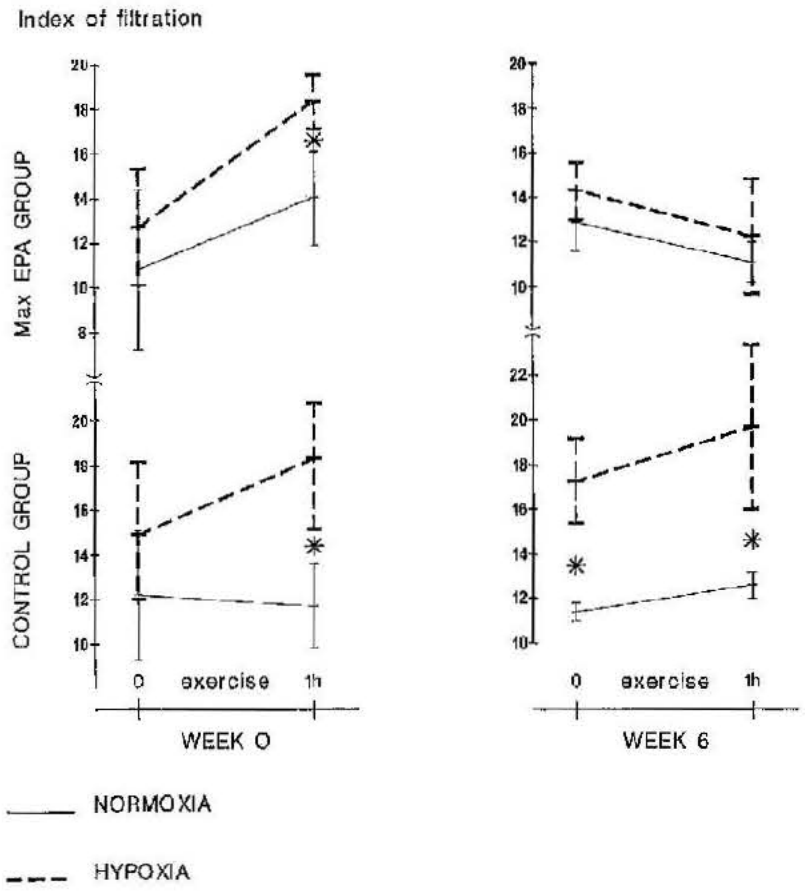


Figure 4. Changes in the index of filtration I (red cell suspension pressure/buffer pressure) following exercise performed in normoxia and hypoxia in control and MaxEPA groups (18).

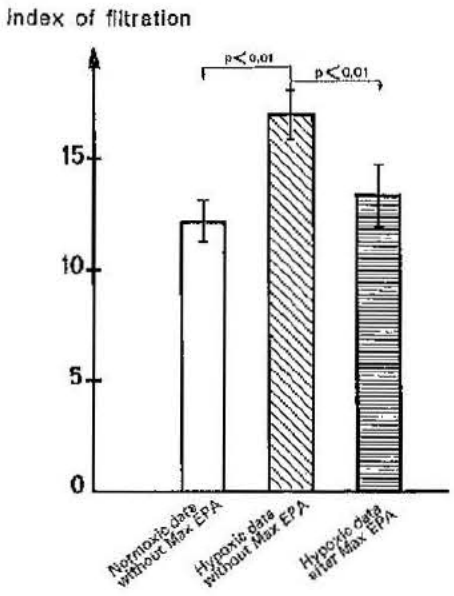


Figure 5. Comparison of the index of filtration I of erythrocytes under normoxic conditions without MaxEPA, hypoxic conditions without MaxEPA, and hypoxic conditions after 6 weeks MaxEPA supplementation (18).

Effect of ω-3 PUFA Supplementation on Erythrocyte Deformability

Chronic supplementation with ω-3 PUFAs (specifically eicosapentaenoic acid, EPA, and docosahexaenoic acid, DHA) is a possible treatment to increase erythrocyte deformability. While an extensive amount of research has been conducted on this topic, there is no data, to our knowledge, examining this treatment in endurance trained athletes.

ω-3 PUFAs are fatty acids with a double bond at the third carbon atom from the end of the carbon chain and contain at least one other double bond in the carbon chain. In humans, ω-3 PUFAs are unable to be synthesized and must come through diet. The most important ω-3 PUFA is α-linolenic acid (18:3ω-3, ALA), which contains 18 carbon atoms with 3 double bonds and is sourced from plants, seeds, and nuts. ALA is considered an essential fatty acid because other ω-3 PUFAs can be synthesized from it,

such as eicosapentaenoic acid (20:5ω-3, EPA) and docosahexaenoic acid (22:6ω-3, DHA). EPA and DHA play an important role in a number of physiological processes and can also be absorbed through the diet from seafood and marine oils.

Early research examining the effects of ω-3 PUFA supplementation on erythrocyte deformability yielded promising results. Terano et al (41) examined 8 healthy adult males before and after 4 weeks of supplementation of EPA (3.6g EPA/day). They found no change in plasma viscosity, a decrease in whole blood viscosity, and an increase in erythrocyte deformability following supplementation (Figure 6). Additionally, there was a positive correlation between erythrocyte deformability and EPA content in the phospholipid membrane of erythrocytes (Figure 7). Cartwright et al [8] found the same results using a similar protocol. They examined 5 healthy adult males before, after 3 weeks, and after 6 weeks of omega-3 EPA supplementation (3.4g EPA/day). Erythrocyte deformability was significantly increased after 3 and 6 weeks of EPA supplementation (Figure 8), resulting in a reduction in whole blood viscosity. Since plasma viscosity was unaffected by supplementation, the increase in erythrocyte deformability was responsible for the decrease in whole blood viscosity. Also, the viscoelastic properties of the erythrocyte membrane are dependent upon EPA content. Therefore, EPA supplementation represents an effective way to increase the elasticity and deformability of erythrocytes.

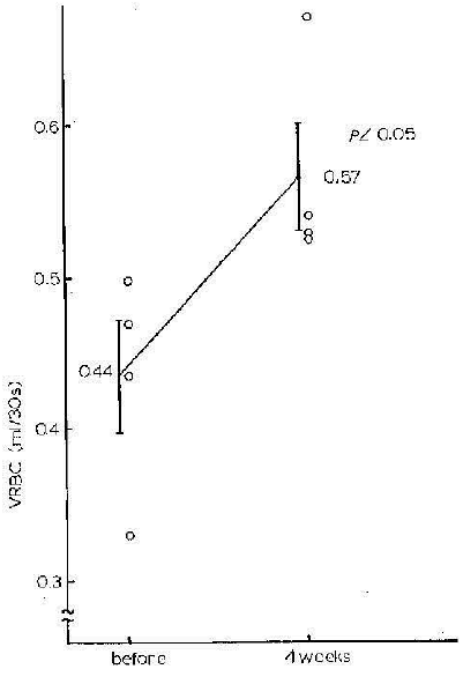


Figure 6. Erythrocyte deformability (V_{RBC}) before and after 4 weeks of EPA supplementation (41).

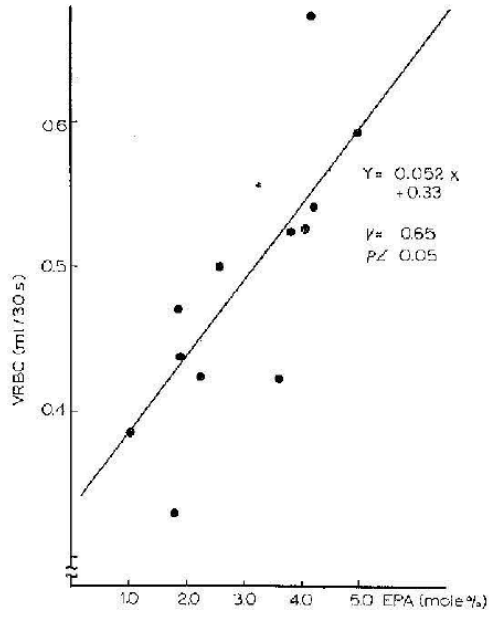


Figure 7. Correlation between erythrocyte deformability (V_{RBC}) and EPA content in erythrocyte phospholipids (41).

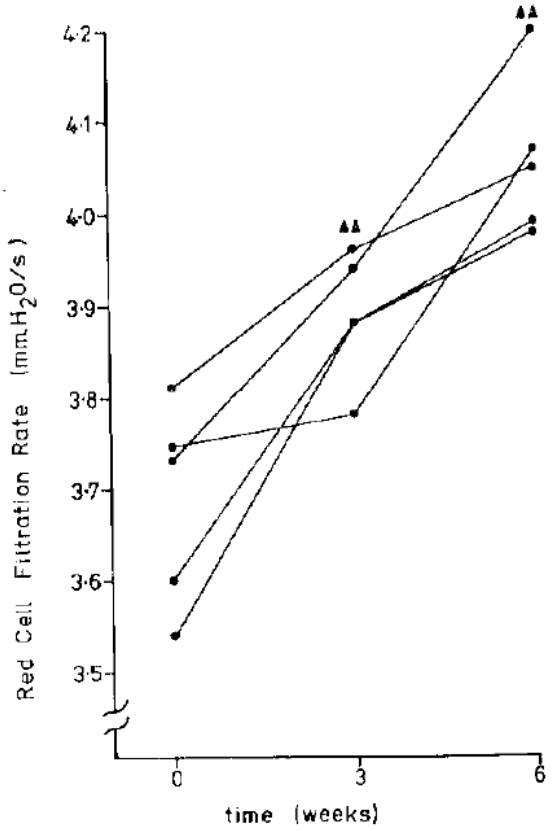


Figure 8. Erythrocyte deformability, expressed as red cell filtration rate (mm·H_2O/s), before (week 0) and after 3 and 6 weeks EPA supplementation (5).

Although some studies have shown an increase in erythrocyte deformability following ω-3 PUFA supplementation, a number of studies have shown no such change. Rillaerts et al (38) gave 20 men with coronary heart disease 6 weeks of ω-3 PUFA supplementation (0.9g EPA, 0.6g DHA). Following supplementation, there was no change in erythrocyte deformability, as shown by a lack of decrease in whole blood viscosity and erythrocyte viscosity. The lower amount of fish oil given (1.5g total compared to 3.6g and 3.4g in the Terano (41) and Cartwright (5) studies, respectively) could explain the differences in the results shown. However, Blonk et al (2) found no

significant difference in erythrocyte deformability following 12 weeks of supplementation with 1.5g, 3.0g, and 6.0g of ω-3 PUFAs, despite finding a dose-response increase in the amount of ω-3 PUFAs incorporated into the phospholipid membrane. Oostenbrug et al (30) and Ho et al (22) showed the same results following 3 weeks and 12 weeks of ω-3 PUFA supplementation, respectively. With the lack of consistency in the methodology, namely the supplementation amount, length, and measurement for erythrocyte deformability, the results are conflicting. It is evident that further research, using a standard procedure for measuring erythrocyte deformability and supplementation length, is needed to create reproducible studies to allow for fair comparisons.

Chapter 3: Methodology

The purpose of this study was to determine the effect of chronic ω-3 PUFA supplementation on skeletal muscle oxygenation, oxygen uptake, and endurance exercise performance in highly trained cyclists. This study utilized a placebo controlled mixed model design, with ω-3 PUFA treatment as a between group factor and pre/post treatment as a within groups factor. Primary dependent variables were erythrocyte elongation index (EI), oxy/deoxy/total-hemoglobin and myoglobin (Hb+Mb; from near infared spectroscopy, NIRS), exercise metabolic measures, and time to exhaustion at a power output equal to 75% of POmax.

Subjects: The study was performed on highly trained cyclists (n=13; sample size from power analysis of (18, 43)). Inclusion criteria for the highly trained cyclists included indication on a questionnaire that they considered themselves to be an endurance trained individual and a $\dot{V}O_2max$ greater than 55 $ml \cdot kg^{-1} \cdot min^{-1}$. Exclusion criteria for all subjects included: current smoker or smoking history in the last 12 months, history of hypertension, cardiovascular, or pulmonary disease, musculoskeletal or other existing health condition which preclude participation in physical activity, abnormal ECG or blood pressure response to physical activity based on the maximal cycle ergometry test, resting systolic BP > 130 mmHg and resting diastolic BP > 90 mmHg, women who are or believe they could be pregnant, who are post-menopausal, or who are taking birth control or hormone replacement therapy, known allergy to ω-3 or ω-6 PUFAs, fish intake > 1 serving per week, fish oil or omega-3 supplementation over the previous 4 weeks, and BMI > 32 kg/m^2.

Study Design: On five separate days, subjects were asked to report to the laboratory to complete testing protocol. On Day 1, subjects were asked to complete the informed consent form at the Human Performance Laboratory. Subjects then performed a maximal oxygen uptake exercise test on a cycle ergometer. Subject candidates were informed of their eligibility to continue their participation in the study ($\dot{V}O_2max > 55$ $mL \cdot kg^{-1} \cdot min^{-1}$). Days 2 and 3 used the same testing procedures. Subjects were asked to complete a submaximal cycle ergometry exercise test (explained in detail later). Subjects breathed either normoxic (room) air or hypoxic (15% O_2) air for exercise trials, randomized between days. After Day 3, subjects underwent supplementation for six weeks (explained in following section). Then, on Days 4 and 5 subjects followed the same testing procedures as Days 2 and 3.

Treatment: Subjects were pair matched by age and baseline EI, and then randomized into ω-3 PUFA and placebo treatment groups. The ω-3 PUFA treatment consisted of a daily dose of 8 gel capsules totaling 2g of (DHA), 3g of (EPA), and 100mg of Vitamin E, taken daily for 6 weeks (Table 2). This dosage and supplementation duration has been shown in healthy subjects to improve both brachial arterial blood flow during forearm exercise (43) and erythrocyte deformability (18). The placebo groups were given iso-caloric doses (matching the ω-3 PUFA doses) of safflower oil capsules, an ω-6 PUFA shown not to affect erythrocyte deformability or endothelial function (Table 3). Subjects were asked to continue their exercise training as normal throughout the duration of the study. Additionally, subjects were asked to provide training logs for 6 weeks prior to participating in the study, as well as during the duration of the study.

Subjects were asked to keep a daily supplement diary and to return the supplement diary, medication containers, and unused pills at their final testing session.

Fatty Acid	Weight %
4:0	0
6:0	0
8:0	0.5
10:0	0.3
12:0	0
14:0	0.4
14:1	0
15:0	0
16:0	2.8
16:1	1.1
18:0	3.7
18:1	10.3
18:2ω-6	1.2
18:3ω-6	0.2
18:3ω-3	0.7
18:4ω-3	1.8
20:0	0.4
20:1	2.5
20:2ω-6	0.3
20:3ω-6	0.3
20:4ω-6	0.2
20:3ω-3	1.6
20:4ω-3	1.5
20:5ω-3	36.5
22:0	0.2
22:1	1.8
22:2ω-6	0
22:4ω-6	0
22:5ω-6	0.7
22:5ω-3	4.1
22:6ω-3	26.3
24:0	0
24:1	0.5

Table 2. Fatty acid composition of ω-3 supplement. The fatty acid composition is described with the following notation X:Yω-B

X: the number of carbon atoms

Y: the number of double bonds

B: the position of double bonds from the terminal carbon

Fatty Acid	Weight %
4:0	0
6:0	0
8:0	1.2
10:0	0.1
12:0	0
14:0	0.1
14:1	0
15:0	0
16:0	10.8
16:1	0.1
18:0	2.8
18:1	23.9
18:2ω-6	53.8
18:3ω-6	0
18:3ω-3	5.5
18:4ω-3	0
20:0	0.2
20:1	0.2
20:2ω-6	0
20:3ω-6	0
20:4ω-6	0
20:3ω-3	0
20:4ω-3	0
20:5ω-3	0
22:0	0.3
22:1	0
22:2ω-6	0
22:4ω-6	0
22:5ω-6	0
22:5ω-3	0.9
22:6ω-3	0
24:0	0
24:1	0

Table 3. Fatty acid composition of placebo supplement. The fatty acid composition is described with the following notation X:Yω-B

X: the number of carbon atoms

Y: the number of double bonds

B: the position of double bonds from the terminal carbon

Exercise Protocols

Maximal Oxygen Uptake Cycle Ergometry Exercise Test: This test measures the maximal rate of oxygen uptake, and is often referred to as a $\dot{V}O_2$max test. This test was performed on an electronically braked cycle ergometer (Velotron, RacerMate Inc., Seattle, WA), where the resistance was automatically adjusted, depending on the pedaling cadence the subject used, to equal the set workload. During the test, subjects were fitted with a fingertip pulse oximeter, heart rate monitor, NIRS sensor, and oro-nasal face mask. All testing equipment was adjusted to maximize subject comfort. The test began with the subject pedaling at a self-selected cadence between 60 and 100 rpm. The workload started at 75W and increased 25W every minute. The test ended when the subject could no longer maintain the required power output or the subject voluntarily stopped (volitional fatigue). Following the test, subjects were allowed to cool down for up to 10 minutes, pedaling at a self-selected intensity.

Cycle ergometry test: Subjects performed three exercise trials on a cycle ergometer. Each trial consisted of 3 min of cycling at 25%, 50%, and 75% of POmax, with each workload preceded by 1 min of unloaded pedaling (to control for any NIRS movement artifact). Between trials, subjects rested for 5 minutes. In the 75% trial, after the 3rd minute subjects continued at this workload until they were unable to maintain power output (fall under 60 rpm) or volitional exhaustion. Time to exhaustion was recorded.

Hypoxic inspirate: Submaximal cycle exercise protocols were performed in normoxia and normobaric hypoxia (inspired O_2 fraction 15%; equivalent to an altitude of

3000m / 9900ft at typical barometric pressure of Bloomington, IN). Subjects were blinded to the inspirate, and the order of the inspirate was randomized and counterbalanced between Days 2-3 and Days 4-5. The hypoxic inspirate was delivered from pre-mixed gas cylinders into a 100 L balloon reservoir, attached to the inspired side of a Hans Rudolph face mask. Fractional oxygen content of each tank was verified by mass spectrometry by the manufacturer and was verified in our laboratory using a metabolic cart (Vmax Encore Metabolic Cart, CareFusion Corporation, San Diego, CA). Subjects breathed the inspirate for 10 minutes at rest prior to the initial cycle exercise workloads, during the complete exercise bout, and during all rest periods between bouts. Arterial oxyhemoglobin saturation was continuously monitored with fingertip pulse oximetry (Model PC-68A, Shenzen Creative Industry Co, Ltd., Shenzen, China).

Near-infrared Spectroscopy (NIRS): NIRS (ISS Model 96208, ISS, Inc., Champaign, IL) was used in vivo to non-invasively determine the volume of heme-O_2 carriers in the exercising muscle microcirculation. Prior to testing, the NIRS sensor was calibrated at two different wavelengths of near-infrared light using a manufacture provided calibration block. For the submaximal cycle exercise trials, the NIRS sensor was placed on the surface of the right vastus lateralis (15 cm proximal from the middle of the proximal border of the patella, then 5 cm lateral). Device probes were secured with a Velcro strap and wrapped with a cloth bandage to prevent movement and light leakage. Position of the device probe was marked with indelible marker and photographed to match placement between trials.

Metabolic measures: For all trials, subjects were fitted with a face mask (#2700, Hans Rudolph Inc., Shawnee, KS) attached to a two-way non-rebreathing valve.

Ventilation and expired gas concentrations were continuously measured, with these variables and oxygen uptake measured on a breath by breath basis.

Erythrocyte deformability: Ektacytometry (RheoScan-D300, Sewon Meditech, Inc., Seoul, Korea) was used to measure the elongation index (EI) of erythrocytes before and after exercise. EI measurements from the RheoScan-D300 have been validated in the literature (39). This device required only 5 µL of blood for processing; therefore finger prick capillary samples were collected. Finger prick samples were collected at rest and immediately following the cycle exercise protocol. Therefore, on each of Days 2, 3, 4, and 5, each subject gave 2 finger prick samples, for a total of 8 for the entire study. Prior to testing, the ekyacytometer was calibrated using an internal calibration procedure.

Statistical analysis: This study utilized multiple 2 x 2 split plot, repeated measures ANOVAs with time (pre, post) as the within subject variable and treatment (ω-3 or placebo) as the between subject variable. Separate ANOVAs were utilized for each dependent variable of interest. Tests of a priori simple main effects were used to determine differences in pre- and post- measures within each treatment group. An alpha of $p<0.05$ was used to indicate statistical significance.

Chapter 4: Results

Subject Characteristics

Table 4. Subject Characteristics

Characteristics	ω-3 PUFA	Placebo
Age (years)	20.3 ± 1.4	21.1 ± 2.4
Mass (kg)	78.9 ± 7.3	76.1 ± 6.4
Height (cm)	177.6 ± 7.7	180.5 ± 4.0
$\dot{V}O_2max$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	60.2 ± 4.5	67.3 ± 2.9
Sex	5M, 1F	7M

Values are presented as mean ± SD.

Erythrocyte Deformability

In hypoxia, post-exercise elongation index was significantly increased from pre- to post-supplementation in the ω-3 PUFA group at 17 Pa (pre: 0.512 ± 0.006; post: 0.519 ± 0.004) and 20 Pa (pre: 0.574 ± 0.004; post: 0.580 ± 0.003) of shear stress (Table 5). No significant pre/post-supplementation differences were seen at any of the other shear stresses within either experimental group. Post-exercise elongation index in the ω-3 PUFA group was significantly greater than placebo following supplementation at 20 Pa, but no significant differences between groups were seen at any of the other shear stresses.

In normoxia, there were no significant differences from pre- to post-supplementation in elongation index within either group at any shear stress (Table 6). Additionally, there were no significant differences in elongation index between groups at any shear stress in normoxia.

Table 5. Elongation Index in hypoxia, post-exercise.

	2 Pa		3 Pa		4 Pa		5 Pa		6 Pa		7 Pa	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
ω-3 PUFA	0.205 ± 0.022	0.219 ± 0.026	0.270 ± 0.022	0.281 ± 0.023	0.305 ± 0.011	0.310 ± 0.011	0.320 ± 0.006	0.323 ± 0.007	0.330 ± 0.005	0.333 ± 0.006	0.340 ± 0.005	0.344 ± 0.006
Placebo	0.213 ± 0.039	0.209 ± 0.026	0.273 ± 0.030	0.274 ± 0.032	0.305 ± 0.015	0.306 ± 0.018	0.331 ± 0.008	0.320 ± 0.010	0.331 ± 0.008	0.331 ± 0.008	0.343 ± 0.009	0.342 ± 0.008
	8 Pa		10 Pa		12 Pa		15 Pa		17 Pa		20 Pa	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
ω-3 PUFA	0.353 ± 0.006	0.357 ± 0.006	0.382 ± 0.006	0.388 ± 0.007	0.416 ± 0.007	0.423 ± 0.007	0.472 ± 0.006	0.480 ± 0.006	0.512 ± 0.006	†0.519 ± 0.004	0.574 ± 0.004	*† 0.580 ± 0.003
Placebo	0.356 ± 0.011	0.355 ± 0.009	0.387 ± 0.013	0.385 ± 0.009	0.421 ± 0.013	0.419 ± 0.009	0.477 ± 0.011	0.475 ± 0.008	0.516 ± 0.009	0.514 ± 0.007	0.576 ± 0.004	0.574 ± 0.006

Pre/Post-supplementation Elongation Index at various shear stresses following experimental trials in hypoxia.

Note: Values are presented as mean ± SD.

Pa, Pascals of shear stress.

* denotes significant difference between groups ($P < 0.05$).

† denotes significant difference in pre/post-supplementation measurements ($P < 0.05$).

Table 6. Elongation Index in normoxia, post-exercise.

	2 Pa		3 Pa		4 Pa		5 Pa		6 Pa		7 Pa	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
ω-3 PUFA	0.214 ± 0.033	0.214 ± 0.030	0.275 ± 0.025	0.277 ± 0.024	0.307 ± 0.012	0.308 ± 0.012	0.321 ± 0.007	0.321 ± 0.007	0.332 ± 0.007	0.332 ± 0.006	0.343 ± 0.008	0.343 ± 0.006
Placebo	0.219 ± 0.041	0.212 ± 0.018	0.278 ± 0.030	0.277 ± 0.014	0.308 ± 0.014	0.308 ± 0.007	0.322 ± 0.008	0.322 ± 0.003	0.333 ± 0.008	0.332 ± 0.003	0.344 ± 0.009	0.342 ± 0.004
	8 Pa		10 Pa		12 Pa		15 Pa		17 Pa		20 Pa	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
ω-3 PUFA	0.356 ± 0.010	0.356 ± 0.007	0.386 ± 0.011	0.386 ± 0.009	0.420 ± 0.012	0.420 ± 0.009	0.476 ± 0.010	0.475 ± 0.009	0.515 ± 0.008	0.513 ± 0.009	0.576 ± 0.003	0.572 ± 0.009
Placebo	0.358 ± 0.011	0.355 ± 0.004	0.389 ± 0.012	0.385 ± 0.005	0.423 ± 0.012	0.419 ± 0.006	0.478 ± 0.010	0.476 ± 0.005	0.516 ± 0.007	0.516 ± 0.004	0.574 ± 0.004	0.578 ± 0.003

Pre/Post-supplementation Elongation Index at various shear stresses following experimental trials in normoxia.

Note: Values are presented as mean ± SD.

Pa, Pascals of shear stress.

* denotes significant difference between groups ($P < 0.05$).

† denotes significant difference in pre/post-supplementation measurements ($P < 0.05$).

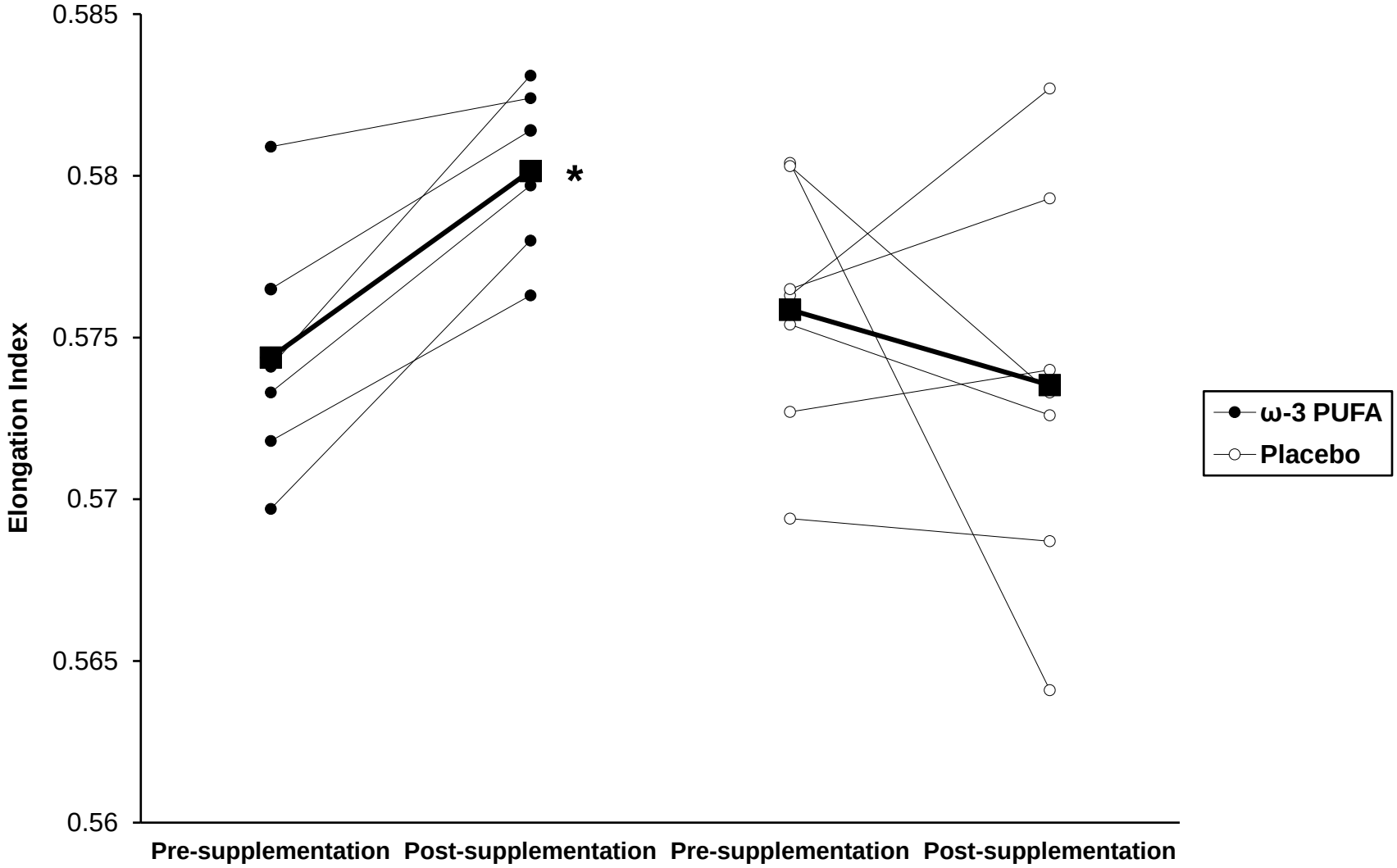


Figure 1. Individual subject pre/post-supplementation elongation index at 20 Pa of shear stress in hypoxia in ω-3 PUFA and placebo groups. * denotes significant difference between pre- and post-supplementation ($P < 0.05$).

Oxygen Consumption

No significant differences were seen in $\dot{V}O_2$ during the post- supplementation 25%, 75%, and TTE trials in hypoxia between the ω-3 PUFA (Figure 2) and placebo groups (Figure 3). In the post-supplementation 50% trial in hypoxia, the ω-3 PUFA group had a significantly higher $\dot{V}O_2$ (post: 40.9 ± 2.8 $mL \cdot kg \cdot min^{-1}$; pre: 38.2 ± 3.6 $mL \cdot kg \cdot min^{-1}$). Conversely, $\dot{V}O_2$ during the post-supplementation 50% trial in hypoxia in the placebo group was significantly lower (post: 38.5 ± 4.4 $mL \cdot kg \cdot min^{-1}$; pre: 41.7 ± 4.7 $mL \cdot kg \cdot min^{-1}$).

During post-supplementation exercise trials in hypoxia, no significant differences in $\dot{V}O_2$ were seen between groups at any workload (Figure 4). Additionally, no significant differences in $\dot{V}O_2$ were seen between or within groups during post-supplementation exercise trials in normoxia (Figure 5).

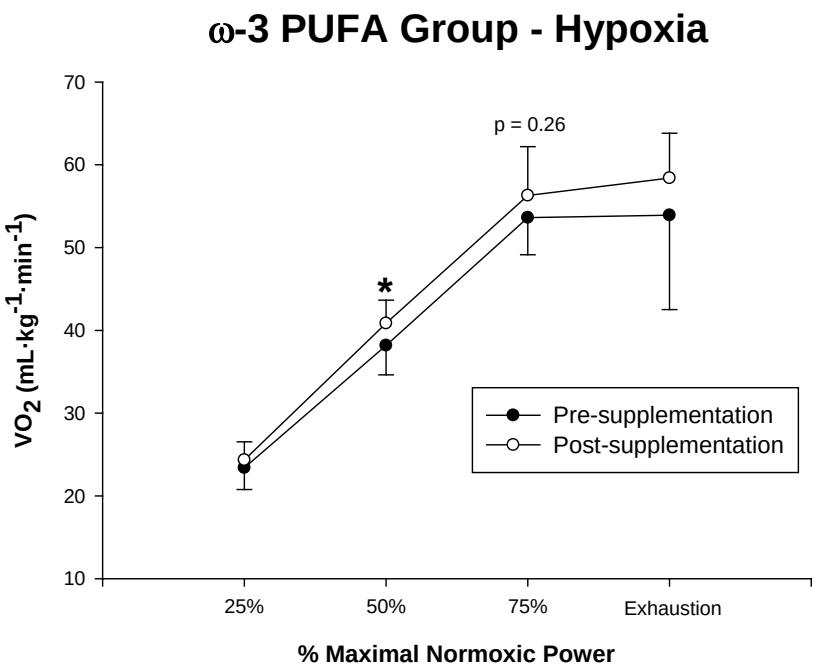


Figure 2. Oxygen Consumption during exercise in hypoxia corresponding to 25%, 50% and 75% of POmax in ω-3 PUFA group pre- and post-supplementation.

*denotes significant difference in pre/post-supplementation measurements ($P < 0.05$).

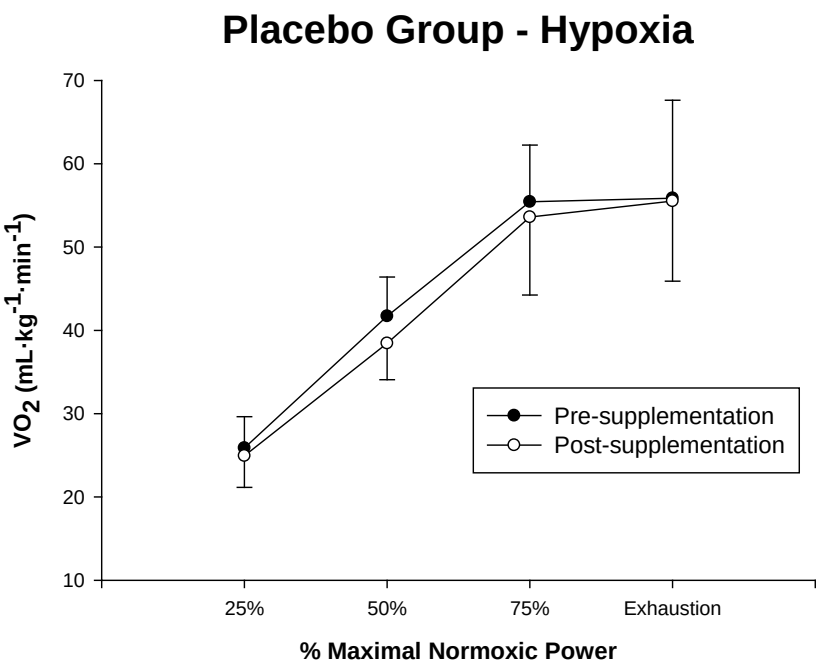


Figure 3. Oxygen Consumption during exercise in hypoxia corresponding to 25%, 50% and 75% of POmax in Placebo group pre- and post-supplementation.

*denotes significant difference in pre/post-supplementation measurements ($P < 0.05$).

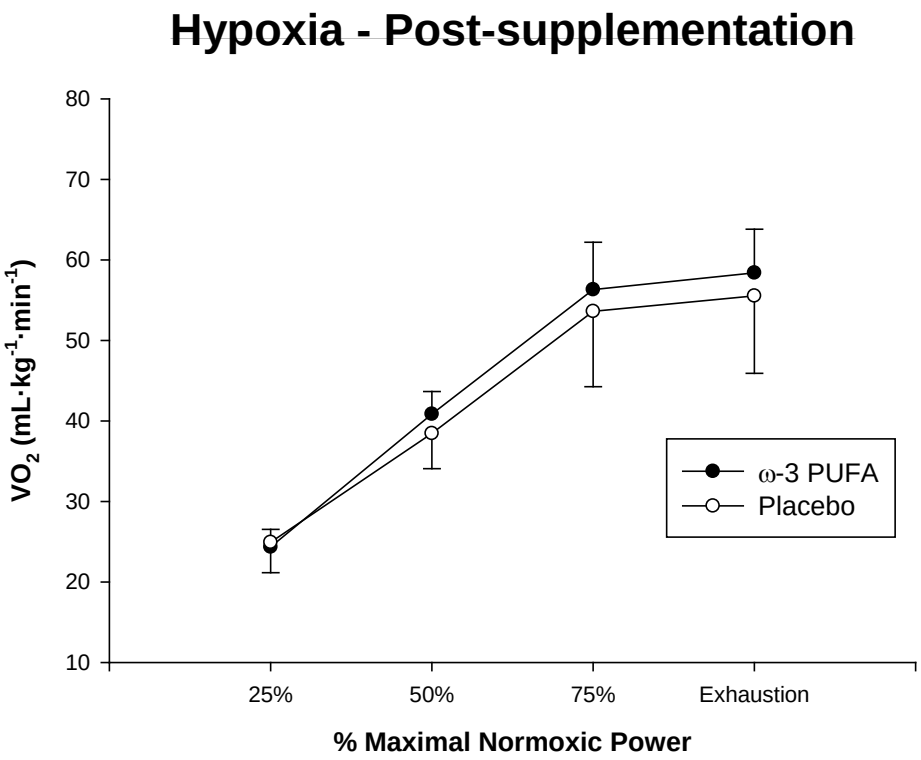


Figure 4. Post-supplementation oxygen consumption between groups during exercise in hypoxia corresponding to 25%, 50% and 75% of POmax.

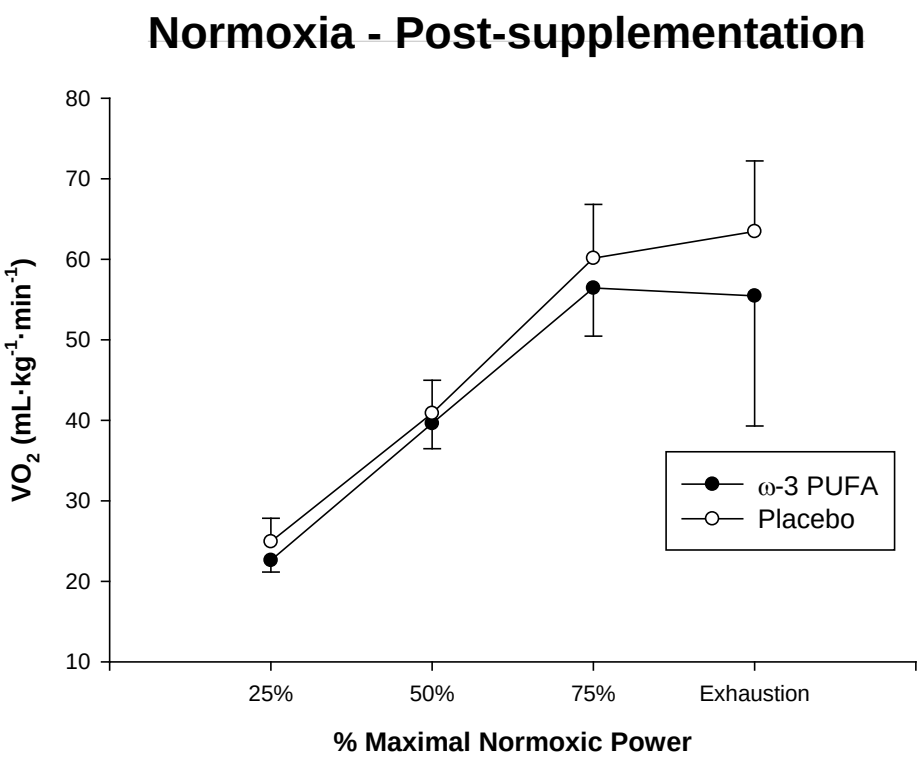


Figure 5. Post-supplementation oxygen consumption between groups during exercise in normoxia corresponding to 25%, 50% and 75% of POmax.

Time to Exhaustion

No significant difference was seen in pre- or post-supplementation time to exhaustion at 75% of POmax between groups in hypoxia (Figure 6) or normoxia (Figure 7). Furthermore, no significant difference was seen between pre- and post-supplementation time to exhaustion within groups in hypoxia or normoxia (Table 7).

	Time to Exhaustion (sec)			
	Pre-Normoxia	Post-Normoxia	Pre-Hypoxia	Post-Hypoxia
ω-3 PUFA	860 ± 176	902 ± 165	358 ± 83	354 ± 82
Placebo	652 ± 200	719 ± 251	344 ± 59	357 ± 113

Table 7. Pre- and post-supplementation time to exhaustion at 75% of POmax during exercise trials in hypoxia and normoxia.

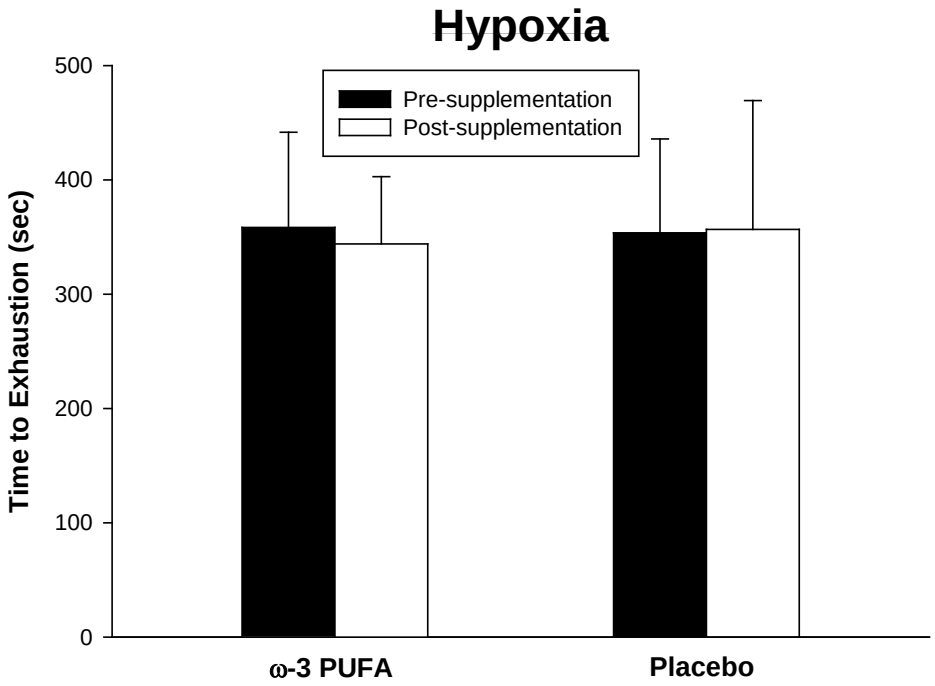


Figure 6. Pre- and post-supplementation time to exhaustion at 75% of POmax in ω-3 PUFA and placebo groups during exercise trials in hypoxia.

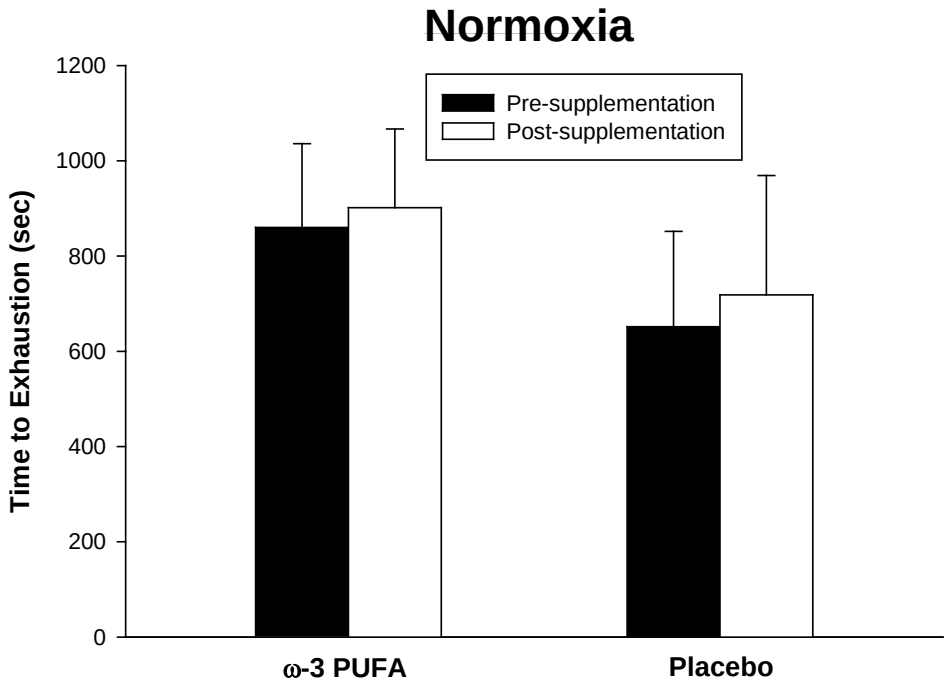


Figure 7. Pre- and post-supplementation time to exhaustion at 75% of POmax in ω-3 PUFA and placebo groups during exercise trials in normoxia.

Chapter 5: Discussion

The aim of this study was to examine the degree to which six weeks of ω-3 PUFA supplementation alters erythrocyte deformability, and if any such changes alter oxygen uptake and time to exhaustion in normoxia and normobaric hypoxia. The primary findings were that 6 weeks of ω-3 PUFA supplementation (1) significantly but marginally increased erythrocyte deformability in hypoxia, but not in normoxia, (2) increased $\dot{V}O_2$ during cycling only at 50% of POmax in hypoxia, but no other workloads or conditions, and (3) did not result in any change in time to exhaustion in normoxia or hypoxia.

Erythrocyte Deformability

Erythrocyte deformability has been shown to decrease at low oxygen pressures (24), after chronic high altitude exposure (31), and after hypoxic exercise (18) in humans, but animal models have not yielded the same results (11, 19, 23). Inconsistent methodology in these data makes it difficult to reach concrete conclusions of these results. Prior to ektacytometry, there was no standard for measuring and reporting erythrocyte deformability, therefore, each of the previous studies measured and reported deformability differently. La Celle measured the negative pressure required to cause an erythrocyte to enter a 2.9μm micropipette (24). Guezennec used the suspension technique and measured deformability as the erythrocyte suspension/buffer pressure ratio (18). Palareti (31) measured filtration time of erythrocytes passed through a 5 μm filter at 20 cm/H_2O pressure, where they were filtered at 37°C, compared to room temperature in the Guezennec study. Doyle (11) and Hakim (19) both measured filtration time through a 4.7 μm membrane, but did not report the pressure at which erythrocytes passed through the membrane. Kaniewski used a very similar ektacytometry protocol as the one used in this study (23). Although the current method of

measuring erythrocyte deformability is ektacytometry, the Kaniewski study remains one of the few studies that have collected deformability data in this manner. Therefore, interpreting our results with previous studies using different methodologies may not be appropriate.

To our knowledge, this is the first study to examine the effect of acute hypoxic exercise on erythrocyte deformability, using the current method of ektacytometry. We found that chronic ω-3 PUFA supplementation significantly, but marginally (1%) increases erythrocyte deformability after acute hypoxic exercise, consistent with previous literature using different methodology to measure deformability (18). However, these results (along with no change in deformability in normoxia) did not have a significant effect on endurance exercise performance, suggesting that increased erythrocyte deformability of this small magnitude is not likely a limiting factor in endurance performance in highly trained athletes.

Despite hypothesizing that deformability would be increased in both hypoxia and normoxia, our data showed no change in normoxia and a marginal increase (1%) in hypoxia. It was thought that a significant increase in erythrocyte deformability with chronic omega-3 supplementation would lead to less resistive capillary blood flow, thereby increasing the rate at which erythrocytes could enter and pass through the capillaries, and as a result, improving oxygen delivery. Although each subject in the omega-3 group increased their deformability at 20 Pa shear stress in hypoxia (Figure 1), deformability was not different at lower shear stresses or at any shear stress in normoxia. This, combined with no significant change in $\dot{V}O_2$ or time to exhaustion, suggests that chronic omega-3 supplementation does not increase erythrocyte deformability in highly-trained endurance athletes at a physiologically relevant shear stress. If deformability was increased at a physiologically relevant range of shear

stresses during exercise, we would expect to see changes in $\dot{V}O_2$ and time to exhaustion. However, no such changes were seen.

Therefore, chronic ω-3 PUFA supplementation may have no practical effect on erythrocyte deformability or there may be something unique to endurance athletes that results in no change. Weed (44) states that erythrocyte deformability is dependent upon three main factors: 1) maintenance of biconcave shape to maintain high surface area ratio, 2) normal internal fluidity of cell, and 3) intrinsic membrane deformability. Normal internal fluidity of the erythrocytes is dependent upon intracellular hemoglobin and intrinsic membrane deformability is dependent intracellular ATP. Since we know that endurance athletes have increased hemoglobin mass compared to untrained populations (21), and generate more ATP at higher $\dot{V}O_2$ (1), endurance training itself may "max out" deformability, to where it cannot increase any further. Our data showing only a marginal increase in deformability at very high shear stress in hypoxia support this claim. If endurance training itself increases deformability, then it is likely that erythrocyte deformability would play no significant role in limiting exercise performance in endurance trained athletes.

Although our data suggest a marginal 1% change in erythrocyte deformability is not large enough to affect endurance exercise performance, further research in this area is needed. There is a lack of data in four key areas. (1) More data is needed examining the effect of omega-3 supplementation on erythrocyte deformability in untrained individuals to fully support the previous claim about endurance athletes. (2) Our study is one of a handful of studies that has examined erythrocyte deformability in trained endurance athletes (18, 30, 35). Most previous research was performed using animal models (11, 19, 23) or used outdated filtration techniques (18). It is relatively unknown how trained athletes' erythrocyte

deformability compares to other, more researched populations. Of the studies examining erythrocyte deformability in relation to performance (30, 35), there is both a lack of homogeneity and variety in the methodology in regards to the exercise being performed. Including this study, all three studies have attempted to examine endurance performance, but have all used different exercise modes and intensities (30, 35). Furthermore, sprint based, anaerobic exercise events have not been studied. (4) Although the effect of hypoxia on erythrocyte deformability has been examined (11, 19, 23, 24, 31), its effect specifically in relation to exercise performance has not been extensively researched (18). With a growing number of trained athletes competing and training at altitude, mitigating any negative effects is crucial for optimal athletic performance.

Oxygen Consumption

Our data suggest that following ω-3 PUFA supplementation, oxygen consumption is significantly increased at submaximal, but unchanged at near maximal workloads in hypoxia, and unchanged at all workloads in normoxia. Our data show that $\dot{V}O_2$ is significantly higher post-supplementation in the ω-3 PUFA group during cycling in hypoxia at 50% of POmax. This finding is difficult to interpret. Since these subjects were performing the same workload during both tests, a higher oxygen consumption suggests that they were less economical, given they required more oxygen to perform the same workload. Since the trial at 50% of POmax occurred submaximally, if the increase in deformability increased O_2 delivery, we would expect O_2 extraction to change proportionally and for $\dot{V}O_2$ to remain the same. Since no change was seen in $\dot{V}O_2$ at exhaustion, the change in deformability likely had no effect on O_2 delivery, and the difference in $\dot{V}O_2$ at 50% of POmax was likely due to changes in

economy. Due to the short duration (3 min) that the subjects cycled for at this workload, it remains difficult to draw a conclusion from the evidence present.

Normoxic $\dot{V}O_2$max has been shown to increase following ω-3 PUFA supplementation (27), but Oostenbrug et al (30) found no change in mean $\dot{V}O_2$ during a fixed workload test in normoxia. Despite Oostenbrug et al's subjects supplementing for 3 weeks compared to 6 weeks in this study, both studies showed no significant change in normoxic $\dot{V}O_2$. This suggests that increasing erythrocyte deformability through ω-3 PUFA supplementation is not beneficial for increasing oxygen consumption in normoxia. Further investigation examining longer duration exercise in hypoxia is needed to determine a conclusion in hypoxia.

Performance

Our finding that time to exhaustion was not improved in normoxia following ω-3 PUFA supplementation lines up with previous research (30, 35). Although erythrocyte deformability was not measured in these studies, our data showing no change in deformability in normoxia following supplementation suggest that their subjects likely did not see any increase in deformability. Although increasing deformability, in theory, should allow for greater O_2 delivery, it does not appear that chronic supplementation with a large dose of ω-3 PUFAs (5g/day) can cause enough of an increase in deformability in endurance trained athletes to result in significant performance benefits in normoxia. Thus, erythrocyte deformability is not likely a limiting factor during endurance exercise in normoxia.

Unlike normoxia, acute hypoxic exposure, as well as prolonged exercise in hypoxia, has been shown to decrease erythrocyte deformability (18). Although a lack of data exist relating this to performance, we hypothesized that this decreased deformability has a negative effect on endurance performance. Therefore, increasing deformability through ω-3

PUFA supplementation could result in improved endurance performance. However, our results showed no change in time to exhaustion in hypoxia following supplementation in the ω-3 PUFA group, despite an increase in deformability. These data suggest that the modest increase in erythrocyte deformability with chronic omega-3 supplementation is not a factor in mitigating the decline in endurance exercise performance in normobaric hypoxia.

Conclusion

Although six weeks of ω-3 PUFA supplementation significantly, but marginally increased erythrocyte deformability (1%), this resulted in no beneficial improvements in time to exhaustion performance or oxygen uptake. 5g/day of omega-3s produced a modest increase in deformability, but does not appear to be enough of a change to improve performance in trained endurance athletes.

References

1. Bassett DR, Howley ET. Limiting factors for maximum oxygen uptake and determinants of endurance performance. *Medicine and science in sports and exercise*. 2000;32(1):70-84.
2. Blonk MC, Bilo HJ, Nauta JJ, Popp-Snijders C, Mulder C, Donker AJ. Dose-response effects of fish-oil supplementation in healthy volunteers. *The American journal of clinical nutrition*. 1990;52(1):120-7.
3. Brooks GA, Fahey, T.D., Baldwin, K.M. Exercise Physiology: Human Bioenergetics and Its Applications. In. New York, New York: Glass, W.R.; 2005.
4. Canham PB, Burton AC. Distribution of size and shape in populations of normal human red cells. *Circulation research*. 1968;22(3):405-22.
5. Cartwright IJ, Pockley AG, Galloway JH, Greaves M, Preston FE. The effects of dietary omega-3 polyunsaturated fatty acids on erythrocyte membrane phospholipids, erythrocyte deformability and blood viscosity in healthy volunteers. *Atherosclerosis*. 1985;55(3):267-81.
6. Chapman RF, Emery M, Stager JM. Degree of arterial desaturation in normoxia influences VO2max decline in mild hypoxia. *Medicine and science in sports and exercise*. 1999.
7. Chapman RF, Emery, M., and Stager, J.M. Degree of arterial desaturation in normoxia influences $\dot{V}O_{2max}$ decline in mild hypoxia. *Medicine and Science in Sports and Exercise*. 1999;31(5):658-63.
8. Chapman RF, Stager JM, Tanner DA, Stray-Gundersen J, Levine BD. Impairment of 3000-m run time at altitude is influenced by arterial oxyhemoglobin saturation. *Medicine and science in sports and exercise*. 2011.
9. Charache S, Conley CL, Waugh DF, Ugoretz RJ, Spurrell JR. Pathogenesis of hemolytic anemia in homozygous hemoglobin C disease. *Journal of Clinical Investigation*. 1967;46(11):1795-811.
10. Dobbe JG, Hardeman MR, Streekstra GJ, Strackee J, Ince C, Grimbergen CA. Analyzing red blood cell-deformability distributions. *Blood cells, molecules & diseases*. 2002;28(3):373-84.
11. Doyle MP, Walker BR. Stiffened erythrocytes augment the pulmonary hemodynamic response to hypoxia. *J Appl Physiol (1985)*. 1990;69(4):1270-5.
12. Driessen GK, Haest CW, Heidtmann H, Kamp D, Schmid-Schonbein H. Effect of reduced red cell "deformability" on flow velocity in capillaries of rat mesentery. *Pflugers Archiv : European journal of physiology*. 1980;388(1):75-8.
13. Forman S, Bischel M, Hochstein P. Erythrocyte deformability in uremic hemodialyzed patients. *Annals of internal medicine*. 1973;79(6):841-3.
14. Gavin TP, Derchak PA, Stager JM. Ventilation's role in the decline in VO2max and SaO2 in acute hypoxic exercise. *Medicine and science in sports and exercise*. 1998.
15. Gore CJ, Hahn AG, Scroop GC. Increased arterial desaturation in trained cyclists during maximal exercise at 580 m altitude. *J Appl Physiol*. 1996.
16. Gore CJ, Hahn, A.G., Scroop, G.C., Watson, D.B., Norton, K.I., Wood, R.J., Campbell, D.P., Emonson, D.L. Increased arterial desaturation in trained cyclists

during maximal exercise at 580 m altitude. *Journal of Applied Physiology*. 1996;80(6):2204-10.
17. Gregersen MI, Bryant CA, Hammerle WE, Usami S, Chien S. Flow Characteristics of Human Erythrocytes through Polycarbonate Sieves. *Science*. 1967;157(3790):825-7.
18. Guezennec CY, Nadaud JF, Satabin P, Leger F, Lafargue P. Influence of polyunsaturated fatty acid diet on the hemorrheological response to physical exercise in hypoxia. *International journal of sports medicine*. 1989;10(4):286-91.
19. Hakim TS, Macek AS. Effect of hypoxia on erythrocyte deformability in different species. *Biorheology*. 1988;25(6):857-68.
20. Harris JW, Brewster HH, Ham TH, Castle WB. Studies on the Destruction of Red Blood Cells. X. The Biophysics and Biology of Sickle-Cell Disease. *Arrch. Intern. Med.* 1956;97(2):145-68.
21. Heinicke K, Wolfarth B, Winchenbach P et al. Blood volume and hemoglobin mass in elite athletes of different disciplines. *International journal of sports medicine*. 2001;22(07):504-12.
22. Ho M, Maple C, Bancroft A, McLaren M, Belch JJ. The beneficial effects of omega-3 and omega-6 essential fatty acid supplementation on red blood cell rheology. *Prostaglandins, leukotrienes, and essential fatty acids*. 1999;61(1):13-7.
23. Kaniewski WS, Hakim TS, Freedman JC. Cellular deformability of normoxic and hypoxic mammalian red blood cells. *Biorheology*. 1994;31(1):91-101.
24. La Celle PL, Weed RI. Low oxygen pressure: : A cause of erythrocyte membrane rigidity. *Journal of Clinical Investigation*. 1970;49(6):54-5.
25. La Celle PL, Weed RI. The contribution of normal and pathologic erythrocytes to blood rheology. *Progress in hematology*. 1971;7(0):1-31.
26. Lawler J, Powers, S.K., and Thompson, T. Linear relationship between VO2 max and VO2 max decrement during exposure to acute hypoxia. *Journal of Applied Physiology*. 1988;64(4):1486-92.
27. Leaf DA, Rauch CR. Omega-3 supplementation and estimated VO2max: a double blind randomized controlled trial in athletes. *Annals of Sports Medicine*. 1989;4(1):37-40.
28. Murphy JR. Hemoglobin CC disease: rheological properties or erythrocytes and abnormalities in cell water. *The Journal of clinical investigation*. 1968;47(7):1483-95.
29. Nakao M, Nakao T, Yamazoe S. Adenosine triphosphate and maintenance of shape of the human red cells. *Nature*. 1960;187:945-6.
30. Oostenbrug GS, Mensink RP, Hardeman MR, De Vries T, Brouns F, Hornstra G. Exercise performance, red blood cell deformability, and lipid peroxidation: effects of fish oil and vitamin E. *Journal of applied physiology*. 1997;83(3):746-52.
31. Palareti G, Coccheri S, Poggi M, Tricarico MG, Magelli M, Cavazzuti F. Changes in the rheologic properties of blood after a high altitude expedition. *Angiology*. 1984;35(7):451-8.
32. Pandolf KB, Sawka, M.N., Gonzalez, R.R. Human Performance Physiology and Environmental Medicine at Terrestial Extremes. In. Natick, Massachusetts: Cooper Publishing Group.
33. Parthasarathi K, Lipowsky HH. Capillary recruitment in response to tissue hypoxia and its dependence on red blood cell deformability. *The American journal of physiology*. 1999;277(6 Pt 2):H2145-57.

34. Ponder E. *Hemolysis and Related Phenomena*. New York: Grune & Stratton; 1948.
35. Raastad T, Hastmark A, Strømme S. Omega-3 fatty acid supplementation does not improve maximal aerobic power, anaerobic threshold and running performance in well-trained soccer players. *Scandinavian journal of medicine & science in sports*. 1997;7(1):25-31.
36. Rifkind JM, Abugo OO. Alterations in erythrocyte deformability under hypoxia: implications for impaired oxygen transport. *Advances in experimental medicine and biology*. 1994;361:345-51.
37. Rifkind RA. Heinz body anemia: an ultrastructural study. II. Red cell sequestration and destruction. *Blood*. 1965;26(4):433-48.
38. Rillaerts E, Van Camp K, Vandewoude MF, Rademakers F, De Leeuw I. Blood viscosity parameters in coronary heart disease: effect of fish oil supplementation. *Acta clinica Belgica*. 1989;44(1):17-23.
39. Shin S, Hou J, Suh J, Singh M. Validation and application of a microfluidic ektacytometer (RheoScan-D) in measuring erythrocyte deformability. *Clinical hemorheology and microcirculation*. 2007;37(4):319-28.
40. Squires RW, and Buskirk, E.R. Aerobic capacity during actue exposure to simulated altitude, 914 to 2286 meters. *Medicine and Science in Sports and Exercise*. 1982;14(1):36-40.
41. Terano T, Hirai A, Hamazaki T et al. Effect of oral administration of highly purified eicosapentaenoic acid on platelet function, blood viscosity and red cell deformability in healthy human subjects. *Atherosclerosis*. 1983;46(3):321-31.
42. Terrados N, Mizuno, M., and Andersen, H. Reduction in maximal oxygen uptake at low altitudes: Role of training status and lung function. *Clincial Physiology (book>?)*. 1985.
43. Walser B, Giordano RM, Stebbins CL. Supplementation with omega-3 polyunsaturated fatty acids augments brachial artery dilation and blood flow during forearm contraction. *European journal of applied physiology*. 2006;97(3):347-54.
44. Weed RI. The importance of erythrocyte deformability. *The American journal of medicine*. 1970;49(2):147-50.
45. Weed RI, LaCelle PL, Merrill EW. Metabolic dependence of red cell deformability. *The Journal of clinical investigation*. 1969;48(5):795-809.
46. West JB. Respiratory physiology. In. Baltimore, MD2008.

Supplemental Materials

Appendix 1. Subject Characteristic data.

SubjectID	Supplement	Age	Sex	Height (cm)	Weight (kg)	$\dot{V}O_2$max (mL/kg/min)	Max Power (W)
1015	Omega-3	20	M	183.0	82.1	68.6	450
1017	Omega-3	18	M	175.5	72.5	61.7	350
1020	Omega-3	20	F	168.7	68.0	56.8	300
1021	Placebo	19	M	184.8	84.2	67.9	450
1022	Omega-3	21	M	177.4	84.2	57.4	325
1024	Placebo	21	M	180.6	71.1	67.1	375
1027	Placebo	21	M	178.7	72.2	69.6	375
1029	Omega-3	21	M	171.2	79.3	56.9	375
1033	Placebo	22	M	182.9	80.7	70.2	425
1034	Omega-3	22	M	189.5	87.1	59.8	475
1035	Placebo	19	M	186.8	86.9	67.6	400
1036	Placebo	20	M	183.6	76.3	67.2	400
1037	Placebo	26	M	175.1	72.1	61.4	300
Omega-3 Mean		20.3		177.55	78.87	60.20	379.2
Omga-3 Sdev		1.4		7.69	7.29	4.54	69.7
Placebo Mean		21.1		181.79	77.64	67.29	389.3
Placebo Sdev		2.4		3.97	6.36	2.86	47.6

Appendix 2. Time to exhaustion, HR, and $\dot{V}O_2$ data.

SubjectID	PrNTTE	PrHTTE	PNTTE	PHTTE	PrNHR	PrHHR	PNHR	PHHR	PrNV25	PrHV25	PNV25	PHV25
1015	1035	482	793	349	-	-	-	-	23.98	24.52	22.88	21.74
1017	565	267	723	463	-	-	-	-	25.03	27.12	23.85	24.95
1020	1023	430	1200	434	-	-	-	-	22.48	24.87	20.17	25.31
1021	896	254	738	246	-	-	-	-	25.83	24.10	23.56	21.91
1022	804	363	848	294	179	174	185	-	24.04	20.10	21.68	26.04
1024	540	368	707	332	193	180	182	169	28.20	28.50	25.40	28.50
1027	456	326	553	224	188	179	185	168	30.53	33.12	30.37	25.90
1029	927	303	927	335	174	174	-	176	24.05	20.98	24.10	21.51
1033	965	447	1261	541	-	179	181	-	28.37	25.67	25.49	31.03
1034	807	305	918	247	200	188	190	169	23.27	22.79	22.99	26.56
1035	485	345	540	464	185	-	194	-	29.78	24.14	25.29	20.44
1036	626	313	641	351	185	185	185	-	24.95	22.77	23.54	22.72
1037	593	355	590	338	188	182	174	174	21.63	22.89	20.87	24.04
Omega-3 Mean	860.2	358.3	901.5	353.7	184.3	178.7	187.5	172.5	23.81	23.40	22.61	24.35
Omga-3 Sdev	175.9	83.3	165.2	82.1	13.8	8.1	3.5	4.9	0.86	2.62	1.47	2.19
Placebo Mean	651.6	344.0	718.6	356.6	187.8	181.0	183.5	170.3	27.04	25.88	24.93	24.93
Placebo Sdev	200.2	58.7	250.5	112.8	3.3	2.5	6.5	3.2	3.11	3.74	2.90	3.78

Pr	Pre-supplementation
P	Post-supplementation
N	Normoxia
H	Hypoxia
TTE	Time To Exhaustion (sec)
HR	Heart Rate (bpm)
V25	$\dot{V}O_2$ (mL/kg/min) during last 30 sec of 25% trial
V50	$\dot{V}O_2$ (mL/kg/min) during last 30 sec of 50% trial
V75	$\dot{V}O_2$ (mL/kg/min) during last 30 sec of 75% trial
V75TTE	$\dot{V}O_2$ (mL/kg/min) during last 30 sec of Time to Exhaustion trial

SubjectID	PrNV50	PrHV50	PNV50	PHV50	PrNV75	PrHV75	PNV75	PHV75	PrNV75TTE	PrHV75TTE	PNV75TTE	PHV75TTE
1015	38.99	41.95	41.78	42.49	61.68	59.21	61.80	62.30	71.70	65.62	72.75	66.13
1017	46.50	42.84	43.46	45.07	71.06	57.64	59.91	62.00	66.21	61.38	68.80	61.63
1020	37.76	35.23	35.70	38.14	48.79	47.16	49.23	50.81	55.38	53.14	52.18	53.71
1021	43.73	38.67	42.22	37.29	61.89	54.68	62.03	54.99	62.58	56.94	67.74	57.88
1022	34.30	34.02	35.94	39.06	48.90	50.55	48.36	53.43	41.20	33.00	27.23	52.55
1024	46.70	47.30	41.80	46.60	65.90	66.10	65.50	70.60	68.80	66.10	65.50	70.60
1027	45.98	47.48	44.56	41.59	65.00	59.67	63.95	59.35	71.34	66.06	75.57	60.61
1029	39.95	37.01	40.70	38.29	58.42	52.64	59.31	49.10	62.75	52.28	59.02	54.97
1033	45.39	42.66	41.46	35.08	58.31	59.77	64.86	41.45	69.01	62.83	69.15	38.99
1034	38.10	38.08	40.10	42.08	58.40	54.48	59.96	60.17	60.76	58.04	52.73	61.38
1035	47.28	42.16	42.76	34.29	51.32	46.46	61.11	48.90	39.41	32.39	61.25	55.06
1036	42.06	39.11	41.34	38.84	58.64	51.40	57.07	51.75	61.74	55.45	54.90	54.67
1037	30.49	34.55	31.89	35.49	41.32	49.95	46.46	48.22	47.98	51.31	50.02	50.85
Omega-3 Mean	39.27	38.19	39.61	40.86	57.88	53.61	56.43	56.30	59.67	53.91	55.45	58.40
Omga-3 Sdev	4.03	3.56	3.15	2.80	8.39	4.48	5.98	5.89	10.56	11.41	16.15	5.42
Placebo Mean	43.09	41.70	40.86	38.45	57.48	55.43	60.14	53.61	60.12	55.87	63.45	55.52
Placebo Sdev	5.84	4.71	4.10	4.38	8.64	6.81	6.67	9.36	12.01	11.76	8.76	9.62

Pr	Pre-supplementation
P	Post-supplementation
N	Normoxia
H	Hypoxia
TTE	Time To Exhaustion (sec)
HR	Heart Rate (bpm)
V25	$\dot{V}O_2$ (mL/kg/min) during last 30 sec of 25% trial
V50	$\dot{V}O_2$ (mL/kg/min) during last 30 sec of 50% trial
V75	$\dot{V}O_2$ (mL/kg/min) during last 30 sec of 75% trial
V75TTE	$\dot{V}O_2$ (mL/kg/min) during last 30 sec of Time to Exhaustion trial

Appendix 3. Elongation index data in normoxia.

		Pre-Supplementation Elongation Index in Normoxia							
		Shear Stress (Pa)							
SubjectID	Supplement	0.5	1	1.5	2	2.5	3	4	5
1015	Omega-3	0.000049	0.007036	0.120780	0.183152	0.215518	0.249197	0.293950	0.313530
1017	Omega-3	0.000041	0.006637	0.123509	0.183965	0.216403	0.250435	0.294754	0.313772
1020	Omega-3	-0.000415	0.124083	0.187103	0.224515	0.261305	0.288149	0.313863	0.324713
1021	Placebo	0.000023	0.004186	0.107309	0.181390	0.213273	0.247994	0.293626	0.312790
1022	Omega-3	-0.000389	0.039028	0.159170	0.198176	0.233352	0.265104	0.302861	0.318976
1024	Placebo	0.000444	0.026034	0.157178	0.196634	0.232939	0.265404	0.302959	0.318837
1027	Placebo	0.000101	0.012988	0.142800	0.190371	0.225224	0.258477	0.299051	0.316412
1029	Omega-3	-0.000430	0.120602	0.187800	0.224550	0.261267	0.288209	0.313990	0.324722
1033	Placebo	-0.001135	0.159136	0.209146	0.250057	0.284204	0.304385	0.320456	0.328549
1034	Omega-3	-0.005563	0.178507	0.223847	0.269012	0.297623	0.311407	0.322653	0.331780
1035	Placebo	-0.002199	0.168749	0.214933	0.258309	0.290525	0.307869	0.321442	0.329801
1036	Placebo	-0.009542	0.188851	0.233469	0.278003	0.303335	0.314430	0.323546	0.332460
1037	Placebo	0.000025	0.004537	0.110006	0.180386	0.213051	0.247975	0.293573	0.313084
	Omega-3 Mean	-0.001118	0.079316	0.167035	0.213895	0.247578	0.275417	0.307012	0.321249
	Omega-3 Sdev	0.002189	0.071662	0.040392	0.032718	0.031882	0.024653	0.011648	0.007150
	Placebo Mean	-0.001755	0.080640	0.167834	0.219307	0.251793	0.278076	0.307808	0.321705
	Placebo Sdev	0.003555	0.086439	0.051608	0.041263	0.039271	0.029600	0.013525	0.008348

		Pre-Supplementation Elongation Index in Normoxia							
		Shear Stress (Pa)							
SubjectID	Supplement	6	7	8	10	12	15	17	20
1015	Omega-3	0.324906	0.335565	0.347531	0.376125	0.409686	0.466412	0.507359	0.572481
1017	Omega-3	0.325014	0.335785	0.347946	0.376912	0.410685	0.467335	0.507952	0.572143
1020	Omega-3	0.334103	0.345190	0.358233	0.388844	0.423431	0.479560	0.518701	0.579018
1021	Placebo	0.323998	0.334820	0.347085	0.376216	0.409922	0.465869	0.505571	0.567670
1022	Omega-3	0.329341	0.339863	0.351949	0.380890	0.414712	0.471613	0.512543	0.577453
1024	Placebo	0.329430	0.340395	0.352971	0.382785	0.417238	0.474601	0.515522	0.579935
1027	Placebo	0.327396	0.338338	0.350751	0.380137	0.414146	0.470855	0.511341	0.575098
1029	Omega-3	0.333945	0.344864	0.357743	0.388041	0.422313	0.477949	0.516738	0.576486
1033	Placebo	0.337978	0.349827	0.363649	0.395129	0.429445	0.483114	0.519351	0.573550
1034	Omega-3	0.343494	0.357506	0.373058	0.406630	0.441456	0.493521	0.527386	0.576413
1035	Placebo	0.340162	0.352925	0.367476	0.399795	0.434218	0.486924	0.521887	0.573372
1036	Placebo	0.344386	0.358620	0.374300	0.407786	0.442112	0.492806	0.525426	0.572201
1037	Placebo	0.324846	0.336258	0.349106	0.379402	0.414325	0.472291	0.513502	0.578146
	Omega-3 Mean	0.331801	0.343129	0.356077	0.386240	0.420381	0.476065	0.515113	0.575666
	Omega-3 Sdev	0.007018	0.008195	0.009508	0.011347	0.011813	0.010096	0.007539	0.002765
	Placebo Mean	0.332599	0.344455	0.357905	0.388750	0.423058	0.478066	0.516086	0.574282
	Placebo Sdev	0.008127	0.009266	0.010515	0.012106	0.012183	0.009724	0.006728	0.004020

		Post-Supplementation Elongation Index in Normoxia							
		Shear Stress (Pa)							
SubjectID	Supplement	0.5	1	1.5	2	2.5	3	4	5
1015	Omega-3	-0.000387	0.061465	0.171642	0.206609	0.242771	0.273466	0.307224	0.321152
1017	Omega-3	-0.000336	0.125292	0.184146	0.220469	0.256808	0.284424	0.312118	0.323711
1020	Omega-3	-0.000750	0.145009	0.199300	0.239603	0.275211	0.298111	0.318058	0.327552
1021	Placebo	-0.001401	0.160339	0.209479	0.251703	0.285429	0.304808	0.320331	0.328795
1022	Omega-3	0.000017	0.003017	0.087659	0.176146	0.206879	0.241300	0.289751	0.311072
1024	Placebo	0.000102	0.013530	0.142969	0.188893	0.223811	0.257067	0.298051	0.316018
1027	Placebo	-0.000234	0.101940	0.174345	0.211085	0.247456	0.277149	0.309028	0.322135
1029	Omega-3	-0.000194	0.063518	0.161801	0.200197	0.235724	0.267108	0.303938	0.319554
1033	Placebo	-0.000616	0.140008	0.196533	0.235602	0.271974	0.296238	0.317466	0.326754
1034	Omega-3	-0.000251	0.048983	0.159638	0.198204	0.233663	0.265386	0.302933	0.318887
1035	Placebo	-0.000240	0.082655	0.171576	0.208160	0.244203	0.274400	0.307673	0.321489
1036	Placebo	-0.000841	0.137847	0.197247	0.236669	0.273066	0.296812	0.317263	0.326468
1037	Placebo	0.000063	0.011091	0.147439	0.191864	0.227145	0.260953	0.300517	0.316754
	Omega-3 Mean	-0.000459	0.084775	0.165866	0.213904	0.248485	0.276529	0.307589	0.321383
	Omega-3 Sdev	0.000554	0.068240	0.044784	0.029109	0.030070	0.024319	0.011847	0.006835
	Placebo Mean	-0.000330	0.083720	0.172654	0.211683	0.247604	0.276864	0.308403	0.321720
	Placebo Sdev	0.000300	0.047119	0.018717	0.017868	0.018293	0.014489	0.006753	0.003775

		Post-Supplementation Elongation Index in Normoxia							
		Shear Stress (Pa)							
SubjectID	Supplement	6	7	8	10	12	15	17	20
1015	Omega-3	0.330916	0.341506	0.353888	0.383497	0.417811	0.474969	0.515734	0.579877
1017	Omega-3	0.332978	0.343544	0.355878	0.384793	0.417453	0.470330	0.507066	0.563409
1020	Omega-3	0.337374	0.349326	0.363218	0.395032	0.430054	0.485490	0.523344	0.580576
1021	Placebo	0.338786	0.351163	0.365433	0.397577	0.432305	0.486225	0.522428	0.576324
1022	Omega-3	0.323115	0.334198	0.346545	0.375818	0.409864	0.466797	0.507481	0.571550
1024	Placebo	0.327353	0.338336	0.350530	0.378870	0.411165	0.464191	0.501500	0.559396
1027	Placebo	0.331550	0.341909	0.354059	0.383083	0.416603	0.472152	0.511558	0.573211
1029	Omega-3	0.329716	0.340188	0.352320	0.381550	0.415880	0.473932	0.515896	0.582784
1033	Placebo	0.336008	0.347422	0.360894	0.392247	0.427252	0.483356	0.522066	0.581142
1034	Omega-3	0.329148	0.339613	0.351683	0.380696	0.414716	0.472142	0.513579	0.579498
1035	Placebo	0.331153	0.341599	0.353801	0.382986	0.416816	0.473150	0.513298	0.576405
1036	Placebo	0.335873	0.347449	0.361024	0.392365	0.427075	0.482267	0.520083	0.577419
1037	Placebo	0.327393	0.338442	0.351143	0.381189	0.415716	0.472765	0.513162	0.576292
	Omega-3 Mean	0.331754	0.343012	0.355915	0.385931	0.419775	0.474667	0.512926	0.571855
	Omega-3 Sdev	0.005954	0.006456	0.007278	0.008693	0.009426	0.009395	0.008958	0.008798
	Placebo Mean	0.331549	0.342375	0.354989	0.384874	0.419151	0.475681	0.515663	0.578107
	Placebo Sdev	0.003295	0.003650	0.004211	0.005153	0.005516	0.004920	0.003950	0.003257

Appendix 4. Elongation index data in hypoxia.

		Pre-Supplementation Elongation Index in Hypoxia							
		Shear Stress (Pa)							
SubjectID	Supplement	0.5	1	1.5	2	2.5	3	4	5
1015	Omega-3	-0.000766	0.027799	0.152989	0.194972	0.229449	0.261571	0.300869	0.317627
1017	Omega-3	-0.000251	0.116204	0.178505	0.214835	0.251306	0.280305	0.310441	0.322725
1020	Omega-3	-0.000191	0.065107	0.163705	0.201415	0.236472	0.267600	0.304180	0.319583
1021	Placebo	0.000006	0.001170	0.047479	0.169269	0.198005	0.231919	0.284935	0.308138
1022	Omega-3	-0.000622	0.136355	0.194869	0.234154	0.270465	0.294940	0.316994	0.326974
1024	Placebo	-0.000224	0.131942	0.180857	0.216207	0.252085	0.280823	0.310662	0.322549
1027	Placebo	0.000021	0.003647	0.097123	0.177742	0.208968	0.243440	0.290840	0.311651
1029	Omega-3	0.000333	0.028156	0.161869	0.199055	0.235766	0.267799	0.303851	0.319188
1033	Placebo	0.000024	0.003480	0.083772	0.172046	0.203471	0.236962	0.286405	0.309431
1034	Omega-3	-0.002097	0.165647	0.212256	0.255720	0.288608	0.306597	0.320773	0.329256
1035	Placebo	0.000119	0.012127	0.136376	0.187799	0.221390	0.254532	0.296905	0.315236
1036	Placebo	-0.000436	0.136767	0.192102	0.230108	0.266569	0.292258	0.315890	0.325801
1037	Placebo	-0.005530	0.181716	0.226291	0.270982	0.298946	0.312207	0.322962	0.332003
	Omega-3 Mean	-0.000341	0.079763	0.153067	0.205142	0.239630	0.269526	0.304680	0.319599
	Omega-3 Sdev	0.000291	0.057175	0.053719	0.022191	0.024854	0.021776	0.011183	0.006451
	Placebo Mean	-0.001081	0.075934	0.158541	0.213350	0.246245	0.273399	0.305375	0.320367
	Placebo Sdev	0.002126	0.081412	0.055478	0.039219	0.038583	0.030486	0.014732	0.008824

		Pre-Supplementation Elongation Index in Hypoxia							
		Shear Stress (Pa)							
SubjectID	Supplement	6	7	8	10	12	15	17	20
1015	Omega-3	0.327965	0.338225	0.349998	0.378366	0.411751	0.468255	0.509085	0.574090
1017	Omega-3	0.331963	0.342418	0.354765	0.384265	0.418263	0.474484	0.514306	0.576544
1020	Omega-3	0.329473	0.339619	0.351374	0.379715	0.412963	0.468997	0.509325	0.573281
1021	Placebo	0.320490	0.331788	0.344529	0.374910	0.410045	0.467974	0.508714	0.571799
1022	Omega-3	0.336670	0.348353	0.361985	0.393451	0.428388	0.484152	0.522500	0.580850
1024	Placebo	0.331339	0.341347	0.353262	0.381925	0.415089	0.470012	0.508916	0.569676
1027	Placebo	0.323730	0.335064	0.347743	0.377816	0.412824	0.471555	0.513709	0.580435
1029	Omega-3	0.329874	0.341100	0.353917	0.383952	0.418223	0.474574	0.514337	0.576289
1033	Placebo	0.321965	0.332762	0.344539	0.372692	0.406125	0.463431	0.505305	0.572672
1034	Omega-3	0.339625	0.352392	0.366973	0.399450	0.434154	0.487482	0.522975	0.575403
1035	Placebo	0.326122	0.336528	0.348291	0.376452	0.409499	0.465294	0.505512	0.569373
1036	Placebo	0.334848	0.345806	0.358778	0.389231	0.423539	0.478970	0.517454	0.576500
1037	Placebo	0.343789	0.357942	0.373675	0.407677	0.442998	0.495895	0.530361	0.580344
	Omega-3 Mean	0.329650	0.340292	0.352652	0.382105	0.416083	0.472312	0.512141	0.574373
	Omega-3 Sdev	0.005371	0.005434	0.005772	0.006401	0.006665	0.006268	0.005503	0.003915
	Placebo Mean	0.331422	0.343085	0.356274	0.386753	0.421052	0.476743	0.515665	0.575859
	Placebo Sdev	0.008277	0.009422	0.010827	0.012889	0.013487	0.011740	0.009024	0.003960

		Post-Supplementation Elongation Index in Hypoxia							
		Shear Stress (Pa)							
SubjectID	Supplement	0.5	1	1.5	2	2.5	3	4	5
1015	Omega-3	-0.000322	0.142139	0.190298	0.227673	0.263752	0.290173	0.315490	0.326040
1017	Omega-3	-0.000305	0.049488	0.163841	0.201395	0.236875	0.268230	0.304616	0.319975
1020	Omega-3	-0.000797	0.145501	0.199911	0.239933	0.275740	0.298664	0.318264	0.327389
1021	Placebo	-0.000461	0.129799	0.189845	0.227521	0.264155	0.290303	0.314868	0.325348
1022	Omega-3	-0.000739	0.146059	0.200718	0.240742	0.276367	0.299221	0.318825	0.327975
1024	Placebo	0.000012	0.002303	0.078038	0.174072	0.204935	0.240242	0.289609	0.310807
1027	Placebo	0.000002	0.000393	0.014970	0.132624	0.180598	0.209561	0.268594	0.299661
1029	Omega-3	-0.000403	0.093574	0.179792	0.217097	0.254472	0.283226	0.312084	0.323978
1033	Placebo	-0.000357	0.112275	0.184548	0.221234	0.257921	0.285693	0.312997	0.324098
1034	Omega-3	-0.002290	0.170383	0.215156	0.258572	0.290697	0.307786	0.320888	0.328982
1035	Placebo	-0.000488	0.115710	0.187155	0.224980	0.262025	0.288831	0.314189	0.324724
1036	Placebo	0.000250	0.018506	0.149107	0.191825	0.226641	0.259690	0.299985	0.317048
1037	Placebo	-0.000728	0.090001	0.183103	0.219446	0.256954	0.285034	0.312318	0.323619
	Omega-3 Mean	-0.000435	0.102548	0.170442	0.218556	0.253637	0.281139	0.310279	0.322922
	Omega-3 Sdev	0.000301	0.061502	0.047197	0.026023	0.027824	0.022973	0.011352	0.006581
	Placebo Mean	-0.000573	0.085835	0.159119	0.209397	0.247044	0.274260	0.305865	0.320301
	Placebo Sdev	0.000823	0.058667	0.066401	0.039082	0.034720	0.031785	0.017559	0.009751

		Post-Supplementation Elongation Index in Hypoxia							
		Shear Stress (Pa)							
SubjectID	Supplement	6	7	8	10	12	15	17	20
1015	Omega-3	0.335183	0.346102	0.359055	0.389694	0.424541	0.481433	0.521312	0.583061
1017	Omega-3	0.330130	0.340691	0.352922	0.382266	0.416547	0.474185	0.515640	0.581391
1020	Omega-3	0.336950	0.348726	0.362494	0.394154	0.429098	0.484505	0.522384	0.579711
1021	Placebo	0.334705	0.345836	0.358909	0.389450	0.423764	0.479114	0.517499	0.576338
1022	Omega-3	0.337566	0.349384	0.363212	0.395046	0.430229	0.486104	0.524362	0.582350
1024	Placebo	0.322979	0.334534	0.347532	0.378233	0.413594	0.472056	0.513418	0.577951
1027	Placebo	0.314658	0.326174	0.338456	0.367856	0.402442	0.460139	0.500916	0.564118
1029	Omega-3	0.333469	0.344403	0.357279	0.387772	0.422604	0.479777	0.520048	0.582695
1033	Placebo	0.333133	0.343709	0.356236	0.385940	0.419806	0.475158	0.513958	0.573999
1034	Omega-3	0.339204	0.351890	0.366398	0.398686	0.433124	0.485909	0.520956	0.572605
1035	Placebo	0.333829	0.344606	0.357287	0.386988	0.420403	0.474284	0.511602	0.568701
1036	Placebo	0.327664	0.338269	0.350428	0.379601	0.413789	0.471477	0.513089	0.579268
1037	Placebo	0.333025	0.343977	0.356820	0.386935	0.420931	0.476007	0.514336	0.573265
	Omega-3 Mean	0.332919	0.344212	0.357354	0.388141	0.422962	0.479566	0.519103	0.580134
	Omega-3 Sdev	0.005527	0.005646	0.006036	0.006643	0.006671	0.005589	0.004247	0.002623
	Placebo Mean	0.330712	0.341861	0.354701	0.384825	0.419014	0.474679	0.513558	0.573522
	Placebo Sdev	0.007828	0.007973	0.008555	0.009367	0.009311	0.007915	0.006602	0.006191

Appendix 5. Informed Consent Form.

INDIANA UNIVERSITY INFORMED CONSENT STATEMENT FORM

The effects of altered erythrocyte deformability on microvasculature oxygenation and exercise tolerance in adults with type 2 diabetes

You are invited to participate in a research study that will help determine the effects of omega-3 fatty acid (fish oil) supplementation on how you complete an exercise task. You were selected as a possible subject because you identified that you were either: a) a type 2 diabetic, b) an individual who does not regularly engage in training / physical activity, or c) a highly endurance trained individual. We ask that you read this form and ask any questions you may have before agreeing to be in the study.

Disclaimer: It is possible that you will not qualify for the study following the completion of the blood test, height, weight, or maximal exercise test.

The study is being conducted by Robert F. Chapman, Ph.D. (Principal Investigator), Timothy Mickleborough, PhD, Chad Wiggins, and Josh Foss in the Department of Kinesiology at Indiana University-Bloomington, and Kieren Mather, MD of the Department of Medicine at Indiana University. This study is funded by a 2013 American Diabetes Association Innovation Award.

STUDY PURPOSE

The purpose of the proposed study is to investigate how six weeks of omega-3 fatty acid (fish oil) supplements affects your ability to complete various exercise tasks.

NUMBER OF PEOPLE TAKING PART IN THE STUDY:

If you agree to participate, you will be one of 60 subjects who will be participating in this research.

PROCEDURES FOR THE STUDY:

If you agree to be in the study, the following items are included:

An invitation will be extended to visit the Human Performance laboratory a total of 6 times over a time period of approximately 10-12 weeks. Each visit will be done at a previously agreed-upon time. The first two visits last about 60 minutes and the remaining four visits last about 75 minutes. You should refrain from drinking caffeine for at least 8 hours prior to your visit. If you are an endurance trained athlete, you should refrain from exhaustive exercise (exercise that causes significant fatigue) for 24 hours prior to each visit. If you are an untrained individual (completing less than 90 minutes of physical activity a week), we ask that you maintain the same low level of physical activity throughout the study.

Visit #1
This visit includes a blood draw to measure your blood cholesterol, blood glucose, and hemoglobin values.

Visit #2

This visit includes the following tests: a) measures of your height, weight, resting pulse, and resting blood pressure, and b) a maximal exercise test on a stationary bicycle.

Visits #3 through #6
These visits include the following tests: a) a forearm handgrip test (i.e. repeated squeezing against a resistance), and b) a submaximal exercise test on a stationary bicycle. For these tests, you will either breathe room air or low oxygen air, simulating an altitude of approximately 9,900 feet, which is equivalent to an altitude you might experience in the Rocky Mountains.

Between Visits #4 and #5, you will be asked to take capsules that are omega-3 (fish oil) supplements or placebos (capsules of safflower oil that look like the fish oil supplement). You will take 8 capsules a day, 4 in the morning and 4 in the evening, each day for 6 weeks (42 consecutive days). You will be asked to record the date and time you take your capsules on a supplement diary sheet, which we will provide you. You may also record any comments you may have on this sheet. You will be asked to return the supplement diary sheet, the supplement containers, and any unused pills with your final visit to the lab (Visit #6).

Each of the tests and supplement routine is described below.

Blood draw (visit #1 only). A small amount of blood will be collected to measure your cholesterol, glucose, and hemoglobin levels. For this test, the inside of your arm, opposite your elbow will be swabbed with alcohol and a sterile needle will be briefly inserted. A small amount of blood (about 4 teaspoons) will be collected into a tube for analysis.

Height, weight, heart electrical activity, and blood pressure measures. Height will be measured by asking you to stand against a wall and a device will be lowered until it touches the top of your head. Weight will be measured by having you sit on a chair, which is placed on a scale. Blood pressure will be measured by placing a cuff around your upper arm. The cuff will be inflated, squeezing your upper arm, and quickly deflated. Adhesive electrodes will be placed on your chest and wires attached to monitor the electrical activity of your heart.

Maximal Cycle Exercise Test (visit #2 only). This exercise test will be completed on a stationary bicycle. Resting measurements will be collected for 5 minutes and followed by a 5 minute warm-up at a pace you select. The test begins with cycling at between 70 - 100 rpm with a light resistance load. Every minute, a small amount of additional resistance will be added until you can no longer maintain the required power output. The goal is for you to exercise for as long as you can, and for most individuals, this is about 8-12 minutes of pedaling.

Forearm Handgrip Test (visits #3 through #6 only). For this test, you will be asked to lie down on a padded table with your dominant arm (the arm you use to throw a ball) held out away from your side, resting on a table. You will be asked to wrap your fingers around a handgrip device, which is similar to a handle on a suitcase. You will be asked to squeeze the handle as hard as you can 3 times, resting between each squeeze. You will then be asked to perform 3 different exercise bouts of repeated squeezing. Repeated squeezing will be 5 seconds of squeezing and 5 seconds of relaxing. An audio recording will tell you when to squeeze and when to relax. The first two exercise bouts of repeated squeezing will be for 3 minutes, separated by 5 minutes of rest. The first bout will be at a low effort (17% of your maximal squeeze force) and the second at a medium effort (33% of maximal squeeze force). The third and last bout of repeated squeezing will be at 50% of your maximal squeeze force, done for as long as you can, or until your force falls to less than 33% of your maximal squeeze force for 3 consecutive squeezes. Normally, this third bout of repeated squeezing lasts about 4 to 7 minutes. You will be able to view a monitor that tells how hard you are squeezing and what target you are trying to achieve.

Submaximal Cycle Exercise Test (visits #3 through #6 only). This exercise test will be completed on a stationary bicycle. You will be asked to complete 3 separate cycle exercise efforts lasting 3 minutes each, separated by 10 minutes of seated rest. Each of the cycle exercise efforts will be at progressively higher resistances, equal to 25%, 50%, and 75% of the peak resistance you achieved in the maximal cycle test. For each of the 3 cycle exercise efforts, resting measurements will be collected for 5 minutes and followed by a 5 minute warm-up at a very light resistance. You will be able to view a screen that shows the time completed.

All of the exercise tests (maximal cycle exercise, forearm handgrip, and submaximal cycle exercise) include wearing a clip that shines light through your index finger, either adhesive electrodes on your chest or a wireless heart rate monitor strap, and breathing either through a rubber mouthpiece while wearing nose clips, or a face mask which covers your nose and mouth. Either room air or low oxygen air simulating an altitude of approximately 9,900ft will flow into and out of your lungs as you breathe through the mouthpiece or face mask. You will not be told which air (room air or low oxygen air) you will be given. Rubber mouthpieces, face masks, heart rate monitor, and nose clips are cleansed in a detergent and antibacterial solution following each use. During each forearm exercise test, a fiber optic cable bundle will be secured by elastic bandages to your exercising forearm (about 1/3rd of the distance from your elbow to your wrist). During the submaximal cycle exercise test, a fiber optic cable bundle will be secured by elastic bandages to your left thigh and left calf. This device uses light waves to measure the oxygen content of your tissues.

Finger prick blood samples. Prior to the first exercise effort and immediately after each exercise effort of the forearm handgrip and submaximal cycle exercise test, we will prick your fingertip with a sterile needle and collect a few drops of blood. This means on each of Visits #3 through #6, you will receive a total of 8 finger pricks.

RISKS OF TAKING PART IN THE STUDY:

While on the study, the risks are:

Both maximal and moderate level exercise tests of healthy individuals, as described by the American College of Sports Medicine, presents little risk to the subject and does not require medical clearance for subjects under 40. For untrained and type 2 diabetic subjects, men over 45 years of age and women over 55 years of age, the risks associated with exercise testing increases. Potential risks and/or discomforts can include episodes of temporary light-headedness, chest discomfort, leg or arm cramps, occasional irregular heartbeats, and abnormal blood pressure responses. The risk of heart attack, although minor, (approximately 1 to 2 in 10,000) does exist, and a heart attack could result in death. During exercise testing you will be closely monitored for any abnormal changes in heart rate or breathing. You are free to indicate any discomfort and discontinue participation at any time.

There is a slight risk of skin discomfort or irritation from the fiber optic bundles and electrodes that will be placed on your skin.

There is a risk of blood pooling in your legs and low blood pressure immediately following the cycle exercise tests. After the tests, you will be allowed to cool down on the exercise bike. If at any time during testing you become light headed, you may have to lie down until you feel normal.

Risks associated with taking blood include excessive bleeding, fainting or feeling lightheaded, and possible infection. All blood samples will be taken by qualified individuals. A reasonable effort will be made to minimize the risks associated with drawing blood through the use of proper procedures and sterile techniques.

Potentially negative side effects could occur while taking the supplements. Common side effects include vomiting, nausea, bloating and burping. Rare side effects consist of easy bleeding/bruising and serious allergic reaction.

Breathing low oxygen air involves the risk of lightheadedness, heavy breathing, dry throat, and irritation of nasal passages due to the dry nature of the gas used.

In type 2 diabetic subjects, there is a risk of hypoglycemia (low blood sugar).

There is a potential risk of loss of confidentiality.

BENEFITS OF TAKING PART IN THE STUDY:

The benefits to participation that are reasonable to expect are information regarding your cholesterol and glucose levels, overall level of fitness, and how omega-3 fatty acid (fish oil) supplements may help you tolerate exercise better. Other than this information, you will gain little benefit. All subjects will be provided with feedback concerning their own results and the general findings of the study upon request.

CONFIDENTIALITY

Efforts will be made to keep your personal information confidential. Data will be stored on password protected computers in locked rooms with limited public access. We cannot guarantee absolute confidentiality. Your personal information may be disclosed if required by law. Your identity will be held in confidence in reports in which the study may be published and databases in which results may be stored.

Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as the study investigator and his/her research associates, the American Diabetes Association, the IU Institutional Review Board or its designees, and (as allowed by law) state or federal agencies, specifically the Office for Human Research Protections (OHRP) and the U.S. Food and Drug Administration (FDA), who may need to access the collected medical and/or research data.

PAYMENT

You will be paid a total of $150 for completing all (6) days of testing. Payment will be made by check and will be delivered by postal mail within approximately 4 weeks of your final testing session. If you withdraw prior to completing all days of testing, you will be paid according the exercise trial(s) you complete or attempt to complete; $30 per exercise trial (Visits 2-6).

COMPENSATION FOR INJURY

In the event of physical injury resulting from your participation in this research, necessary medical treatment will be provided to you at your own expense. Costs not covered by your health care insurer will be your responsibility. Also, it is your responsibility to determine the extent of your health care coverage. There is no program in place for other monetary compensation for such injuries. However, by signing this form you are not giving up any legal rights or benefits to which you are otherwise entitled.

CONTACTS FOR QUESTIONS OR PROBLEMS

For questions about the study or a research-related injury, contact the researcher Robert Chapman, Ph.D. at (812) 856-2452 or rfchapma@indiana.edu. If you cannot reach the researcher during regular business hours (i.e. 8:00AM-5:00PM), please call the IU Human Subjects Office at (812) 856-4242 or (800) 696-2949.

For questions about your rights as a research participant or to discuss problems, complaints or concerns about a research study, or to obtain information, or offer input, contact the IU Human Subjects Office at (812) 856-4242 or (800) 696-2949.

VOLUNTARY NATURE OF STUDY

Taking part in this study is voluntary. You may choose not to take part or may leave the study at any time. Leaving the study will not result in any penalty or loss of benefits to which you are entitled. Your decision whether or not to participate in this study will not affect your current or future relations with the investigators or Indiana University.

Your participation may be terminated by the investigator without regard to your consent in the following circumstances: blood pressure, ECG, cholesterol, height and weight, or maximal exercise test results that do not meet the criteria for inclusion in the study, an abnormal response to exercise testing, or an inability to complete the exercise tests.

SUBJECT'S CONSENT

In consideration of all of the above, I give my consent to participate in this research study.

I will be given a copy of this informed consent document to keep for my records. I agree to take part in this study.

Subject's Printed Name:_____

Subject's Signature:_____**Date**:_____
(must be dated by the subject)

Printed Name of Person Obtaining Consent:_____

Signature of Person Obtaining Consent:_____**Date**:_____

For IRB Office Use ONLY

IRB Approval Date:

Expiration Date:

Appendix 6. Modified Physical Activity Readiness Questionnaire (PAR-Q).

Modified Physical Activity Readiness Questionnaire (PAR-Q)

Name			Date
DOB	**Age**	**Home Phone**	**Work Phone**

Regular exercise is associated with many health benefits, yet any change of activity may increase the risk of injury.
Please read each question carefully and answer every question honestly:

Yes	**No**	**1. Has your doctor ever said that you have a heart condition and that you should only do physical activity recommended by a doctor?**
Yes	**No**	**2. Do you feel pain in your chest when you do physical activity?**
Yes	**No**	3. **In the past month, have you had chest pain when you were not doing physical activity?**
Yes	**No**	**4. Do you lose your balance because of dizziness or do you ever lose consciousness?**
Yes	**No**	**5. Do you have a bone or joint problem that could be made worse by a change in your physical activity?**
Yes	**No**	**6. Is your doctor currently prescribing drugs (for example, water pills) for your blood pressure or heart condition?**
Yes	**No**	**7. Do you know of any other reason you should not do physical activity?**
Yes	**No**	**8. Has your doctor ever told you that you have diabetes?**
Yes	**No**	**9. Has your doctor ever told you that you have high blood pressure?**
Yes	**No**	**10. Has your doctor ever told you that you have high cholesterol?**
Yes	**No**	**11. Has your doctor ever told you that you have high blood sugar?**
Yes	**No**	**12. Do you smoke?**
Yes	**No**	**13. Are you currently inactive?**
Yes	**No**	**14. Do you have a father, brother or son with heart disease before the age of 55 years old or a mother, sister or daughter with heart disease before the age of 65 years old?**

15. Measure height and weight to determine BMI: **16. Measure resting BP and pulse**
Height:_____ **BP:_____ Pulse:_____**
Weight:_____

Participant Signature	**Date**

Appendix 7. General Study Questionnaire.

General Study Questionnaire

Name	**Date**

On average over the last 8 weeks, how many minutes per week did you exercise?	
Do you consider yourself to be a highly endurance trained individual?	**(Circle one) YES NO**

Participant Signature	**Date**

Appendix 8. Omega-3 Food Frequency Questionnaire.

Omega-3 Food Frequency Questionnaire

Department of Kinesiology

Human Performance Laboratory

Indiana University

Food Frequency Questionnaire from: Hanson JA, Lin Y, Strandjord SE, and Hibbeln JR. The Relationship between Omega-3 HUFA Score and Dietary Intake of EPA and DHA among U.S. Soldiers. *Journal of the Academy of Nutrition and Dietetics* 113: A26, 2013

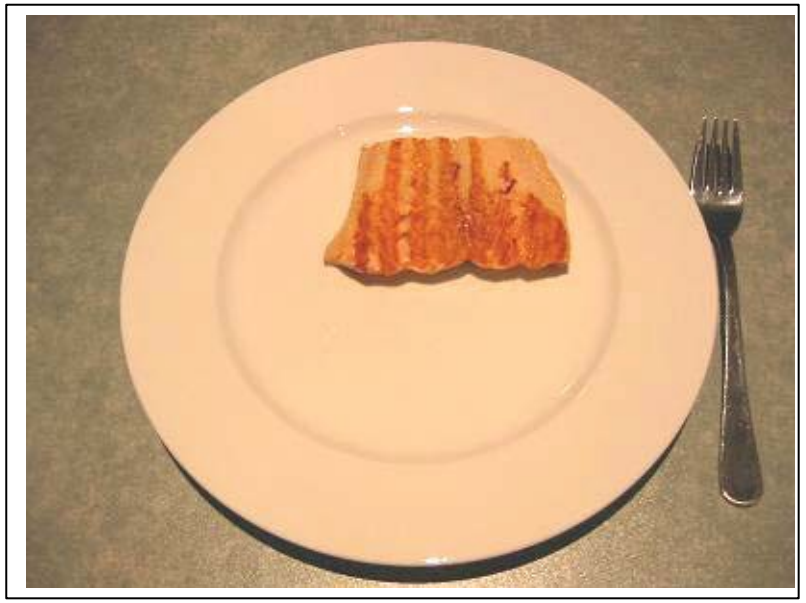
Instructions:

This questionnaire is about how much and how often you ate foods containing high levels of omega-3 fatty acids. It is also about how much and how often you consume different beverages. When answering, think about what you usually ate and drank during the last six months. Please remember to include foods you ate in restaurants, as takeout food, and fish you or someone you know caught. Complete **PARTS 1-3**.

Part 1 Instructions:
Step 1: Mark the column with an "X" to show how often on average you ate the food.
Step 2: Mark your usual serving size with an "X", as small (S), medium (M), or large (L).

Please note:

- A small serving is about one-half (1/2) the medium serving size, or less.
- A large serving is about one-and-a half (1 ½) times the medium serving size or more.
- If you never ate a food, mark "never" and omit the serving size.
- Please do not skip any foods or leave blanks.
- 4 ounces of cooked fish looks like the picture on the right :



Example: This person never ate sushi, but ate a medium serving of baked or broiled white fish once a week.

	NEVER or less than once per month	1 per month	2-3 per month	1 per week	2 per week	3-4 per week	5-6 per week	1 per day	2 + per day	Medium Serving size	S	M	L
Sushi	X									1 roll			
Baked or broiled white fish (such as snapper, cod, halibut, sole)				X						4 ounces		X	

Food Frequency Questionnaire from: Hanson JA, Lin Y, Strandjord SE, and Hibbeln JR. The Relationship between Omega-3 HUFA Score and Dietary Intake of EPA and DHA among U.S. Soldiers. *Journal of the Academy of Nutrition and Dietetics* 113: A26, 2013

PART 1

How often did you eat these foods during the last 6 months?

	NEVER or less than once per month	1 per month	2-3 per month	1 per week	2 per week	3-4 per week	5-6 per week	1 per day	2 + per day	Medium Serving size	S	M	L
Canned tuna, tuna salad, tuna sandwich, tuna casserole										1 can or 1 cup casserole			
Fried fish, fish sandwich, fish sticks										4 ounces or 1 sand-witch			
Shellfish (shrimp, clams, oysters, lobster, crayfish)										4 ounces			
Sardines										1 can			
Baked, broiled, or grilled white fish (such as snapper, cod, halibut, sole)										4 ounces			
Baked, broiled, or grilled dark or oily fish (such as salmon, mackerel, and bluefish)										4 ounces			
Sushi Please write type _____										2 rolls			
Beef										4 ounces			
Pork										4 ounces			
Dark chicken meat										2 pieces			
Eggs with yolks										2 egg			

Food Frequency Questionnaire from: Hanson JA, Lin Y, Strandjord SE, and Hibbeln JR. The Relationship between Omega-3 HUFA Score and Dietary Intake of EPA and DHA among U.S. Soldiers. *Journal of the Academy of Nutrition and Dietetics* 113: A26, 2013

PART 2

See the display for examples of foods enriched with DHA and/or other omega-3 fatty acids. Did you eat any DHA or omega-3 enriched foods during the last 6 months? (Circle) **YES** or **NO**

If NO skip to Part 3. If YES, write in: 1) the food, 2) your usual serving size, and 3) how often eaten.

Food	Usual Serving Size	NEVER or less than once per month	1 per month	2-3 per month	1 per week	2 per week	3-4 per week	5-6 per week	1 per day	2 + per day
Example : Horizon DHA milk	8 oz			X						

Food Frequency Questionnaire from: Hanson JA, Lin Y, Strandjord SE, and Hibbeln JR. The Relationship between Omega-3 HUFA Score and Dietary Intake of EPA and DHA among U.S. Soldiers. *Journal of the Academy of Nutrition and Dietetics* 113: A26, 2013

PART 3

Did you take any nutritional supplements during the last 6 months? (Circle) **Yes** or **No**
If NO, skip to end. If YES, how much and how often?

Supplement	How much did you normally take?
Cod liver oil, fish oil, or omega -3 supplements Please Specify 1._____ 2._____	 1._____ 2._____
Multivitamin Please Specify 1._____ 2._____	 1._____ 2._____
Other vitamin, mineral or nutritional supplements Please Specify 1._____ 2._____	 1._____ 2._____

END

Food Frequency Questionnaire from: Hanson JA, Lin Y, Strandjord SE, and Hibbeln JR. The Relationship between Omega-3 HUFA Score and Dietary Intake of EPA and DHA among U.S. Soldiers. *Journal of the Academy of Nutrition and Dietetics* 113: A26, 2013