CAFFEINE EFFECTS ON HIGH AND LOW CAFFEINE RESPONDERS AND ON DIFFERENT AEROBIC FITNESS LEVEL ATHLETES: BIOLOGICAL RESPONSES AND EXERCISE PERFORMANCE

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DECLARATION

I hereby declare that this thesis has been conducted by myself, that the work of which it is a record has been done by myself except where assistance and help have been acknowledged, which it has not been submitted in any previous application for a higher degree and that all sources of information have been specifically acknowledged by mean of references.

Some of the results obtained in this thesis have been presented as follows:

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THESIS SUMMARY

The purpose of the present series of experiments was to compare the effects of caffeine ingestion on biological responses and on exercise performance in athletes a) with different caffeine responses levels and b) with high and medium aerobic fitness level during a simulated soccer game protocol on treadmill in well-trained soccer players.

In Experiment 1 (EXP1), for differentiating the participants according to their physiological responses to caffeine, the changes in resting mean arterial blood pressure (MAP), plasma glycerol, non-esterified fatty acid (NEFA) and epinephrine concentrations at rest have been taken into account. Based on the existing literature, these four biological parameters usually are influenced by caffeine at rest. Thus, the responses to caffeine ingestion at rest were calculated as the difference between the changes from before to 60 minutes following caffeine ingestion and the corresponding change following placebo ingestion and then individual changes to the mean changes were compared. Those participants who had a response above the mean in two or more variables were categorized as high responders, while the rest were categorized as low responders. The results during this experiment revealed that caffeine ingestion improved the time to fatigue and CMJ height but the RT did not change in both high and low caffeine responders groups. Caffeine ingestion (6 $mg \cdot kg^{-1}$) elevated MAP, heart rate (HR) and plasma glucose and lactate concentrations in both high and low responders to caffeine during a simulated soccer game protocol on treadmill. A reduction in rating of perceived exertion (RPE) was observed only in high responders group following caffeine ingestion relative to placebo. Although this advantage did not contribute to statistically significant improve time-to-fatigue in high responders to caffeine group. However, it should not be underestimated that following caffeine ingestion, the high responders improved the time to fatigue by 63.5 % relative to 39.5 % of the low responders to caffeine group.

In Experiment 2 (EXP2), the participants were divided to high or medium aerobic fitness group based on their VO_2max values. The results revealed that caffeine was effective in improving endurance performance in both high and medium aerobic fitness levels athletes. However, caffeine seems to be effective in improving muscular explosiveness and reducing RPE, in athletes with high aerobic fitness level only. Caffeine elevates MAP, HR and plasma glucose and lactate concentrations in both athletes with high and medium aerobic fitness levels during a simulated soccer game protocol on treadmill.

Both experiments revealed that performance improvements with caffeine ingestion in participants with different caffeine responses and in participants with different fitness levels could be attributed to CNS and neuromuscular factors (reduced RPE and improved CMJ performance, respectively), since no differences were observed in substrate utilization. In addition, a potential caffeine-induced an elevation in anaerobic ATP production system contributing in enhancing endurance high-intensity exercise performance, should not be ignoring.

ΠΕΡΙΛΗΨΗ

Αποστολίδης Ανδρέας: **Επιδράσεις της καφεΐνης σε αθλητές με διαφορετικές φυσιολογικές αποκρίσεις στην καφεΐνη και διαφορετική αερόβια ικανότητα: βιολογικές αποκρίσεις και αθλητική απόδοση**

(Με την επίβλεψη του Δρ. Χατζηχαραλάμπους Μάριου, Αναπλ. Καθηγητή)

Εισαγωγή: Υπάρχουν διφορούμενα αποτελέσματα σχετικά με τις επιδράσεις της καφεΐνης στην αθλητική απόδοση και στις βιολογικές αποκρίσεις. Οι σκοποί της έρευνας ήταν να συγκρίνει την επίδραση της καφεΐνης στις βιολογικές αποκρίσεις και στην αθλητική απόδοση μεταξύ α) υψηλά αποκρινόμενων και χαμηλά αποκρινόμενων στην καφεΐνη και β) ατόμων με υψηλή και μέτρια αερόβια ικανότητα κατά τη διάρκεια παρατεταμένης διακοπτόμενης άσκησης μέχρι εξάντλησης σε αποκρινόμενους και μη αποκρινόμενους στην καφεΐνη (1^{ο} πείραμα) και σε αθλητές με υψηλή και μέτρια αερόβια ικανότητα (2^{ο} πείραμα). **Μεθοδολογία**: Είκοσι πολύ καλά προπονημένοι ποδοσφαιριστές κατανάλωσαν σε μορφή χαπιού καφεΐνη (6 mg·kg^{-1}) ή εικονική καφεΐνη με διπλά τυφλή και αντισταθμισμένη μέθοδο. Ο διαχωρισμός με βάση τις αποκρίσεις έγινε σύμφωνα με την μεταβολή της αρτηριακής πίεσης, της γλυκερόλης, των λιπαρών οξέων και της επινεφρίνης στην ηρεμία. Ο διαχωρισμός με βάση την αερόβια ικανότητα πραγματοποιήθηκε με τον αρχικό έλεγχο της μέγιστης πρόσληψης οξυγόνου. Ακολούθως, η αθλητική απόδοση και οιφυσιολογικές και μεταβολικές αποκρίσεις είχαν αξιολογηθεί. **Αποτελέσματα**: Στο 1^{ο} πείραμα, ο χρόνος άσκησης μέχρι εξάντλησης και το ύψος των κατακόρυφων αλμάτων βελτιώθηκαν με καφεΐνη και στις δυο ομάδες (p < 0.05). Η καρδιακή συχνότητα, η αρτηριακή πίεση, η συγκέντρωση γλυκόζης και γαλακτικού στο πλάσμα αυξήθηκαν κατά την άσκηση μετά από την λήψη της καφεΐνης και στις δυο ομάδες (p < 0.05), ενώ δεν υπήρχε σημαντική διαφορά μεταξύ των ομάδων. Η μείωση στην υποκειμενική αντίληψη κόπωσης μετά από την λήψη καφεΐνης παρατηρήθηκε μόνο στους υψηλά αποκρινόμενους (p < 0.05). Στο 2^{ο} πείραμα, ο χρόνος άσκησης μέχρι εξάντλησης, η καρδιακή συχνότητα, η αρτηριακή πίεση, η συγκέντρωση γλυκόζης και γαλακτικού στο πλάσμα αυξήθηκαν μετά από την λήψη καφεΐνης και στις δυο ομάδες (p < 0.05), ενώ δεν υπήρχε σημαντική διαφορά μεταξύ των ομάδων. Η βελτίωση στο ύψος των κατακόρυφων αλμάτων και η μείωση στην υποκειμενική αντίληψη κόπωσης με καφεΐνη παρατηρήθηκε μόνο στους δοκιμαζόμενους με υψηλή αερόβια ικανότητα (p < 0.05). Ο χρόνος αντίδρασης, η συγκέντρωση γλυκερόλης, λιπαρών οξέων, επινεφρίνης στο πλάσμα και η οξείδωση των υδατανθράκων και λιπών δεν

επηρεάστηκαν από την καφεΐνη (p > 0.05). **Συμπεράσματα**: Η καφεΐνη βελτιώνει την αντοχή σε υψηλής ένταση άσκηση σε αθλητές με υψηλή με χαμηλή απόκριση στην καφεΐνη και σε αθλητές με υψηλή και μέτρια αερόβια ικανότητα. Οι αθλητές με υψηλή και χαμηλή απόκριση στην καφεΐνη (1ο πείραμα) και οι αθλητές με υψηλή αερόβια ικανότητα (2ο πείραμα) βελτίωσαν την μυϊκή εκρηκτικότητα με καφεΐνη.

Λέξεις κλειδιά: εργογόνο βοήθημα, προσομοίωση αγώνα ποδοσφαίρου, εκρηκτικότητα, επίδοση αντοχής, βιοχημικές αποκρίσεις

ABSTRACT

Apostolidis Andreas: **Caffeine effects**
on high and low caffeine responders to caffeine
and on different aerobic fitness level athletes:
biological responses and exercise performance
(Under the supervision of Dr. Hadjicharalambous Marios, Associate Professor)

Introduction: There are controversial findings concerning the effects of caffeine ingestion on exercise performance and biological parameters. The aims of the present thesis were to examine the influence of caffeine ingestion on performance and biological responses a) in participants with high and low responses to caffeine and b) in participants with high and medium aerobic fitness level during prolonged intermittent exercise to exhaustion in high and low responders to caffeine (EXP 1) and in high and medium aerobic fitness levels athletes (EXP 2). **Methodology**: Twenty well-trained male soccer players ingested either caffeine (6 $mg \cdot kg^{-1}$) or placebo in a double-blind, counterbalanced design. The participants were categorized to either high or low responders to caffeine, on the basis of mean arterial blood pressure (MAP), plasma glycerol, non-esterified fatty acid (NEFA) and epinephrine responses at rest. The aerobic fitness level categorization was performed on the basis of the results obtained during their initial VO_2max test. Performance, physiological and metabolic responses were evaluated. **Results**: In EXP 1, time to fatigue and countermovement jumps (CMJ) improved with caffeine in both high and low to caffeine responders groups ($p < 0.05$). Caffeine increased heart rate (HR), MAP, plasma glucose and lactate during exercise in both groups ($p < 0.05$); no differences observed between groups. Caffeine significantly reduced rating of perceived exertion (RPE) only in the high responders ($p < 0.05$). In EXP 2, time to fatigue, HR, MAP, plasma glucose and lactate increased with caffeine in both groups ($p < 0.05$); no differences observed between groups. The CMJ improved and RPE reduced with caffeine in the high aerobic fitness level group ($p < 0.05$). Reaction time (RT), plasma glycerol, NEFA and epinephrine, and fuel oxidation were not affected by caffeine ($p > 0.05$). **Conclusions**: Caffeine was effective in improving endurance performance during high-intensity exercise in pre-distinguished high and low caffeine responder athletes and in athletes with high and medium aerobic fitness levels. Muscular explosiveness improvement was observed in high and low responders to caffeine athletes (EXP 1) and in athletes with high aerobic fitness level only (EXP 2).

Key words: ergogenic aid, simulated soccer-game on treadmill, explosiveness, endurance performance, biochemical responses

ABBREVIATIONS AND SYMBOLS

°	degrees
°C	celsius
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
MAP	mean arterial blood pressure
C.V.	coefficient of variation
Ca^{2+}	calcium ion
cAMP	cyclic adenosine monophosphate
cm	centimeters
CMJ	countermovement jump
CNS	central nervous system
CO_2	carbon dioxide
CoA	coenzyme A
CP	phosphocreatine
EE	energy expenditure
ES	effect size
F	F-ratio (test statistic used in ANOVA)
FAD^+	flavin adenine dinucleotide ion
FADH	flavin adenine dinucleotide
g	gram
GLUT	glucose transporter
H^+	hydrogen ion
HR	heart rate
HRmax	maximum heart rate
IU	international units
kcal	kilocalorie
kg	kilogram
km	kilometers
l	liter

Mg^{2+}	magnesium ion
mg	milligram
min	minute
ml	milliliters
mm	millimeter
mmol	millimole
n	sample size
NAD^{+}	nicotinamide adenine dinucleotide ion
NADH	nicotinamide adenine dinucleotide
NEFA	non-esterified fatty acid
ng	nanogram
O_2	oxygen
P	probability or significance of a test
Pi	phosphorous
RER	respiratory exchange ratio
RPE	rating of perceived exertion
rpm	rates per minute
RT	reaction time
SD	standard deviation
VCO_2	carbon dioxide production
VO_2	oxygen consumption
VO_2max	maximum oxygen uptake
WADA	Word Anti-Doping Agency

CHAPTER ONE:

GENERAL INTRODUCTION

1.1. Caffeine as ergogenic aid and delivery methods

Ergogenic aids are substances which enhance the athletic performance by influencing physiological, metabolic and/or psychological processes (Ping et al., 2010). Coffee is the most concentrated dietary source of caffeine used by athletes prior to athletic competitions (Desbrow and Leveritt, 2006). According to Coso et al. (2011) three out of four elite athletes regularly consume caffeine prior to their athletic competitions in an attempt to improve their exercise performance. Caffeine was previously controlled, by the Word Anti-Doping Agency (WADA), as an illegal substance but athletes were still allowed to use caffeine up to the urinary 12 $mg \cdot kg^{-1}$ levels of caffeine being detected. Caffeine however, has been removed from the prohibited list according to WADA since 2004 (Spriet, 2014). Following the exclusion of caffeine from the WADA list of prohibited substances, there was an increased interest in freely using caffeine, particularly by endurance athletes, as an ergogenic aid supplement (Chester and Wojek, 2008).

This particular ergogenic aid can be found in several consumable products such as coffee and chocolate beans, tea leaves and Kola nuts, gels, bars, chewing gums and/or can be artificially synthesized and added to food and drink; and also in specific pharmaceutical caffeine tablets/capsules (Spriet, 2014; Del Coso et al., 2012; Kamimori et al., 2002). Plasma caffeine concentration rises up to 15-20 $mmol \cdot L^{-1}$ with low caffeine dose (3 $mg \cdot kg^{-1}$), while with caffeine dose around 6 $mg \cdot kg^{-1}$, plasma caffeine concentration rises up to 40 $mmol \cdot L^{-1}$. In addition, when caffeine dose increases up to 9 $mg \cdot kg^{-1}$, plasma caffeine concentration is around 60-70 $mmol \cdot L^{-1}$ (Graham and Spriet, 1995).

From the above mentioned caffeine consumable products, caffeine appearance in the blood was found to be slightly delayed with gels and bars compared with ingestion in coffee or tablets/capsules (Spriet, 2014; Del Coso et al., 2012). In addition, Wickham and Spriet (2018) stated that bars and gels with 100 mg caffeine improved cognitive function, time to exhaustion and time trial performance. Graham et al. (1998) however, suggested that caffeine in capsule form is more effective than all the other forms that have been described above. Kamimori et al. (2002) however, revealed that the rate of caffeine absorption (50, 100 and 200 mg) was significantly faster with caffeine-gum compared to the capsules, suggesting that the increased caffeine absorption through the mouth was due to the buccal mucosa (Wickham and Spriet, 2018; Kamimori et al., 2002; Graham et al., 1998). Further to this, Spriet (2014) suggested that for rapid caffeine absorption, mostly during sport situations, chewing gum may be the desired form of delivery, while plasma caffeine levels can be maintained at desired concentrations when caffeine is delivered in gum with repeated doses

(Syed et al., 2005). According to the literature however, the caffeine in the gum seems not to improve the endurance performance during cycling at 85 % of VO_2max (Ryan et al., 2012), and during maximal repeated cycling sprints (Paton et al., 2010). Apart from the forms of caffeine delivery described above, an alternative delivery form of caffeine is mouth rinsing. According to this method, small volumes of fluid containing high concentrations of caffeine could be mouth-rinsed for 5 – 10s periods, since it was hypothesized that caffeine is sensed in the mouth and signals are sent to the Central Nervous System (CNS; Spriet, 2014). However, the results of the study conducted from Doering et al. (2014) revealed no time-trial performance improvement nor plasma caffeine increase when this method of delivery was employed. On the other hand, De Pauw and colleagues (2015) identified increased activity in the dorsolateral prefrontal cortex and the orbitofrontal cortex, which are brain regions responsible for problem solving and reward. Wickaham and Spriet (2018) suggested therefore that, there is minimal evidence to support the effects of caffeine mouth rinsing on cognitive performance, while it appears that short-duration, high-intensity, repeated-bout sprinting is improved with mouth rinsing in normal and glycogen-depleted states. The same authors, also stated that according to the current literature, there is no support for possible ergogenic effects of caffeine supplementation administrated in the form of energy drinks. Consequently, it was assumed that the best method for caffeine delivery in order to achieve high-intensity performance improvement is through capsules and/or tablets (Wickham and Spriet, 2018).

1.2. Caffeine ingestion and metabolism

Caffeine is metabolized by the liver to form dimethyl and monomethylxanthines, dimethyl and monomethyl uric acids, 3-methyl and dimethylallantoin, and uracil derivatives, while the demethylation, C-8 oxidation and uracil formation occur mostly in liver microsomes (Arnaud, 1987, 1993). In humans the 3-methyl demethylation leads mostly to the formation of paraxanthine (Fredholm et al., 1999). Following ingestion, in the human body caffeine is mostly transformed by the liver into paraxanthine (80 %), theobromine (11 %) and theophylline (5 %), and since it has lipid solubility, it crosses easily the blood-brain barrier (Fredholm et al., 1999), while the remaining 4 % is eliminated in urine without transformation (Graham, 2001; Harland, 2000). Paraxanthine, theobromine and theophylline are taken into account when considering the biological actions of caffeine-containing beverages (Nieman et al., 2018; Fredholm et al., 1999). In the brain, caffeine is metabolized via specific, local enzymatic pathways, leading to high CNS concentrations of theophylline

(Nieman et al., 2018; Fredholm et al., 1999), thus provoking brain effects (Camandola et al., 2018). Peak plasma concentration is evident 1 hour post ingestion (McArdle et al., 2007; Powers and Howley, 2004), while the half-life for elimination ranges from 2.5 to 10 hours in humans (Magkos and Kavouras, 2005). It is absorbed through the gastrointestinal tract (McArdle et al., 2007), moves through cellular membranes and excreted from the body by the kidneys through the urine (Magkos and Kavouras, 2005; Harland, 2000) on a rate of about 50-75 % within 3-6 hours of ingestion (Sokmen at al., 2008; McArdle et al., 2007).

1.3 Caffeine biological responses

In the human body caffeine inflames several biological responses, depending on the caffeine doses consumed. Although higher caffeine doses (10-13 $mg \cdot kg^{-1}$) provoke biological responses to exercise, including increased heart rate (HR), higher blood lactate, non-esterified fatty acids (NEFA), glycerol and catecholamines (epinephrine and norepinephrine) concentrations, they produce severe side effects (Graham and Spriet, 1995; Spriet et al. 1992). These include gastrointestinal discomfort and mental confusion, an inability to focus and disturbed sleep in some participants, which may negatively affect performance particularly in participants who are non-frequent caffeine consumers (Graham and Spriet, 1995). Furthermore, it seems that high-caffeine volume energy drink consumption significantly alters the systolic blood pressure when compared with caffeine alone (Fletcher et al., 2017). In addition, lower doses (3 $mg \cdot kg^{-1}$) did not reveal any physiological effects on the HR, plasma catecholamines, lactate, glycerol and NEFA concentrations (Graham and Spriet, 1995), while Van Nieuwenhoven et al. (2000) revealed that lower doses of caffeine (285 mg in total) had no effect on gastric emptying, gastric pH, orocecal transit time and intestinal permeability. However, when the participants consumed caffeine doses between 5-6 $mg \cdot kg^{-1}$ there was a reduction on the side effects producing positive physiological responses which indicate that this substance could be characterized as an ergogenic aid and which affects therefore the CNS (Spriet, 2014).

Since caffeine seems to provide biological effects through the CNS and thus elevating plasma catecholamines and cortisol levels (Spriet, 2014), it should be noted that catecholamines are secreted into the blood flow by the adrenal medulla and are active until they diffuse into the liver where they are broken down by catechol-Omethyltransferase. Norepinephrine mainly stimulates organs with alpha receptors and lesser beta receptors, while epinephrine stimulates both receptors in a similar manner (Guyton and Hall, 2006). Since epinephrine has a greater impact on organs with beta receptors, it causes a greater

effect on cardiac stimulation than norepinephrine; while norepinephrine increases the total peripheral resistance, thus elevating the arterial pressure, epinephrine elevates arterial pressure to a lesser extent (Guyton and Hall, 2006). In addition, epinephrine provides 5 to 10 times greater metabolic effects in comparison to norepinephrine (Guyton and Hall, 2006). Cortisol (a steroid hormone) also is released when the human body is under stress and when blood glucose concentration is low provoking an increase of blood sugar through gluconeogenesis and improves carbohydrate, fat and protein metabolism (Guyton and Hall, 2006). Cortisol stimulates the formation of glucose in the liver from certain amino acids, (mainly from the muscles) by increasing the release of a series of enzymes and improves the gluconeogenesis process (development of glucose from lactate, glycerol and glucogenic amino acids). When gluconeogenesis process is active, such as in cases of exercise, cortisol allows epinephrine to mobilize glucose and NEFA from the liver and adipose tissue respectively, into the active muscles. Further information regarding the metabolism and the human body energy systems during exercise are described in more details on chapter 1.4.

According to Spriet (2014) there are many references which examine the potential ergogenic effects of caffeine using various exercise protocols, caffeine dosage and supplementation methods employed and untrained, well-trained and/or elite athletes reported conflicting results. During the late 1970s, a study conducted in David Costill's laboratory revealed that trained cyclists improved their times to exhaustion while exercising at 80 % of VO_2max from 75 minutes after placebo ingestion to 96 minutes after caffeine ingestion (Costill et al., 1978). Graham and Spriet (1995) also revealed that endurance running performance was enhanced by 22 % after 3 and 6 $mg \cdot kg^{-1}$ of caffeine respectively when compared with the placebo ingestion, but improved only 11 % and non-significantly after a 9 $mg \cdot kg^{-1}$ caffeine dose. In addition, Kovacs et al. (1998) stated that moderate dose of caffeine (4.5 $mg \cdot kg^{-1}$) can be characterized as the most effective ergogenic dosage during time-trial performance tests in well-trained cyclists and triathletes. A newer study conducted by Guest and colleagues, revealed that caffeine ingestion (2 and 4 $mg \cdot kg^{-1}$ respectively) revealed endurance improvement during 10-Km cycling (Guest et al., 2018), while Bandyopadhyay et al. (2011) concluded that combined and acute supplementation of caffeine and panax ginseng improved the endurance running performance in a hot and humid environment.

Jenkins et al. (2008) revealed that performance during cycling for a 15 minute time period significantly increased after caffeine ingestion, while Wiles et al. (1992) demonstrated that ingesting 200 $mg \cdot kg^{-1}$ of caffeine 1 hour prior to exercise improved the

1500 m running performance in well-trained runners. Moreover, Bridge and Jones (2006) reported 1.8 % improvement in run time of well-trained male runners during an 8 km run after 3 mg·kg^{-1} caffeine ingestion 1 hour prior to exercise on a track, while Lane et al. (2014) indicated that caffeine (3 mg·kg^{-1}) administered in the form of a caffeinated gum increased cycling time-trial performance. Two further studies aimed to investigate the effects of an energy drink containing caffeine (3 mg·kg^{-1}) on volleyball performance in females and males (Pérez-López et al., 2015; Del Coso et al., 2014). The players completed a series of volleyball performance tests (ball velocity in a spike test, several jump tests and the time to complete an agility test) followed by a simulated match (measuring the number of successful volleyball actions). On both occasions energy drink with caffeine or caffeine-free was consumed. The results revealed that both physical performance and the accuracy of the volleyball skills significantly improved 65 minutes after ingesting an available energy drink containing caffeine. Some years earlier, the study from Gant et al. (2010) demonstrated that, after 4 mg·kg^{-1} caffeine ingestion prior to the experimental trials during a soccer match, improved the sprint performance and the countermovement jump (CMJ) performance. It also revealed a significant improvement on the offset of the fatigue-induced decline in the self-selected components of performance. Furthermore, it was demonstrated that co-ingesting caffeine (4 mg·kg^{-1}) with carbohydrate (1.2 g·kg^{-1}), prior to a rugby game, significantly improved the motor skills and the 15 m sprints when compared with the placebo ingestion (Roberts et al., 2010). Performance improvement was also demonstrated during weight lifting after a 7 mg·kg^{-1} caffeine ingestion (Jacobson et al., 1992), while a greater peak of power was attained during the Wingate test, following the ingestion of 5 mg·kg^{-1} of caffeine (Woolf et al., 2008). In addition, it seems from the literature that caffeine ingestion during resistance exercise, enhances the lipolysis at rest and increases fat oxidation rates 30 minutes following resistance exercise (Ratamess et al., 2016). Short-term high intensity cycling was also improved after caffeine ingestion of similar quantities (Wiles et al., 2006; Doherty et al., 2004).

The use of caffeine, apart from exercise performance improvement, has been also examined during vigilance, alertness, mood, executive control and related parameters, mainly during situations where maintaining vigilance is essential as in military and other professions where people are awake for long periods of time (Spriet, 2014). Leiberman et al. (1987) revealed that vigilance and reaction time (RT) significantly improved after caffeine ingestion in all caffeine doses given (32, 64, 128 and 256 mg). Moreover, Olson et al. (2010) and Brunyé et al. (2010) stated that the optimal dose of caffeine, in order to maintain elevated

vigilance, the mood, the alerting, the orienting and the executive control levels seems to be maintained with 200 mg. The significant effect of caffeine in improving the cognitive functions (such as memory, psychomotor and attention tasks) before and immediately after strenuous exercise was also observed from Hogervorst et al. (1999). However, some years later the results of a more recent study (Hogervorst et al., 2008) revealed that cognitive functions (mainly during rapid visual information processing tests) significantly improved following caffeine dose ingestion of 300 mg, after 140 minute of cycling. The results demonstrated that caffeine improves the ability to concentrate and make decisions during and even immediately after exhaustive exercise, situations which happen very often in the sporting community.

Another mechanism behind the positive effects of caffeine is the reduction of the rating of perceived exertion (RPE). Caffeine increases the secretion of β-endorphins allowing the participants to tolerate the discomfort associated with fatigue during exhaustive exercise thus improving the performance mainly during endurance exercise (Doherty and Smith, 2005; Laurent et al., 2000).

In contrast, there is an indication that caffeine impairs the immune system, by affecting the natural killer cells (Fletcher and Bishop, 2011). Natural killer cells are located in the lymphocytes in the blood (compromising about 5-20 % of all the cells) with a major role in the defense against infectious agents and viral infection and they are also tumor immune surveillance. The natural killer cells play an important role in the human body defenses since they do not need prior sensitization or require specific antigen recognition to attack target cells (Antoniou et al., 2006). Elite athletes provide high incidence of viral respiratory infection due to the decrease of natural killer cells after intense exercise (Gleeson, 2007). The reduction of the natural killer cells observed during and after intense exercise, might be due to the fact that since the natural killer cells have A_1 and A_2 adenosine receptors, the increased adenosine and epinephrine concentrations due to exercise, reduce the natural killer cell levels in the blood (Spriet, 2014). Since caffeine was found to be an adenosine receptor antagonist, high and low caffeine doses ingestion (6 $mg \cdot kg^{-1}$ and 2 $mg \cdot kg^{-1}$ respectively) found to increase the natural state of activation of natural killer cells, as well as the antigen-stimulated natural killer cell activation 1 hour after high-intensity prolonged cycling, suggesting therefore that caffeine positively influence the natural killer-cell activation (Fletcher and Bishop, 2011).

Caffeine may positively impairs the biological adaptations of the human body by its ability to alter intracellular cAMP; the actions of adenosine via A_{2a} adenosine receptors have

been shown to inhibit natural killer cell function via activation of adenylate cyclase and increased intracellular cAMP concentration, while the actions of adenosine via adenosine A_1 adenosine receptors have been shown to enhance natural killer cell function via suppression in cAMP (Goto et al., 1983). While caffeine was found to be adenosine receptor antagonist (Spriet, 2014), it was stated that caffeine impede the intracellular cAMP concentration increase in natural killer cells via antagonism of A_{2a} adenosine receptors (Raskovalova et al., 2006) thus affecting the immune function and elevating the performance as well as the defense mechanisms of the human body (Fletcher and Bishop, 2011).

Apart from the caffeine effects on the defense mechanisms of the human body, there is an indication that caffeine consumption affects the iron absorption. It should be noted here, that iron is responsible for the transport, storage and use of oxygen for the aerobic energy production, as it is part of the myoglobin and hemoglobin (Mougios, 2008). According to the literature, caffeine consumption inhibits iron absorption following an iron-rich meal in a concentration-dependent fashion (Morck et al., 1983), while the study conducted from Dewey et al. (1997) revealed that coffee consumption prevents the utilization of supplemental iron. Reduction of VO_2max in nonanemic population with iron depletion was likely caused by factors related to reduce body iron storage, but was unrelated to decreased oxygen-transport capacity of the blood (Zhu and Haas, 1997). However, based on the current literature, there is no documented effect of caffeine on iron storage status prior and during exercise indicating possible effect during prolonged exercise.

On the other hand, there are some indications that provide opposite views regarding the effects of this substance. It was suggested for example that 67 % of the participants improved their time-trial performance after caffeine ingestion, while the remaining 33 % improved their performance due to the carbohydrate ingestion, without any biological responses to exercise (Cox et al., 2002). This suggests that the beneficial effects of caffeine were through the CNS late in exhaustive exercise (Cox et al., 2002). This was supported also by Talanian and Spriet (2016). The authors revealed that the well-trained cyclists improved their time to exhaustion, after caffeine ingestion, without observing any influence of caffeine in the biological responses (HR, respiratory exchange ratio, RER; and epinephrine, glucose, lactate, glycerol and NEFA levels) during the initial 120 minutes of submaximal exercise and prior to the time trials, when compared with the placebo ingestion. One year later, the study conducted by Arcoverde et al. (2017), revealed that caffeine ingestion (5 $mg \cdot kg^{-1}$) before submaximal bouts did not affect supramaximal oxygen demand, concluding therefore no positive effects on the anaerobic capacity and performance.

In addition, the results from the study of Desbrow et al. (2009) revealed no significant improvements in time-trial performance, while Van Nieuwenhoven et al. (2005) demonstrated a lack of performance improvement during an 18 km run after caffeine ingestion. An absence of performance improvement was identified from the study of Schubert et al. (2013) after energy drink ingestion (containing 80 or 140 mg of caffeine respectively) where the participants had to perform a 5 km treadmill time trial. Furthermore, the study conducted from Astorino et al. (2010) reported no effect of ingesting 2 $mg \cdot kg^{-1}$ caffeine on peak torque or knee extension/flexion total work power, when compared with the 5 $mg \cdot kg^{-1}$ caffeine. Astorino et al. (2011) also demonstrated no effect of caffeine ingestion (5 $mg \cdot kg^{-1}$) on pain perception during high-intensity exercise; and Ganio et al. (2011a), where the participants ingested 3 $mg \cdot kg^{-1}$ of caffeine 1 hour prior to exercise and another 3 $mg \cdot kg^{-1}$ caffeine dose 45 minutes into the 2 hours submaximal cycling, followed by a 15-minutes performance trial, revealed reduction of the leg pain by 27 % when exercising in the heat (33 °C) but had no effect in a cool environment (12 °C). Ganio et al. (2011a) and Ganio et al. (2011b) indicated that caffeine ingestion (6 $mg \cdot kg^{-1}$) during exercise in the heat is ergogenic. Roelands et al. (2011) and Cohen et al. (1996) on the other hand, demonstrated that exercising in a hot environment after caffeine ingestion (of doses 5, 6 or 9 $mg \cdot kg^{-1}$) did not reveal any improvement in endurance performance neither any biological parameters where affected. Finally, Irwin et al. (2011) concluded that habitual caffeine-user athletes experienced a significant performance improvement after caffeine ingestion, when compared with the non-caffeine users.

In sum up, caffeine ingestion seems to influence HR and MAP and elevate several blood metabolites including plasma glucose, lactate, NEFA, glycerol and epinephrine concentrations, as well as improve cognitive functions and pain perception. In addition, caffeine ingestion seems to enhance the immune system, thus affecting the human body's defense system, by influencing the natural killer cells. On the other hand, there are some indicators revealing that caffeine does not provide biological responses, thus indicating controversial results regarding the ergogenic effects of this substance.

1.3.1 Biochemical actions of caffeine in the brain

Once caffeine crosses the blood-brain barrier, xanthines can influence 5-nucleotidase and alkaline phosphatase (Fredholm et al., 1978). The only known mechanism however, that is significantly affected by caffeine is the binding to, and the antagonism action of, adenosine receptors (Fredholm, 1980; 1995). According to Fredholm et al. (1999), adenosine is a

normal cellular constituent. The intracellular level is regulated by the balance of several enzymes. Adenosine is formed by the action of an AMP-selective 5-nucleotidase, and the rate of adenosine formation via this pathway is mainly controlled by the amount of AMP. Therefore, the rate of adenosine formation via this pathway is the relative rates of ATP breakdown and synthesis, which in turn are determined by the rate of energy utilization and the availability of the metabolizable substrate.

Adenosine kinase and adenosine deaminase are the two enzymes that constitute the major pathways of adenosine removal. Blockade of adenosine kinase has a much larger effect on the rate of adenosine release than does the blockade of adenosine deaminase (Lloyd and Fredholm, 1995). In addition, the enzyme S-adenosylhomocysteine hydrolase sets the equilibrium between S-adenosylhomocysteine and adenosine + L-homocysteine. When the level of the amino acid is low, this enzyme generates the adenosine. On the other hand, when the level of L-homocysteine is raised, it can trap adenosine formed via AMP breakdown as S-adenosylhomocysteine inside the cell (Fredholm et al., 1999), demonstrating the bulk of the adenosine formed by energy deprivation or electrical field stimulation in hippocampal slices is formed intra- rather that extracellularly (Lloyd et al., 1993).

The extracellular ATP is rapidly hydrolyzed to adenosine and other metabolites from neuronal cells, such as an intercellular signal, providing extracellular adenosine (Fredholm et al., 1999). However, when relatively low frequency stimulation is used or following hypoxia/hypoglycemia, the extracellular ATP is not the major source of adenosine released from the brain slices (Fredholm et al., 1999), since the agents that block extracellular AMP hydrolysis significantly fail to affect the rate of adenosine release (Lloyd et al., 1993), and the intracellular adenosine formation being the most important (Fredholm et al., 1999). Intra- and extracellular adenosine concentrations are kept in equilibrium by means of equilibrative transporters, whereas these transporters are blocked by several agents such as nitrobenzylthioinosine, propentofylline, dipyridamole and dilazep, along with sodium-dependent concentrating transporters that move extracellular adenosine into cells (Fredholm et al., 1999). When inhibitors of equilibrative transport are given, there is an elevator of adenosine in the CNS despite the decrease in the release of adenosine metabolites such as inosine and hypoxanthine (Andinè et al., 1990). When adenosine is released, it is metabolized by the cells into inosine and hypoxanthine (Fredholm et al., 1999). It was also stated that transport inhibitors block the overall release of adenine nucleotide breakdown products (Jonzon and Fredholm, 1985), whereas the addition of L-homocysteine in the presence of transport inhibitors leads to a very substantial reduction in the efflux of

adenosine. Excess of L-homocysteine forces the S-adenosylhomocysteine hydrolase reaction to occur in reverse and intracellular adenosine levels reduce, leading to reduction of the extracellular levels (Fredholm et al., 1999).

It was therefore pointed out that adenosine levels in the extracellular fluid should be increased whenever there is a discrepancy between the rate of ATP consumption and ATP synthesis (Fredholm et al., 1999). Drugs such as caffeine, which interfere with the key enzymes and with the transporters should affect adenosine levels (Fredholm et al., 1999). In humans, there are adenosine receptors that are activated not only by the high adenosine levels in ischemia, but also by the low physiological levels (Fredholm et al., 1999). The A_{2b} receptor has been shown to require higher concentrations of adenosine for activation, thus inhibition of adenosine actions at this receptor is unlikely to provide an explanation for the actions of caffeine under physiological conditions. Under pathophysiological conditions however, A_{2b} receptors that are activated by endogenous adenosine and caffeine may splendidly act on these receptors (Fredholm et al., 1999). In addition, A_1 and A_{2a} receptors, which are activated at low basal adenosine concentrations, are the major targets for caffeine and theophylline (Fredholm et al., 1999). It was indicated that theophylline is 3 to 5 times more potent as an inhibitor to both adenosine A_1 and A_{2a} receptors than caffeine and paraxanthine was indicated that is at least as potent as caffeine (Benowitz et al., 1995). A_1 receptors activation may cause inhibition of adenylyl cyclase and of at least some types of voltage-sensitive Ca^{2+} -channels such as the N- and the Q- channels, and activation of several types of K^+- channels, phospholipase C and phospholipase D (Fredholm et al., 1994a). Activation of A_{2a} receptors activates the adenylyl cyclase and perhaps also activates some types of voltage-sensitive Ca^{2+} -channels, especially the L-channel (Fredholm et al., 1999).

Adenosine A_1 receptors are localized in almost all brain areas, with the highest levels in hippocampus, cerebral and cerebellar cortex, and certain thalamic nuclei (Goodman and Snyder, 1982), as well as on nerve terminals (Johansson et al., 1993a). Adenosine A_{2a} receptors are concentrated in the dopamine-rich regions of the brain (Premont et al., 1979).

1.3.2. Caffeine, brain effects and exercise performance

As it was stated above, it seems that caffeine influences the CNS by acting as adenosine receptor antagonism, which in turn reduces the influence of adenosine and produces motor-activating and arousing effects (Graham-Paulson et al., 2016; Fredholm et al., 1999). Caffeine therefore, positively influence the subjective feelings, such as RPE, mood and cognitive performance (Doherty and Smith, 2005; Smit and Rogers, 2000).

Following exercise, it seems that caffeine provokes protective effect on oculomotor control during prolonged exercise to fatigue, by influencing the central and catecholaminergic neurotransmission (Connell et al., 2017). Manipulation of these neurotransmitter systems via norepinephrine-dopamine reuptake inhibition also prevented fatigue-related impairments to the peak velocity of muscles during exercise (Connell et al., 2017).

In addition, it was revealed that caffeine ingestion reduces the RPE during submaximal or higher workload exercise (Santos et al., 2013; Stadheim et al., 2013; Cureton et al., 2007), as well as produces hypoalgesic effects during submaximal cycling in males and females (Motl et al., 2006; O'Connor et al., 2004), while the adenosine receptor inhibition following caffeine ingestion could influence motor unit recruitment or have direct effect on muscles (Warren et al., 2010; Fredholm et al., 1999). Graham-Paulson et al (2016) stated that a combination of factors may contribute in improving the endurance performance following caffeine ingestion, while caffeine seems to influence the CNS. The authors provoked that caffeine ingestion reduced the RPE during handcycling. However, it has been suggested that caffeine is unable to have hypoalgesic effect during heavy-severe fixed intensity exercise (Black et al., 2015). It was therefore stated that nociceptive stimulus contributing to the peripheral muscle pain during heavy exercise may be too great for the antagonism of adenosine receptors to reduce RPE and pain, thus improving the performance (Graham-Paulson et al., 2016). Similar results have been obtained from the study conducted from Ratamess et al. (2015), in which the RPE was elevated during greater volume load of resistance exercise following combination of caffeine ingestion treatment.

Doherty and Smith (2005) on the other hand, stated that caffeine ingestion improves the performance, through reductions of perception of effort, allowing the participants to tolerate the discomfort associated with fatigue during exercise. In addition, it seems that the performance improvement observed due to caffeine ingestion, might be associated with the altered perceptual response during exercise, which in turn allows the participants to centrally recruit and engage more motor units, thus increasing the power output during time-trial protocols (Cole et al., 1996).

It seems from the literature review, that the possible influence of caffeine on RPE might be associated with the physical conditions of the participants taken part. Participants with quite good physical condition (e.g. trained vs untrained) tended to have the largest reduction in RPE during exercise (LeBlanc et al., 1985). The authors assumed that the reduction observed in RPE within the trained participants might be due to the increased release of epinephrine. In addition, Motl et al. (2003) revealed that perceived exertion ratings

were more reduced within the participants with greater fitness levels, while it was speculated that highly trained participants reveal greater benefit from caffeine. It seems therefore that high fit individuals may have muscles and other tissues that are more responsive to caffeine stimulus (Graham, 2001).

Perceptual response to exercise is the detection and interpretation of signals sent from the body during exercise (Noble and Robertson, 1996). These signals are mainly located in the respiratory-metabolic mediators, as well as in peripheral and nonspecific physiological mediators (Noble and Robertson, 1996). In addition, psychological functions, such as motivation, memory and associated decision-making components seem to play a crucial role in the human body exercise interpretation (St Clair Gibson et al., 2003). Ventilation for example is an important sensory signal, related with the aerobic fitness level of the individuals, while they are able to monitor consciously during exercise (Noble et al., 1973). Since ventilation was found to be stimulated with caffeine during exercise (Powers et al., 1985), Supinski et al. (1986) indicated that the RPE was reduced during inspiratory muscle contractions, while the respiratory was enhanced. It was therefore assumed that a more efficient respiratory system alters the blood flow to the working muscles, thus reduces the RPE related to the effort of breathing (Doherty and Smith, 2005). Also it seems that caffeine ingestion provokes alteration in happiness, calmness and alertness during exercise, reducing therefore the perceived exertion (Backhouse et al., 2004).

Another possible mechanism behind the CNS caffeine effects would be the increase of central dopamine release, since caffeine was found to antagonize the inhibition of adenosine A_1 and A_{2a} receptors on dopamine activity, thus reducing the effort perception during exercise (Davis et al., 2003). It seems from the literature that a high serotonin-dopamine ratio elevates the effort perception and central fatigue, while low serotonin-dopamine ratio increases the arousal and motivation (Davis and Balley, 1997; Davis et al., 1993). Hadjicharalambous et al. (2010) however, revealed reduction in RPE following caffeine ingestion, while the exercise performance was not affected. The authors stated that the absence of performance improvement among with the reduction in RPE might be associated with the lack of difference in plasma prolactin between trials. It was therefore assumed that prolactin secretion would be expected to be lower during exercise following caffeine ingestion (Hadjicharalambous et al., 2010), since caffeine directly attenuates brain serotonin synthesis and/or enhances dopamine release (Davis et al., 2003). It was therefore assumed that the reduction in effort perception due to caffeine ingestion, might be also associated with lack of plasma neuroendocrine prolactin secretion, particularly in trained

participants and when exercising in relatively cold environment (Hadjicharalambous et al., 2010). According to the information stated above, it seems therefore that caffeine ingestion alters several biochemical reactions through the brain, thus indicating possible caffeine CNS effects, which in turn provides performance implications due to caffeine ingestion.

1.4. Caffeine, energy production systems and exercise performance

1.4.1. Energy production systems

ATP-CP system: According to Mougios (2008), there are 3 main systems providing energy to the human body during exercise. The ATP-CP system which provides energy for ~7 sec during maximal effort. This system regenerates the ATP (the molecular unit of currency) from the CP, according to the equation:

$CP + ADP + H^+ \leftrightarrow ATP + C$

whereas C is the creatine and Pi is the phosphorus.

During exercise, the muscle ADP and Pi as well as the AMP increase providing extra ATP during muscle contraction according to the equation:

$2ADP \leftrightarrow AMP + ATP$

Glycogen metabolism: Apart from the ATP-CP system, the human body is supplied with energy through the glycogen metabolism. Glycogen is stored mainly in the liver (3-7 % of liver mass) and muscles (1-1.5 % of muscle mass) and is derived from the diet of carbohydrates, (starch, sucrose, glucose etc.) after being transformed into glucose, with the glycogen synthase the main enzyme, during the glycogen synthesis. During exercise, glucose units are removed from the glycogen, thus producing 1-glucose phosphate, with the phosphorylase and the glycogen debranching enzymes taking part, in order for the glycogenolysis process to be completed. Glycogenolysis is activated when there are enhanced Pi due to the ATP hydrolysis; when there is enhanced AMP according to the equation:

$2ADP \leftrightarrow AMP + ATP;$

When there is an increase of lysate Ca^{2+} (related with phosphorylase kinase) and when there is enhanced epinephrine blood secretion, the phosphorylase enzyme and the adenylate cyclase acid enzyme through the cAMP are stimulated. It should be noted here that even the expectation of exercise, excites the sympathetic system, innervating the adrenals, thus elevating the epinephrine secretion through the blood flow to the β adrenergic receptor. Furthermore, as a result of exercise, blood flow to the active muscles increases; the

activity of the glucose transporters (GLUT) from the intracellular vesicles into the cytoplasmic membrane also increases; among with the activation of the phosphofructokinase enzyme and of the pyruvic acid kinase enzyme (glycolysis catalysts, with the AMP and ADP respectively the activator of the aforementioned enzymes), enhancing the muscle glycolysis.

The 1-glucose phosphate, after being isomerized into 6-glucose phosphate, is transformed into pyruvic acid. Pyruvic acid is then transferred into the mitochondria, where among with the coenzyme A (which consists adenine, ribose, phosphates, and vitamins), produces acetyl-CoA, since the pyruvic acid is oxidized from NAD^+ with the carboxyl group being detached as CO_2. Dehydrogenase enzyme of pyruvic acid controls the pyruvic acid oxidation. In the absence of exercise, the phosphorylation of kinase inhibits and deactivates the dehydrogenase enzyme (due to the enhanced ATP and acetyl-CoA and the reduced Mg^{2+}), while exercise activates the phosphatase of dehydrogenase enzyme (due to the increased Ca^{2+}, Mg^{2+} and CoA). Citric acid cycle, a 9 enzyme reaction process, takes place over the acetyl-CoA thus producing CO_2, with the NAD^+ and FAD^+ the carriers of H^+ and the O_2 the carrier of H^+ through the electron transport chain.

In cases were the exercise intensity is over a limit, a cohesion occurs regarding the production rate of NADH and FADH and the O_2 recruitment. In the case where no H^+ transporters (NAD^+ and FAD^+) are available, glycolysis discontinues. Pyruvic acid however, becomes the H^+ replenish from NADH and FADH, converted into lactate acid through the lactate acid dehydrogenase, while lactate acid would then be converted into pyruvic acid according to the equation:

$$\text{lactate acid} + NAD^+ \leftrightarrow \text{pyruvic acid} + NADH + H^+$$

However, since there are no available NAD^+ and FAD^+ in the active muscles, the lactate acid is transported through the blood flow into other human body organs (inactivated skeletal muscles, heart, liver, brain and kidneys). Glucose would then be reconstructed from pyruvic acid, a process called gluconeogenesis.

According to gluconeogenesis, glucose is synthesized from pyruvic acid, lactate acid, glycerol and amino acids. This process takes place in the lysate, whereas pyruvic acid is converted into 1,6-diphosphate fructose, and after dephosphorization and isomerization glucose is attributed. The 1,6-diphosphate fructose enzyme catalyzes the gluconeogenesis process. Gluconeogenesis occurs in the kidneys and in the liver, but not in the muscles, due to the absence of the phosphatase of the 6-glucose phosphate, whereas the 6-glucose phosphate is finally converted into glucose. The muscles, on the other hand, convert the

lactate acid into 6-glucose phosphate and later into 1-glucose phosphate in order to be used during the glycogen synthesis process.

Fat metabolism: The main human body fatty cells are called adipocytes and are stored in the adipose tissue as triglycerides. Each triglyceride is synthesized from the glycerol activated form (called 3-glycerol phosphate, derived from glucose diet and catalyzed from the glyceraldehyde 3-phosphate dehydrogenase) and from the fatty acid activated form (called acyl-CoA, derived from the adhesion of acyl to CoA and catalyzed from the acyl-CoA synthetase). Two acyl groups (transferred from two acyl-CoA) cohered with the 3-glycerol phosphate (catalyzed from acyl transferase glycerophosphate), generate phosphatidic acid, thus generating triglyceride through hydrolysis and phosphate group extraction with cohesion of one extra acyl group from acyl-CoA. The two final reactions are catalyzed from the triglyceride synthase complex.

Fat metabolism (called lipolysis) occurs in the lysate among with the triglycerides lipase and monoacylglycerol lipase. This provides three fatty acid molecules and one glycerol molecule for each triglyceride molecule. Lipolysis is activated mainly during exercise, due to the increased secretion of epinephrine from the adrenals, activating the cAMP. This in turn enhances the lipolysis through the phosphorylation of lipase, in order to produce three fatty acid molecules and one glycerol molecule. Even with further increase of exercise, lipolysis decreases. This is probably due to the desensitization of the β adrenergic receptor, as a cell protector mechanism of over sensitization.

Fatty acid and glycerol (attached with plasma protein, called albumin) molecules are transferred through the blood flow. Plasma glycerol is recruited from the liver and converted into glycerol 3-phosphate, by the action of glycerol kinase according to the equation:

$$\text{glycerol} + \text{ATP} \leftrightarrow \text{glycerol 3-phosphate} + \text{ADP} + H^+$$

Glycerol 3-phosphate is transformed into dihydroxyacetone phosphate, which can either follow the glycolysis or mainly the gluconeogenesis path.

Plasma fatty acids (derived from adipose tissue) on the other hand, are recruited from the liver for triglycerides synthesis. Most of them (derived from adipose tissue and myocyte triglycerides) are recruited from the muscles producing ATP through the β oxidation path after being converted into acyl-CoA. However, the acyl-CoA crosses the external mitochondrial membrane but not the internal. Carnitine distracts the acyl from the acyl-CoA producing CoA and acyl-carnitine (catalyzed from the protein carnitine acyltransferase I). After being transferred (acyl-carnitine) into the internal mitochondrial membrane, the acyl-

CoA is reproduced (catalyzed from the protein carnitine acyltransferase II), while carnitine returns into the intermembrane space in order the process to be continued. The cycle is completed when the acetyl-CoA enters the citric acid cycle and oxidized into CO_2, with the NAD^+ and FAD^+ the carriers of H^+ and the O_2 the carrier of H^+ through the electron transport chain.

1.4.2 Caffeine, metabolism and exercise performance

Several studies have attempted to explain the multiple mechanisms behind caffeine's effect on metabolic responses and how this may subsequently influence exercise performance. These studies were mainly located on the physiological and psychological mechanisms of energy production and exercise decision respectively and how these mechanisms (aerobic and anaerobic energy production systems) affected the metabolism through the human body and thus altered the exercise performance.

1.4.2.1 Caffeine, aerobic metabolism and exercise performance

Caffeine has attracted the attention of many competitive and noncompetitive athletes as a legal ergogenic aid (Kim et al., 2016). The positive effects in athletic performance during endurance events has been consistently reported. Graham and Spriet (1991) for example, revealed an endurance performance improvement during running and cycling to exhaustion following caffeine ingestion. In addition, Costill et al. (1978) reported running time improvement as a result of stimulated lipolysis. Additionally Bruce et al. (2000) reported significant performance improvement during rowing after ingesting 6 and 9 mg/kg caffeine as a result of NEFA elevation.

Caffeine therefore, has shown to increase fat oxidation (Spriet et al., 1992), and improve endurance performance (Hodgson et al., 2013; Hogervorst et al., 2008) by promoting carbohydrate sparing (Spriet et al., 1992). Caffeine increases the concentration of plasma catecholamine (Van Soeren and Graham, 1998; Essig et al., 1980), which inhibits lipogenesis, stimulating lipolysis and muscle glycolysis and liver and muscle glycogenolysis. These results may enhance the release of plasma glucose, glycerol and NEFA concentrations (Van Soeren and Graham, 1998) and their availability to the active muscles (Essig et al., 1980). This extra availability of NEFA to the contracting muscle during exercise contributes in sparing intramuscular glycogen (Spriet, et al., 1992). The increased lipolysis is probably due to the inhibition of the cyclic nucleotide phosphodiesterase (Butcher et al., 1968), the enzyme responsible for degrading cyclic adenosine monophosphate (cAMP) to AMP (Leijten and van Breemen, 1984). Following caffeine ingestion however, elevates the cAMP

concentration and activates the sympathetic nervous system, which in turn alters the lipolysis (Acheson et al., 1980; Butcher et al., 1968).

The performance enhancement however, indicated by the aforementioned studies might not be due to the traditional carbohydrate sparing effects of caffeine through an enhancement in fat oxidation, since it was indicated that the ergogenic effects of caffeine reflect a stimulant action on the CNS (Tarnopolsky, 2008; Davis et al., 2003; Fredholm et al. 1999). According to this assumption, it seems that caffeine alters the adenosine receptor antagonism mechanism (Fredholm, 1980; 1995), which in turn causes perception effort reduction, elevating therefore the arousal as well as producing muscle pain reduction (Graham-Paulson et al., 2016; Doherty and Smith, 2005; Smit and Rogers, 2000; Fredholm et al., 1999).

1.4.2.2 Caffeine, anaerobic metabolism and exercise performance

On the other hand, anaerobic exercise performance reduction might be associated with the endogenous carbohydrate depletion. Lanqfort et al. (1997) reported reduction in performance during Wingate test due to the endogenous carbohydrate reduction, which reduces the contribution of the anaerobic energy system. This assumption was also confirmed from the study conducted from Miura et al. (Miura et al., 2000).

While low carbohydrate availability seems to reduce the contribution of the anaerobic energy production system during exercise, acute caffeine ingestion seems to have opposite results (Doherty, 1998; Collomp et al., 1991). It was therefore indicated that caffeine ingestion alters the anaerobic energy supply, thus enhancing the athletic performance (Doherty, 1998). In addition, Bell et al. (2001) reported significant time to exhaustion improvement following caffeine ingestion, while similar results have been obtained from Simmonds et al. (2010). Furthermore, it was revealed that caffeine ingestion provides positive effects during time-trials (Astorino et al., 2012; Kilding et al., 2012; Wiles et al., 2006), while it was reported that caffeine elevates the muscular explosiveness (Kammerer et al., 2012; Tarnopolsky, 2008; Doherty and Smith, 2005; Kalmar and Cafarelli, 2004) by enhancing the peak muscle velocity contraction and subsequently the jump height (McCann et al., 2012).

The anaerobic contribution elevation therefore, during exercise among with the performance improvement, might be associated with the inhibitory action of caffeine on adenosine receptors (Fredholm, 1980; 1995). Caffeine was found to increase the activity of the enzyme phosphofructokinase, which in turn increases the anaerobic glycolysis (Simmonds et al., 2010; Bridge and Jones, 2006), thus improving the anaerobic performance

(Silva-Cavalcante et al., 2013). In addition, caffeine seems to affects the CNS, leading to an increase in motivational drive and neuromuscular excitability, thus lowering the effort perception (Doherty and Smith, 2005) and improving the neuromuscular function, results which have been obtained via electromyography activity (Bazzucchi et al., 2011). The study conducted by Silva-Cavalcante et al. (2013) confirmed the above notion, in which despite the intramuscular glycogen depletion, the participants adopted a more aggressive pacing strategy when consumed caffeine compared with placebo during cycling.

Furthermore, the primary mechanism which is responsible for the performance improvement observed during muscular explosiveness (Kammerer et al., 2012; Tarnopolsky, 2008; Doherty and Smith, 2005; Kalmar and Cafarelli, 2004) might be associated with the alteration of intramuscular ion (increased Ca^{2+}) handling within the intracellular space (Magkos and Kavouras, 2005). This was attributed to a potentiation of calcium release at lower stimulation frequencies, promoting a greater influx of Ca^{2+} in the presence of caffeine (Tarnopolsky and Cupido, 2000).

1.5. Responsiveness levels of caffeine and exercise performance: A methodological consideration

Based on the existing literature, there are strong evidence available supporting that caffeine is in general effective in enhancing plasma glycerol, NEFA (Graham and Spriet, 1995; Spriet et al., 1992) and catecholamine (Van Soeren and Graham, 1998; Essig et al., 1980) concentrations and the mean arterial blood pressure (MAP; Wardle et al., 2012; Riksen et al., 2009; Papamichael et al., 2005).

Previous studies however, revealed that while caffeine ingestion improved endurance performance, it did not impose any significant effect on cardio-respiratory parameters (Ping et al., 2010). In addition, Hadjicharalambous et al. (2010) speculated that the lack of performance improvement might be due to the inter-individual variability to caffeine, since while caffeine ingestion revealed significant reduction in RPE and enhanced concentrations of plasma metabolites, the rates of fuel oxidation and endurance performance were not affected. In addition, Cox et al. (2002) revealed an increase in endurance performance without supporting a carbohydrate sparing effect of caffeine, while Wells et al. (1985) indicated that caffeine ingestion (6 $mg \cdot kg^{-1}$) resulted in no differences in plasma NEFA or glucose concentrations from placebo, without any significant effect on endurance performance. The equivocal metabolic, physiological, RPE and exercise performance results reported in the literature (e.g. Hadjicharalambous et al. 2006; 2010; Skinner et al., 2010;

Wiles et al., 2006; Doherty et al., 2004) may partially explain by this inter-individual variability effects of caffeine on human's biological systems. Del Coso et al. (2012) therefore, suggested that participants who did not show any response to caffeine should be categorized as non-responders. Hence, it seems necessary to investigate the effects of caffeine on participants with different responses and adaptations to caffeine, providing therefore further information regarding the potential ergogenic effects of this substance through the human body.

Evaluating therefore initially a method of identifying the levels of responsiveness of caffeine in human biological systems and investigating then, the effects of caffeine on physiological and metabolic responses and on exercise performance, would provide more elaborating evidence of the actual effects of caffeine on biological and performance parameters during exercise-stress conditions. In addition, by accomplishing the above pre-distinguished levels of responsiveness methodology, the type II statistical error potentially evident in previous caffeine studies might be eliminated.

Wardle et al. (2012) proposed a method of determining responsiveness to caffeine according to MAP effects of caffeine at rest. However, it seems that caffeine ingestion affects not only MAP (Wardle et al., 2012) but also plasma glycerol, NEFA and epinephrine concentrations (Van Soeren and Graham, 1998; Spriet et al., 1992). Thus, taking into consideration the four above markers of biological response to caffeine (MAP, glycerol, NEFA and epinephrine) at rest, would highlight any potential inter-individual variability with regard to caffeine sensitivity.

1.6. Caffeine, different fitness level athletes and performance: A methodological consideration

Reviewing the relevant literature, it is evident that several previous caffeine studies did not get into consideration whether the different aerobic fitness levels of the evaluated participants could interfere the obtained biological as well as performance results. Furthermore, studies examined the influence of caffeine on repeated sprints and on single Wingate tests, revealing equivocal results (Greer et al., 1998; Collomp et al., 1991). In addition, Collomp et al. (1992) investigated any possible caffeine effects during swimming between trained and untrained participants, while Schneiker et al. (2006) attempted to examine any possible influence of caffeine during repeated sprints in well-trained team sport players. Caffeine ingestion enhances the athletic performance during time-trials (Astorino et al., 2012) as well as the neuromuscular explosiveness (Ali et al., 2016). On the other hand,

it was indicated that while caffeine enhances the athletic performance, it did not reveal any influence on any physiological parameters (Woolf et al., 2008). The work done by Woolf and coworkers however, aimed to investigate the influence of caffeine ingestion between competitive and non-competitive athletes. In addition, the study from Stuart et al. (2005) revealed that caffeine positively influence single Wingate bouts in well-trained team-sports athletes.

Previous studies examined the possible caffeine effects in trained and untrained participants (Talanian and Spriet, 2016; Ping et al., 2010; Woolf et al., 2008), during single Wingate (Stuart et al., 2005) and repeated sprints (Schneiker et al., 2006), jump tests (Ali et al., 2016) as well as during time-trials (Astorino et al., 2012). No studies however, were found to evaluate the effects of caffeine between participants with high and medium aerobic fitness levels during high-intensity prolonged intermitted exercise. It was therefore assumed that any discrepancies regarding the ergogenic effects of caffeine identified from the above studies, might be due to the different aerobic fitness levels of the participants taken part and due to the exercise protocols employed. The aerobic fitness level can be identified through the VO_2max values obtained during the VO_2max test. According to Figueira et al. (2008) there are three different aerobic fitness levels based on values obtained from the VO_2max evaluation. Participants with high aerobic fitness level have VO_2max above 62 $ml \cdot kg^{-1} \cdot min^{-1}$, whereas VO_2max between 43 and 58 $ml \cdot kg^{-1} \cdot min^{-1}$ indicate that the participants have medium aerobic fitness level and finally low aerobic fitness level have the participants with VO_2max below 38 $ml \cdot kg^{-1} \cdot min^{-1}$.

Pre-distinguishing therefore the participants with high and medium aerobic fitness levels and then evaluate the effects of caffeine in participants with high and medium aerobic fitness level would highlight possible ergogenic effects of caffeine during exercise stress conditions, simulating a soccer game, eliminating the type II error.

1.7. The aims of this study

The outcome of the above literature review suggests that the biological responses, as well as the performance influence following caffeine ingestion are equivocal. The discrepancies observed regarding the effects of this substance to human body and to exercise performance might be due to the a) caffeine dose effect, b) environmental conditions (evaluations in cool or hot environment), c) the exercise protocols employed, d) participants' inter-individual variability regarding the levels of responsiveness to caffeine and e) inter-individual physical fitness conditions variability of the participants taking part. However, no

studies so far examined whether 1) the inter-individual variability of the responsiveness levels of caffeine and 2) the different aerobic fitness levels of the participants could potentially interfere the collected data differently. The aims therefore of the present thesis were to: 1) initially distinguish the participants according to their responsiveness to caffeine, 2) distinguish the participants into high and medium aerobic fitness level athletes and 3) examine the effects of caffeine on biological responses and on exercise performance in athletes who were pre-distinguished as high and low responders to caffeine and in athletes with high and/or medium aerobic fitness level during a simulated soccer game protocol on treadmill. It was assumed that caffeine ingestion would provoke biological effects and would improve exercise performance and neuromuscular explosiveness, while these effects would be more apparent within the high responders to caffeine athletes and within the athletes with high aerobic fitness level.

CHAPTER TWO:

GENERAL METHODS

2.1. Participants

Twenty male soccer players (age 22 ± 4 years; body height 1.78 ± 0.05 m; body mass 74.2 ± 7.7 kg; body fat 11.5 ± 3.7 %; VO_2max 60.8 ± 4 $ml \cdot kg^{-1} \cdot min^{-1}$; daily caffeine consumption with median 72 mg and range 0-391 mg) took part in the study voluntarily. The study was approved by the Cyprus National Bioethics Committee and conformed to the Code of Ethics of the World Medical Association (Declaration of Helsinki). The participants had previous professional or semi-professional soccer experience of at least 5 years with regular training and participation in official national league soccer games. Following explanation of the test and procedures, as well as the nature, benefits and risks of the study during the preliminary session, the participants gave their written consent, after which medical history, life style questionnaires and daily caffeine consumption questionnaires were completed. Participants were excluded if they had any history of cardiovascular disease, metabolic, renal, hepatic, or musculoskeletal disorders or were taking any other medication as determined by the medical history questionnaire (Church et al., 2015). None of them was on any medication and/or drug which could influence physical performance during the study. No participant had a history of any disease or evidence of musculoskeletal injury before and during the study period.

2.2. Anthropometric characteristics

During the preliminary session, the participants' body mass and height were identified using a calibrated weight and free standing height scale (Tanita, Tokyo, Japan). Skinfold thickness (Harpenden skinfold caliper) was measured at seven sites (triceps, subscapular, mid-axillary, anterior suprailiac, pectoral, abdomen and thigh) in duplicate and the mean was used to estimate body fat percentage according to Jackson and Pollock (1978). Measurements were performed by the same trained person for all subjects.

2.3. VO_2max testing

The participants' VO_2max was determined at their third visit to the laboratory. Each participant ran on a treadmill (h/p cosmos Mercury, Nussdorf, Germany) at zero incline, starting with a speed of 8 $km \cdot h^{-1}$, which was increased by 1 $km \cdot h^{-1}$ every minute until volitional exhaustion. Oxygen uptake was measured breath-by-breath (Quark CPET, Cosmed, Rome, Italy), HR (Cosmed wireless HR monitor) was continuously assessed, and RPE was recorded every three minutes using the Borg scale (Borg, 1982). The participants were deemed to have achieved maximal effort when i) HR was at least 90 % of age predicted HR max (220-

age), ii) RER was at least 1.10 and iii) RPE was 20 (Taylor et al., 1955). The configuration of the flow-meter calibration was performed using the 10-stroke method to measure ventilation, verified with a calibration syringe of known volume (3 ml). Oxygen and carbon dioxide gas analyzers were calibrated with known standard gas concentration.

2.4. Experimental design

During all tests, the laboratory temperature was set between 20 and 24°C, with relative humidity between 45 and 66 %. Prior to the two main exercise protocols, the participants underwent two familiarization trials separated at least 96 hours including all treadmill protocol and study experimental procedures, for familiarizing themselves with the soccer game protocol on treadmill and experimental procedures, including MAP assessment, cannulation, and blood sample collection. During these familiarization trials, the participants consumed either caffeine or placebo capsules, as described below, in a double blind manner for elimination of the learning effect (McArdle et al., 2007). 15 minutes prior executing any treadmill procedure, the participants performed a standard soccer warm up session, including running, skipping, jumping and stretching (Owoeye et al., 2014).

At least 96 hours following the familiarization trials, the participants underwent two identical exercise trials separated by at least 96 hours, for allowing full recovery (Del Coso et al., 2012). All trials were carried out between 08:00 and 14:00 hours, since caffeine ingestion in the evening (18:00) has been reported to have little effect on neuromuscular performance with high rates of negative side-effects (Mora-Rodríguez et al. 2015). A high carbohydrate meal (70 % with prescribed values between 1650-1850 kcal based on participants anthropometric characteristics; software: Diet 200A, Science Technology) intended to maximize liver and muscle glycogen, was prescribed to the participants 3 hours prior to each protocol (Figure 1). The participants were asked to refrain from strenuous physical activity and from alcohol or caffeine consumption for 48 and 72 hours respectively, prior to each protocol (Hadjicharalambous et al., 2010).

Upon arrival at the laboratory, 95 minutes prior to the exercise test, the participants were seated comfortably and 5 minutes later resting MAP was recorded (OMRON M6, Omron Healthcare, Milton Keynes, UK). Thereafter, their right hand and forearm were immersed in water at 42 – 44 °C for 15 minutes for achieving arterialization of the venous blood (Forster et al., 1972). A Veflon cannula (20 G) with 3-way function was introduced into a superficial vein on the ventral surface of the heated arm and a resting blood sample (5 ml) was obtained. The cannula was kept patent by slow infusion (3 ml) of heparin (50 IU /5 ml) between samples

(Hadjicharalambous et al., 2010). The participants then consumed, in a crossover double-blind and counterbalanced manner, capsules containing either 6 $mg \cdot kg^{-1}$ caffeine or placebo. Prior to any subsequent blood sample, 3 ml of blood were discarded in order to remove any heparin residues from the vein. A second resting blood sample was obtained and MAP measured 60 minutes after capsule ingestion at rest.

Following 15 minutes of standard warm-up, including running, skipping, jumping and stretching (Owoeye et al., 2014), the participants performed two CMJ (OptoJump Next, Microgate, Bolzano, Italy) starting from the standing position and two squat jumps starting with the knee angle at 90 °. Both types of jumps were performed with an arm swing. During the CMJ, the participants were asked to jump vertically and land with both feet simultaneously for measuring the best jump height, while during the squat jumps, they were asked to jump vertically as fast as possible after an optical stimulus for measuring the best RT. Between jumps one minute of recovery was allowed. Following this first battery of CMJ and RT tests, the participants started performing a treadmill protocol aimed to simulate a soccer game (Drust et al. 2000). The protocol consisted of three 22.5 minutes periods, followed by steady-state running. The treadmill incline was set at zero during the whole treadmill protocol, while the speed of the treadmill was periodically set at 6, 12, 15, 18 or 21 $km \cdot h^{-1}$, with shorter times at high speeds and longer times at low speeds (Figure 2). The duration and the frequency of each treadmill speed was the same for all participants.

At the end of the first period of 22.5 minutes blood sampling, MAP measurement, and a second battery of CMJ and RT tests were performed as before exercise within 5 min. This was repeated at the end of the second period, which was separated from the third one by 15 min, corresponding to the interval between half-times in a soccer game. Blood sampling, MAP measurement, and a third battery of CMJ and RT tests were repeated right before and after the third period. Immediately following that, the participants were connected to the gas analyzer by wearing a face mask and embarked on the fourth period, consisting in running at the speed corresponding to 75 % of VO_2max (on the basis of the VO_2max test) and exercised to volitional fatigue (exhaustion). HR was continuously recorded, while RPE was determined at the beginning, middle and end of each period of the treadmill protocol.

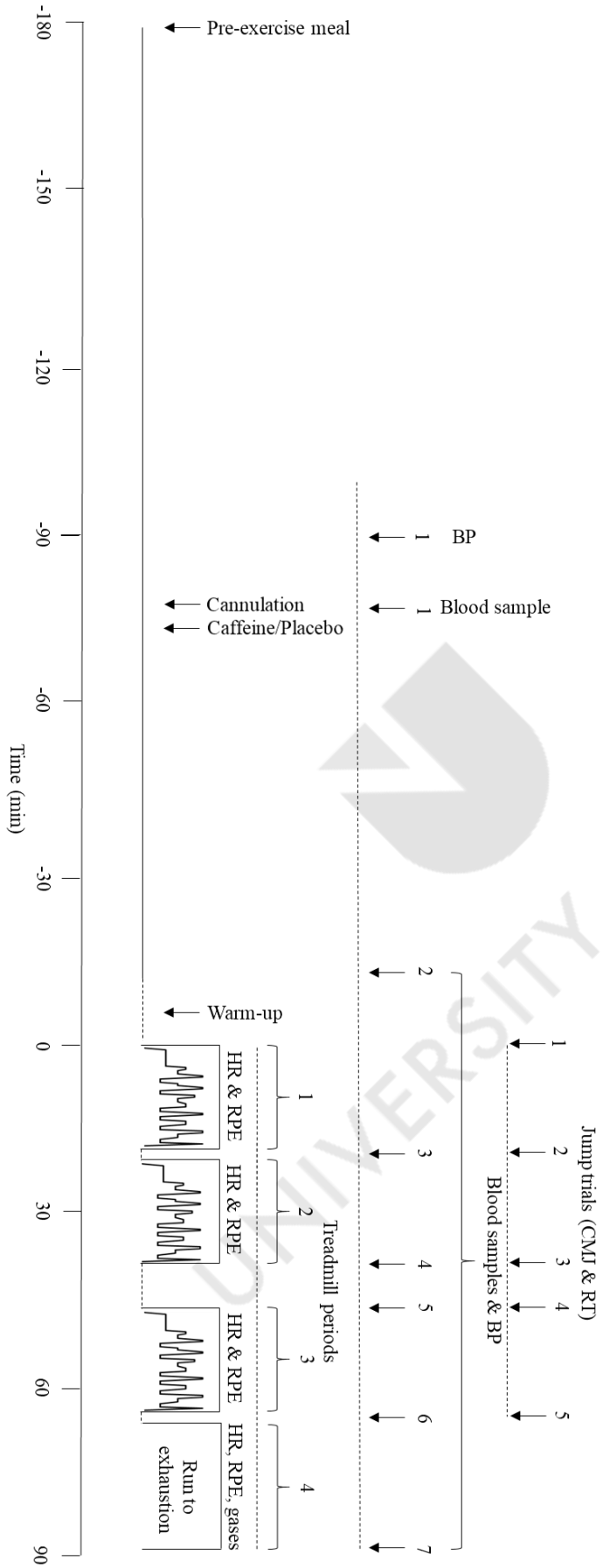

Figure 1. Schematic illustration of the experimental design.

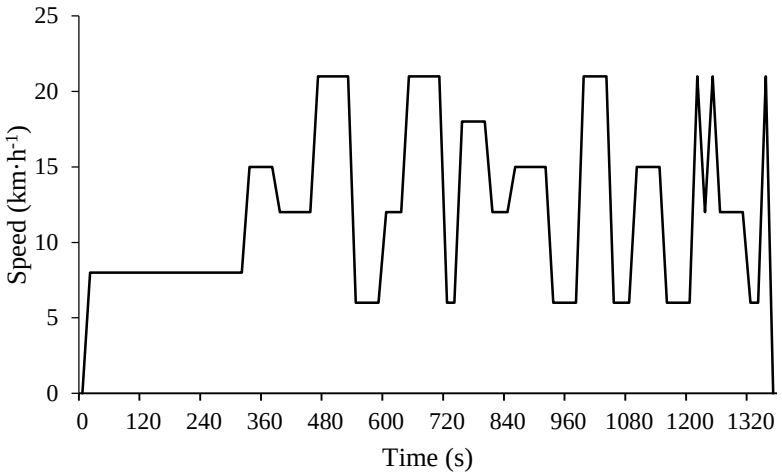

Figure 2. Diagrammatic representation of one treadmill period (22.5 min) of the soccer game protocol

2.5. Energy expenditure and substrate oxidation

Oxygen uptake (VO_2, $l \cdot min^{-1}$), carbon dioxide production (VCO_2, $l \cdot min^{-1}$) and RER were measured during the first 5 minutes of the fourth period (Hadjicharalambous et al., 2010). Energy expenditure and rates of fat and carbohydrate oxidation were estimated as follows. Initially, raw breath-by-breath data were examined for outliers and any breaths with VO_2, VCO_2 and RER values that lay more than four standard deviations away from the mean response were excluded (Lamarra et al., 1987). Then energy expenditure ($kcal \cdot min^{-1}$) was calculated using the following equation (Ravussin et al., 1985):

Energy expenditure = {4.686 + [(RER - 0.707) / 0.293] x 0.361} x VO_2

Additionally, the rates of fat and carbohydrate oxidation ($g \cdot min^{-1}$) were calculated using the following equations (Alkahtani, 2014):

Fat oxidation = (1.67 x VO_2) - (1.67 x VCO_2)

Carbohydrate oxidation = (4.55 x VCO_2) - (3.21 x VO_2)

2.6. Blood treatment and biochemical analyses

Blood samples were drawn from the vein using disposable 3 ml and 5 ml BD syringes (Cocate et al., 2011). Blood samples were immediately transferred into K_2EDTA-containing tubes, using disposable 5 ml BD syringes and BD Precision-Glide needles (0.80 x 25 mm) (Cocate et al., 2011) and centrifuged (Rotofix 32A, Hettich, Germany) at 4000 rpm for 10 minutes to obtain plasma (Horton et al., 2002). Plasma samples were immediately transferred into 2 ml cryotubes (Greiner Bio-One, Austria) and stored at -80 ºC until assayed (Bilet et al., 2011) for later assessment of plasma glucose, glycerol and NEFA (Greiner Bio-One, Frickenhausen, Germany) and lactate (DiaSys Diagnostic Systems, Holzheim, Germany)

using standard enzymatic methods (Bilet et al., 2011) in an automatic spectrophotometric analyzer (COBAS MIRA Plus CC, Roche). Additionally, epinephrine (Tecan Group Ltd, Mannedorf, Switzerland) was measured using a microplate absorbance reader (TECAN Sunrise with ELISA microplate reader; Horton et al., 2002).

2.7. Classification of participants into high and low caffeine responders

For each participant the MAP (Wardle et al., 2012) , glycerol, NEFA and epinephrine (Van Soeren and Graham, 1998; Spriet et al., 1992) responses to caffeine ingestion at rest were calculated as the difference between the change from before to 60 minutes following caffeine ingestion and the corresponding change following placebo ingestion(Wardle et al., 2012), that is (caffeine60 – caffeine0) – (placebo60 – placebo0). Then the means for the four responses were calculated. Those participants who had a response above the mean in two or more variables (n = 11) were categorized as high responders, while the rest (n = 9) were categorized as low caffeine responders.

2.8. Statistical analysis

The SPSS statistical package (version 22) was used for all the statistical computations. All data are expressed as the mean and SD, while data regarding daily caffeine consumption among the participants are expressed in median and range. Characteristics of the two groups were compared by Mann-Whitney test. All test variables were analyzed by mixed ANOVA with one between factor (high or low response to caffeine during experiment 1; high or medium aerobic fitness level group during experiment 2) and one or two within factors (time and/or treatment), as appropriate. Mauchly's test of sphericity was performed for all test variables, and Greenhouse-Geisser correction for within-subject effects was employed in cases where the assumption of sphericity was violated. Significant interactions were followed up by pairwise comparisons through simple main effect analysis with Sidak correction for multiple comparisons. Effect sizes *(ES)* of caffeine supplementation were calculated as the difference between means corresponding to caffeine and placebo, divided by the SD corresponding to placebo (Field, 2009). For all statistical analyses a value of $p \leq 0.05$ was considered statistically significant.

2.9. Coefficient of Variation (C.V.)

C.V. is the SD expressed as a proportion or percentage of the mean (Field, 2009). Consequently, the intra-assay C.V. was calculated (see equation below) from the SD of the

difference between double measurements of the sample expressed as a percentage of the total mean sample (Table 1). When the cost of the duplicate analysis exceeded €1 per sample, the C.V. was determined from at least 30 aliquots of the same assay (Table 1).

$$C.V.\ \% = (SD \cdot mean^{-1}) \times 100$$

Table 1. Coefficient of Variation of plasma assays

Assay	Method	n	C.V.
Glucose	Colorimetric method, Roche Diagnostics	280	0.6
Lactate	Colorimetric method, Roche Diagnostics	35	1.6
Glycerol	Colorimetric method, Roche Diagnostics	134	2.2
NEFA	Colorimetric method, Roche Diagnostics	44	1.6
Epinephrine	Manual enzyme immunoassay, ELISA IBL	280	0.3

CHAPTER THREE:

EXPERIMENT ONE

Caffeine ingestion effects on high and low caffeine responders

3.1. Introduction

Caffeine ingestion prior to athletic competitions has always been a very popular ergogenic aid method utilized by several athletes in an attempt to improve exercise performance (Desbrow and Leveritt, 2006). According to Coso et al. (2011), three out of four elite athletes have been reported to employ this practice in anticipation of improved performance. For several years, pre-athletic competition caffeine ingestion was considering as an illegal substance when the amount of urinary caffeine samples exceeded 12 $mg \cdot kg^{-1}$ (Spriet, 2014). However, the World Anti-doping Agency has removed caffeine from the list of prohibited substances in 2004 increasing the interest in freely using caffeine as an ergogenic aid supplement by many athletes (Chester and Wojek, 2008).

Several studies have attempted to explain the multiple mechanisms behind caffeine's effect on biological responses and athletic performance. Caffeine has been found to increase plasma catecholamine concentrations thus inhibiting lipogenesis, stimulating lipolysis and consequently enhancing the release of glycerol and NEFA from adipose tissue into the plasma (Van Soeren and Graham, 1998), as well as their availability to the active muscles (Essig et al., 1980), resulting in carbohydrate sparing (Spriet et al., 1992). Costill et al. (1978) for example, reported a running time improvement (90.2 vs 75.5 minutes) with caffeine (300 mg) compared with placebo due to stimulating lipolysis. Bruce et al. (2000) found an improvement in endurance performance on an air-baked ergometer due to plasma NEFA elevation following caffeine ingestion. Erickson et al. (1987), reported decreased muscle glycogen utilization following caffeine ingestion (5 $mg \cdot kg^{-1}$) during cycling at 65-75 % VO_2max for 90 min, while Cruz et al. (2015) revealed increased whole-body fat oxidation during cycling with constant-load tests of 30 minutes following 6 $mg \cdot kg^{-1}$ caffeine ingestion. It was assumed however that the increase lipolysis *per se* does not necessarily affect the endurance performance due to increase fat and carbohydrate oxidation sparing.

A significant number of studies, suggested an increase in endurance performance without supporting the carbohydrate sparing effect of caffeine (e.g. Cox et al., 2002; Greer et al., 2000; Laurent et al., 2000; Graham and Spriet, 1995). Wells et al. (1985) on the other hand, indicated that caffeine ingestion (6 $mg \cdot kg^{-1}$) resulted in no differences in plasma NEFA or glucose concentrations from placebo, without any significant effect on endurance performance. Hadjicharalambous et al. (2006; 2010) however, revealed significant reduction in rating of RPE and enhanced concentrations of plasma metabolites, while the rates of fuel oxidation and endurance performance were not affected by caffeine. These authors speculated

that the lack of performance improvement might be due to the inter-individual differences in physiological/metabolic responses to caffeine.

Indeed, there was an indication of performance improvement of some participants, while in others performance was not affected due to caffeine (Skinner et al., 2010; Wiles et al., 2006; Doherty et al., 2004). It is plausible therefore that caffeine may differently influence biologically various individuals. A previous study, suggested that caffeine might be physiologically and metabolically more effective in athletes who are not daily caffeine users, while caffeine users revealed no significant differences between the caffeine and placebo trials in the duration and magnitude of the caffeine ergogenic effect (Bell and McLellan, 2002). In contrast, Talanian and Spriet (2016) indicated that caffeine did not affect biological variables in well-trained cyclists and triathletes who were not caffeine users. Even mild also caffeine users were found not to be physiologically influenced by caffeine (Cox et al. 2002). In addition, Irwin et al. (2011) found that regular caffeine users had significant performance improvement compared with non-caffeine users, even when the former had withdrawn from caffeine four days prior the experimental trials. A recent study, however (Gonçalves et al., 2017), revealed that performance effects during a 30-minutes cycling protocol following caffeine supplementation (6 $mg \cdot kg^{-1}$) were not influenced by the level of habitual caffeine consumption; while the blood lactate and RPE where not different between the trials (caffeine, placebo and no supplement) and between the habitual caffeine consumption level. In addition, the study conducted by Del Coso et al. (2012) showed that 89 % of the participants improved their performance with caffeine, while the remaining 11 % improved their performance after consuming a decaffeinated drink, suggesting therefore that those participants who did not reveal any response to caffeine should be categorized as non-responders to this drug. Therefore, the necessity emerges to test the effects of caffeine on participants with different responses to this substance in order to provide further information regarding its potential ergogenic effects.

It is however, difficult to accurately reveal whether someone is responsive to caffeine action or not. To our knowledge only one clinical study was located in examining the responsiveness or not of caffeine on human physiology using, however, just one evaluating variable. Wardle et al. (2012) proposed a method of determining the responsiveness to caffeine on the basis of its cardiovascular effects and, in particular, on increase in MAP. According to this method, the participants' responses to caffeine can be identified by calculating the MAP through the following equation: MAP = systolic + (2 x diastolic)/3. The response measures were produced by calculating by change of MAP from baseline to the time

before and the time after the effort expenditure for rewards task. The average change scores were then calculated, producing the average MAP change for the caffeine session and for the placebo session. The average scores for placebo were subtracted from their respective average scores for caffeine producing the marginal mean estimates of MAP response for each person. Although caffeine is generally considered to raise MAP (Wardle et al., 2012; Papamichael et al., 2005), there are several studies which revealed opposite results (Riksen et al., 2009; Salvaggio et al., 1990). Thus, it might be risky to base the estimate of responsiveness to caffeine on MAP measurements only. Other variables, such as metabolic ones, might have to be taken into account as well. This might allow a more elaborate examination of the ergogenic effects of caffeine. It was therefore speculated that the method for investigating the responsiveness or not of caffeine on human physiology should include not only the MAP estimates. Metabolic variables such as glycerol, NEFA and epinephrine concentrations should be taken into account as well.

There are no studies however, investigating human responsiveness to caffeine using methods which take into consideration the subjects' MAP, glycerol, NEFA and epinephrine concentrations due to caffeine supplementation. Thus, the aims of the present experiment were to: a) identify the levels of responses to caffeine on the basis of four biological parameters that usually respond to caffeine at rest and b) compare the effects of caffeine supplementation on fatigue and biological responses between high and low caffeine responders during a simulated soccer game protocol on treadmill.

3.2. Materials and Methods

3.2.1. Participants

Twenty (n = 20) male soccer players (age 22 ± 4 years; body height 1.78 ± 0.05 m; body mass 74.2 ± 7.7 kg; body fat 11.5 ± 3.7 %; VO_2max 60.8 ± 4 $ml \cdot kg^{-1} \cdot min^{-1}$; daily caffeine consumption with median 72 mg and range 0-391 mg) voluntarily took part in the present experiment, which was approved by the National Bioethics Committee, conformed to the Code of Ethics of the World Medical Association (Declaration of Helsinki). For more information regarding participants' medical screening and study approval see chapter 2.

3.2.2. Anthropometric characteristics

The participants' anthropometric characteristics identification are described in chapter 2.

3.2.3. VO_2max testing

The participants' VO_2max determination is described in chapter 2.

3.2.4. Experimental design

Prior to the two main soccer game protocols on treadmill, the participants underwent two familiarization trials for familiarizing themselves with the soccer game protocol on treadmill and experimental procedures, including MAP assessment, cannulation and blood samples collection. The experimental design is described in further details in chapter 2.

3.2.5. Energy expenditure and substrate oxidation

For details regarding the EE and rate of substrate oxidation calculations and pulmonary gas exchange collection and analysis see chapter 2.

3.2.6. Blood treatment and biochemical analyses

For details regarding the blood handling and biochemical analyses see chapter 2.

3.2.7. Classification of participants into high and low caffeine responders

For the classification method of high responders and low responders to caffeine please see chapter 2.

3.2.8. Statistical analysis

All test variables were analyzed by mixed ANOVA with one between factor (high or low response to caffeine at rest) and one or two within factors (time and/or treatment), as appropriate. Further details regarding the statistical analysis are described in chapter 2.

3.3. Results

3.3.1. High and low responders to caffeine

The separation of the participants into high and low caffeine responders on the basis of their MAP and plasma glycerol, NEFA, and epinephrine responses to caffeine at rest are presented in Table 2. Eleven participants were categorized as high responders and daily caffeine consumption with median 73 mg and range 0-391 mg and nine participants as low responders and daily caffeine consumption with median 71 mg and range 15-336 mg. The anthropometric and physiological characteristics of the two groups, are presented in table 3. Groups did not significantly differ in any of these characteristics.

Table 2. Separation of high and low caffeine responders

Participant No.	MAP (mm Hg)	Glycerol ($mmol \cdot l^{-1}$)	NEFA ($mmol \cdot l^{-1}$)	Epinephrine ($ng \cdot ml^{-1}$)	Group
1	-0.67	**0.56**	**1.60**	-0.45	High
2	**1.07**	**0.15**	-0.06	-0.12	High
3	**0.78**	-0.19	-0.64	**0.39**	High
4	-1.07	**2.00**	**1.92**	-0.15	High
5	**1.99**	**1.62**	**1.67**	**0.02**	High
6	-0.76	**0.49**	**0.28**	-0.27	High
7	**0.08**	-0.79	-1.45	**3.41**	High
8	**0.67**	**0.36**	**0.01**	**0.30**	High
9	-0.82	**0.42**	**0.96**	**0.07**	High
10	**2.14**	-0.49	**0.24**	**1.26**	High
11	-0.29	**1.54**	**1.29**	-0.43	High
12	-1.41	-2.24	-1.15	-0.45	Low
13	**1.03**	-0.48	-0.40	-1.13	Low
14	-0.15	**0.38**	-0.47	-0.18	Low
15	-1.15	-0.96	-0.30	**0.24**	Low
16	-0.13	-0.59	-1.28	-1.71	Low
17	-0.92	-0.34	-0.57	**0.18**	Low
18	-0.06	**0.08**	-0.43	-0.02	Low
19	-0.11	-0.43	-0.45	-0.33	Low
20	-0.21	-1.10	-0.77	-0.63	Low
Mean response	-0.05	0.13	0.08	0.14	

Values are differences of each participant's response to caffeine over placebo from the mean response of all 20 participants. Positive values (that is, responses above the mean) are marked in boldface. Participants with at least two positive values were classified as high responders. MAP: mean arterial blood pressure, calculated as systolic + (2 x diastolic)/3; NEFA: non-esterified fatty acids.

Table 3. Characteristics of the high and low caffeine responders (mean ± SD).

Group	Age (years)	Body height (m)	Body mass (kg)	Body fat (%)	VO_2max ($ml \cdot kg^{-1} \cdot min^{-1}$)
High responders (n = 11)	22 ± 4	1.79 ± 0.07	74.1 ± 8.1	10.7 ± 3.4	61.8 ± 4.6
Low responders (n = 9)	21 ± 4	1.78 ± 0.04	74.2 ± 7.7	12.4 ± 3.9	59.4 ± 3.8

3.3.2. Time to fatigue

There was a significant main effect of treatment (caffeine vs placebo) on the time to fatigue during the steady state running (fourth period) [F (1, 18) = 23.66, $p < 0.001$]. Time to fatigue (Figure 3) was longer with caffeine compared with placebo in both high responders (63.5 %; 797 ± 201 vs 487 ± 258 s; *ES* 1.20) and low responders (39.8 %; 625 ± 357 vs 447 ± 198 s; *ES* 0.90), with only one high responder and two low responders exhibiting shorter times. However, time to fatigue was not statistically different between groups, and there was no treatment-by-group interaction.

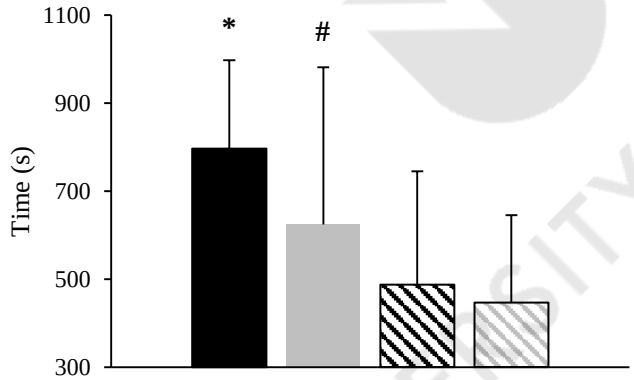

Figure 3. Mean and SD of time to fatigue with caffeine (solid bars) and placebo (hatched bars) of high responders (bars in black) and low responders (bars in gray). * Significant difference between caffeine and placebo of high responders. # Significant difference between caffeine and placebo of low responders.

3.3.3. Explosives performance and reaction time

There were significant main effects of treatment [F (1, 18) = 24.06, $p < 0.001$] and time [F (4, 72) = 16.16, $p < 0.001$] on CMJ height (Figure 4*a*). In both high and low responders, CMJ was higher with caffeine compared with placebo (marginal mean 42.1 ± 5.5 cm vs 40.5 ± 5.7 cm, *ES* 0.28, in high responders and 41.0 ± 3.8 cm vs 38.8 ± 4.6 cm, *ES* 0.49, in low responders), with only one participant from each group exhibiting a lower average CMJ height. There was no significant main effect or interaction in RT (Figure 4*b*). RT was similar

with caffeine and with placebo in both high responders (marginal mean 0.60 ± 0.06 vs 0.59 ± 0.05 s, *ES* 0.14) and low responders (0.61 ± 0.08 vs 0.63 ± 0.10 s, *ES* -0.21).

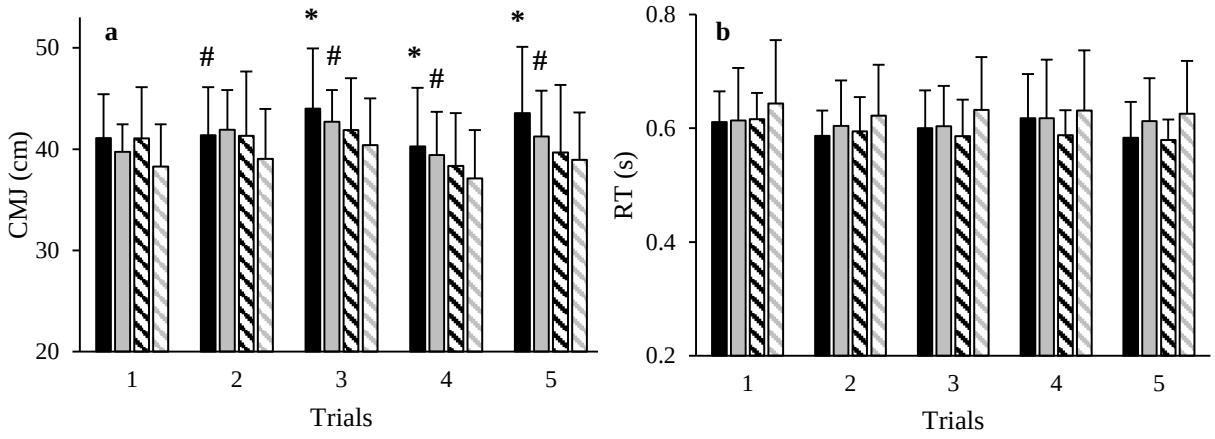

Figure 4. Means and SD of countermovement jump height *(a)* and reaction time *(b)* with caffeine (solid bars) and placebo (hatched bars) of high responders (bars in black) and low responders (bars in gray) at the five trials indicated in Fig. 1. Trial 1 corresponds at time 0 min, trial 2 at time 22.5 min, trial 3 at time 45 (1st half) min, trial 4 at time 45 (2nd half) min and trial 5 at time 67.5 min. * Significant difference between caffeine and placebo of high responders. # Significant difference between caffeine and placebo of low responders.

3.3.4. Cardiovascular responses and perception of effort

MAP (Figure 5) exhibited significant main effects of treatment [F (1, 16) = 17.46, p = 0.001] and time [F (2.60, 41.66) = 25.01, p < 0.001], as well as significant interactions of treatment by time [F (6, 96) = 2.75, p = 0.017] and treatment x time x response to caffeine [F (6, 96) = 3.24, p = 0.006]. MAP was higher with caffeine in both groups (marginal mean 99 ± 9 mm Hg vs 94 ± 10 mm Hg, *ES* 0.52, in high responders and 96 ± 10 mm Hg vs 92 ± 9 mm Hg, *ES* 0.49, in low responders). Pairwise comparisons showed that the only significant difference between groups following caffeine ingestion was at the 2nd time point (before warm-up), MAP being higher in the high compared to the low responders (98 ± 5 mm Hg vs 90 ± 8 mm Hg, p = 0.023), in accordance with the separation of the participants into these groups.

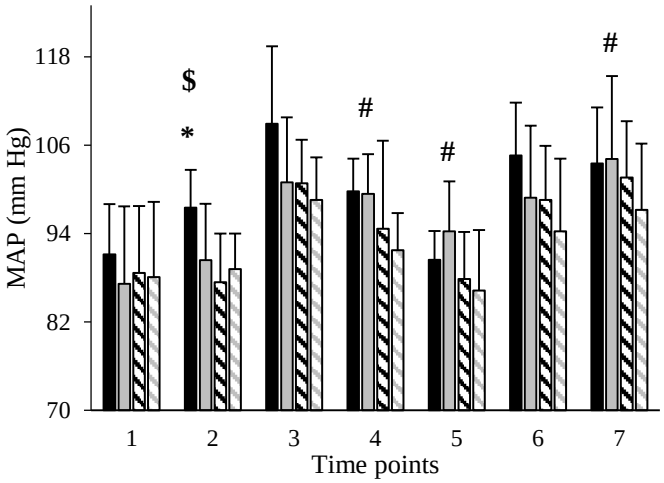

Figure 5. Means and SD of MAP with caffeine (solid bars) and placebo (hatched bars) of high responders (bars in black) and low responders (bars in gray) at the five trials indicated in Fig. 1. Time point 1 corresponds to sample at time -75 min, time point 2 at time -15 min, time point 3 at time 22.5 min, time point 4 at time 45 (1st half) min, time point 5 at time 45 (2nd half) min, time point 6 at time 67.5 min and time point 7 at exhaustion. * Significant difference between caffeine and placebo of high responders. # Significant difference between caffeine and placebo of low responders. $ Significant difference between high and low caffeine responders.

Average HR during each of the four periods of the treadmill protocol (Table 4) showed significant main effects of treatment [F (1, 16) = 18.18, p = 0.001] and time [F (2.04, 32.68) = 12.79, p < 0.001], as well as a significant interaction treatment x time [F (3, 48) = 3.22, p = 0.031]. HR was higher with caffeine than placebo in both high responders (marginal mean 161 ± 12 bpm vs 154 ± 12 bpm, *ES* 0.56) and low responders (164 ± 15 bpm vs 160 ± 16 bpm, *ES* 0.28).

There were significant main effects of treatment [F (1, 18) = 11.63, p = 0.003] and time [F (3, 54) = 116.77, p < 0.001] on RPE (Table 4). RPE was lower with caffeine than placebo in both high responders (marginal mean 12.4 ± 2.0 vs 13.3 ± 2.1, *ES* – 0.42) and low responders (12.7 ± 3.0 vs 13.3 ± 2.8, *ES* – 0.21). There was also a significant interaction between response x time [F (3, 54) = 3.91, p = 0.013]. Pairwise comparisons revealed that high responders differed from low responders in that RPE was lower with caffeine than placebo during treadmill periods 3 and 4 only in the former (p = 0.009 and 0.004, respectively).

Table 4. Average HR and RPE for each treadmill period during EXP. 1 (mean ± SD)

			Treadmill period			
Treatment	**Variable**	**Group**	**1**	**2**	**3**	**4**
Caffeine	HR (bpm)	High responders	153 ± 8	159 ± 8	157 ± 11	174 ± 10 *
		Low responders	160 ± 14	164 ± 14	162 ± 17	172 ± 15 #
	RPE	High responders	11 ± 1.3	12.4 ± 1.7	12.4 ± 1.6 *	14.4 ± 1.4 *
		Low responders	10 ± 1.5	12.3 ± 2.5	12.8 ± 2.7	15.7 ± 2.1
Placebo	HR (bpm)	High responders	151 ± 9	156 ± 7	151 ± 9	159 ± 16
		Low responders	152 ± 19	164 ± 15	160 ± 15	165 ± 13
	RPE	High responders	11.2 ± 1.1	12.8 ± 1.6	13.7 ± 1.5	15.7 ± 1.2
		Low responders	10.3 ± 1.7	12.9 ± 2.4	13.8 ± 2	16.1 ± 1.8

* Significant difference between caffeine and placebo of high responders. # Significant difference between caffeine and placebo of low responders.

3.3.5. Energy expenditure and fuel oxidation

No significant statistical outcomes were obtained with regard to RER, energy expenditure, fat oxidation, or carbohydrate oxidation during the first 5 minutes of the fourth period ($p > 0.05$). RER was similar with caffeine and with placebo in both high responders (marginal mean 0.81 ± 0.03 vs 0.79 ± 0.04, *ES* 0.39) and low responders (0.80 ± 0.03 vs 0.80 ± 0.03, *ES* -0.05). Energy expenditure was similar with caffeine and with placebo in both high responders (marginal mean 16 ± 2.1 vs 16.3 ± 2.4 kcal·min^{-1}, *ES* -0.12) and low responders (15.5 ± 1.2 vs 15.1 ± 1.4 kcal·min^{-1}, *ES* 0.32). Fat oxidation was also similar with caffeine and with placebo in both high responders (marginal mean 1.1 ± 0.2 vs 1.2 ± 0.3 g·min^{-1}, *ES* -0.31) and low responders (1.1 ± 0.1 vs 1 ± 0.2 g·min^{-1}, *ES* 0.62). Carbohydrate oxidation was similar with caffeine and with placebo in both high responders (marginal mean 1.6 ± 0.6 vs 1.3 ± 0.6 g·min^{-1}, *ES* 0.38) and low responders (1.3 ± 0.3 vs 1.4 ± 0.5 g·min^{-1}, *ES* -0.27).

3.3.6. Blood metabolites

Plasma glucose (Figure 6*a*) exhibited significant main effects of treatment [F (1, 18) = 12.78, p = 0.002] and time [F (3.34, 60.11) = 13.17, $p < 0.001$], as well as an interaction between treatment x time [F (2.49, 44.83) = 5.01, p = 0.007]. Plasma glucose was higher with caffeine than with placebo in both high responders (marginal mean 5.7 ± 1.0 mmol·l^{-1} vs 5.3 ± 0.8 mmol·l^{-1}, *ES* 0.48) and low responders (5.5 ± 0.8 mmol·l^{-1} vs 5.3 ± 0.8 mmol·l^{-1}, *ES* 0.25).

Likewise, there were significant main effects of treatment [F (1, 18) = 8.05, p = 0.011] and time [F (1.76, 31.71) = 59.52, p < 0.001], as well as an interaction between treatment x time [F (3.17, 57.00) = 4.24, p = 0.008] in plasma lactate (Figure 6*b*). Plasma lactate was higher with caffeine than placebo in both high responders (marginal mean 3.2 ± 1.9 mmol·l^{-1} vs 2.8 ± 1.9 mmol·l^{-1}, *ES* 0.20) and low responders (3.3 ± 2.2 mmol·l^{-1} vs 2.9 ± 2.0 mmol·l^{-1}, *ES* 0.19).

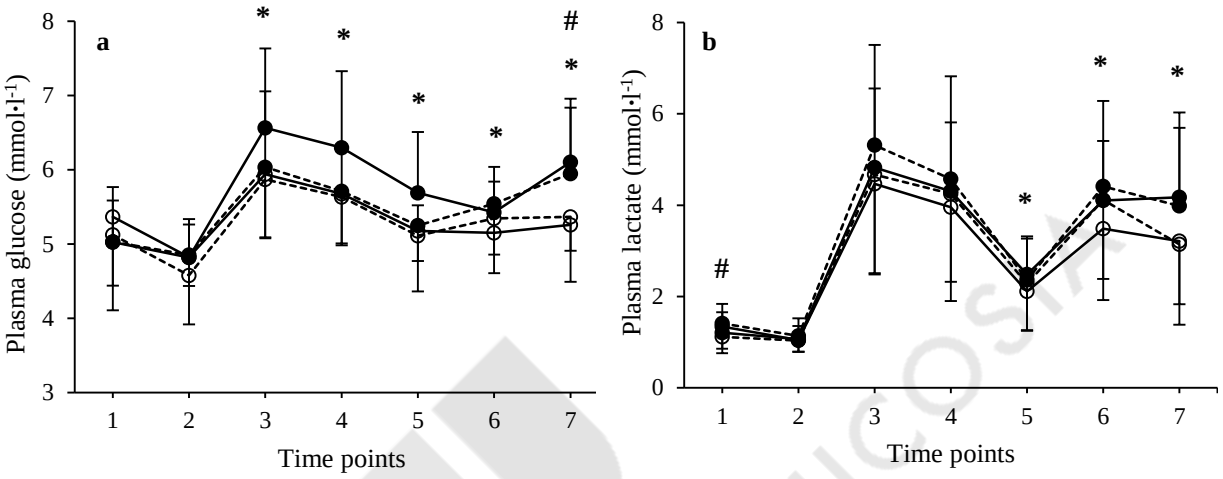

Figure 6. Means and SD of plasma glucose (*a*) and lactate (*b*) with caffeine (full circles) and placebo (open circles) of high responders (solid lines) and low responders (dashed lines) at the seven blood sampling time points indicated in Fig. 1. Time point 1 corresponds to sample at time -75 min, time point 2 at time -15 min, time point 3 at time 22.5 min, time point 4 at time 45 (1st half) min, time point 5 at time 45 (2nd half) min, time point 6 at time 67.5 min and time point 7 at exhaustion. * Significant difference between caffeine and placebo of high responders. # Significant difference between caffeine and placebo of low responders.

With regard to plasma glycerol (Figure 7*a*), NEFA (Figure 7*b*), and epinephrine (Figure 8), the only statistically significant outcome of the factorial ANOVA was a main effect of time [F (1.96, 35.31) = 128.39, F (2.47, 44.52) = 112.87, and F (2.83, 50.91) = 21.21, respectively, p < 0.001 for all]. Plasma glycerol was similar with caffeine and with placebo in both high responders (marginal mean 0.1 ± 0.04 vs 0.1 ± 0.05 mmol·l^{-1}, *ES* 0.11) and low responders (0.1 ± 0.05 vs 0.09 ± 0.03 mmol·l^{-1}, *ES* 0.15). Plasma NEFA was similar with caffeine and with placebo in both high responders (marginal mean 0.7 ± 0.4 vs 0.7 ± 0.4 mmol·l^{-1}, *ES* 0.16) and low responders (0.6 ± 0.4 vs 0.6 ± 0.2 mmol·l^{-1}, *ES* 0.04). Plasma epinephrine was similar with caffeine and with placebo in both high responders (marginal mean 2.3 ± 0.2 vs 2.2 ± 0.2 ng·ml^{-1}, *ES* 0.27) and low responders (2.2 ± 0.3 vs 2.2 ± 0.3 ng·ml^{-1}, *ES* 0.23).

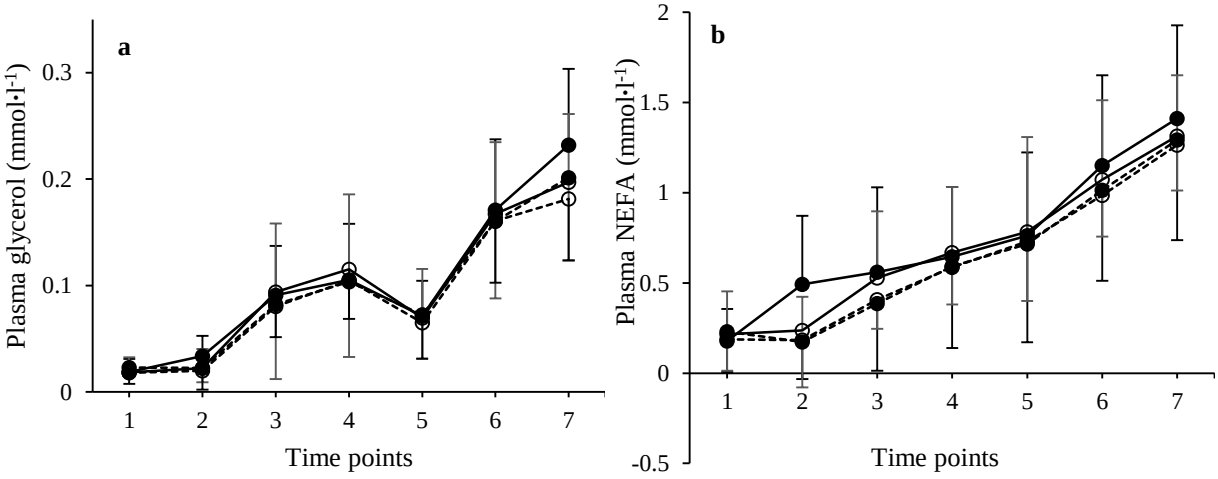

Figure 7. Means and SD of plasma glycerol (left panel) and plasma NEFA (right panel) with caffeine (full circles) and placebo (open circles) of high responders (solid lines) and low responders (dash lines) at the seven blood sampling time points indicated in Fig. 1. Time point 1 corresponds to sample at time -75 min, time point 2 at time -15 min, time point 3 at time 22.5 min, time point 4 at time 45 (1st half) min, time point 5 at time 45 (2nd half) min, time point 6 at time 67.5 min and time point 7 at exhaustion.

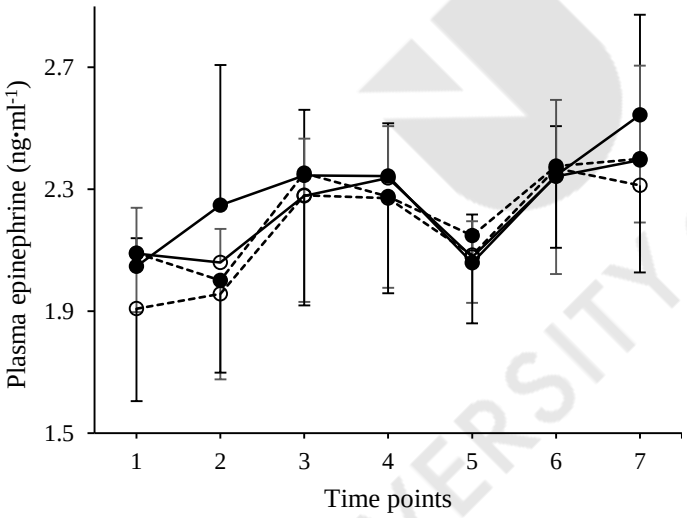

Figure 8. Means and SD of plasma epinephrine with caffeine (full circles) and placebo (open circles) of high responders (solid lines) and low responders (dash lines) at the seven blood sampling time points indicated in Fig. 1. Time point 1 corresponds to sample at time -75 min, time point 2 at time -15 min, time point 3 at time 22.5 min, time point 4 at time 45 (1st half) min, time point 5 at time 45 (2nd half) min, time point 6 at time 67.5 min and time point 7 at exhaustion.

3.4. Discussion

This was the first study which attempted to initially differentiate the potential high responders and low responders to caffeine and then evaluate the effect of caffeine on biological responses and on exercise performance during a simulated soccer game protocol on treadmill in well-trained soccer players. This was achieved by having the participants exercising in a well-controlled laboratory regime, while monitoring also their pre-experimental meal and co-ingesting either caffeine capsules or placebo (6 $mg \cdot kg^{-1}$), in a double-blind manner. The major finding of the present experiment was that caffeine is effective in improving muscular explosiveness (CMJ) and enhancing endurance in high-intensity exercise during a soccer-specific exercise protocol of trained male soccer players irrespectively of whether they are high or low responders to caffeine.

However, to our knowledge, no studies so far attempted to pre-differentiate the participants into high responders and low responders to caffeine prior to examining its actual effect on biological responses and on exercise performance. Only one clinical study was located that had previously used MAP evaluation for indenting high responders and low responders to caffeine (Wardle et al., 2012). According to this method, the participants' responses to caffeine can be identified by calculating the mean arterial pressure (Wardle et al., 2012).

In the present experiment on the other hand, three more metabolic parameters (plasma glycerol, NEFA and epinephrine concentration) responses to caffeine and/or placebo ingestion, were taken into consideration for the above differentiation. To distinguish therefore between high and low responders to caffeine, we introduced a method that takes into consideration the changes in resting mean arterial pressure and plasma glycerol, NEFA, and epinephrine concentrations 65 minutes following caffeine compared to placebo ingestion, and then compares individual changes to the mean change. We detected profound inter-individual differences, with a variety of different combinations. Those participants who exhibited a change above the mean in at least two of the four variables were categorized as high responders, while the rest were considered low responders. This method expands the criteria used by Wardle and coworkers (2012), who based their assessment of responsiveness to caffeine on MAP evaluation solely. We feel that examining more parameters ensures a more accurate classification into high and low responders. From the twenty participants who took part in this experiment, and according to the four biological criteria used for this classification attempt, eleven were categorized as high responders and nine as low responders to caffeine.

Several reports provided evidence of potential different biological effects (enhancing or decreasing) of caffeine on the aforementioned variables (Wardle et al., 2012; Van Soeren and Graham, 1998; Graham and Spriet, 1995; Spriet et al., 1992; Salvaggio et al., 1990; Essig et al., 1980) suggesting that there is a mean increase in plasma glycerol, NEFA and epinephrine concentration following caffeine ingestion (Wardle et al., 2012; Van Soeren and Graham, 1998). However, there is an inter-individual variability in the response within the studies. This may imply the different responses induced by caffeine in individuals and this variability may interfere the obtained data. However, no studies were located to previously employ any biochemical basis method in an attempt to pre-differentiate the high responders and low responders to caffeine prior to examining its actual effect on biological responses and on exercise performance. Our data justified this selection, since these four were the only measured parameters at rest that increased at 65 minutes after caffeine compared to placebo ingestion (even though the latter three were not affected by caffeine during exercise). Specifically, MAP, glycerol, NEFA, and epinephrine increased by 6 %, 23 %, 69 %, and 3 %, respectively. The other three measured parameters (HR, glucose, and lactate) decreased with caffeine compared to placebo at rest. Thus, we feel confident that we have chosen four appropriate markers of biological response to caffeine, which can be a useful tool in examining the potential dependence of the metabolic and/or ergogenic effects of caffeine on inter-individual variability with regard to caffeine sensitivity. Naturally, this battery of variables should be validated through additional studies.

Although there was no significant difference between the high and low caffeine responders according to their daily caffeine consumption as well as in any of the performance, physiological, or biochemical parameters that were monitored during the exercise protocol and were influenced by caffeine, the high responders exhibited greater effect sizes (even by 0.01) in all parameters except CMJ. In addition, in the only parameter (RPE) that displayed an interaction of caffeine response and time, high responders experienced a more favorable effect of caffeine than low responders during the latter part of the simulated soccer-game protocol. This difference may be responsible for the considerably larger *ES* (by 0.30) and numerical improvement in time to fatigue (by 2.2 min) with caffeine in the high vs low responders. These findings suggest that high caffeine responders may enjoy a partial, or quantitative, ergogenic advantage over low responders. Nevertheless, the "big picture" coming out of this experiment is that caffeine seems to provide ergogenic effects regardless of high or low biological responses to its intake at rest, with the response criteria, subject characteristics, exercise protocol, and performance tests employed in the present experiment.

The results of the present experiment revealed that while there were no significant differences in exercise performance between the high responders and low responders groups, the high responders group revealed a greater percentage (63.5 %) of improvement in time to fatigue relative to the low responders (39.8 %) compared to their respective placebo trials. The absence, however, of any significant differences between the two groups regarding the performance enhancement due to caffeine ingestion, enhances the assumption that caffeine seems to provide ergogenic effect through the human body irrespectively whether someone can be categorized as a responder or not to this substance. The exact mechanism however, for this exercise improvement following caffeine ingestion remains relatively unknown. Caffeine easily crosses the blood-brain barrier (Fredholm et al., 1978), and takes about 6 hours (Sokmen at al., 2008; McArdle et al., 2007) to be excreted from the body by the kidneys through the urine (Magkos and Kavouras, 2005; Harland, 2000). Since all the participants of this experiment had withdrawn from any caffeine-containing products for 72 hours prior each test (Hadjicharalambous et al., 2010), it was assumed that any ergogenic effects due to caffeine would be similar among the participants.

Muscular power can be considered as a major fitness component during a soccer game. In the present experiment, muscular power assessed through CMJ height, was found to be higher in both high responders and low responders groups following caffeine ingestion, results that are in accordance with previous reports (Ali et al., 2016; Lara et al., 2014; Kammerer et al., 2012; McCann et al., 2012). A potential explanation regarding the CMJ improvement with caffeine is the increase in voluntary activation due to caffeine ingestion which in turn increases the strength and power regardless of contraction mode (Behrens et al., 2015; McCann et al., 2012). This improvement in performance is probably due to the caffeine's direct antagonism of adenosine receptors (A_1 and A_{2a}) on the sarcolemma, which improves excitation-contraction coupling (Tarnopolsky, 2008) via greater release of Ca^{2+} from the sarcoplasmatic reticulum (Tallis et al., 2012) and/or improved Na^+/K^+ ATPase pump activity (Mohr et al., 2011).

Caffeine supplementation has also been associated with enhanced ability in alertness tasks, RT and attention during physical activity (Church et al., 2015; Beaumont et al., 2004; Leiberman et al., 2002). However, the present experiment did not reveal any significant improvement in RT with caffeine. The absence of RT improvement observed in this experiment might be due to the protocol employed, since the participants performed their RT tests mainly following high intensity bouts. This, in turn, may have reduced reaction ability to optical stimulus tests due to excessive feeling of tiredness independent of caffeine

supplementation. The nature of the protocol *per se* reduces the reaction ability on optical stimulus tests due to the feeling of excessive tiredness by the participants, whether those were responders or non-responders to caffeine. Therefore, it seems that caffeine does not improve RT under controlled laboratory conditions that imitate real soccer game. However, the feeling of tiredness was inevitable not to be evident since the main purpose of this experiment was to imitate a real soccer game environment in a controlled laboratory regime and evaluate whether caffeine may have any influence in RT of the players during the game as well.

Most of the measured parameters related to exercise metabolism (that is, energy expenditure; fat and carbohydrate oxidation; and plasma glycerol, NEFA, and epinephrine) were not affected by caffeine supplementation. These findings provide little support for a metabolic basis of the ergogenic effects of caffeine. Since therefore carbohydrate and fat oxidation rates were not different between the caffeine and placebo trials the improvement in time to fatigue observed in both caffeine groups, cannot be attributed to the metabolic theory effect of caffeine. Consequently, there are three potential explanations driving to this improvement. The first one, might be associated with the direct ergogenic effect of caffeine on the central (CNS) and peripheral nervous systems, since caffeine easily crosses the blood-brain barrier, acting as an adenosine receptor antagonist (A_1 and A_{2a}), thus affecting the central and peripheral nervous systems and stimulating the HR (Tarnopolsky, 2008; Doherty and Smith, 2005; Kalmar and Cafarelli, 2004). However, the exact mechanism behind this potential ergogenic effect of caffeine remains unclear. The second explanation may be associated with the extra glucose availability observed also in both caffeine groups, without however supporting the theory that caffeine increased plasma glucose concentration providing energy to the active muscle through the aerobic energy production system (Van Soeren and Graham, 1998). It was assumed that the pre-exercise meal, given to the participants 3 hours prior to each protocol (Hadjicharalambous et al., 2006), might affect the glucose availability. The pre-exercise meal however, was exactly same during both conditions (caffeine and placebo). It is possible therefore, that this extra glucose availability (see further below) to the active muscle contributed to the anaerobic energy production system. Caffeine provokes insignificant plasma epinephrine elevation (Mohr et al., 2011), elevating the cAMP, thus increasing the protein kinase, suspending the enzyme phosphocreatine 2 and activating the 2,6-fructose diphosphate phosphatase enzyme, which in turn prevents the glycolysis and enhances the gluconeogenesis (Mougios, 2008). Gluconeogenesis therefore is the glucose formation from pyruvic, lactate and glycerol and takes place in the liver and kidneys (Mougios, 2008). A recent study suggested that caffeine increases anaerobic glycolysis thus

improving anaerobic performance, since it promotes the activity of the enzyme phosphofructokinase (Silva-Cavalcante et al., 2013), while Hadjicharalambous et al. (2010) revealed plasma glucose elevation following caffeine ingestion. The higher also plasma lactate results (see further below) observed in this experiment following caffeine ingestion, especially at the later stages of the exercise protocol may further support the above assumption. Finally, the third explanation might be due to the reason that caffeine may cause a greater release/mobilization of Ca^{2+} from the sarcoplasmatic reticulum as well as may produce phosphodiesterase inhibition improving the contraction velocity and endurance and/or to the increased sodium-potassium (Na^{+}/K^{+}) pump and ATPase activation (Hodgson et al., 2013). The later may also be explained the anaerobic performance improvement effect, observed in previous studies, following caffeine ingestion.

The significant increase in plasma lactate, observed in both caffeine groups during the second half of the treadmill protocol, might be resulting from the enhanced skeletal muscle glycogenolysis and the inability of the mitochondria to absorb the high pyruvate concentration providing more substrate for anaerobic glycolysis and therefore more lactate production (Hadjicharalambous et al. 2006). In previous studies, for example, a higher blood pyruvate and lactate were observed during endurance exercise following caffeine ingestion supporting the above notion (Hadjicharalambous et al. 2010; 2006). Alternatively, the increased plasma lactate concentration may indicate an inhibition of lactate uptake by non-exercising muscles and/or by the liver (Hadjicharalambous et al., 2006).

Several previous studies reported an increase in plasma epinephrine concentration following caffeine ingestion (e.g. Greer et al., 2000; Van Soeren and Graham, 1998; Essig et al., 1980). However, this experiment does not support this caffeine-induced mean epinephrine enhancement at rest and during exercise; although, there was an inter-individual variability in enhancing plasma epinephrine concentration following caffeine ingestion at rest. This is probably due to the aerobic fitness level of the participants taken part. According to the literature review, caffeine ingestion elevates the plasma epinephrine concentration (Van Soeren and Graham, 1998; Greer et al., 2000). The participants taken part in the aforementioned studies had mean VO_2max values between 54.5 and 57.5 $ml \cdot kg^{-1} \cdot min^{-1}$. However, the participants of this study had a mean VO_2max 60.8 ± 4 $ml \cdot kg^{-1} \cdot min^{-1}$. Therefore, the absence of significant plasma epinephrine elevation following caffeine ingestion in both experiments is probably due to the aerobic fitness level of the participants taken part, while it was assumed that plasma epinephrine elevation due to caffeine ingestion depends on participants' aerobic fitness level. Hence, it was assumed that participants with low aerobic

fitness level would provoke enhanced plasma epinephrine concentration due to caffeine ingestion, while participants with higher VO_2max values would not reveal detectable plasma epinephrine elevation. Furthermore, any absence of epinephrine elevation might be due to the negligible effect of theophylline (caffeine metabolic product) on epinephrine (Vestal et al., 1983). In addition, it was stated that any increase in plasma epinephrine levels, due to caffeine ingestion, revealed no detectable metabolic effects (Van Soeren and Graham, 1998), whereas in cases of caffeine-induced biological effects there was not any significant increase in catecholamines (Mohr et al., 2011). Consequently, since caffeine did not significantly influence plasma epinephrine concentration, any modification in plasma glycerol and NEFA concentration during exercise following caffeine ingestion should not be expected either.

It was consistently reported that caffeine ingestion enhances the epinephrine concentration (Van Soeren and Graham, 1998; Essig et al., 1980) and combined with the adenosine receptor antagonism may react synergistically to stimulate muscle glycolysis and liver and muscle glycogenolysis, enhancing the release of glycerol and NEFA (Van Soeren and Graham, 1998). The absence of significant plasma glycerol and NEFA elevation due to caffeine ingestion is probably due to the absence of plasma epinephrine elevation, since epinephrine promotes the release of glycerol and NEFA from the adipose tissue (Van Soeren and Graham, 1998). Epinephrine promotes the formation of cyclic AMP in the cells, initiating several chemical reactions, thus activating the phosphorylase, enhancing the blood glucose concentration (Guyton and Hall, 2006). The results however, revealed no significand plasma epinephrine elevation, while the high responders' group revealed consistently higher glucose levels between caffeine and placebo sessions. The low responders on the other hand, revealed higher plasma glucose levels during the last blood sample, indicating that caffeine as adenosine receptor antagonism (Tarnopolsky, 2008; Doherty and Smith, 2005; Kalmar and Cafarelli, 2004) along with the caffeine muscle glycolysis and liver and muscle glycogenolysis function (Van Soeren and Graham, 1998) may play an important role to the plasma glucose elevation in the high responders rather than in the low responders. The results however of this experiment indicated no significant differences between the two groups in either epinephrine or glucose concentrations. This supports the notion that, while there were no significant differences between participants with different caffeine responses, plasma glucose concentration seems to be elevated in participants who provide enhanced responses to caffeine. The exact mechanism however needs to be further examined.

This experiment indicated that caffeine enhances the MAP in both groups, results which are in agreement with previous studies (Wardle et al., 2012; Papamichael et al., 2005),

probably due to the increase of epinephrine secretion. However, as it was stated previously, the results of this experiment revealed no significant plasma epinephrine elevation. Previous studies revealed caffeine-induced biological effects with no significant increase in catecholamines (Mohr et al., 1985), indicating therefore that caffeine elevates the MAP in high responders and low responders to caffeine, since even insignificant plasma epinephrine elevation causes biological effects, such as MAP and HR elevation (Mohr et al., 2011; Van Soeren and Graham, 1998). The results of this experiment, revealed that MAP was significantly higher mainly during the first half of the simulated soccer game protocol on treadmill after caffeine ingestion in the high responders group, while the low responders revealed higher MAP values after caffeine ingestion during the second half. During this experiment however, all the participants were required to refrain from alcohol and caffeine-containing products ingestion for 72 hours prior each protocol (Hadjicharalambous et al., 2010). In addition, there was a significant increase in HR during the last treadmill period in both groups. The increased HR during the last treadmill period in both groups might be due to the protocol employed, since during the last treadmill period the participants had to exercise until exhaustion, thus elevating their HR. It is also possible that the pre-exercise caffeine ingestion elevates the secretion of β-endorphins allowing the participants to tolerate the discomfort associated with fatigue (Doherty and Smith, 2005; Laurent et al., 2000), and therefore to exercise for a longer period of time, thus elevating their HR.

Previous studies revealed that pre-exercise caffeine ingestion provokes a decline in the RPE during exercise (Hadjicharalambous et al., 2006; 2010; Tarnopolsky, 2008; Davis et al., 2003), indicating a direct ergogenic effect of caffeine on the CNS. The study, however, by Hadjicharalambous et al. (2006) revealed no differences in exercise performance between caffeine and placebo trials, despite the decline in RPE observed in the caffeine trial. On the other hand, this experiment suggested that high responders to caffeine provided a significant reduction in RPE during the second half of the soccer game protocol on treadmill than in the low responders group without any significant differences however, in exercise performance. The reduction in RPE observed in the high responders group is probably due to the direct inhibition effect of caffeine on the brain adenosine A_1 and A_{2a} receptors (Tarnopolsky, 2008; Doherty and Smith, 2005; Kalmar and Cafarelli, 2004) by increasing the secretion of β-endorphins (Doherty and Smith, 2005; Laurent et al., 2000). Caffeine ingestion was also found to improve motor recruitment (Tarnopolsky, 2008) and stimulation-contraction (Tallis et al., 2012; Mohr et al., 2011) attenuating muscle sensory signals to the brain by decreasing the threshold of activation of motor neurons thus delaying the muscle soreness due to exercise

(Doherty et al., 2004) among with improvement of jump height (Kammerer et al., 2012). It is plausible therefore that the high responders are more sensitive to this substance in comparison to the low responders, since plasma glucose and lactate results within the high responders group were consistently higher in more time-points of blood samples during the protocol employed, compared with the low responders group. Supporting this, the almost 3 minutes increase in time to fatigue performance observed in the high responders group compared with low responders should not be underestimated, as well as the 63.5 % in time to fatigue improvement in high responders and the 39.8 % in low responders comparing with their respective placebo trials (Figure 3). Consequently, the significant reduction in RPE observed in the high responders group relative to low responders may have endowed high responders with this almost 3 minutes advantage over low responders.

3.5. Conclusions

In conclusion, the results of the present experiment indicate that caffeine is effective in improving endurance in high-intensity exercise and neuromuscular performance in both high and low responders to caffeine (determined on the basis of cardiovascular and biochemical responses at rest). Nevertheless, the higher reduction in RPE during the second half of the simulated soccer game and the considerably higher improvement in time to fatigue may endow high responders with an advantage over low responders. Thus, inter-individual differences in biological responses to caffeine at rest seem to play a minor role in the ergogenic effect of caffeine supplementation. Performance improvements with caffeine ingestion could be attributed to the central and/or neuromuscular factors, since no differences in substrate utilization were found. However, a potential caffeine-induced an elevation in anaerobic ATP production system contributing in enhancing endurance performance, should not be ignored.

CHAPTER FOUR:

EXPERIMENT TWO

The effects of caffeine on high and medium aerobic fitness level athletes

4.1. Introduction

Caffeine is becoming a very popular ergogenic aid method in the sport community in an attempt to improve athletic performance (Del Coso et al., 2012). Previous studies revealed that caffeine ingestion may increase fat oxidation (Spriet et al., 1992) and improve endurance performance (Hodgson et al., 2013; Hogervorst et al., 2008) by promoting intramuscular carbohydrate sparing (Spriet et al., 1992). However, caffeine was found to increase anaerobic performance (O'Rourke et al. 2008), where glycogen depletion is not the primary cause of muscular fatigue. According to Silva-Cavalcante et al., (2013) caffeine-induced anaerobic performance improved by elevating the activity of the enzyme phosphofructokinase, which in turn increases the anaerobic glycolysis. Caffeine has also be shown to increase the plasma catecholamine concentration, which stimulate muscle glycolysis and liver and muscle glycogenolysis (Van Soeren and Graham, 1998; Essig et al., 1980), enhancing therefore the availability of the intramuscular glycogen during the later stages of exercise enhancing the performance (Spriet, et al., 1992, Astorino et al. 2012, Kilding et al. 2012, Wiles et al. 2006). Other studies also found an improvement in exercise performance following caffeine ingestion due to its effect in reducing rating of perceived exertion (RPE) *per se*. This RPE reduction following caffeine ingestion was correlated with the increased secretion of *β*-endorphins allowing the participants to tolerate the discomfort associated with fatigue during exhaustive exercise (Doherty and Smith, 2005). However, Hadjicharalambous et al. (2006; 2010), observed a significant reduction in RPE and enhanced concentrations of plasma metabolites, while the rates of fuel oxidation and incremental and endurance steady-state performance were not affected by caffeine.

Based on the above, there was an indication of performance improvement in some studies, while in others performance was not affected by caffeine (Skinner et al., 2010; Wiles et al., 2006; Doherty et al., 2004). Simmonds et al. (2010) and Doherty (1998) revealed an improvement in anaerobic performance during high-intensity exercise, while some studies found positive effects of caffeine ingestion during time-trials (Astorino et al., 2012; Kilding et al., 2012; Wiles et al., 2006) and neuromuscular explosiveness (Ali et al., 2016; Kammerer et al., 2012). Ping et al. (2010) however, provoked that caffeine ingestion did not impose any significant effect on cardio-respiratory parameters, while Talanian and Spriet (2016) indicated that caffeine did not physiologically affect well-trained cyclists and triathletes although their time-trial performance was improved with caffeine. In addition, Cox et al. (2002) stated that caffeine supplementation did not biologically influence the participants taken part. A study

conducted from Hadjicharalambous et al. (2010) revealed that while caffeine ingestion reduced the RPE, there were no effects on performance during endurance exercise, while the total fat and carbohydrate oxidation did not differ between the caffeine and placebo trials. It is therefore plausible that the equivocal results concerning exercise performance following caffeine ingestion are due to the inter-individual differences in aerobic fitness level of the differing participants taking part as well as the protocols employed in the various studies.

According therefore to Figueira et al. (2008), individuals with low aerobic fitness level have VO_2max below 38 ml·kg^{-1}·min^{-1}, while VO_2max between 43 and 58 ml·kg^{-1}·min^{-1} indicates that the individuals have medium aerobic fitness level. On the other hand, individuals with high aerobic fitness level have VO_2max above 62 ml·kg^{-1}·min^{-1}. However, there is limited available information regarding the ergogenic effects of caffeine ingestion in participants who have different aerobic fitness levels. Studies for example, examined the effects of caffeine on swimming sprinting performance in trained and untrained participants (Collomp et al. 1992), on strength and muscular endurance in resistance-trained female (Goldstein et al. 2010), on repeated (Greer et al. 1998) and single Wingate tests (Collomp et al., 1991), and on all-out high intensity 60s bouts (Crowe et al. 2006) reporting equivocal results. Crowe et al. (2006) and Collomp et al., (1991), concluded that caffeine had no significant effect on peak power, work output and on RPE during two 60s maximal cycling bouts and did not influence peak power and total work performance during a single Wingate test respectively. Woolf et al. (2008) suggested that competitive athletes achieved greater peak power output than non-competitive athletes during the Wingate test following moderate dose (5 mg/kg) of caffeine ingestion. Caffeine was also found to positively influence single Wingate bouts (Stuart et al. 2005) and repeated sprints performance (Schneiker et al. 2006) in well-trained team-sports participants. However, all the above mentioned studies, examined well-trained individuals and/or compared between trained and untrained groups, following caffeine ingestion, in a single high intensity bouts (e.g. Wingate test) or during two repeated 60s all out bouts, reveling an advantage to trained relative to untrained individuals.

However, no studies so far examined the effects of caffeine on biological responses and on exercise performance in team-sport participants with different aerobic fitness levels. The aim therefore of this experiment study was to investigate the effects of caffeine on biological responses and on soccer-specific exercise performance during a simulated soccer-game protocol on treadmill in athletes with high and medium aerobic fitness level.

4.2. Materials and Methods

4.2.1. Participants

Twenty (n = 20) male soccer players (age 22 ± 4 years; body height 1.78 ± 0.05 m; body mass 74.2 ± 7.7 kg; body fat 11.5 ± 3.7 %; VO_2max 60.8 ± 4 $ml \cdot kg^{-1} \cdot min^{-1}$; daily caffeine consumption with median 72 mg and range 0-391 mg) voluntarily took part in the present experiment, which was approved by the National Bioethics Committee, conformed to the Code of Ethics of the World Medical Association (Declaration of Helsinki). For more information regarding participants' medical screening and study approval see chapter 2.

4.2.2. Anthropometric characteristics

The participants' anthropometric characteristics identification are described in chapter 2.

4.2.3. VO_2max testing

The participants' VO_2max determination is described in chapter 2.

4.2.4. Experimental design

Prior to the two main soccer game protocols on treadmill, the participants underwent two familiarization trials for familiarizing themselves with the soccer game protocol on treadmill and experimental procedures, including MAP assessment, cannulation and blood samples collection. The experimental design is described in further details in chapter 2.

4.2.5. Energy expenditure and substrate oxidation

For details regarding the EE and rate of substrate oxidation calculations and pulmonary gas exchange collection and analysis see chapter 2.

4.2.6. Blood treatment and biochemical analyses

For details regarding the blood handling and biochemical analyses see chapter 2.

4.2.7. Statistical analysis

All test variables were analyzed by mixed ANOVA with one between factor (high or medium aerobic fitness level) and one or two within factors (time and/or treatment), as appropriate. Further details regarding the statistical analysis are described in chapter 2.

4.3. Results

4.3.1. High and medium aerobic fitness level

From the twenty male soccer-players that took part in this study, nine of them were reported as participants with high aerobic fitness level ($VO_2max \geq 62$ ml·kg^{-1}·min^{-1}) and daily caffeine consumption with median 59 mg and range 17-373 mg and seven with medium aerobic fitness level (VO_2max between 43 and 58 ml·kg^{-1}·min^{-1}) and daily caffeine consumption with median 166 mg and range 0-351 mg. Groups did not significantly differ regarding the daily caffeine consumption. The remaining participants having VO_2max between 58 and 62 ml·kg^{-1}·min^{-1} and were excluded from the statistical analysis. The anthropometric and physiological characteristics of the participants are presented on table 5.

Table 5. Characteristics of the high and medium aerobic fitness level group (mean ± SD).

Group	Age (years)	Body height (m)	Body mass (kg)	Body fat (%)	VO_2max (ml·kg^{-1}·min^{-1})
High (n = 9)	22 ± 4	1.78 ± 0.04	71.3 ± 6.4	9.6 ± 2.7	64.7 ± 2.3*
Medium (n = 7)	23 ± 4	1.77 ± 0.07	76.9 ± 10.3	13.7 ± 3.5	56 ± 1.3

* Difference between high and medium: $p < 0.001$

4.3.2. Time to fatigue

There was a significant main effect of treatment (caffeine vs placebo) on the time to fatigue during the steady state running (fourth period) [F (1, 14) = 13.45, $p < 0.001$]. Time to fatigue was longer with caffeine compared with placebo in both high (33 %; 725 ± 313 s vs 508 ± 227 s; *ES* 0.95, $p = 0.016$) and medium aerobic fitness level group (30 %; 667 ± 341 s vs 449 ± 264 s; *ES 0.83*, $p = 0.027$). However, time to fatigue was not different between groups, and there was no treatment-by-group interaction. ($p > 0.05$).

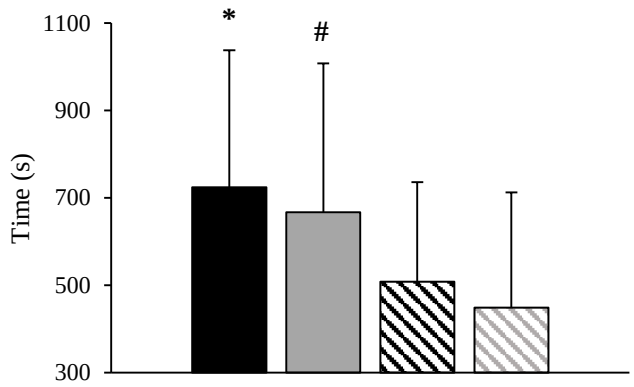

Figure 9. Mean and SD of time to fatigue with caffeine (solid bars) and placebo (hatched bars) of participants with high (bars in black) and medium (bars in gray) aerobic fitness level. * Significant difference between caffeine and placebo of participants with high aerobic fitness level. # Significant difference between caffeine and placebo of participants with medium aerobic fitness level.

4.3.3. Explosives performance and reaction time

There were significant main effects of treatment [F (1, 14) = 16.39, $p < 0.001$] and time [F (4, 56) = 10.98, $p < 0.001$] on CMJ (Figure 10*a*). In the high aerobic fitness level group, CMJ was significantly higher with caffeine ($p < 0.05$) when compared with placebo (marginal mean 42.3 ± 1.7 cm vs 40.1 ± 1.5 cm, *ES* 0.67); as well as over-time in either caffeine or placebo ($p < 0.05$). In the medium aerobic fitness level group, CMJ was not different between caffeine ($p > 0.05$) and placebo trials (marginal mean 41.1 ± 1.2 cm vs 39.3 ± 1.1 cm, *ES* 0.26) ; while there was a significant effect of time, revealing that CMJ was higher over-time in either caffeine or placebo ($p < 0.05$). There was no significant main effect or interaction in RT. (Figure 10*b*). RT was similar with caffeine and with placebo in both high responders (marginal mean 0.59 ± 0.07 vs 0.59 ± 0.05 s, *ES* -0.04) and low responders (0.64 ± 0.08 vs 0.65 ± 0.10 s, *ES* -0.14).

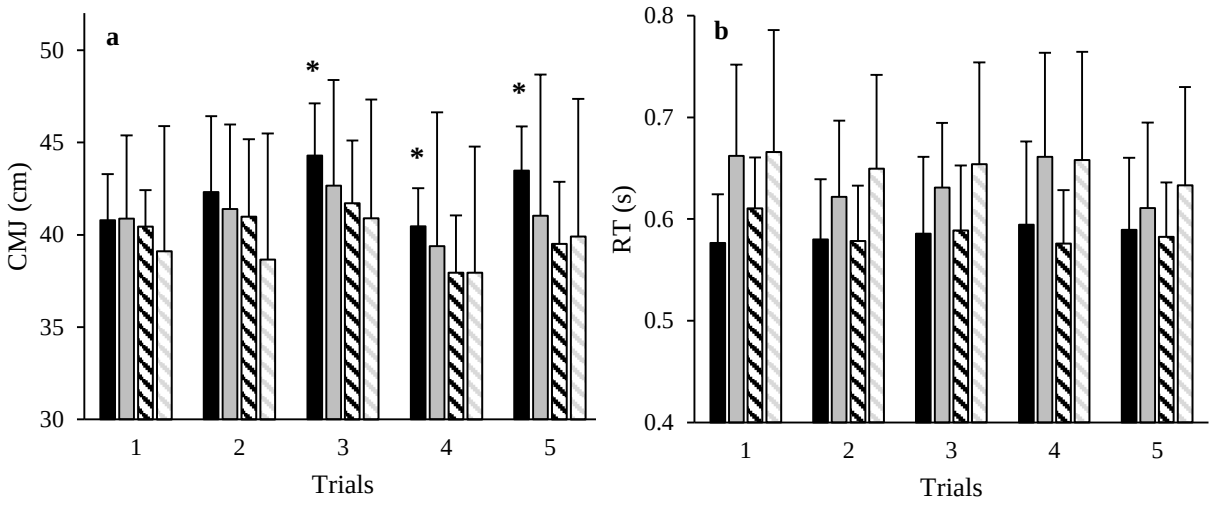

Figure 10. Means and SD of countermovement jump height *(a)* and reaction time *(b)* with caffeine (solid bars) and placebo (hatched bars) of participants with high (bars in black) and medium aerobic fitness level group (bars in gray) at the five trials indicated in Fig. 1. Trial 1 corresponds at time 0 min, trial 2 at time 22.5 min, trial 3 at time 45 (1st half) min, trial 4 at time 45 (2nd half) min and trial 5 at time 67.5 min. * Significant difference between caffeine and placebo of participants with high aerobic fitness level.

4.3.4. Cardiovascular responses and perception of effort

MAP (Figure 11) exhibited significant main effects of treatment [F (1, 12) = 19.86, p < 0.001] and time [F (2.59, 31.12) = 22, p < 0.001], as well as significant interactions of treatment x time [F (6, 72) = 2.44, p = 0.033] and treatment x time x group of fitness level [F (3.46, 41.53) = 3.78, p = 0.002]. MAP was higher with caffeine in both groups (marginal mean 98 ± 8 mm Hg vs 90 ± 6 mm Hg, *ES* 0.81, in high aerobic fitness level group; and 100 ± 5 mm Hg vs 96 ± 6 mm Hg, *ES* 0.77, in medium aerobic fitness level group). Pairwise comparisons showed that the significant differences between groups following caffeine ingestion were at the 4th and 5th time point, MAP being higher in the medium compared to the high aerobic fitness level group following placebo (98 ± 3 mm Hg vs 89 ± 11 mm Hg, p = 0.046 and 91 ± 1 mm Hg vs 82 ± 7 mm Hg, p < 0.001, respectively) in accordance with the separation of the participants into these groups. For over-time MAP results within groups and treatment, see Figure 11.

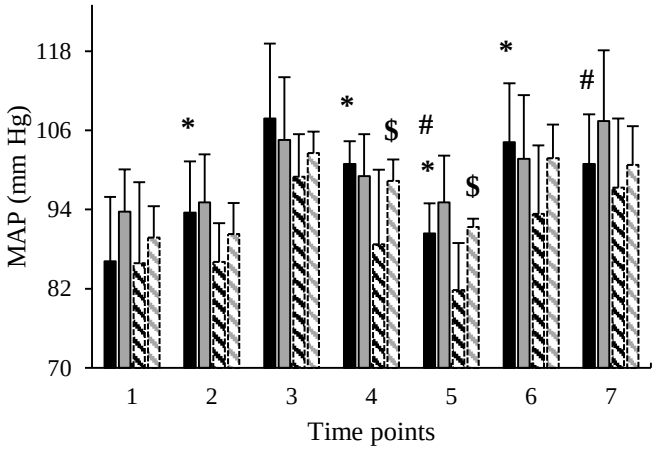

Figure 11. Means and SD of MAP with caffeine (solid bars) and placebo (hatched bars) of high aerobic fitness level group (bars in black) and medium aerobic fitness level group (bars in gray) at the five trials indicated in Fig. 1. Time point 1 corresponds to sample at time -75 min, time point 2 at time -15 min, time point 3 at time 22.5 min, time point 4 at time 45 (1st half) min, time point 5 at time 45 (2nd half) min, time point 6 at time 67.5 min and time point 7 at exhaustion. * Significant difference between caffeine and placebo of participants with high aerobic fitness level. # Significant difference between caffeine and placebo of participants with medium aerobic fitness level. $ Significant difference between high and medium aerobic fitness level group.

Average HR during each of the four periods of the treadmill protocol (Table 6) showed significant main effects of treatment [F (1, 12) = 20.56, $p < 0.001$] and time [F (2.06, 24.72) = 9.88, $p < 0.001$]. HR was higher with caffeine than placebo in both high (marginal mean 161 ± 11 bpm vs 155 ± 11 bpm, *ES* 0.52) and medium aerobic fitness level group (164 ± 15 bpm vs 156 ± 16 bpm, *ES* 0.47).

There were significant main effects of treatment [F (1, 4) = 9.48, $p < 0.001$] and time [F (3, 42) = 67.47, $p < 0.001$] on RPE (Table 6). RPE was lower with caffeine than placebo in both high (marginal mean 12.1 ± 2.1 vs 12.9 ± 1.8, *ES* – 0.45) and medium aerobic fitness level group (marginal mean 13.4 ± 1.4 vs 14.0 ± 1.5, *ES* – 0.40). Pairwise comparisons revealed that the high aerobic fitness level group differed from medium aerobic fitness level group in that RPE was lower with caffeine than placebo during treadmill period 3 only (p = 0.015). In the medium aerobic fitness level group, RPE was not significantly different between caffeine and placebo trials during the treadmill protocol ($p > 0.05$). For over-time RPE results within both groups and treatments, see Table 6.

Table 6. Average HR and RPE for each treadmill period during EXP. 2 (mean ± SD)

Treatment	Variable	Group	Treadmill period			
			1	2	3	4
Caffeine	HR (bpm)	High	154 ± 11	159 ± 11	158 ± 15	173 ± 7 *
		Medium	159 ± 14	164 ± 13	160 ± 15	172 ± 17 #
	RPE	High	10.1 ± 1.6	11.7 ± 2.4	11.7 ± 2.4 *	14.9 ± 1.9
		Medium	11.4 ± 1.1	13.2 ± 1.3	13.6 ± 1.6	15.5 ± 1.7
Placebo	HR (bpm)	High	152 ± 13	156 ± 12	151 ± 13	162 ± 5
		Medium	149 ± 18	159 ± 12	157 ± 10	161 ± 23
	RPE	High	10.4 ± 1.8	12.5 ± 2.2	13.1 ± 1.6	15.6 ± 1.4
		Medium	11.5 ± 1.1	13.5 ± 1.9	14.6 ± 1.7	16.5 ± 1.5

* Significant difference between caffeine and placebo of participants with high aerobic fitness level.
# Significant difference between caffeine and placebo of participants with medium aerobic fitness level.

4.3.5. Energy expenditure and fuel oxidation

No significant statistical outcomes were obtained with regard to RER, energy expenditure, fat oxidation, or carbohydrate oxidation during the first 5 minutes of the fourth period. RER was similar with caffeine and with placebo in both high (marginal mean 0.8 ± 0.04 vs 0.79 ± 0.04, *ES* 0.27) and medium aerobic fitness level group (0.79 ± 0.02 vs 0.80 ± 0.01, *ES* -1.16). Energy expenditure was similar with caffeine and with placebo in both high (marginal mean 16 ± 2.3 vs 16 ± 2.1 kcal·min^{-1}, *ES* -0.02) and medium aerobic fitness level group (15.3 ± 1.6 vs 15 ± 1.8 kcal·min^{-1}, *ES* 0.18). Fat oxidation was also similar with caffeine and with placebo in both high (marginal mean 1.1 ± 0.1 vs 1.2 ± 0.3 g·min^{-1}, *ES* -0.36) and medium aerobic fitness level group (1.1 ± 0.2 vs 1 ± 0.1 g·min^{-1}, *ES* 0.77). Carbohydrate oxidation was similar with caffeine and with placebo in both high (marginal mean 1.5 ± 0.7 vs 1.3 ± 0.7 g·min^{-1}, *ES* 0.32) and medium aerobic fitness level group (1.2 ± 0.4 vs 1.4 ± 0.2 g·min^{-1}, *ES* -1.27).

4.3.6. Blood metabolites

Plasma glucose (Figure 12*a*) exhibited significant main effects of treatment [F (1, 14) = 13.80, $p < 0.001$] and time [F (3.11, 43.56) = 9.59, $p < 0.001$], as well as an interaction between treatment x time [F (2.68, 37.49) = 4.95, $p < 0.001$]. Plasma glucose was higher with caffeine than placebo in both high (marginal mean 5.6 ± 0.6 mmol·l^{-1} vs 5.2 ± 0.5 mmol·l^{-1}, *ES* 0.71) and medium aerobic fitness level group (5.6 ± 0.6 mmol·l^{-1} vs 5.4 ± 0.5 mmol·l^{-1}, *ES* 0.35).

Likewise, there were significant main effects of treatment [F (1, 14) = 6.89, p = 0.02] and time [F (1.81, 25.31) = 50.96, p < 0.001], as well as an interaction between treatment x time [F (3.04, 42.51) = 2.95, p = 0.043] in plasma lactate (Figure 12*b*). Plasma lactate was higher with caffeine than placebo in both high (marginal mean 2.8 ± 1.4 mmol·l^{-1} vs 2.5 ± 1.2 mmol·l^{-1}, *ES* 0.28) and medium aerobic fitness level group (3.8 ± 2.0 mmol·l^{-1} vs 3.3 ± 1.8 mmol·l^{-1}, *ES* 0.35).

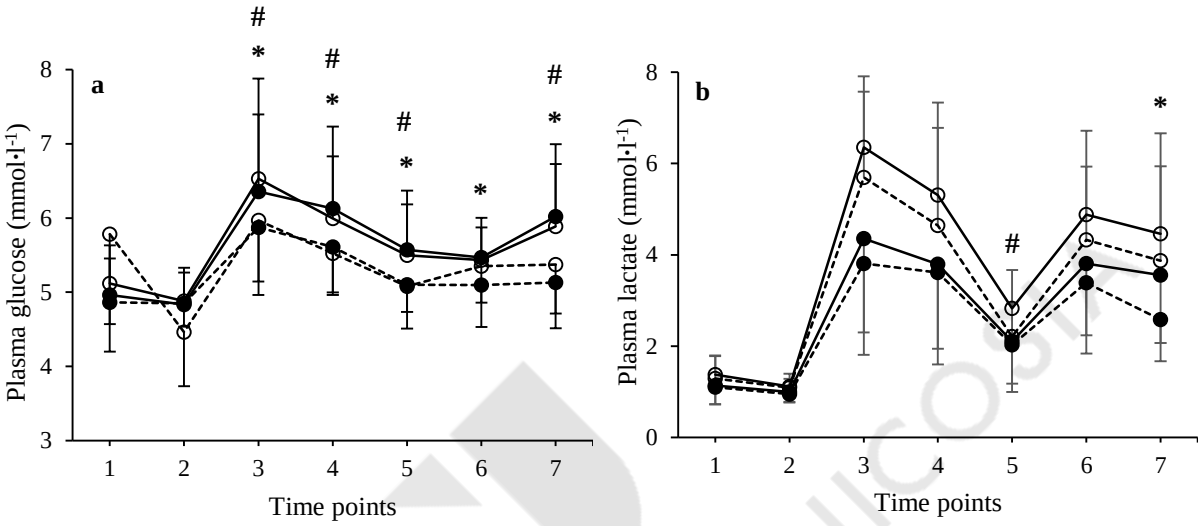

Figure 12. Means and SD of plasma glucose (*a*) and lactate (*b*) with caffeine (full circles) and placebo (open circles) of high aerobic fitness level group (solid lines) and medium aerobic fitness level group (dashed lines) at the seven blood sampling time points indicated in Fig. 1. Time point 1 corresponds to sample at time -75 min, time point 2 at time -15 min, time point 3 at time 22.5 min, time point 4 at time 45 (1st half) min, time point 5 at time 45 (2nd half) min, time point 6 at time 67.5 min and time point 7 at exhaustion. * Significant difference between caffeine and placebo of participants with high aerobic fitness level. # Significant difference between caffeine and placebo of participants with medium aerobic fitness level.

With regard to plasma glycerol (Figure 13*a*) and NEFA (Figure 13*b*), the only statistically significant outcome of the factorial ANOVA was a main effect of time [F (1.85, 25.93) = 86.86, and F (2.56, 35.80) = 81.55 respectively, p < 0.001 for all]. Plasma glycerol was similar with caffeine and with placebo in both high (marginal mean 0.1 ± 0.04 vs 0.1 ± 0.05 mmol·l^{-1}, *ES* 0.03) and medium aerobic fitness level group (0.1 ± 0.07 vs 0.08 ± 0.03 mmol·l^{-1}, *ES* 0.91). Plasma NEFA was similar with caffeine and with placebo in both high (marginal mean 0.7 ± 0.4 vs 0.7 ± 0.3 mmol·l^{-1}, *ES* 0.04) and medium aerobic fitness level group (0.7 ± 0.5 vs 0.6 ± 0.2 mmol·l^{-1}, *ES* 0.88).

Plasma epinephrine (Figure 14) exhibited significant main effects of treatment [F (1, 14) = 5.31, p = 0.037] and time [F (2.95, 41.25) = 24.45, p < 0.001]. Plasma epinephrine was similar with caffeine and placebo in both high (marginal mean 2.2 ± 0.2 ng·ml^{-1} vs 2.5 ± 0.2

ng·ml^{-1}, *ES* 0.18) and medium aerobic fitness level group (2.3 ± 0.1 ng·ml^{-1} vs 2.2 ± 0.1 ng·ml^{-1}, *ES* 0.59).

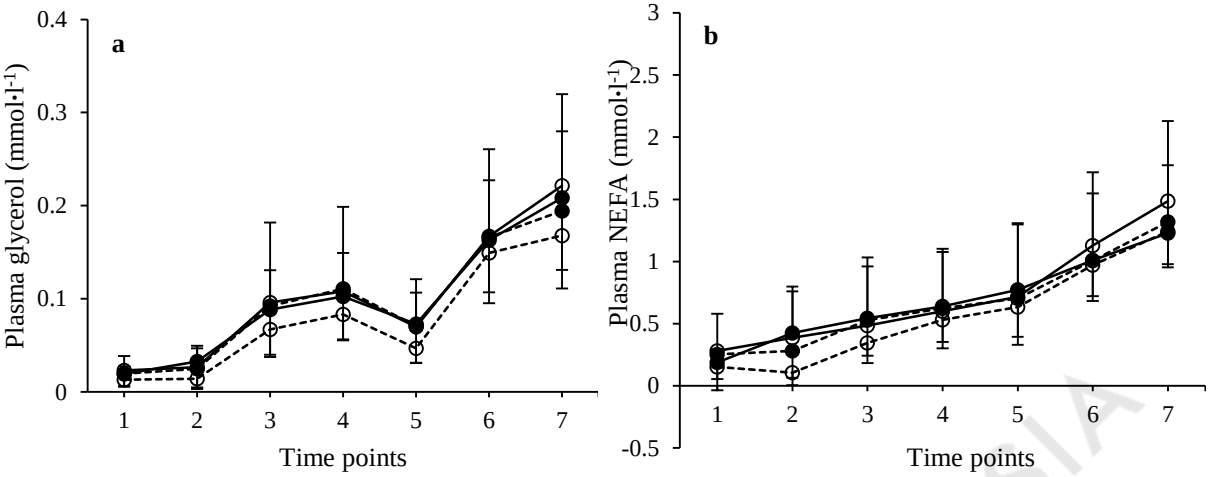

Figure 13. Means and SD of plasma glycerol (left panel) and plasma NEFA (right panel) with caffeine (full circles) and placebo (open circles) of high aerobic fitness level group (solid lines) and medium aerobic fitness level group (dash lines) at the seven blood sampling time points indicated in Fig. 1. Time point 1 corresponds to sample at time -75 min, time point 2 at time -15 min, time point 3 at time 22.5 min, time point 4 at time 45 (1st half) min, time point 5 at time 45 (2nd half) min, time point 6 at time 67.5 min and time point 7 at exhaustion.

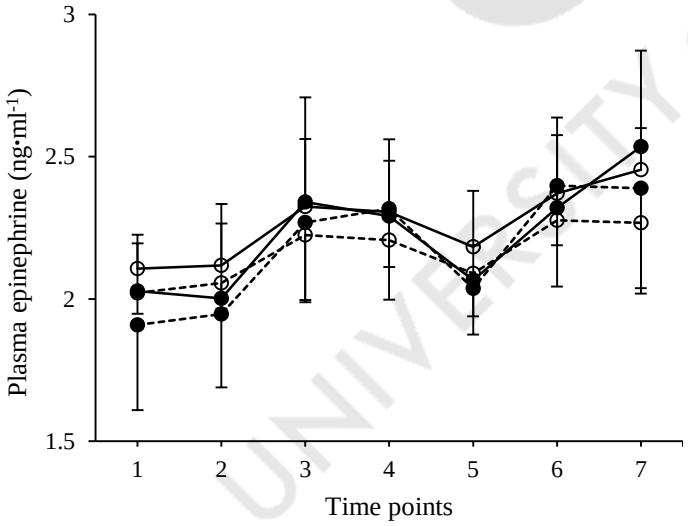

Figure 14. Means and SD of plasma epinephrine with caffeine (full circles) and placebo (open circles) of high aerobic fitness level group (solid lines) and medium aerobic fitness level group (dash lines) at the seven blood sampling time points indicated in Fig. 1. Time point 1 corresponds to sample at time -75 min, time point 2 at time -15 min, time point 3 at time 22.5 min, time point 4 at time 45 (1st half) min, time point 5 at time 45 (2nd half) min, time point 6 at time 67.5 min and time point 7 at exhaustion.

4.4. Discussion

This experiment examined the effects of caffeine ingestion on biological responses and on soccer-specific exercise performance during a simulated treadmill soccer game protocol in athletes pre-distinguished with high and medium aerobic fitness level according to their VO_2max test values. From the twenty participants taken part in this experiment, nine of them revealed high aerobic fitness level values (VO_2max $\geq$ 62 $ml \cdot kg^{-1} \cdot min^{-1}$) and were included into the high aerobic fitness level group. Seven of them revealed medium aerobic fitness level values (VO_2max between 43-58 $ml \cdot kg^{-1} \cdot min^{-1}$) and were included into the medium aerobic fitness level group (Figueira et al., 2008). The four participants who revealed values between the medium and high aerobic fitness level values were excluded from the analysis. The novel findings of the present study were that caffeine was effective in improving time-to-fatigue, which was measured following the 65 minutes of a simulated soccer-specific treadmill protocol, irrespectively whether someone has a high or a medium aerobic fitness level (30 % and 33 % improvement respectively relative to placebo). In addition, caffeine was effective in enhancing muscular explosiveness (CMJ) and reducing RPE during a simulated soccer-specific exercise protocol on the treadmill in participants with high aerobic fitness level only.

This experiment revealed that caffeine enhances high intensity endurance performance in both high and medium aerobic fitness level groups, results which are in accordance with previous studies (Silva-Cavalcante et al., 2013; Kammerer et al., 2012), while a previous study on the other hand, (Chesley et al., 1998) reported no influence of caffeine ingestion during cycling at 85 % of VO_2max in untrained participants. Although caffeine was effective in improving endurance exercise performance in both high and medium aerobic fitness level groups, this improvement in time-to-fatigue cannot be attributed to the metabolic theory effect of caffeine since substrate utilization were not different between the caffeine and placebo trials. Consequently, there are three potential explanations leading to this improvement. It might be associated with the direct ergogenic effect of caffeine on the central (CNS) and peripheral nervous systems, since caffeine can easily crosses the blood-brain barrier, acting as an adenosine receptor antagonist (A_1 and A_{2a}), positively affecting the central motivation to exercise and the peripheral neuromuscular system activation and stimulating the heart rate, the myocardial oxygen consumption regulation and the blood flow (Tarnopolsky, 2008; Doherty and Smith, 2005; Kalmar and Cafarelli, 2004). However, the exact mechanism behind this potential ergogenic effect of caffeine remains unclear. The second explanation may be associated with the extra glucose availability observed also in both groups, without however

supporting the theory that caffeine increased plasma glucose concentration providing energy to the active muscles through the aerobic energy production system (Van Soeren and Graham, 1998). It is possible therefore, that this caffeine-induced extra glucose availability to the active muscles to contribute in donating the anaerobic energy production system. A recent study for example suggested that caffeine increases the anaerobic glycolysis, thus improving anaerobic performance (Silva-Cavalcante et al., 2013). The higher also plasma lactate results (see further below) observed in this experiment following caffeine ingestion may further support the above assumption. Third, caffeine may enhance the mobilization of calcium (Ca^{2+}) from the sarcoplasmatic reticulum (Tallis et al., 2012) and the sodium-potassium (Na^{+}/K^{+}) pumps and ATPase activation and it may produce phosphodiesterase inhibition improving therefore the contraction velocity and muscular endurance (Hodgson et al., 2013). This may also explain the anaerobic performance improvement effect, observed in previous studies, following caffeine ingestion (O'Rourke et al. 2008).

In addition, this experiment revealed a CMJ improvement observed only within the high aerobic fitness level group following caffeine ingestion relative to placebo. A potential explanation regarding the improvement in CMJ with caffeine in the increase in voluntary activation, which in turn, increases the strength and power regardless of the contraction mode (Behrens et al., 2015; McCann et al., 2012), contributing in enhancing neuromuscular explosiveness (Ali et al., 2016; Lara et al., 2014; Kammerer et al., 2012; McCann et al., 2012). This improvement in performance is probably due to the caffeine's direct antagonism of adenosine receptors (A_1 and A_{2a}) on the sarcolemma, which improves excitation-contraction coupling (Tarnopolsky, 2008) via greater release of Ca^{2+} from the sarcoplasmatic reticulum (Tallis et al., 2012) and/or improved Na^{+}/K^{+} ATPase pump activity (Mohr et al., 2011). These results attenuated the muscle sensory processing increasing motor units recruitment (Doherty et al., 2004) elevating therefore the force for a given maximum stimulus (Spriet and Howlett, 2000). It is plausible therefore that the high aerobic fitness level athletes to be more sensitive to this substance in comparison to the medium aerobic fitness level athletes. Goldstein et al. (2010) for example stated that caffeine increased anaerobic maximum performance (e.g. a single Wingate bout) in well aerobic-trained relative to untrained individuals. Woolf et al. (2008) also suggested that competitive athletes achieved greater peak power output than non-competitive athletes during the Wingate test following moderate dose (5 mg/kg) of caffeine ingestion. Caffeine was also found to positively influence single Wingate bouts (Stuart et al. 2005) and repeated sprints performance (Schneiker et al. 2006) in well-trained team-sports participants than in non-well-trained counterparts.

Caffeine has also been associated with enhanced ability to alertness tasks, RT and attention during physical activity (Church et al., 2015; Santos et al. 2014; Beaumont et al., 2004; Leiberman et al., 2002). However, this study has not shown any significant effect of caffeine on optical stimulus tests (RT) in both groups. Regarding the post time-to-fatigue evaluation of the RT, the results are consistent with a previous study conducted by Santos et al. (2014) who observed that caffeine does not influence RT when the participants are in exercise-fatigued condition. On the other hand, in a recent review, it is pointed out that caffeine is effective in improving RT when measured under resting conditions (McLellan et al. 2016). However, this experiment failed to show any influence of caffeine in the RT tests evaluated at resting conditions, prior to exercise protocol. One possible explanation is that caffeine is more effective in positively influencing RT in non-well-trained individuals. Perhaps, the high fitness level of the participants evaluated in this study may have overcome the potential positive threshold effect of caffeine on RT ability. In other words, the high physical fitness level of the participants *per se* did not allow any potential improvement of RT due to caffeine ingestion. Strong evidence for example indicates a larger amplitude and shorter responses to a stimulus appearing frequently on the monitor (latency P3s test) across a variety of cognitive tasks in a group with high aerobic fitness level compared with unfit individuals (Hillman et al. 2008). This may suggest a greater amount of aerobic fitness is favorable to cognitive processes that are associated to the allocation of attentional resources and faster cognitive processing during stimulus encoding (Hillman et al. 2008). It is possible therefore that caffeine is more effective in non-exercise-fatigued individuals as well as in unfit participants.

Several previous studies reported that caffeine ingestion increased plasma epinephrine concentration (Greer et al., 2000; Van Soeren and Graham, 1998; Essig et al., 1980) and synergistically with the adenosine receptor antagonism effects of caffeine, stimulates muscle glycolysis and liver and muscle glycogenolysis, enhancing the glycerol and NEFA release (Van Soeren and Graham, 1998) and its availability to the active muscles (Essig et al., 1980). The absence of significant plasma glycerol and NEFA elevation due to caffeine ingestion is probably due to the absence of plasma epinephrine elevation, since epinephrine promotes the release of glycerol and NEFA from the adipose tissue (Van Soeren and Graham, 1998). Furthermore, this experiment does not support this caffeine-induced a significant mean epinephrine elevation at rest and during exercise. Following ingestion, in the human body, caffeine is mostly transformed by the liver into paraxanthine (80 %), theobromine (11 %) and theophylline (5 %), and since it has lipid solubility, it crosses easily the blood-brain barrier

(Fredholm et al., 1999), while the remaining 4 % is eliminated in urine without transformation (Graham, 2001). In the brain, caffeine is metabolized via specific, local enzymatic pathways, leading to high CNS concentrations of theophylline, thus affecting epinephrine elevation (Fredholm et al., 1999). It was indicated that theophylline is 3 to 5 times more potent as an inhibitor to both adenosine A_1 and A_{2a} receptors than caffeine, and paraxanthine was indicated that is at least as potent as caffeine (Benowitz et al., 1995). This study revealed no significant plasma epinephrine elevation. This is probably due to the aerobic fitness level of the participants taken part. According to the literature review, caffeine ingestion elevates the plasma epinephrine concentration (Van Soeren and Graham, 1998; Greer et al., 2000). The participants taken part in the aforementioned studies had mean VO_2max values between 54.5 and 57.5 $ml \cdot kg^{-1} \cdot min^{-1}$. However, the participants of this study had mean VO_2max 60.8 ± 4 $ml \cdot kg^{-1} \cdot min^{-1}$. Therefore, the absence of significant plasma epinephrine elevation following caffeine ingestion in both experiments is probably due to the aerobic fitness level of the participants taken part, while it was assumed that plasma epinephrine elevation due to caffeine ingestion depends on participants' aerobic fitness level. Hence, it was assumed that participants with low aerobic fitness level would provoke enhanced plasma epinephrine concentration due to caffeine ingestion, while participants with higher VO_2max values would not reveal detectable plasma epinephrine elevation. Furthermore, any absence of epinephrine elevation might be due to thenegligible effect of theophylline (caffeine metabolic product) on epinephrine (Greer et al., 2000; Vestal et al., 1983). Previous studies stated that the increase in plasma epinephrine concentration following caffeine ingestion revealed no detectable metabolic effects (Van Soeren and Graham, 1998), while there was no significant catecholamines elevation in cases of caffeine-induced biological effects (Mohr et al., 2011).

Epinephrine however, was found to promote the formation of cyclic AMP in the cells which initiates several chemical reactions such as activating the phosphorylase. During exercise, glucose units are removed from the glycogen, thus producing 1-glucose phosphate, with the phosphorylase and the glycogen debranching enzymes taking part, in order for the glycogenolysis process to be completed. Glycogenolysis is activated when there are enhanced Pi due to the ATP hydrolysis and when there is enhanced AMP, thus elevating the blood glucose concentration (Guyton and Hall, 2006). In this experiment both caffeine groups revealed higher plasma glucose levels relative to placebo sessions. This may indicate that caffeine as adenosine receptor antagonism (Tarnopolsky, 2008; Doherty and Smith, 2005; Kalmar and Cafarelli, 2004) along with the caffeine muscle glycolysis and liver and muscle glycogenolysis effects (Van Soeren and Graham, 1998) contributed in increasing plasma

glucose concentration in both caffeine groups. It is possible therefore that the insignificant caffeine-induced increased in plasma epinephrine secretion to produce a significant elevation in plasma glucose concentration. The significant increase in plasma lactate, observed in both caffeine groups during exercise might be resulting from the enhanced skeletal muscle glycogenolysis but the inability however, of the mitochondria to absorb the high pyruvate concentration providing more substrate for anaerobic glycolysis and therefore more lactate production (Hadjicharalambous et al., 2006). In previous studies for example a higher blood pyruvate and lactate concentrations were observed during endurance exercise following caffeine ingestion supporting the above notion (Hadjicharalambous et al., 2006; 2010). Alternatively, the increased in plasma lactate concentration may indicate an inhibition of lactate uptake by non-exercising muscles and/or by the liver (Hadjicharalambous et al., 2006).

This experiment indicated that caffeine enhances the MAP in both caffeine groups, results which are in agreement with several previous studies (Wardle et al., 2012; Riksen et al., 2009; Papamichael et al., 2005). Greer et al. (2000) attributed this MAP elevation to caffeine-induced elevation in epinephrine secretion. However the results of this study revealed no significant plasma epinephrine elevation. Previous studies revealed caffeine-induced metabolic and physiological effects without observing any significant influence in plasma catecholamine concentration (Mohr et al., 2011). Since therefore insignificant plasma epinephrine elevation causes biological effects due to caffeine ingestion, such as MAP and HR elevation (Mohr et al., 2011; Van Soeren and Graham, 1998), it was therefore assumed that the high and medium aerobic fitness level athletes might revealed similar MAP and HR elevation. In addition, a significant increase in HR during the last treadmill period in both caffeine groups was also observed during this experiment. This might be occurred since the mean HR was evaluated during the last treadmill (time-to-fatigue) period and the participants in both caffeine groups were lasted longer than those in the placebo groups.

In this experiment a reduction in RPE was observed within the high aerobic fitness level group only following caffeine ingestion relative to placebo. This reduction in RPE observed during this experiment due to pre-exercise caffeine ingestion, and observed in previous studies (Hadjicharalambous et al., 2010; 2006; Tarnopolsky, 2008; Davis et al., 2003), may be probably due to the increased of the secretion of β-endorphins ameliorating the muscular discomfort experienced during prolonged high intensity exercise (Doherty and Smith, 2005). Alternatively, this may be due to the direct inhibition effect of caffeine on the brain adenosine A_1 and $_{A2a}$ receptors (Tarnopolsky, 2008; Doherty and Smith, 2005; Kalmar and Cafarelli, 2004) and subsequently to the elevation of brain dopamine (DA) concentration

leading to the reduction of central fatigue by reducing the brain serotonin (5-HT):DA ratio favors in increasing arousal and central motivation (Davis et al., 2003). In both above explanations this reduction effect of caffeine in RPE is obviously more pronounced in higher aerobic fitness level athletes than in lower counterparts; but the exact mechanism of why caffeine is more effective in reducing RPE only in higher aerobic fitness level athletes is not cleared yet. The significant improvement in muscular explosiveness observed in the high aerobic fitness level group, while the medium aerobic fitness level group revealed no significant improvement (Figure 9), should not be undervalued. Therefore, the significant reduction in RPE observed in the high aerobic fitness level group in comparison to the medium aerobic fitness level group may have endowed high aerobic fitness level athletes with an advantage over the medium aerobic fitness level athletes during soccer games, whereas enhanced muscular explosiveness ability is an essential skill for success.

4.5. Conclusions

In conclusion, the results of this experiment, indicate that caffeine was effective in improving endurance in high-intensity exercise in both pre-distinguished groups with high and medium aerobic fitness levels. However, caffeine was effective in improving neuromuscular performance and reducing RPE in athletes with high aerobic fitness level only. Both high intensity endurance and muscular explosiveness performance improvements with caffeine ingestion could be attributed to the CNS positive (e.g. reduction in RPE) and neuromuscular factors since no differences were observed in aerobic substrate utilization. Similar with EXP 1 however, caffeine-induced an elevation in anaerobic ATP production system, donating the endurance performance may also be taken into consideration, since in both high and medium aerobic groups plasma glucose and lactate were higher with caffeine than with placebo.

CHAPTER FIVE:

GENERAL DISCUSSION

The aims of the present study were: a) to initially distinguish the participants according to their responses to caffeine levels, b) to distinguish the participants into high and medium aerobic fitness level athletes and c) to examine the effects of caffeine on biological responses and on exercise performance in athletes who are pre-distinguished as high and low responders to caffeine and as high and medium aerobic fitness level during a simulated soccer game protocol on treadmill. This was achieved by having the participants exercising in a well-controlled laboratory regime, while their pre-experimental meal was monitored and co-ingesting either caffeine capsules or placebo (6 $mg \cdot kg^{-1}$) in a double blind and counterbalanced manner. Additionally, imitating real soccer-game running patterns on a treadmill, contributed even further towards eliminating the potential interferes of the results, since several environmental variables were controlled.

The major findings of the present study are the following:

Caffeine is effective 1) in improving intermittent high-intensity endurance performance, irrespectively of whether someone a) is either a high or low responder to caffeine and b) has high or medium aerobic fitness level, 2) in enhancing muscular explosiveness (CMJ) in high and low caffeine responder athletes and in athletes with high aerobic fitness level only and 3) in reducing RPE, during the treadmill protocol, in the high responders and high aerobic fitness level athletes relative to the low responders and medium aerobic fitness level athletes respectively.

5.1. Responsiveness of caffeine

This was the first study which attempted to initially differentiate the high and low caffeine responders and then to evaluate the effect of caffeine on biological responses and on exercise performance during a simulated soccer game protocol on treadmill in well-trained soccer players. The results regarding the possible ergogenic effects of caffeine on exercise performance were equivocal. Similarly, there are several studies revealing blood catecholamine, NEFA and glycerol elevation following caffeine ingestion (Van Soeren and Graham, 1998; Spriet et al., 1992; Essig et al., 1980); while some others suggested no differences in blood NEFA or glucose concentrations between from placebo ingestion (Wells et al., 1985). In addition, while several studies revealed MAP elevation following caffeine ingestion (Wardle et al., 2012; Riksen et al., 2009), opposite results have been published (Salvaggio et al. 1998). However, why some studies revealed positive, some neutral and some other negative exercise performance and blood metabolites concentrations results following caffeine ingestion?

The reason perhaps of the above inconsistencies regarding the effect of caffeine ingestion on MAP is the different time of recording MAP during the day across studies. In other words, due to physiological circadian variation, MAP results might differ among the aforementioned studies, since physiological adaptations are affected by the circadian/ultradian rhythms, which vary by the time of day (Heishman et al., 2017). The MAP for example during the study conducted by Wardle et al. (2012) was recorded following caffeine ingestion at noon, thus revealing MAP elevation. Salvaggio et al. (1998) however reported MAP reduction during evening recordings. The enzyme cytochrome P450 1A2 responsible for the caffeine metabolism, has been shown to have higher activity levels during sleeping and following the initial hours after waking up compared to the evening (Kalow and Tanq, 1991). In the current thesis, in order therefore to eliminate the circadian variation on MAP and to use those recordings into the formula for the caffeine responsiveness classification, the recordings of MAP for all the participants have been obtained at resting condition, in the morning (at 08:00 am).

In addition, for improving the method for discrimination in high and low caffeine responders, other variables such as plasma glycerol, NEFA and epinephrine concentrations were taken into account as well. As it was stated above, caffeine was found to elevate the resting levels of the plasma epinephrine, glycerol and NEFA concentrations (Hadjicharalambous et al. 2006; 2010; Greer et al., 2000; Van Soeren and Graham, 1998; Essig et al., 1980). It seems therefore, that using these four variables into the differentiation method could be a useful tool in separating the participants into high and low caffeine responders, since caffeine seems to either elevate or reduce the aforementioned biological variables.

For differentiating therefore the participants into high and low responders to caffeine, a specific method was developed, which takes into consideration the resting responses modifications of MAP, plasma glycerol, NEFA and epinephrine concentrations, measured before and 60 minutes following caffeine and placebo ingestions. Those participants who had a change above the mean in two or more of the above variables were categorized as high responders, while the rest of them were categorized as low responders. In addition, these four variables increased at rest after caffeine compared to placebo ingestion, while other measured parameters such as HR, glucose and lactate, decreased with caffeine compared to placebo at rest. The four variables used for classifying the participants into high and low responders can be an effective tool in investigating the potential dependence of the metabolic and/or ergogenic effects of caffeine on inter-individual variability with regard to caffeine sensitivity.

Furthermore, this method expands the criteria used by Wardle and coworkers (2012), who based their assessment of responsiveness to caffeine on MAP evaluation solely. From the twenty participants therefore who took part in this experiments, eleven were categorized as high responders and nine as low responders to caffeine.

5.2. Time to fatigue, explosives performance and reaction time

Fatigue is defined as the inability of producing a force-generating capacity for maintaining a required power output during exercise; it might be developed at several sites along the pathway from CNS to the neuromuscular junction influencing the contracting properties of the skeletal muscles during exercise (Nybo and Nielsen, 2001). There are several studies which indicate that caffeine ingestion seems to contribute in overcoming fatigue incidences enhancing the athletic performance such as during time-trials (Astorino et al., 2012; Kilding et al., 2012; Wiles et al., 2006). Supporting this, the current study indicated that caffeine enhances endurance performance in both high and low responders to caffeine athletes as well as in athletes with high and medium aerobic fitness level. Although, there was no significant difference in time to fatigue between the high and low responders in caffeine trials (EXP 1), the high responders group revealed a greater percentage (63.5 %) of improvement in time to fatigue relative to the low responders (39.8 %) compared to their respective placebo trials. Furthermore, the two aerobic fitness level groups (EXP 2) revealed similar improvements due to caffeine ingestion (30 % and 33 %). Consequently, since carbohydrate and fat oxidation were not different between the caffeine and placebo trials, the improvement in time to fatigue observed in all caffeine groups, cannot be attributed to the aerobic metabolic theory effect of caffeine.

There are three potential explanations leading to this improvement:

1) It might be associated with the direct ergogenic effect of caffeine on the central and peripheral nervous system. Caffeine can easily crosses the blood-brain barrier, acting as an adenosine receptor (A_1 and A_{2a}) antagonist. This may positively affect the central motivation to exercise (Tarnopolsky, 2008; Doherty and Smith, 2005; Kalmar and Cafarelli, 2004) irrespectively of the levels of the responsiveness of caffeine or the aerobic fitness levels of the evaluated participants. The lower RPE results observed in both EXP 1 and 2, in all trials following caffeine ingestion strongly support the above assumption. Perceptual response to exercise is the detection and interpretation of signals sent from the CNS and the periphery during exercise (Noble and Robertson, 1996). These signals are filtrated from the CNS, mainly located in the respiratory-metabolic mediators, as well as in peripheral and nonspecific

physiological mediators (Noble and Robertson, 1996). In addition, since caffeine directly attenuates brain serotonin synthesis and/or enhances dopamine release (Davis et al., 2003) by reducing the prolactin secretion (Hadjicharalambous et al., 2010), thus reducing the peripheral muscle pain during heavy exercise (Graham-Paulson et al., 2016). Hence since caffeine provokes alteration in happiness, calmness and alertness during exercise, contributes in reducing the perceived exertion enhancing exercise performance (Backhouse et al., 2004). Alternatively, in recent study, caffeine was found to improve high-intensity exercise tolerance (during five sets of intense single-leg knee extensor trial), despite a greater phosphocreatine depletion and H^+ accumulation with caffeine than with placebo (Bowtell et al., 2018). The later, greater H^+ accumulation result with caffeine, is in accordance with this thesis. Bowtell et al. (2018) suggested that caffeine is effective in enhancing central motor drive activation and corticospinal excitability function contributing in improving high-intensity exercise tolerance. They suggested that, "this may have been induced by the afferent feedback of the greater disturbance of the muscle milieu, resulting in a stronger inhibitory input to the spinal and supraspinal motor neurons" (Bowtell et al., 2018). Consequently, based on the study by Bowtell et al. (2018), the enhancement of central motor drive and corticospinal excitability function, may both contribute in reducing RPE and enhancing performance during intense exercise.

2) The second explanation may be associated with the extra glucose availability observed in all caffeine-supplemented groups, without however supporting the theory that caffeine increased plasma glucose concentration providing energy to the active muscles through the aerobic energy production system (Van Soeren and Graham, 1998). It is possible therefore that this extra glucose availability to the active muscles contributed in donating the anaerobic energy production system equally in all caffeine groups. As Silva-Cavalcante et al. (2013) suggested, caffeine may improve anaerobic performance by increasing the activity of the enzyme phosphofructokinase, leading to enhanced anaerobic glycolysis (Simmonds et al., 2010; Bridge and Jones, 2006) and consequently anaerobic performance improvement (Silva-Cavalcante et al., 2013). The higher plasma lactate results (see further below) observed in this study, in all caffeine groups, especially at the later stages of the exercise protocol, may further support the above assumption. In addition, according to Miura et al. (2000) and Lanqfort et al. (1997) even anaerobic exercise performance reduction, might be associated with the endogenous carbohydrate depletion. Following caffeine ingestion on the other hand, the athletic performance was enhanced, since caffeine alters the anaerobic energy supply

(Doherty, 1998). The anaerobic performance improvement following caffeine ingestion was also observed from the study conducted by Simmonds et al. (2010) and Bell et al. (2001).

3) Finally, the third explanation is that caffeine may enhance the mobilization of Ca^{2+} from the sarcoplasmatic reticulum and the sodium-potassium (Na^{+}/K^{+}) pumps and ATPase activation (Hodgson et al., 2013) and may produce phosphodiesterase inhibition improving therefore the contraction velocity and muscular endurance (Hodgson et al., 2013). In addition, it was indicated that caffeine ingestion produces hypoalgesic effects during submaximal workloads (Santos et al., 2013; Stadheim et al., 2013; Cureton et al., 2007; Motl et al., 2006; O'Connor et al., 2004), while the adenosine receptor inhibition following caffeine ingestion could influence motor unit recruitment or have direct effect on muscles (Warren et al., 2010; Fredholm et al., 1999). The later may positively influence the peripheral neuromuscular system activation, thus explaining the higher CMJ results observed in the relevant caffeine groups. This may also clarify the anaerobic performance improvement effect, observed in previous studies, following caffeine ingestion (O'Rourke et al. 2008).

Concerning CMJ, several reports indicated CMJ performance improvement following caffeine ingestion (Kammerer et al., 2012; Tarnopolsky, 2008; Doherty and Smith, 2005; Kalmar and Cafarelli, 2004). Supporting the above studies, the current thesis also revealed muscular explosiveness (CMJ) improvement in both high and low responders to caffeine athletes, as well as in athletes with high aerobic fitness level only. A potential explanation to this CMJ improvement following with caffeine is the positive effect of caffeine on excitation contraction coupling (Behrens et al., 2015; McCann et al., 2012) contributing to the enhanced neuromuscular explosiveness (Ali et al., 2016; Lara et al., 2014; Kammerer et al., 2012; McCann et al., 2012). It was reported that caffeine promotes CNS effects, elevating the motivational drive and neuromuscular excitability, thus lowering the effort perception (Doherty and Smith, 2005) and improving the neuromuscular function (Silva-Cavalcante et al., 2013; Bazzucchi et al., 2011). The muscular explosiveness improvement observed during the CMJ trials might be due to the caffeine's direct antagonism of adenosine receptors (A_1 and A_{2a}) on the sarcolemma (Tarnopolsky, 2008; Fredholm, 1995; 1980) via greater release of Ca^{2+} from the sarcoplasmatic reticulum (Tallis et al., 2012) thus elevating the intramuscular Ca^{2+} handling within the intracellular space and consequently improving the neuromuscular function (Magkos and Kavouras, 2005). Furthermore, it seems that caffeine promotes phosphodiesterase inhibition, improving the contraction velocity and/or the increase in the activity of the sodium-potassium (Na^{+}/K^{+}) pump and ATPase activity (Hodgson et al., 2013; Mohr et al., 2011). These results attenuated the muscle sensory processing increasing motor

units recruitment (Doherty et al., 2004) elevating therefore the force for a given maximum stimulus (Spriet and Howlett, 2000). Alternatively, the recent study contacted by Bowtell et al. (2018) may also strongly explain this CMJ improvement with caffeine. They suggested that caffeine improved performance during five-sets of maximal single-leg knee extensor trials to fatigue attributing these outcome to the positive effect of caffeine in improving afferent feedback by attenuating the disturbance of the muscle milieu, resulting in a stronger inhibitory input to the spinal and supraspinal motor neurons.

On the other hand, the lack of significant improvement in muscular explosiveness (CMJ) observed in the medium aerobic fitness level group might be due to the caffeine's sensitivity in the medium aerobic fitness level athletes in comparison to the high aerobic fitness level and to the high and low caffeine responders. Caffeine effects for example, through the CNS are associated with the physical conditions of the participants taken part, since participants with relatively high physical condition tended to have largest RPE reduction during exercise (LeBlanc et al., 1985), results that are supported by this thesis. Graham (2001) also, stated that high fit individuals may have muscles and other tissues that are more responsive to caffeine stimulus. Furthermore, Goldstein et al. (2010) suggested that caffeine increased anaerobic maximum performance (e.g. a single Wingate bout) in well aerobic-trained relative to untrained individuals. Woolf et al. (2008) also suggested that competitive athletes achieved greater peak power output than non-competitive athletes during the Wingate test following moderate dose (5 mg/kg) of caffeine ingestion. Caffeine was found to positively influence single Wingate bouts (Stuart et al., 2005) and repeated sprints performance (Schneiker et al., 2006) in well-trained team-sports participants than in non-well-trained counterparts. Consequently, the lower fitness levels of the participants in the medium aerobic fitness level group than the high aerobic fitness level group did not allow any potential caffeine-induced improvement in CMJ.

Caffeine supplementation has also been associated with enhanced ability in alertness tasks, RT, and attention during physical activity (Church et al., 2015; Beaumont et al., 2004; Leiberman et al., 2002). Regarding the post time to fatigue evaluation of the RT, the results are consistent with a previous study conducted by Santos et al. (2014) who observed that caffeine does not influence RT when the participants are in exercise-fatigued condition. On the other hand, in a recent review, it is pointed out that caffeine is effective in improving RT when measured under resting conditions (McLellan et al. 2016). However, this thesis failed to show any influence of caffeine in the RT tests evaluated at resting conditions, prior to exercise protocol. Therefore, it seems that the high physical fitness level of the participants

per se did not allow any further improvement of RT due to caffeine ingestion. Strong evidence for example, indicates a larger amplitude and shorter responses to a stimulus appearing frequently on the monitor (latency P3s test) across a variety of cognitive tasks in a group with high aerobic fitness level compared with unfit individuals (Hillman et al. 2008). This may suggest a greater amount of aerobic fitness is favorable to cognitive processes that are associated to the allocation of attentional resources and faster cognitive processing during stimulus encoding (Hillman et al. 2008). It is possible therefore that caffeine is more effective in non-exercise-fatigued individuals as well as in unfit participants.

5.3. Cardiovascular responses and perception of effort

This study revealed significant MAP and HR elevation due to caffeine supplementation (Wardle et al., 2012; Riksen et al., 2009; Papamichael et al., 2005) during the soccer game protocol on treadmill employed. The results indicated that caffeine enhances the MAP in the high and low responders to caffeine, as well as in the participants with high and medium aerobic fitness level, indicating therefore that the MAP elevation due to caffeine ingestion is not related with the different responses to this substance nor to the aerobic fitness level of the athletes. The MAP elevation observed due to caffeine supplementation, might be due to the increase of epinephrine secretion (Van Soeren and Graham, 1998). However the results of this study revealed no significant plasma epinephrine elevation (see further below). A previous study revealed caffeine-induced biological effects with no significant increase in catecholamines (Mohr et al., 2011), indicating therefore that caffeine elevates the MAP in high and low responders to caffeine and in high and medium aerobic fitness level athletes with insignificant influence of caffeine on plasma epinephrine concentration. Several studies for example revealed that even insignificant observed plasma epinephrine elevation may produce significant elevation in MAP and HR (Mohr et al., 2011; Van Soeren and Graham, 1998).

The results of this thesis, revealed that following caffeine ingestion, MAP was significantly higher during the first half of the treadmill protocol, in the high responders group, while the low responders and the athletes with medium aerobic fitness level, higher MAP values observed during the second half. In addition, the high responders and the high aerobic fitness level athletes reveal lower RPE values than the low responders and medium aerobic fitness level athletes during the treadmill protocol employed. It was thus assumed that the low and medium aerobic fitness level athletes reached an exertion phase earlier within the protocol, therefore making more effort to complete it, elevating their MAP. In addition, there was a significant increase in HR during the last treadmill period in the high and low responders

groups, as well as in the high and medium aerobic fitness level groups during the caffeine trial relative to the placebo trial. The increased HR observed during the last treadmill period in the above groups might be due to the protocol employed, since during the last treadmill period the participants had to exercise until exhaustion, thus a physiological elevation in HR was expected.

Previous studies revealed that pre-exercise caffeine ingestion provokes a decline in the RPE during exercise (Hadjicharalambous et al., 2006; 2010; Tarnopolsky, 2008; Davis et al., 2003; Nybo and Nielsen, 2001), indicating a direct ergogenic effect of caffeine on the CNS. The study, however, by Hadjicharalambous et al. (2006) revealed no differences in exercise performance between caffeine and placebo trials, despite the decline in RPE observed in the caffeine trial. On the other hand, this thesis suggested that high responders to caffeine athletes as well as the high aerobic fitness level athletes provided a significant reduction in RPE during the second half of the simulated soccer game protocol than the low responders' athletes and the medium aerobic fitness level groups. Further to this, the reduction in RPE observed in the high caffeine responders group and in the high aerobic fitness level group is probably due to the direct inhibition effect of caffeine on the brain adenosine A_1 and A_{2a} receptors (Tarnopolsky, 2008; Doherty and Smith, 2005; Kalmar and Cafarelli, 2004), which in turn reduces the influence of adenosine and produces motor-activating and arousing effects (Graham-Paulson et al., 2016; Fredholm et al., 1999). This may subsequently increase the secretion of β-endorphins (Doherty and Smith, 2005; Laurent et al., 2000) and elevate the brain dopamine (DA) concentration leading to the reduction of central fatigue by reducing the brain serotonin (5-HT):DA ratio favors in increasing arousal and central motivation (Davis et al., 2003). In addition, it was stated that caffeine improves motor recruitment and stimulation-contraction (Bowtell et al. 2018; Tallis et al., 2012; Mohr et al., 2011; Tarnopolsky, 2008) attenuating muscle sensory signals to the brain by decreasing the threshold of activation of motor neurons thus delaying the muscle soreness due to exercise (Doherty et al., 2004). Caffeine therefore, positively influence the CNS and consequently the subjective feelings, such as RPE, mood and cognitive performance (Doherty and Smith, 2005; Smit and Rogers, 2000), by influencing the central and catecholaminergic neurotransmission (Connell et al., 2017). Manipulation of these neurotransmitter systems via norepinephrine-dopamine reuptake inhibition also prevented fatigue-related impairments to the peak velocity of muscles during exercise (Connell et al., 2017).

In addition, the adenosine receptor inhibition following caffeine ingestion could influence motor unit recruitment or have direct effect on skeletal muscles (Warren et al., 2010;

Fredholm et al., 1999), allowing the participants to centrally recruit and engage more motor units (Doherty et al., 2004), thus increasing the power output (Cole et al., 1996) and elevating the force for a given stimulus (Spriet and Howlett, 2000). Furthermore, caffeine reduces RPE during exercise by affecting the respiratory-metabolic mediators (Noble and Robertson, 1996), alters the respiratory system function (Supinski et al., 1986), stimulates the HR, the myocardial oxygen consumption regulation and thus the blood flow to the working muscles (Tarnopolsky, 2008; Doherty and Smith, 2005). St Clair Gibson et al. (2003) stated that during exercise, the human body is able to interpret the exercise itself by psychological functions, such as motivation, memory and associated decision-making components. It should also not be underestimated that the CNS stimulation function due to caffeine ingestion is affected from the exercise intensity. It was stated therefore that the caffeine stimulation function on the CNS during heavy-severe intensity exercise was found to be neglected (Black et al., 2015).

Moreover, it seems that participants with very good physical condition reveal greater reduction in RPE compared with untrained participants, due to the increased release of epinephrine (LeBlanc et al., 1985). Highly trained participants revealed reduced perceived exertion ratings (Motl et al., 2003), thus indicating that participants with very good physical condition may have muscles and other tissues that are more responsive to caffeine stimulus (Graham, 2001). Therefore, the participants taken part in the study conducted by Van Soeren and Graham (1998) and Spriet et al. (1992) had VO_2max values less than 56 $ml \cdot kg^{-1} \cdot min^{-1}$. It is plausible therefore that the high responders to caffeine athletes, as well as the athletes with high aerobic fitness level are more sensitive to this substance in comparison to the low responders to caffeine athletes and to the medium aerobic fitness level athletes respectively.

In support of this, the almost 3 minute increase in time to fatigue performance observed in the high responders group compared with low responders; (63.5 % in time to fatigue improvement in high responders and 39.8 % in low responders) should not be underestimated when compared with their respective placebo trials (Figure 3). The time to fatigue improvement however observed within the high and medium aerobic fitness level group due to caffeine ingestion was similar (30 % and 33 % respectively). The significant improvement in muscular explosiveness observed in the high aerobic fitness level along with the absence of significant improvement in the medium aerobic fitness level group, should not however be underestimated. Consequently, the significant reduction in RPE observed in the high responders to caffeine group relative to low responders one may have endowed high responders with these almost 3 minutes advantage over low responders during high intensity endurance performance. On the other hand, the higher RPE observed in the medium aerobic

fitness level group during the second half of the protocol, indicates that these athletes were making more effort in order to overcome the protocol, thus elevating their exertion rates, in comparison to the high aerobic fitness level athletes.

5.4. Energy expenditure and fuel oxidation

The results revealed no significant effect of caffeine on energy expenditure and on fat and carbohydrate oxidation during the time to fatigue period of the treadmill protocol. Similar results were obtained in previous studies (Hadjicharalambous et al., 2006; 2010; Tarnopolsky, 2008). The results support the notion that the performance improvement during a simulated soccer game protocol on treadmill observed, is unlikely to be due to aerobic substrate utilization potential effects of caffeine as previously observed (Spriet et al., 1992). In the current thesis, the absence of significant caffeine effect on substrate utilization might be due to the prolonged intermittent high-intensity protocol employed. Spriet et al. (1992) for example, revealed that caffeine ingestion prior to exercise elevates the intramuscular triacylglycerol and/or extramuscular NEFA at the first 15 minutes of a steady-steady cycling, (~80 % of VO_2max) to volitional exhaustion. In this thesis, the energy expenditure, and the fat and carbohydrate oxidation date were obtained during the last treadmill period of the protocol employed, where the participants had to run at 75 % of VO_2max until exhaustion, followed by 75 minutes of an intermitted high-intensity exercise. As Horton and Beisel (1994) stated, fuel utilization during exercise is influenced from several factors, including the intensity and the duration of the exercise and the physical condition of the participants. Exercising at about half the maximum aerobic capacity requires 50/50 mixture of glucose and free fatty acids, with amino acid oxidation still supplying only 1-2 % of the energy. When exercise intensity reaches 75 % or greater of the maximum aerobic capacity, glucose oxidation becomes the main fuel supply during exercise. Then, as exercise persists, blood free fatty acid concentrations increase, while muscles gradually shifts over to burning more fatty acids and less glucose. In addition, athletes with quite good physical condition tend to oxidize fatty acids more effectively and thus to spare both muscle and liver glycogen. Prior to the last treadmill period (time to fatigue) however, the participants had to complete three identical high-intensity treadmill periods, where each period was designed to last 22.5 minutes leading therefore to glycogen depletion. Therefore, the absence of any significant caffeine effect on substrate utilization observed during this thesis, might be due to the fact that the participants had already reached their tolerance limits prior the last treadmill period, in which substrate utilization was measured.

5.5. Blood metabolites

According to Guyton and Hall (2006) epinephrine stimulates the HR and MAP and prepares the body for the upcoming exercise. It was previously reported that caffeine ingestion enhanced the plasma epinephrine concentration (Essig et al., 1980; Van Soeren and Graham, 1998; Greer et al., 2000) and combined with the adenosine receptor antagonism effect of caffeine may work synergistically to stimulate a) both muscle glycolysis and liver and muscle glycogenolysis and b) the release of glycerol and NEFA (Van Soeren and Graham, 1998) and its availability to the active muscles (Essig et al., 1980). However, this thesis does not support this caffeine-induced mean epinephrine elevation at rest and/or during exercise. However, the study of Mohr et al. (1998) revealed that caffeine induced biological effects, but did not cause any significant increase in epinephrine in tetraplegic humans at rest and during functional electrical stimulation of their paralyzed limbs to the point of fatigue. The results however of this thesis, while revealed significant RPE reduction among with performance improvement, an absence of significant plasma epinephrine elevation and induced biological effects following caffeine ingestion in both experiments was observed. It should be noted that following ingestion, caffeine is transformed by the liver into paraxanthine, theobromine and theophylline and crosses the blood-brain barrier (Fredholm et al., 1999). Vestal et al. (1983) indicated that theophylline produces negligible effect on epinephrine, promoting therefore an absence of significant elevation. Consequently, the absence of significant epinephrine elevation following caffeine observed in both experiments during this thesis might be due to the negligible effect of theophylline on epinephrine, which in turn provokes undetectable plasma epinephrine elevation (Vestal et al., 1983).

Likewise, caffeine has shown to increase fat oxidation (Spriet et al., 1992) and stimulate muscle glycolysis and liver and muscle glycogenolysis. Consequently enhancing the release of plasma glycerol and NEFA concentrations (Van Soeren and Graham, 1998) and their availability to the contracting muscles during exercise (Essig et al., 1980) contributes in sparing intramuscular glycogen (Spriet, et al., 1992). The absence of significant plasma glycerol and NEFA elevation due to caffeine ingestion in this thesis is probably due to the protocol employed. It was indicated that caffeine ingestion promotes plasma glycerol and NEFA elevation during exercise whereas steady state protocols have been employed (Hadjicharalambous et al., 2006). On the other hand, there are several reports indicating that plasma glycerol and NEFA concentration remained either unchanged or reduced during exercise, while at rest and prior exercise significantly elevated following caffeine (Van Soeren and Graham, 1998; Spriet, et al., 1992). During this thesis however, the results regarding the

blood metabolites were obtained at rest and following high intensity intermittent exercise, indicating therefore that the absence of significant plasma glycerol and NEFA elevation might be due to the protocol designed.

In addition, as it was previously stated, epinephrine promotes the formation of cyclic AMP in the cells, initiating several chemical reactions, thus activating the phosphorylase. It is possible therefore that the insignificant caffeine-induced increased in plasma epinephrine secretion to produce a significant elevation in plasma glucose concentration. During exercise, glucose units are removed from the glycogen, thus producing 1-glucose phosphate, with the phosphorylase and the glycogen debranching enzymes taking part, in order for the glycogenolysis process to be completed. Glycogenolysis is activated when there are enhanced Pi due to the ATP hydrolysis and when there is enhanced AMP, thus elevating the blood glucose concentration (Guyton and Hall, 2006). The high and low caffeine responders as well as the group with high and medium aerobic fitness level revealed significantly enhanced plasma glucose concentration following caffeine. Consequently, these results indicate that caffeine induced muscle glycolysis and liver and muscle glycogenolysis effects (Van Soeren and Graham, 1998) contributed in increasing plasma glucose concentration in all groups.

According to Mougios (2008), it is very crucial the human body to be supplied with energy during exercise. Glycogen, which is mainly stored in the liver and muscles, is transformed into glucose and later on into pyruvic acid which is then transferred into the mitochondria for oxidation, thus producing ATP which is the human body energy currency. Furthermore, during high intensity exercise, pyruvic acid is converted into lactate acid which is transported through the blood flow into other human body organs (inactivated skeletal muscles, heart, liver, brain and kidneys) for glucose production, a process called gluconeogenesis. Therefore, exercise metabolism obeys the need for increased energy supply to the contracted muscles, whereas the main energy provider during prolonged and high intensity exercise (such as during soccer games) is due to the carbohydrate metabolism. Based on what was stated above, elevated plasma glucose concentration would ensure the required energy supply during exercise, thus maintaining a steady ATP production into the working muscles in participants with different caffeine responses and/or different fitness levels

Along with the significant plasma glucose elevation, this study revealed significant increase in plasma lactate with caffeine, observed in both high and low responders to caffeine, as well as in high and medium aerobic fitness level groups. The plasma lactate elevation observed, might result from the enhanced skeletal muscle glycogenolysis and the inability of the mitochondria to absorb the high pyruvate concentration, therefore providing more

substrate for anaerobic glycolysis and therefore more lactate production (Astorino et al., 2012; Kilding et al., 2012; Hadjicharalambous et al. 2006; Wiles et al., 2006). In previous studies for example a higher blood pyruvate and lactate were observed during endurance exercise following caffeine ingestion supporting the above notion (Hadjicharalambous et al. 2006; 2010). Alternatively, the increased in plasma lactate concentration may indicate an inhibition of lactate uptake by non-exercising muscles and/or by the liver (Hadjicharalambous et al., 2006).

5.6. General conclusions

In conclusion, the results of this series of experiments indicate that caffeine ingestion enhances the endurance exercise performance irrespectively of whether someone is high or low responder to caffeine and he/she has high or medium aerobic fitness level. Although, not statistically significant, the almost 3 minutes higher time to fatigue performance observed in the high responders group compared with the low responders should not be underestimated. These 3 minutes better performance in high responders group can be attributed the reduction in RPE endowing high responders to caffeine athletes with an advantage over low responders. This study revealed also that caffeine enhances muscular explosiveness (CMJ) in both pre-distinguished high and low responders to caffeine and in high aerobic fitness level group only. Both, time to fatigue and muscular explosiveness are essential elements for success during a soccer game.

Caffeine ingestion elevates plasma glucose and lactate concentrations, HR and MAP in the high and low responders and in the high and medium aerobic fitness level groups. These results may suggest that the performance improvements with caffeine ingestion could be attributed to the CNS and neuromuscular factors (reduced RPE and improved CMJ performance, respectively) since no differences were observed in aerobic substrate utilization. Alternatively, the caffeine-induced an elevation in anaerobic ATP production system during exercise donating in endurance performance enhancement should not be ignored, since both blood glucose availability but particularly plasma lactate concentration were higher with caffeine than with placebo. It seems therefore that there is a direct stimulant action of caffeine on the central (RPE reduction) and peripheral (neuromuscular junction) nervous system as well as an elevation in glycolytic system function and therefore in anaerobic ATP production.

5.7. Limitations of the study

Although this study offers novel insight into the ergogenic effects of caffeine on biological responses and exercise performance, some limitations of this thesis should be reported:

1. Although performing a series of tests imitating a soccer game in the laboratory offer the advantage of strict control over the test variables, it should not be underestimated that results during actual game might be different due to uncontrolled variables (e.g., weather, tactics, opponents, and ball skills).
2. Another limitation could be considered the relatively small sample size of the two groups. This was imposed by the inability to find more well-trained professional soccer players and by the cost of the analyses.

5.8. Directions for future research

Direction for future research:

1. The relationship between the genotypes (related with the action and metabolism of caffeine) and the biological effects of caffeine providing further information regarding the potential ergogenic effects of caffeine during exercise. The participants could be categorized as caffeine responders and non-responders based on genetics variables.
2. It would be also interesting to investigate the biological effects of overdose caffeine supplementation in the non-athlete population along with further cardiorespiratory parameters being investigated (such as cardiogram and echo) for possible cardiovascular caffeine side effects. This would highlight possible potential risk factors due to caffeine overdose ingestion and provide further information regarding the caffeine overconsumption by the population.
3. The ergogenic effects of caffeine in athletes with relatively low aerobic fitness level during low-intensity exercise should be also examined.
4. It would be also interesting to investigate the biological effects of caffeine during a simulated soccer game protocol on treadmill, where ball skills along with mental activities would be included. The later would highlight any potential stimulant action of caffeine on the central and peripheral (neuromuscular junction) nervous system.

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APPENDICES

APPENDIX A: Inform consent forms

ΕΝΤΥΠΑ ΣΥΓΚΑΤΑΘΕΣΗΣ για συμμετοχή σε πρόγραμμα έρευνας (Τα έντυπα αποτελούνται συνολικά από 6 σελίδες)
Σύντομος Τίτλος του Προγράμματος στο οποίο καλείστε να συμμετάσχετε
Επιδράσεις της καφεΐνης σε αθλητές με διαφορετικές φυσιολογικές αποκρίσεις στην καφεΐνη και διαφορετική αερόβια ικανότητα: βιολογικές αποκρίσεις και αθλητική απόδοση

Καλείστε να συμμετάσχετε σε ένα ερευνητικό πρόγραμμα. Πιο κάτω (βλ. «Πληροφορίες για Ασθενείς ή/και Εθελοντές») θα σας δοθούν εξηγήσεις σε απλή γλώσσα σχετικά με το τι θα ζητηθεί από εσάς ή/και τι θα σας συμβεί σε εσάς, εάν συμφωνήσετε να συμμετάσχετε στο πρόγραμμα. Θα σας περιγραφούν οποιοιδήποτε κίνδυνοι μπορεί να υπάρξουν ή ταλαιπωρία που τυχόν θα υποστείτε από την συμμετοχή σας στο πρόγραμμα. Θα σας επεξηγηθεί με κάθε λεπτομέρεια τι θα ζητηθεί από εσάς και ποιος ή ποιοι θα έχουν πρόσβαση στις πληροφορίες ή/και άλλο υλικό που εθελοντικά θα δώσετε για το πρόγραμμα. Θα σας δοθεί η χρονική περίοδος για την οποία οι υπεύθυνοι του προγράμματος θα έχουν πρόσβαση στις πληροφορίες ή/και υλικό που θα δώσετε. Σε περίπτωση που θέλετε να λάβετε τα αποτελέσματα της έρευνας θα πρέπει να δηλώσετε το email σας πιο κάτω. Θα σας επεξηγηθεί τι ελπίζουμε να μάθουμε από το πρόγραμμα σαν αποτέλεσμα και της δικής σας συμμετοχής. Επίσης, θα σας δοθεί μία εκτίμηση για το όφελος που μπορεί να υπάρξει για τους ερευνητές ή/και χρηματοδότες αυτού του προγράμματος. Δεν πρέπει να συμμετάσχετε, εάν δεν επιθυμείτε ή εάν έχετε οποιουσδήποτε ενδοιασμούς που αφορούν την συμμετοχή σας στο πρόγραμμα. Εάν αποφασίσετε να συμμετάσχετε, πρέπει να αναφέρετε εάν είχατε συμμετάσχει σε οποιοδήποτε άλλο πρόγραμμα έρευνας μέσα στους τελευταίους 12 μήνες. Εάν αποφασίσετε να μην συμμετάσχετε και είστε ασθενής, η θεραπεία σας δεν θα επηρεαστεί από την απόφασή σας. Είστε ελεύθεροι να αποσύρετε οποιαδήποτε στιγμή εσείς επιθυμείτε την συγκατάθεση για την συμμετοχή σας στο πρόγραμμα. Εάν είστε ασθενής, η απόφασή σας να αποσύρετε την συγκατάθεση σας, δεν θα έχει οποιεσδήποτε επιπτώσεις στην θεραπεία σας. Έχετε το δικαίωμα να υποβάλετε τυχόν παράπονα ή καταγγελίες, που αφορούν το πρόγραμμα στο οποίο συμμετέχετε, προς την Επιτροπή Βιοηθικής του Πανεπιστημίου Λευκωσίας που ενέκρινε το πρόγραμμα ή ακόμη και στην Εθνική Επιτροπή Βιοηθικής Κύπρου.
Πρέπει όλες οι σελίδες των εντύπων συγκατάθεσης να φέρουν το ονοματεπώνυμο και την υπογραφή σας.

Σύντομος Τίτλος του Προγράμματος στο οποίο καλείστε να συμμετάσχετε
Επιδράσεις της καφεΐνης σε αθλητές με διαφορετικές φυσιολογικές αποκρίσεις στην καφεΐνη και διαφορετική αερόβια ικανότητα: βιολογικές αποκρίσεις και αθλητική απόδοση
Υπεύθυνος του Προγράμματος στο οποίο καλείστε να συμμετάσχετε
Δρ. Χατζηχαραλάμπους Μάριος

Επίθετο:		Όνομα:	
Υπογραφή:		Ημερομηνία:	

ΕΝΤΥΠΑ ΣΥΓΚΑΤΑΘΕΣΗΣ για συμμετοχή σε πρόγραμμα έρευνας (Τα έντυπα αποτελούνται συνολικά από 6 σελίδες)
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Δίδετε συγκατάθεση για τον εαυτό σας ή για κάποιο άλλο άτομο;	
Εάν πιο πάνω απαντήσατε για κάποιον άλλο, τότε δώσετε λεπτομέρειες και το όνομα του.	

Ερώτηση	ΝΑΙ ή ΟΧΙ
Συμπληρώσατε τα έντυπα συγκατάθεσης εσείς προσωπικά;	
Τους τελευταίους 12 μήνες έχετε συμμετάσχει σε οποιοδήποτε άλλο ερευνητικό πρόγραμμα;	
Διαβάσατε και καταλάβατε τις πληροφορίες για ασθενείς ή/και εθελοντές;	
Είχατε την ευκαιρία να ρωτήσετε ερωτήσεις και να συζητήσετε το Πρόγραμμα;	
Δόθηκαν ικανοποιητικές απαντήσεις και εξηγήσεις στα τυχόν ερωτήματά σας;	
Καταλαβαίνετε ότι μπορείτε να αποσυρθείτε από το πρόγραμμα, όποτε θέλετε;	
Καταλαβαίνετε ότι, εάν αποσυρθείτε, δεν είναι αναγκαίο να δώσετε οποιεσδήποτε εξηγήσεις για την απόφαση που πήρατε;	
(Για ασθενείς) καταλαβαίνετε ότι, εάν αποσυρθείτε, δεν θα υπάρξουν επιπτώσεις στην τυχόν θεραπεία που παίρνετε ή που μπορεί να πάρετε μελλοντικά;	
Συμφωνείτε να συμμετάσχετε στο πρόγραμμα;	
Με ποιόν υπεύθυνο μιλήσατε;	

Επίθετο:		Όνομα:	
Υπογραφή:		Ημερομηνία:	

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Στοιχεία έρευνας

Κύριος ερευνητής: Αποστολίδης Ανδρέας, διδακτορικός φοιτητής στο Πανεπιστήμιο Λευκωσίας στο Πρόγραμμα Επιστήμη της Άσκησης και της Φυσικής Αγωγής

Δοκιμαζόμενοι: ποδοσφαιριστές ηλικίας 18-28, υψηλού και μέτριου επιπέδου
Αριθμός επισκέψεων: 5

Προκαθορισμένη διατροφή (3 ώρες πριν την έναρξη των δοκιμασιών), αποφυγή κατανάλωσης καφεΐνης και αλκοόλ (72 ώρες πριν την έναρξη των δοκιμασιών) και έντονης άσκησης (48 ώρες πριν την έναρξη των δοκιμασιών).

Επίσκεψη 1η: Καταγραφή σωματικού βάρους, ύψους καθώς και μέτρηση σωματικού λίπους με δερματοπτυχόμετρο σε 7 σημεία (τρικέφαλο, δικέφαλο, κοιλιακό, υπερλαγώνιο, υποπλάτιο, στήθος και τετρακέφαλο), με σκοπό τον υπολογισμό του ποσοστού λίπους. Μέτρηση αρτηριακής πίεσης. Τοποθέτηση cannula (πεταλούδα) στο χέρι των δοκιμαζομένων. Η cannula θα παραμείνει στο χέρι των δοκιμαζομένων καθ' όλη τη διάρκεια της επίσκεψής τους. Λήψη αίματος. Στη συνέχεια θα γίνει χορήγηση καφεΐνης ή εικονικής καφεΐνης σε μορφή χαπιού (6 $mg \cdot kg^{-1}$ σωματικού βάρους). Ούτε οι δοκιμαζόμενοι, αλλά ούτε και οι ερευνητές θα γνωρίζουν το είδος του χαπιού. Επανάληψη λήψης αίματος μετά από 1 ώρα και μέτρηση της αρτηριακής πίεσης. Εξάσκηση στο πρωτόκολλο. Διάρκεια επίσκεψης: περίπου 2 ώρες.

Επίσκεψη 2η: Μέτρηση αρτηριακής πίεσης. Τοποθέτηση cannula στο χέρι των δοκιμαζομένων. Η cannula θα παραμείνει στο χέρι των δοκιμαζομένων καθ' όλη τη διάρκεια της επίσκεψής τους. Λήψη αίματος. Χορήγηση εικονικής καφεΐνης ή καφεΐνης σε μορφή χαπιού (6 $mg \cdot kg^{-1}$). Ούτε οι δοκιμαζόμενοι, αλλά ούτε και οι ερευνητές θα γνωρίζουν το είδος του χαπιού. Λήψη αίματος και μέτρηση αρτηριακής πίεσης. Επανάληψη λήψης αίματος μετά από 1 ώρα και μέτρηση της αρτηριακής πίεσης. Εξάσκηση στο πρωτόκολλο. Σκοπός της 1ης και 2ης επίσκεψης είναι να εξασκηθούν οι δοκιμαζόμενοι στο πρωτόκολλο και στις διαδικασίες που θα εφαρμοστούν. Διάρκεια επίσκεψης: περίπου 2 ώρες.

Επίθετο:		Όνομα:	
Υπογραφή:		Ημερομηνία:	

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ΠΛΗΡΟΦΟΡΙΕΣ ΓΙΑ ΑΣΘΕΝΕΙΣ ή/και ΕΘΕΛΟΝΤΕΣ, συνέχεια:

Επίσκεψη 3η: Έλεγχος μέγιστης πρόσληψης οξυγόνου και γαλακτικό κατώφλι. Οι δοκιμαζόμενοι θα εξασκηθούν στο δαπεδοεργόμετρο, του οποίου η αρχική ταχύτητα θα είναι ρυθμισμένη στα 8 $km \cdot h^{-1}$ και θα αυξάνεται σταδιακά κάθε λεπτό κατά 1 $km \cdot h^{-1}$ μέχρι εξάντλησης. Η κλίση του διαδρόμου θα είναι ρυθμισμένη συνεχώς στο μηδέν. Καθ' όλη τη διάρκεια της δοκιμασίας θα φοράνε ειδική μάσκα για να ελέγχεται ο εκπνεόμενος αέρας και ζώνη στο στήθος για να καταγράφεται η καρδιακή συχνότητα. Χρησιμοποιώντας κλίμακα που μετρά την υποκειμενική αντίληψη της κόπωσης (Borg scale) θα ενημερώνουν τους ερευνητές για το επίπεδο κούρασης τους κάθε 3 λεπτά. Σκοπός της 3ης επίσκεψης είναι να βρεθούν τα άτομα που έχουν υψηλή και μέτρια αντοχή. Διάρκεια επίσκεψης: περίπου 30 λεπτά.

Επίθετο:		Όνομα:	
Υπογραφή:		Ημερομηνία:	

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Επίσκεψη 4η και 5η (πρωτόκολλο): Μεταξύ της 4ης και της 5ης επίσκεψης θα μεσολαβεί περίοδος 96 ωρών. Οι δοκιμαζόμενοι θα πρέπει να βρίσκονται στο εργαστήριο 105 λεπτά πριν την έναρξη των δοκιμασιών. Στη συνέχεια θα γίνει μέτρηση της αρτηριακής πίεση και ακολούθως τοποθέτηση της cannula στο χέρι. Η cannula θα παραμείνει στο χέρι των δοκιμαζομένων καθ' όλη τη διάρκεια της επίσκεψής τους. Ακολούθως θα γίνει λήψη αίματος. Θα χορηγηθεί στους δοκιμαζόμενους καφεΐνη ή εικονική καφεΐνη σε μορφή χαπιού (6 $mg \cdot kg^{-1}$). Ούτε οι δοκιμαζόμενοι, αλλά ούτε και οι ερευνητές θα γνωρίζουν το είδος του χαπιού. Η λήψη αίματος θα επαναληφθεί 15 λεπτά πριν την έναρξη των δοκιμασιών (60 λεπτά μετά την χορήγηση του χαπιού). Οι δοκιμαζόμενοι ακολούθως θα πραγματοποιήσουν συγκεκριμένο πρόγραμμα προθέρμανσης. Στη συνέχεια οι δοκιμαζόμενοι θα εκτελέσουν 4 κατακόρυφα άλματα με κίνηση χεριών. Δύο από αυτά θα είναι ελεύθερα με σκοπό την καταγραφή του ύψους άλματος, ενώ τα άλλα δύο θα γίνουν μετά από οπτικό ερέθισμα με σκοπό την καταγραφή του χρόνου αντίδρασης. Ακολούθως, θα εξασκηθούν σε ειδικό πρόγραμμα στο δαπεδοεργόμετρο (οι ταχύτητες και η διάρκειά τους θα αυξομειώνονται αυτόματα, 6, 12, 15, 18 και 21 $km \cdot h^{-1}$) συνολικής διάρκειας 22.5 λεπτά. Αφότου ολοκληρώσουν το πρόγραμμα στο δαπεδοεργόμετρο, θα γίνει λήψη αίματος και καταγραφή της αρτηριακής πίεσης και θα επαναλάβουν το ίδιο πρόγραμμα όπως προηγουμένως (κατακόρυφα άλματα και τρέξιμο για άλλα 22.5 λεπτά). Στη συνέχεια θα γίνει λήψη αίματος/καταγραφή πίεσης και θα εκτελέσουν για τρίτη φορά τα κατακόρυφα άλματα. Μετά από μια περίοδο ξεκούρασης 15 λεπτών, θα γίνει λήψη αίματος/καταγραφή πίεσης και θα εκτελέσουν ξανά τα κατακόρυφα άλματα, και ακολούθως θα εξασκηθούν στο ίδιο ειδικό πρόγραμμα του δαπεδοεργομέτρου. Στη συνέχεια θα γίνει λήψη αίματος/καταγραφή πίεσης και θα εκτελέσουν για τελευταία φορά τα κατακόρυφα άλματα. Ακολούθως, οι δοκιμαζόμενοι θα εξασκηθούν στο δαπεδοεργόμετρο (φορώντας όμως την ειδική μάσκα καταγραφής εκπνεόμενου αέρα) με σκοπό την ανάλυση της πρόσληψης οξυγόνου και υπολογισμού της ποσότητας κατανάλωσης υδατανθράκων και λιπών. Η ταχύτητα του δαπεδοεργομέτρου σε αυτό το στάδιο θα είναι σταθερή (75 % της VO_2max) και οι δοκιμαζόμενοι θα πρέπει να τρέξουν μέχρι εξάντλησης. Στο τέλος θα γίνει λήψη αίματος και καταγραφή της πίεσης. Κατά τη διάρκεια ολόκληρου του πρωτοκόλλου, θα καταγράφεται η καρδιακή συχνότητα και το επίπεδο κούρασης. Οι αιματολογικές εξετάσεις θα γίνουν με σκοπό την ανάλυση της ποσότητας επινεφρίνης, γλυκερόλης, ελεύθερων λιπαρών οξέων, γλυκόζης και γαλακτικού οξέος στο αίμα. Διάρκεια επίσκεψης: περίπου 4 ώρες.

Επίθετο:		Όνομα:	
Υπογραφή:		Ημερομηνία:	

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Πιθανοί κίνδυνοι και παρενέργειες από την συμμετοχή στο ερευνητικό πρόγραμμα:

1) Η κατανάλωση καφεΐνης πιθανόν να προκαλέσει γαστρεντερικές διαταραχές και ήπιας μορφής πνευματική σύγχυση

2) Οι δοκιμαζόμενοι πιθανόν να νοιώσουν άβολα κατά την τοποθέτηση της cannula (ήπιας μορφής ενόχληση ή πόνο). Θα υπάρχει συνεχής παρουσία νοσηλευτικού και ιατρικού προσωπικού για παροχή πρώτων βοηθειών όταν και αν κριθεί απαραίτητο

3) Οι δοκιμαζόμενοι πιθανόν να νοιώσουν κούραση κατά τη διάρκεια των δοκιμασιών και γι' αυτό τον λόγο θα υπάρχουν περίοδοι ξεκούρασης

Με το τέλος της έρευνας, εάν επιθυμείτε να σας σταλούν ηλεκτρονικά τα αποτελέσματά σας, παρακαλούμε σημειώστε την διεύθυνση του ηλεκτρονικού σας ταχυδρομείου:

Email:……

Αν έχετε οποιαδήποτε απορία ή ανησυχία για την έρευνα, μπορείτε να καλέσετε τον Δρ. Χατζηχαραλάμπους Μάριο στο 22461566. Για επιπλέον πληροφορίες που αφορούν την ανθρώπινη συμμετοχή σε έρευνα μπορείτε να καλέσετε την Επιτροπή Βιοηθικής του Πανεπιστημίου Λευκωσίας στο 22841675. Σε περίπτωση που επιθυμείτε να υποβάλετε οποιοδήποτε παράπονο σχετικά με την ερευνητική διαδικασία ή να ζητήσετε την ανεξάρτητη γνώμη κάποιου λειτουργού του Πανεπιστημίου Λευκωσίας σε σχέση με την έρευνα που λαμβάνετε μέρος, σας παρακαλούμε να μη διστάσετε να επικοινωνήσετε με τον Δρ. Αδαμίδη Κωνσταντίνο στο 22841675.

Επίθετο:		Όνομα:	
Υπογραφή:		Ημερομηνία:	

APPENDIX B: Medical and life style history questionnaire

Ονοματεπώνυμο:	Ημερομηνία:
Λαμβάνεις κάποιο φάρμακο; …….. Αν ναι, δήλωσε το είδος φαρμάκου: α)……β)..……γ)...……..	
Έχεις διαγνωσθεί με: Υπέρταση;.....Καρδιαγγειακά;.....Διαβήτη;.....Νεφρική ανεπάρκεια;.....Άλλο;.....	
Καπνίζεις; …… Αν ναί, πόσα τσιγάρα την ημέρα;……..…	

Να καταγράψεις το είδος και την ποσότητα τροφίμων/ποτών που κατανάλωσες τις τελευταίες 48 ώρες:

Ώρα	Τρόφιμα/Ποτά	Ποσότητα (γρ/φλ/κγ/κσ)	Τρόπος μαγειρέματος (τηγανητό/βραστό/άλλο)

(γρ=γραμμάρια, φλ=φλιντζάνι, κγ=κουταλάκι γλυκού, κσ=κουτάλι σούπας)

Να καταγράψεις το είδος της φυσικής δραστηριότητάς σου τις τελευταίες 48 ώρες:

Ώρα	Είδος φυσικής άσκησης	Διάρκεια	Ένταση (χαμηλή/μέτρια/έντονη)

Επίθετο:		Όνομα:	
Υπογραφή:		Ημερομηνία:	

APPENDIX C: Daily caffeine consumption questionnaire

		Κατά μέσον όρο κατανάλωση τα τελευταία 2 χρόνια								
ΜΕΡΙΔΑ	Τρόφιμα & Ποσότητες	6+ φορές την ημέρα	4-6 φορές την ημέρα	2-3 φορές την ημέρα	1 φορά την ημέρα	5-6 φορές / εβδ.	2-4 φορές / εβδ.	1 φορά / εβδ	1-3 φορές / μήνα	Λίγες φορές το χρόνο ή ποτέ
	Καφεϊνούχα Τρόφιμα και Ποτά									
	Καφές φίλτρου (1 φλιτζάνι)									
	Καφές στιγμιαίος (1 φλιτζάνι)									
	Καφές ελληνικός (1 φλιτζάνι)									
	Καφές εσπρέσο (1 φλιτζάνι)									
	Καφές ντεκαφεϊνέ (1 φλιτζάνι)									
	Άλλο είδος καφέ (1 φλιτζάνι)									
	Τσάι (1 φλιτζάνι)									
	Τσάι πράσινο (1 φλιτζάνι)									
	Καφεϊνούχα αναψυκτικά π.χ. Coca Cola (1 φλιτζάνι)									
	Καφεϊνούχα ενεργειακά ποτά π.χ. Red Bull (1 φλιτζάνι)									
	Σοκολατούχα ροφήματα (1 φλιτζάνι)									
	Σοκολάτα (1 μικρή)									
	Προσθήκη καφέ σε κάποιο τρόφιμο π.χ. στο γάλα (1 κουταλάκι γλυκού)									
	Άλλο καφεϊνούχο ρόφημα (1 φλιτζάνι) ή τρόφιμο (1 τραπουλόχαρτο)									

Ονοματεπώνυμο:……Ημερομηνία:……/……/……..

APPENDIX D: Plasma glucose method

Plasma glucose concentration was evaluated through colorimetric method (COBAS MIRA, Roche Diagnostics) with reagents obtained from Randox Laboratories Ltd (A.C. Medlab Solutions Ltd). The product used was intended for quantitative in vitro determination of glucose in blood, serum and plasma, suitable for use on the Cobas Mira analyzer.
For the purpose of this study the following reagents for plasma glucose were used:

1) Reagent 1a: Buffer
2) Reagent 1b: GOD-PAP reagent (one vial of Reagent 1b with a portion of Buffer Reagent 1a and then entire contents transferred to bottle 1)
3) Calibrator: Standard
4) Glucose control: Human assayed Multi-sera-level 2 was used as assayed quality control material for monitoring assay performance

APPENDIX E: Plasma lactate method

Plasma lactate concentration was evaluated through colorimetric method (COBAS MIRA, Roche Diagnostics) with reagents obtained from DiaSys Diagnostic Systems (Medisell Company Ltd). The product used was intended for quantitative in vitro determination of lactate in plasma and CSF suitable for use on photometric systems.
For the purpose of this study the following reagents for plasma lactate were used:

1) Reagent 1: Buffer (pH 9.0, 500 $mmol \cdot l^{-1}$) and LDH (≥ 25 $kU \cdot L^{-1}$)
2) Reagent 2: NAD (20 $mmol \cdot l^{-1}$)
3) Calibrator: Multi-calibrator used
4) Lactate control: TruLab N and TruLab P used as assayed quality control material for monitoring assay performance

APPENDIX F: Plasma glycerol method

Plasma glycerol concentration was evaluated through colorimetric method (COBAS MIRA, Roche Diagnostics) with reagents obtained from Randox Laboratories Ltd (A.C. Medlab Solutions Ltd). The product used was intended for quantitative in vitro determination of glycerol in serum and plasma, suitable for use on the Cobas Mira analyzer.
For the purpose of this study the following reagents for plasma glycerol were used:

1) Reagent 1a: Buffer
2) Reagent 1b: One vial of Reagent 1b with 4.5 ml of Buffer
3) Calibrator: Standard
4) Glycerol control: Quality control of glycerol assays on clinical chemistry systems for the control of accuracy and precision

APPENDIX G: Plasma NEFA method

Plasma NEFA concentration was evaluated through colorimetric method (COBAS MIRA, Roche Diagnostics) with reagents obtained from Randox Laboratories Ltd (A.C. Medlab Solutions Ltd). The product used was intended for quantitative in vitro determination of NEFA in serum and plasma, suitable for use on the Cobas Mira analyzer.
For the purpose of this study the following reagents for plasma glycerol were used:

1) Reagent 1a: Buffer
2) Reagent 1b: Enzyme/Coenzymes (one vial of Enzyme/Coenzymes reagent 1b were reconstituted with 14 ml of Buffer reagent 1a)
3) Reagent 2a: Enzyme diluent
4) Reagent 2b: Maleimide (one bottle of reagent 2b was reconstituted with the entire contents of one bottle of Enzyme diluent reagent 2a)
5) Reagent 2c: Enzyme reagent (one vial of Enzyme reagent 2c was reconstituted with 11 ml of Maleimide reagent 2b)
6) Calibrator: Standard
5) NEFA control: Human assayed Multi-sera-level 2 was used as assayed quality control material for monitoring assay performance

APPENDIX H: Plasma epinephrine method

Plasma epinephrine concentration was evaluated through manual immunoassay enzymatic method on microplate absorbance reader (TECAN Sunrise with ELISA microplate reader) with reagents obtained from IBL International GmbH (Medisell Company Ltd). The product used was intended for quantitative in vitro determination of epinephrine in plasma and urine suitable for use on manual and automated enzyme immunoassay systems.
For the purpose of this study the following reagents for plasma epinephrine were used:

1) Calibrator: epinephrine: 0; 1.5; 5.0; 15; 50; 150 $ng \cdot ml^{-1}$, Norepinephrine: 0; 5.0; 15; 50; 150; 500 $ng \cdot ml^{-1}$, Dopamine: 0; 60; 180; 585; 2300; 11470 $ng \cdot ml^{-1}$, 0.1 moles HCL
2) Control 1+2: epinephrine, Norepinephrine, Dopamine (biologically active), 0.1 moles HCL
3) Enzyme Conjugate: Streptavidin alkaline phosphatase, Tris buffer, HCL, 0.01 % NaN_3
4) Extraction Buffer: 0.016 % NaN_3
5) COMT lyophilized: Catechol-O-methyltransferase (porcine liver), NaN_3
6) Coenzyme Solution: S-Adenosyl-L-Methionine, Stabilizers
7) Enzyme Buffer: Tris buffer, HCL, Stabilizers
8) Release Buffer: 0.1 moles HCL, Indicator
9) Acylation reagent: Dimethylformamide, Ethanol
10) Wash Buffer: Tris buffer, HCL, Tween, 0.2 % NaN_3
11) COMT Additive: Human plasma, Stabilizers, 0.01 % Thimerosal
12) Epinephrine Antiserum: Antibodies against Adrenalin (rabbit), Buffer, Stabilizers
13) PNPP Substrate Solution: p-nitrophenyl phosphate (PNPP)
14) PNPP Stop Solution: 1 mole NaOH, 0.25 moles EDTA