Effects of Cold-Water Immersion (CWI) on Intermittent Fasting (IF) induced Ketogenesis.

Thesis

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By

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Abstract

The effects of cold-water immersion (CWI) and intermittent fasting (IF)/ketosis have been extensively investigated independently. However, the primary objective of this study is to explore potential links between the two. Cold-water immersion is known for diverse benefits, such as aiding athlete recovery, enhancing cognitive function, and contributing to mental health improvement. This research aimed to assess ketone levels in six individuals (four males and two females) across various fasting states (8- and 12-hours, and 8-hours with a ketone ester supplement), exposing them to a 15-minute head-out immersion at 50ºF (10ºC). Although no significant differences in ketone levels were observed across the three trials, this pilot study, involving six participants, unveils potential trends between CWI and capillary ketone levels during a fasted state. Additionally, the introduction of a ketone ester supplement resulted in elevated ketone levels. It is important to note that further research and data are required to draw conclusive insights into the observed 38% increase in our pilot trial.

Acknowledgments

To Alex Buga,

Thank you, Alex Buga, for being the first one in the lab to really push me to be the best and to perform beyond what I thought I could do through research. Without your initial trust in me to help with your study, your constant feedback on how to improve, and conversation on your vast knowledge on various topics I wouldn't know how to push myself to become the best I can be.

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A big thank you to Dr. Volek for having the best lab with the best people. I am extremely grateful for you giving an undergraduate the resources and the space to conduct a novel research protocol and to execute it. Without that kind of open-mindedness and trust none of this would have happened.

Vita

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Plans after Graduation

Armed with a college diploma and a Bachelor of Science in biology, my immediate post-graduation objective is to secure employment in Columbus. Ideally, I aim to work in proximity to my hometown, Upper Arlington, enabling regular visits to my family. This job will serve as a means to accrue and save funds, with the future goal of supporting my aspirations for medical school. Simultaneously, I plan to dedicate time to studying for and taking the MCAT in early 2024, intending to submit any last-minute applications. In addition to pursuing medical school, I will explore institutions offering postbaccalaureate programs to enhance my chances of acceptance and potentially obtain a master's degree. Furthermore, I intend to reengage with Ohio State, contributing to research endeavors when possible. Whether continuing with my thesis or providing assistance where needed, I aspire to remain actively involved in the academic and research community at the university.

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Chapter 1: Introduction.

Cold-water immersion (CWI) has a long history, dating back to 3500 BC, with ancient Greeks using cold water for therapies, relaxation, and socialization. Hippocrates documented its medicinal benefits in the fourth century BC, and later, James Currie explored its impact on human physiology. In the 1960s, D. H. Clarke's work marked a turning point, focusing on CWI for post-exercise recovery. CWI involves submerging the body in cold water, triggering the 'cold shock' phenomenon and activating the sympathetic nervous system. Despite initial shock, the body responds with thermogenesis, using shivering and non-shivering mechanisms.

The study investigates the impact of CWI and intermittent fasting (IF) on blood ketones. IF shifts the body's energy source from glucose to ketones, derived from fatty acids during fasting. The study aims to evaluate the effects of varying intermittent fasting durations with and without prefasting exogenous ketone supplementation and CWI on blood ketone concentrations. The hypothesis suggests that a 12-hour fast with CWI will result in higher ketone levels than an 8-hour fast. Additionally, the intervention of a ketone ester supplement before CWI is expected to surpass both fasting durations. Ten participants underwent prefasting trials, involving different fasting durations and ketone ester ingestion, followed by CWI. Measurements included capillary ketone and glucose levels, blood pressure, heart rate, and tolerability assessments.

Adipose tissue, particularly brown adipose tissue (BAT), plays a crucial role in non-shivering thermogenesis. BAT, activated by cold stress, releases energy through UCP1, promoting heat production. Similar effects are observed in intermittent fasting and ketogenic diets (KDs). The study explores the impact of ketone esters on BAT, aiming to bridge insights from animal models to humans. Plasma ketone levels, influenced by diet composition, are discussed, with ketone esters transiently increasing concentrations. The study hypothesizes that combining CWI, intermittent fasting, and ketone esters can enhance ketosis and brown fat activation, offering potential health benefits. The research looks to elucidate the relationship between IF, CWI and ketosis.

Chapter 2: Literature Review.

Cold-water immersion (CWI) has a rich historical background, dating back to as early as 3500 BC. In ancient Greece, cold-water was not only used for therapies but also served as a means of relaxation and socialization. Hippocrates, in the fourth century BC, documented the medicinal uses and analgesic benefits of cold water in his work "On airs, waters and places," stating that "water can cure everything". Centuries later, physiologist James Currie explored the impact of cold water on human physiology, conducting experiments on body temperature, pulse, and respiration. His documented records of human temperatures in various conditions, conducted at his "water cure establishment," aimed to validate the value of hydrotherapy.

The shift towards investigating the benefits of cold-water immersion for post-exercise recovery occurred in the 1960s, spurred by the work of D. H. Clarke (Clarke, 1963; Clarke and Stelmach, 1966). This marked a pivotal moment in understanding and exploring the therapeutic potential of CWI in the context of recovery from physical exertion (Peterson et al, 2021). The practice involves submerging the body in water below a specific temperature, proving effective in enhancing insulin sensitivity and elevating plasma dopamine levels, while simultaneously reducing plasma adrenaline, cortisol, and insulin concentrations (Espeland et al, 2022; Srámek et al, 2000). This immersion triggers the 'cold shock' phenomenon, marked by hyperventilation, activation of the sympathetic nervous system (SNS), an increase in heart rate, and elevated blood pressure (Srámek et al, 2000; Espeland et al, 2022). Despite the initial shock, the body responds through thermogenesis, employing shivering and non-shivering thermogenesis to generate heat and activate brown adipose tissue (BAT) (Espeland et al, 2022).

Adipose tissue is used for various physiological mechanisms. There are two types of adipose tissues, white adipose tissue (WAT) and brown adipose tissue (BAT), which have differing functions. Within humans, WAT is the most prevalent of the two, and is primarily used for energy storage during fasted states. BAT, while being the lesser of the two, is the focus of this study due to its crucial role in non-shivering thermogenesis. What makes BAT different from WAT is the higher presence of an inner mitochondrial membrane within the tissue. Uncoupling protein 1 (UCP1) is the specific coupling transfer protein for BAT that gives it is thermogenic abilities. While under cold stress, the sympathetic nervous system (SNS) releases norepinephrine (NE). Cyclic adenosine monophosphate (cAMP) increases due to the activation of beta-adrenergic receptors because of the increased NE. Due to the increase in cAMP, protein kinase A is activated, which induces lipolysis releasing free fatty acids (FFA) that promote the expression of UCP1 in the inner mitochondrial membrane. UCP1 allows for active transport of protons outside of the mitochondrial matrix through the electron transport chain releasing energy. Active UCP1 then promotes proton movement back into the cell causing a production of heat through the transporter protein.

Intermittent fasting (IF), akin to CWI, is another historical practice that offers comparable health advantages through timed caloric restriction. IF is widely embraced as a method to reduce calorie intake and facilitate weight loss. During fasting periods, the body's primary energy source transitions from glucose to ketones, which are derived from fatty acids in adipose tissue (de Cabo et al, 2019). Certain fasting regimens activate BAT within white adipose tissue, mirroring the effects of CWI. This activation leads to significant improvements in conditions such as obesity, insulin resistance, and hepatic steatosis. Beyond its positive impact on obesity-related issues, IF also presents compelling evidence for potential benefits in delaying neurodegenerative disorders and extending lifespan in animals (de Cabo et al, 2019).

Similar to IF, ketogenic diets (KDs) featuring high fat and low carbohydrate content elevate UCP1 protein levels in the BAT of mice, correlating with increased energy expenditure. Further exploration includes comparing KD effects on BAT with a diet supplemented with ketone ester (KE), which has shown increased BAT mitochondrial biogenesis and UCP1 levels. This research investigates ultrastructural changes in brown adipocytes, highlighting both similarities and differences between KD and the KE diet. Plasma ketone levels are usually <0.3 mmol/L when carbohydrates are the major macronutrient of the diet, 0.5-4 mmol/L when carbohydrates are restricted to less than 50 g/day (i.e., a nutritional ketosis)(Foster, 1967; Kackley et al., 2020; Stubbs et al., 2020b; Volek et al., 2014), and between 5-8 mmol/L after prolonged starvation (G. Cahill, 2006). Ingestion of a KE can transiently increase plasma ketone concentrations similar to those achieved during prolonged fasting, 3-5 mM via either a ketone monoester or diester compound (Cox et al., 2016; Stubbs et al., 2017; Vandoorne et al., 2017). Substituted diets in a rat model shows the addition of a KE supplement in a 1-month trial yielded more mitochondria and double the number of UCP1 in the electron transport chain (Srivastava, et. al, 2012). The increase of these two directly affect BAT being that they are both crucial in activation and release of energy during cold exposure. There is more to be elucidated in humans.

This study aims to investigate the impact of variable levels of cold-water immersion (CWI) and intermittent fasting on changes in blood ketones. In a typical diet, carbohydrates serve as the primary energy source for the body. However, during fasting, the body, lacking access to carbohydrates, seeks an alternative energy source, often found in fatty tissue. As free fatty acids break down, ketone bodies are released. Emerging evidence suggests that ketones offer distinctive cognitive and physical benefits compared to carbohydrates, prompting exploration into the potential advantages unlocked by intermittent fasting. We aim to examine the relationship between cold-water immersion and blood ketones, as CWI has been linked to brown fat loss, and ketone bodies are released from the breakdown of free fatty acids (FFA). This study seeks to evaluate the effects of varying levels of intermittent fasting with and without prefasting exogenous ketone supplementation and CWI on the concentration of ketone bodies in the blood.

With these facts in mind, we hypothesize that undergoing a 12 hour fast and completing cold water immersion (CWI) will cause ketone levels to be higher than that of an 8 hour fast. Furthermore, the intervention of a ketone ester supplement 8 hours before the CWI will be greater than both 8 and 12 hour fast with no supplement. This can be concluded due to the body using more of its energy stores with a longer fasting period from gluconeogenesis so the body will be further into ketosis in a 12 hour fast than that of an 8 hour. The intervention of a ketone supplement, the body will metabolically switch to using ketones instead of carbohydrates. This leads us to believe that the body will already be primed to undergo lipolysis and during CWI the body will be able to react faster to using BAT.

Chapter 3: Methods.

Participants

This was a 3-way crossover study design. For the primary aim, we planned to randomize 10 participants into 3 different fasting regiments prior to a CWI trial. A total of six participants, encompassing both male and female individuals aged between 18 and 65 (22.2 ± 4.06), with a body mass index ranging from 18 to 35 kg/m^2, were enrolled in this trial. Participant characteristics can be found in **Table 1**.

Table 1 | Participant Characteristics

(n=6)						
ID	**001**	**002**	**003**	**004**	**005**	**006**
Sex	Male	Female	Male	Female	Male	Male
Age (years)	21	31	22	20	19	20
Weight (kg)	95	55	80	56	82	69
BMI (kg/m^2)	29.3	21.5	24.7	24.2	24.5	23.9
Ketones (mmol/L)	0.1	0.3	0.2	0.1	0.3	0.1
Glucose (mg/dL)	99	100	114	80	94	103
HeartRate (BPM)	74	104	95	74	61	76
Blood Pressure (mm Hg)	136/70	128/78	122/68	108/62	120/78	114/74

Participants were randomly assigned to complete three prefasting trials, each involving different fasting durations: an 8-hour fast, a 12-hour fast, and an 8-hour fast with the ingestion of a ketone ester before fasting (**Figure 1**). The protocol included a 15-minute head-out water submersion, followed by a thirty-minute warming period. Throughout the experiment, participants wore bathing suits or equivalent attire. CWI took place in a

Portable Bathtub Ice Bath Tub, 29.5" (Bui Yuen, China). The water temperature, initially set to 50°F (10°C), was meticulously monitored using a thermometer, with adjustments made as needed to maintain the desired temperature.

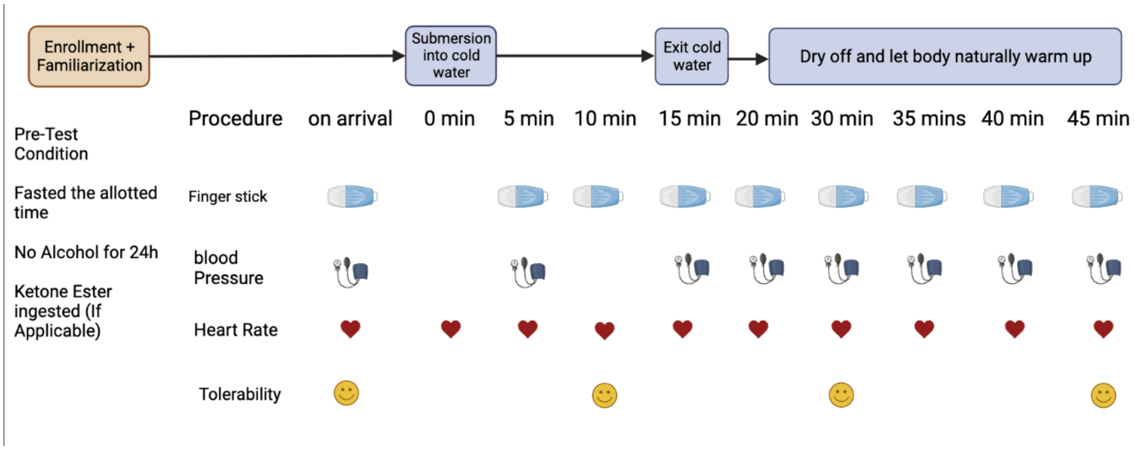

Figure 1 | Experimental Design

Baseline Measurements: Before entering the ice bath, baseline measurements were taken, including participant height, weight, and hydration levels. Pretesting protocol was confirmed, such as fasting and abstaining from alcohol or intense exercise 24 hours before the trial. Additionally, participants completed a tolerability assessment to evaluate their perceptions of physiological and cognitive factors, covering temperature, discomfort, alertness, and tiredness.

Physiological Assessments: Following baseline measurements, participants' capillary ketone (mmol/L) and glucose (mg/dL) levels were obtained through fingersticks using a KetoMojo device. Blood pressure and heart rate were recorded using a synced Polar watch and puck.

Cold-Water Immersion and Out of Tub Warming Procedure: The 15-minute CWI exposure timer began when participants fully submerged their bodies into the ice bath, leaving only their heads above water. At each 5-minute interval, participants removed their arms from the bath for the recording of heart rate, blood pressure, capillary ketone, and glucose concentrations. After the 15-minute immersion, participants exited the tub, dried themselves, and sat in a chair for 30 minutes to facilitate natural body warming. Heart rate, blood pressure, and ketones/glucose were recorded every 5 minutes throughout the warming period.

Tolerability Assessments: Tolerability assessments were administered at ten, thirty, and post-test intervals to observe potential adaptations to cold-water immersion and any associated cognitive changes.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism version [insert version number]. Descriptive statistics, presented as mean ± standard deviation (SD) unless otherwise specified, were employed to analyze baseline characteristics, including age, body mass index (BMI), and hydration levels. Categorical variables such as gender were summarized using frequencies and percentages. For the prefasting trials (8-hour fast, 12-hour fast, and 8-hour fast with ketone ester supplementation), one-way analysis of variance (ANOVA) with post hoc Tukey tests for pairwise comparisons was utilized for continuous variables, while chi-squared tests were applied for categorical variables. Changes in capillary ketone and glucose levels, as well as blood pressure and heart rate, were assessed using repeated measures analysis of variance (RM-ANOVA) with appropriate post hoc tests for within-group variations over time. Statistical significance was set at $p < 0.05$ for all analyses, and data visualization was performed using GraphPad Prism.

Chapter 4: Results.

Post Hoc Analysis for all results can be found in **Supplementary Table 1**.

Capillary Ketone Levels

There was a noticeable spike in ketone levels for all trials during the first 5-10 minutes of the trial. In Trials 2 and 3, which involved a 12-hour fast and an 8-hour fast with a supplement, there was an increase in ketone levels observed from the 20-25 minute mark until the end of the trial. No significance was noted. Individual ketone responses are added in **Supplementary Figures 1-6.**

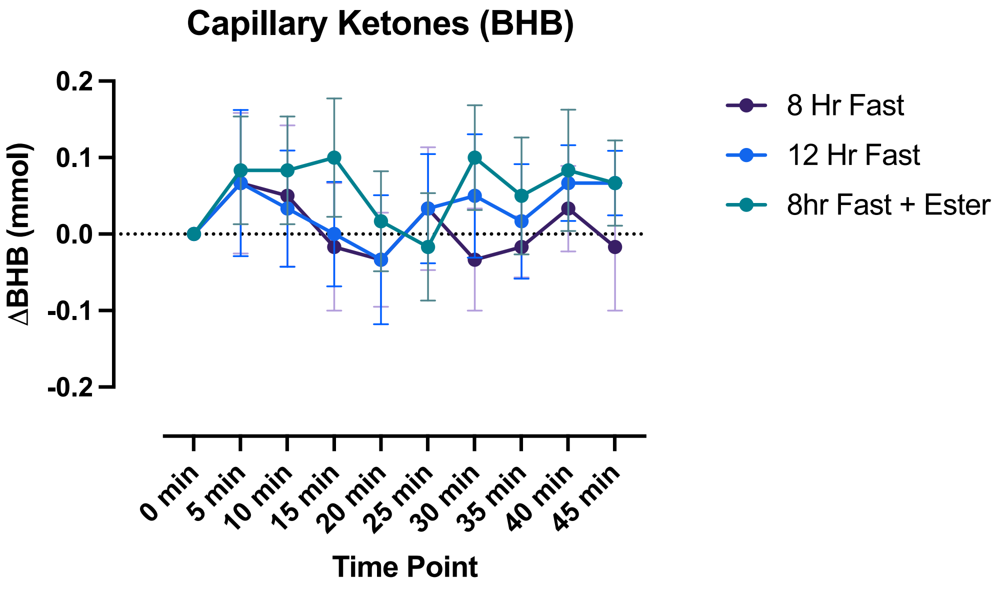

Figure 2 | Capillary Ketones Levels

Capillary Glucose Levels

There was a noticeable spike in ketone levels for all trials during the first 5-10 minutes of the trial. In Trials 2 and 3, which involved a 12-hour fast and an 8-hour fast with a supplement, there was an increase in ketone levels observed from the 20-25 minute mark until the end of the trial. No significance was noted.

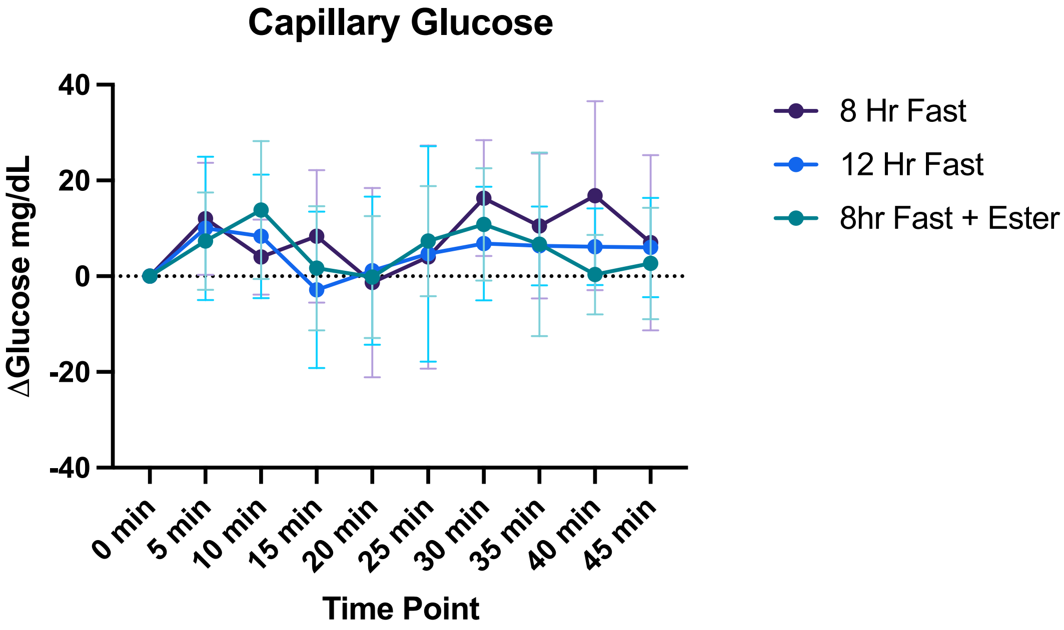

Figure 3 | Capillary Glucose Levels

Heart Rate

The heart rate initially decreased upon entering the cold tub, experienced a subsequent increase upon exiting the tub, and eventually stabilized at the 35-minute mark, with the last 20 minutes spent out of the tub (**Figure 4**). When separating HR during the CWI and post CWI, a main effect of time was seen in both blocks of testing (CWI, *p=0.023*; Post CWI *p= 0.012*, respectively) (**Figure 5 & 6**).

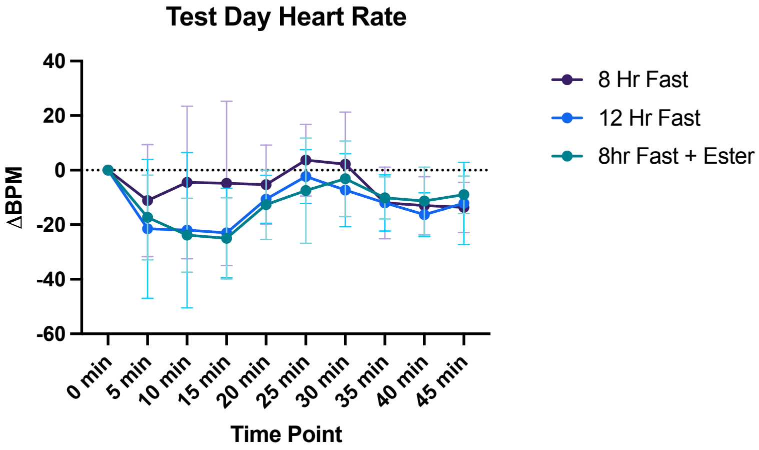

Figure 4 | Test Day Heart Rate

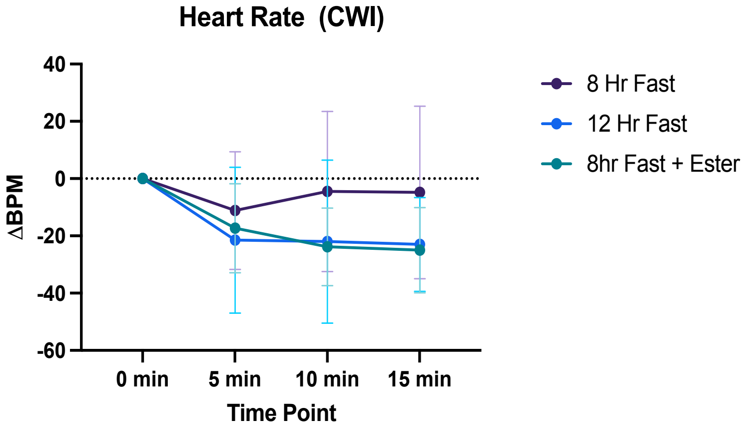

Figure 5 | CWI Heart Rate

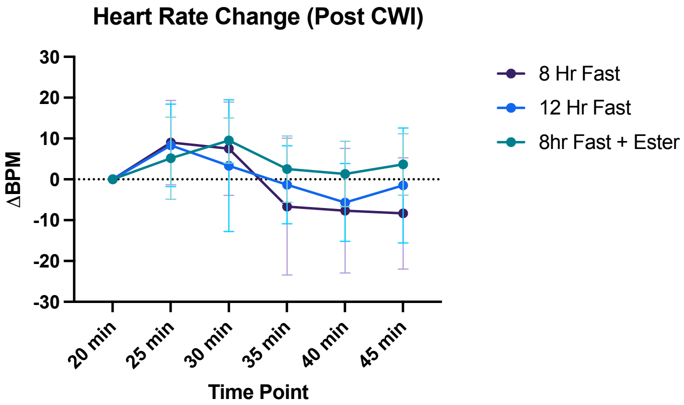

Figure 6 | Post CWI Heart Rate

Blood Pressure

A trial effect was seen in systolic blood pressure (p=0.024); however, no effects were found in diastolic blood pressure.

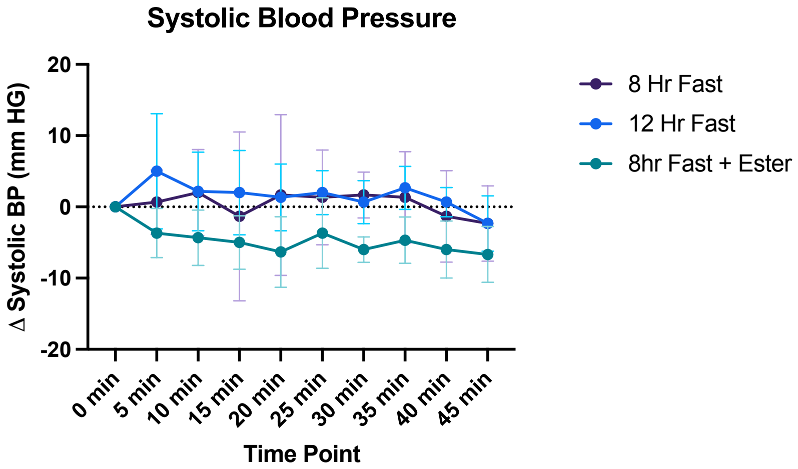

Figure 7 | Systolic Blood Pressure

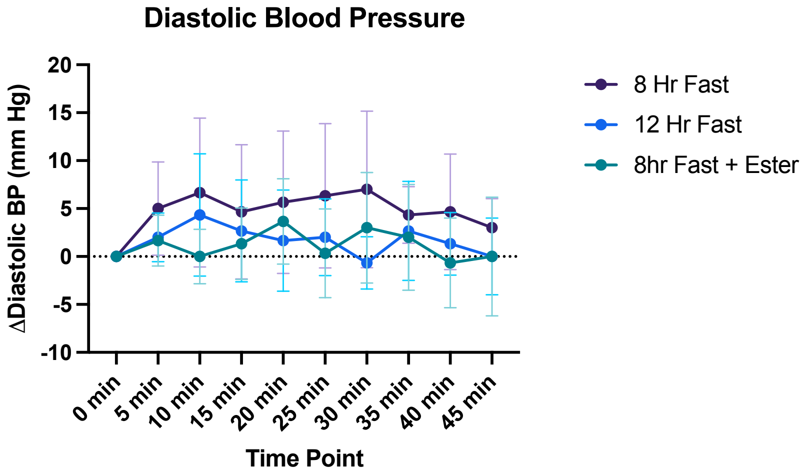

Figure 8 | Diastolic Blood Pressure

Chapter 5: Discussion.

Numerous studies have independently explored the effects of CWI and IF, with and without KE consumption, but there is limited research on their potential interconnection. This study seeks to contribute to this gap by initiating a novel hypothesis regarding the potential synergistic benefits of these two practices. Our findings revealed an increase in ketone levels within the first 5-10 minutes of a head-out cold plunge, both with and without supplementation. While these results align with our hypothesis, statistical significance is currently lacking due to limited data.

The study revealed a noticeable spike in ketone levels across all trials during the initial 5-10 minutes. Interestingly, Trials 2 and 3, involving a 12-hour fast and an 8-hour fast with a supplement, exhibited an increase in ketone levels from the 20-25 minute mark until the conclusion of the trial. A trend was found in the glucose concentration response to the CWI ($p=1.03$). Higher enrollment may elucidate a significant response to a gluconeogenic effect during CWI, independent of fasting and ketone levels. Despite these trends, no statistical significance was observed. It is worth noting that there was also a substantial fluctuation in ketone levels between the male and female participants during exposure to cold water, with males showing a significant ketone spike, while females either maintained consistency or experienced a decline. This gender-specific reaction warrants further investigation in subsequent studies.

In terms of physiological responses, the heart rate exhibited an initial decrease upon entering the cold tub, followed by an increase upon exiting and eventual stabilization at the 35-minute mark, with the last 20 minutes spent out of the tub. Further analysis, particularly during and post CWI revealed significant time-dependent effects on heart rate. Additionally, systolic blood pressure showed a trial effect, with variability observed within the 6 participants. The study emphasizes the importance of addressing limitations, such as the relatively small sample size and heterogeneity, and proposes incorporating advanced techniques like DEXA scans, PET-CT imaging, and gender-specific considerations in future research to enhance the precision and comprehensiveness of findings in the intriguing intersection of cold-water immersion and intermittent fasting.

The intentional use of broad inclusion criteria aimed to capture a diverse population, fostering variability and trend identification despite demographic differences. Although our participants skewed towards being younger and leaner, noticeable variability persisted among them. Constraints on variability arose from factors such as the size of the tub used and time constraints in participant recruitment. Future studies stand to benefit from the incorporation of tools like DEXA scans for assessing body fat percentage and PET scans to measure brown adipose tissue activation. These tools can provide a more nuanced understanding of each participant, allowing for potential adjustments to optimize outcomes related to CWI.

Participants in our study underwent three fasting trials in random order, and compliance was solely dependent on participant honesty, as continuous monitoring was unfeasible. Notably, the majority of participants were male, revealing a sex-linked disparity in ketone levels during exposure to cold water. Males exhibited a significant ketone spike, while females either maintained consistency or experienced a decline. Investigating these gender-specific reactions during CWI should be a focal point in subsequent studies.

Considering the pivotal roles of body composition, particularly body fat percentage and brown adipose tissue, in individuals' responses to cold water exposure, future research must prioritize a deeper understanding of these factors. Incorporating tools such as DEXA scans can offer a more detailed analysis of the impact of body composition on responses to CWI. Additionally, to delve into the mechanistic aspects of brown adipose tissue activation, the utilization of PET-CT imaging is recommended. This advanced imaging technique can provide detailed insights into the metabolic processes within adipose tissue, enhancing our understanding of observed physiological changes.

Despite the valuable insights gained from this study, certain limitations, such as the relatively small sample size and the heterogeneity of the population, warrant acknowledgment. Addressing these limitations is crucial for future research to enhance robustness and applicability. Firstly, there is a clear need to homogenize the sample by narrowing inclusion criteria, minimizing confounding variables that could impact internal validity. Expanding the sample size is also crucial to achieving greater statistical power and reliability. With a larger and more diverse participant pool, researchers can better discern trends, correlations, and potential subgroup variations, enhancing the overall validity of the study's outcomes.

Furthermore, future studies should take into account core temperature variations to gain a more comprehensive understanding of the physiological responses during CWI and intermittent fasting. Monitoring and analyzing core temperature data could provide valuable insights into how different individuals regulate their body temperature during these interventions. Incorporating advanced techniques such as DEXA body composition scanning can contribute significantly to the study's precision. Distinguishing between lean and fat mass allows for a more nuanced analysis of the impact of body composition on responses to CWI and fasting.

To delve deeper into the mechanistic aspects of brown adipose tissue activation, utilizing PET-CT imaging for adipose browning activity is recommended. This advanced imaging technique can provide detailed information about the metabolic processes occurring within adipose tissue, offering a more sophisticated understanding of the observed physiological changes.

Additionally, recognizing and investigating gender differences is crucial for refining our understanding of the varied responses to cold-water immersion and intermittent fasting. Future studies should intentionally include a balanced representation of both male and female participants and systematically explore potential gender-specific effects.

In conclusion, this exploratory study suggests a potential link between cold-water immersion and intermittent fasting. While initial data is promising, further research and trials are imperative for a comprehensive and statistically relevant analysis. Integrating new tools and technology can offer additional insights into individual variations, aiding in more robust conclusions about the validity of our hypothesis. Moreover, these advancements could pave the way for new studies and discoveries in this intriguing field.

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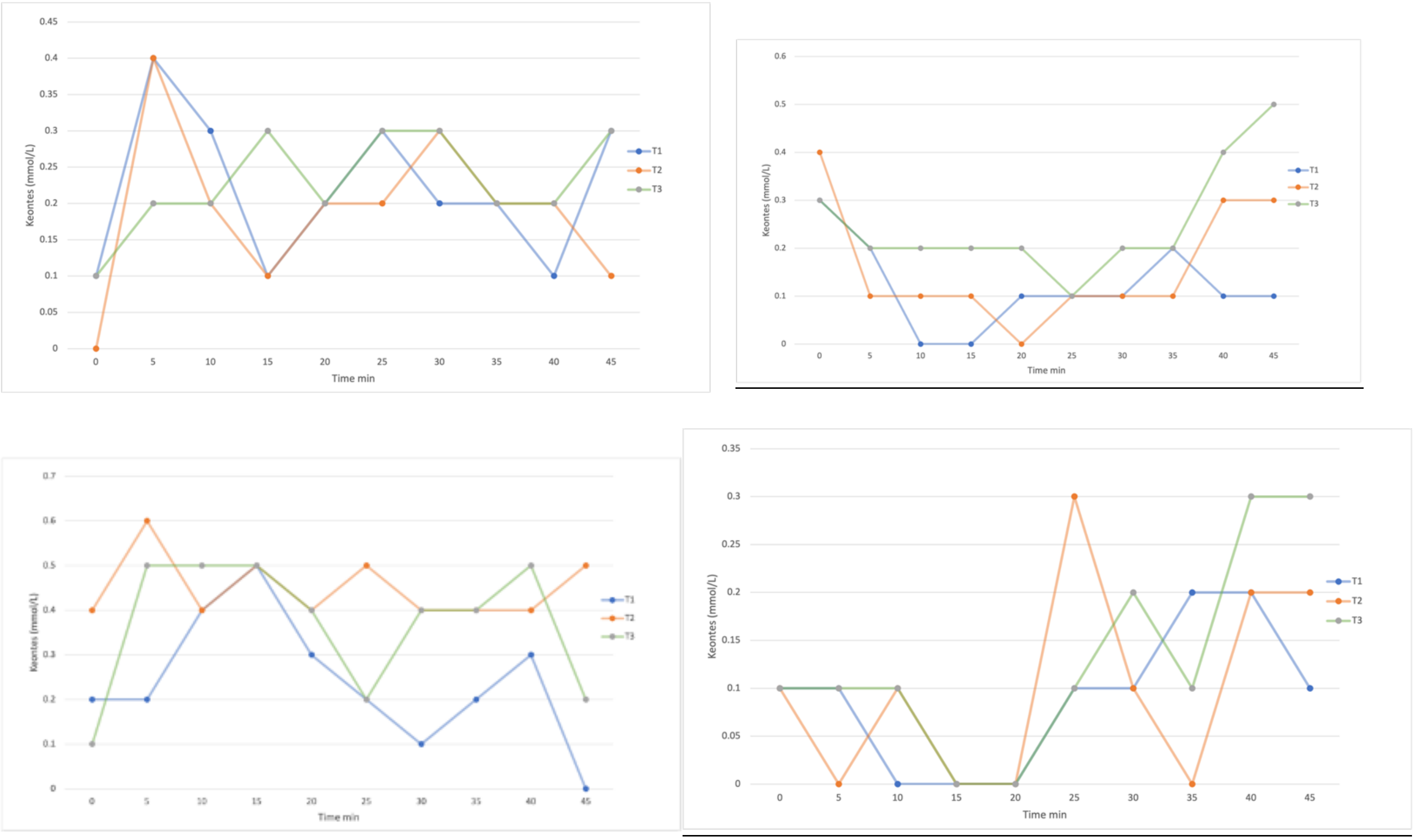
Appendix A. Supplemental Tables.

Supplemental Table 1| Post Hoc Analysis

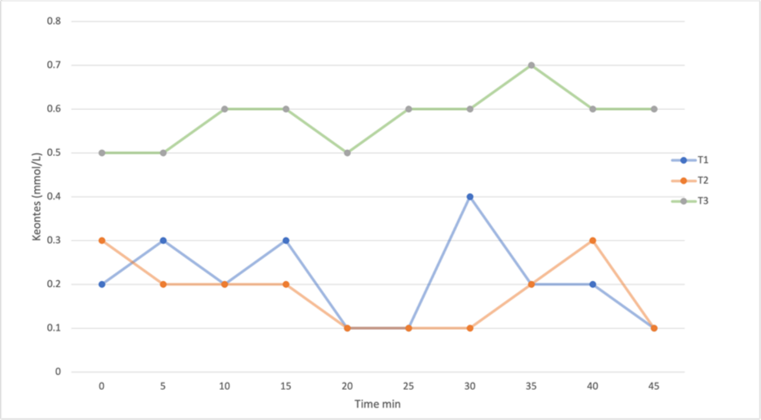
Variable	**Week**	Mean (SD)		Trial	Time	Interaction
Capillary Ketones(mmol)	T1	0.21	(0.06)	.672	.474	.551
	T2	0.35	(0.05)			
	T3	0.35	(0.05)			
Capillary Glucose(mg/dL)	T1	105	(6.47)	0.312	0.103	0.297
	T2	101	(5.46)			
	T3	103	(5.94)			
Heart Rate (BPM)	T1	74.97	(6.41)	0.397	0.192	0.380
	T2	69.10	(8.06)			
	T3	69.17	(8.117)			
Systolic BP (mm Hg)	T1	121.7	(1.53)	**0.024**	0.164	0.621
	T2	120.8	(1.91)			
	T3	117.0	(1.95)			
Diastolic BP (mm Hg)	T1	76.40	(2.05)	0.218	0.145	0.413
	T2	77.27	(1.50)			
	T3	74.67	(1.44)			
CWI Heart Rate (BPM)	T1	75.71	(4.59)	0.287	**0.023**	0.267
	T2	65.21	(11.10) †			
	T3	63.63	(11.53)			
POST CWI Heart Rate (BPM)	T1	74.47	(7.79)	0.720	**0.012**	0.364
	T2	71.69	(4.79)			
	T3	71.19	(3.36)			

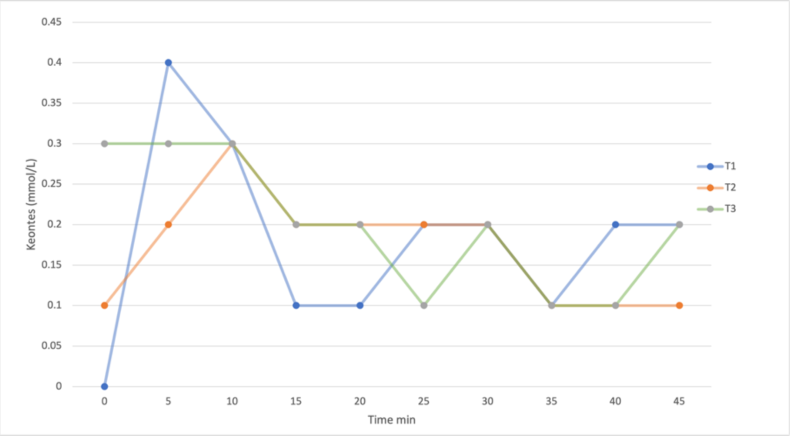
Data reported as mean (SD). Bold face values denote significant effect or interaction ($p < 0.05$).
KE, Ketone Ester; T1, 8 hr fast; T2, 12 hr fast; T3, 8 hr fast with KE. Bonferroni correction was applied to all post-hoc compariso

Appendix B. Supplemental Figures.



Supplemental Figures 1-4 | Individual Participant Ketone Levels





Supplemental Figures 5-6 | Individual Participant Ketone Levels