AMERICAN UNIVERSITY OF BEIRUT

COMBINED EFFECT OF DIETARY PHOSPHORUS AND STRUCTURED EXERCISE ON BODY COMPOSITION, ENERGY BALANCE, AND PROTEIN SYNTHESIS INITIATION IN RATS

by
SALLY WADIH SAWAYA

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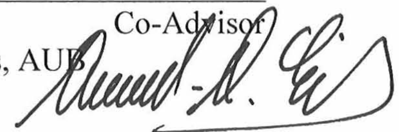

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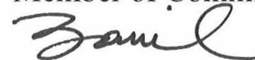

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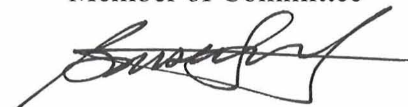

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AN ABSTRACT OF THE THESIS OF

Sally Wadih Sawaya for Doctor of Philosophy
Major: Biomedical Sciences/Nutrition

Title: Combined Effect of Dietary Phosphorus and Structured Exercise on Body Composition, Energy Balance, and Protein Synthesis Initiation in Rats

Individually, both phosphorus (P) ingestion and physical exercise (E) have been found to alter body composition measures and energy balance parameters. Additionally, regular E was reported to be associated with energy compensation either through an increase in energy intake (EI) and/or a reduction in total energy expenditure (TEEx). However, it is not clear whether the ability of dietary P to stimulate energy expenditure (EE) would affect energy compensation following structured E. Accordingly, one of the prominent aims of this study is to assess the combined effect of dietary P and moderate running exercise routine on body composition, energy balance and energy compensation.

P has been associated with the availability of cellular energy for protein synthesis, while regular physical E has been reported to activate protein synthesis in skeletal muscle, but not necessarily so in other tissues. Yet, to date, there are no studies investigating the combined effect of P and E on the mTOR pathway and markers of protein synthesis initiation in various tissues. Correspondingly, the second major aim of this research is to evaluate the impact of P and E on liver and skeletal muscle protein synthesis initiation factors and investigate the signaling pathway involved.

After receiving approval from Institutional Animal Care and Use Committee of the American University of Beirut, two experiments were performed, Low Phosphorus (LP) (0.1%P, 0.2%P, and 0.3% P) and High Phosphorus (HP) (0.3%P, 0.6%P, and 1.2% P) diets. In each experiment, male rats were randomly divided into 3 groups (n=8), in which a sedentary or a moderate-intensity exercise routine (30 minutes 5 days a week) was implemented. EI, body weight and composition, TEEx, energy efficiency, and energy stores were monitored for 6 weeks. Following sacrifice, signaling proteins involved in initiation of protein synthesis translation were measured in liver and gastrocnemius muscle.

In the LP experiment, EI and weight gain were the lowest in the 0.1%P and 0.2%P as compared to the 0.3%P. In the HP experiment, EI was highest in the high P (0.6%P and 1.2%P) groups, while weight gain was reduced. In both experiments, E was able to reduce body fat accumulation and to maintain a higher %LBM. In the LP experiment,

the similarity in TEEx between the sedentary and E groups suggests the probability of a reduction in normal daily activities, which indicates the presence of compensation for the energy expended during exercise by a subsequent reduction in EE. In contrast, the elevated TEEx in the HP exercising groups (0.6%P and 1.2%P) argue against the presence of energy compensation. Therefore, high dietary P decreases the body's capability to compensate for the energy deficit induced by E, consequently maintaining an elevated TEEx.

In reference to protein synthesis, the degree of activation of signaling proteins varied between liver and gastrocnemius muscle. Mammalian target of rapamycin (mTOR) activation increased in the liver as dietary P level increased, while no changes were detected in downstream expression of phosphorylated eukaryotic initiation factor 4E-binding protein-1 (4E-BP1) and ribosomal protein p70S6 kinase (S6K1). The eukaryotic initiation factor 4E (eIF4E) was activated in the exercising groups as the level of P increased. Essentially, E did not result in a change in phosphorylated AMP-activated protein kinase (AMPK) levels, which implies that P availability may have prevented the activation of AMPK in liver, thus maintained mTOR activation and resultant protein synthesis initiation stimulation.

In gastrocnemius muscle, AMPK activation in the 1.2%P exercising group may have resulted in lowering the levels of phosphorylated mTOR and eIF4E, yet, may not have affected protein synthesis considerably. Phosphorylated mTOR was highly expressed in the 0.6%P exercising group which may indicate a greater activation in muscle protein synthesis, though translational initiation factors other than 4E-BP1 and S6K1, which remained unchanged. Further, an mTOR- independent pathway may be responsible for the effect of high P intake (1.2%) on eIF4E activation. Hence, both an mTOR-dependent and mTOR-independent pathway may be involved in protein synthesis in response to P and/or E at the level of skeletal muscle.

In conclusion, these collective results demonstrate that P ingestion above standard level of 0.3%P combined with regular structured moderate-intensity E favours improvement in body composition measures and energy balance outcomes, and may lead to enhancement in protein synthesis through activation of various initiation factors in liver and muscle.

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ABBREVIATIONS

>	Greater than
<	Less than
-	Minus
+	Plus
/	Per
%	Percent
x	Times
Δ	Change in
4E-BP1	eukaryotic initiation factor 4E-binding protein-1
AM	Before Noon
AMP	Adenosine monophosphate
AMPKα	AMP-activated protein kinase
ANOVA	Analysis Of Variance
ATP	Adenosine Triphosphate
AUB	American University of Beirut
BW	Body Weight
CRP	C-reactive protein
d	Day
dl	Deciliter
EAR	Estimated Average Requirements
DRI	Dietary Reference Intake
E	Exercise
EDTA	Ethylenediaminetetraacetic acid
EE	Energy Expenditure
EEf	Energy Efficiency
eEF2	eukaryotic elongation factor 2
EI	Energy Intake
eIF4E	eukaryotic initiation factor 4E
eIF4G	eukaryotic initiation factor 4G
eIF2B	eukaryotic initiation factor 2B
E_{st}	Energy Stores
et al	and others
Fat Mass	FM
Fat_{st}	Fat Stores
FAO	Food and Agriculture Organization
FGF-23	Fibroblast Growth Factor-23
g	Gram
h	Hour
HDL	High Density Lipoprotein
HP	High Phosphorus
IACUC	Institutional Animal Care and Use Committee
Kcal	Kilocalories
kg	Kilogram
L	Liter
LBM	Lean Body Mass
LBM_{st}	Lean Body Mass Stores

LDL	Low Density Lipoprotein
LP	Low phosphorus
mg	Milligram
mmol	Millimol
mRNA	messenger RNA
mTOR	Mammalian Target of Rapamycin
NEAT	Non-exercise activity thermogenesis
NRC	National Research Council
NMR	Nuclear magnetic resonance
p-	Phosphorylated
P	Phosphorus
P	p value
PI3-Kinase	Phosphoinositide 3-Kinase
PKB	Protein Kinase B
PTH	Parathyroid Hormone
PUN	Plasma Urea Nitrogen
RMR	Resting Metabolic rate
RDA	Recommended Dietary Allowance
S	Sedentary
sFRP-4	Secreted frizzled related protein-4
SD	Standard Deviation
S6K1	ribosomal protein p70S6 kinase
TEEx	Total Energy Expenditure
TG	Triglycerides
UL	Tolerable Upper Intake Level
USDA	United States Department of Agriculture

To my darling youngsters who are not so young anymore: Selina, Angela and Elie.

CHAPTER I

LITERATURE REVIEW

A. Phosphorus Overview

1. Phosphorus, an Essential Mineral

Phosphorus (P) is a mineral that is necessary for all living things and essentially required by every cell in the human body for normal function. It is the second most abundant mineral accounting for 500-700g of the body, and constituting ~1% of total body weight [1, 2]. Structurally, about 85% of bodily P is stored in the skeleton (bones and teeth) mainly as part of the hydroxyapatite [3], and 14% is found in muscles and soft tissues [4]. P is also a structural component of DNA and RNA, lipid cell membranes and lipoproteins. In the extracellular compartment, less than 1% of unbound inorganic phosphate is metabolically active and is believed to be maintained within a tight serum concentration range of 2.5–4.5 mg/dl [1, 4].

Functionally, P is a vastly active molecule; it is required for the transduction of cell-signaling pathways, transcription of genetic material, maintenance of normal acid-base balance, phosphorylation of various compounds especially glucose and its metabolites, and activation of enzymes and water-soluble vitamins. Furthermore, P produces and stores energy in phosphate bonds of adenosine triphosphate (ATP), the body's main energy currency, hence playing an important role in cellular metabolism and regulation of subcellular processes [5].

Consequently, an adequate P intake and homeostasis are crucial to support both structural and functional processes in the body, as both, low levels or a surplus of P can

have detrimental health effects. In both humans and experimental animals, hypophosphatemia and intracellular deficiency of inorganic phosphate trigger a dysfunction in several organ systems, resulting in rhabdomyolysis, respiratory failure, heart failure, erythrocyte hemolysis, left ventricular dysfunction, central nervous system abnormalities, skeletal myopathy, and muscle weakness due to its impact on the formation of ATP [5]. On the other hand, metabolically-induced hyperphosphatemia was found to have deleterious effects on bone formation and homeostasis [6], and is associated with vascular calcification [7] and all-cause mortality, specifically in patients with kidney disease [8, 9].

2. *Phosphorus Homeostasis*

In adults, the regulation of P metabolism includes maintaining equal amounts of P lost in urine to the net P absorbed in the intestine, and equivalent amounts deposited and resorbed from bone [2]. In fact, the control of P metabolism is done through an interchange between intestinal absorption, renal excretion, bone resorption, and, to some extent, cellular exchange [10].

The kidney remains the main functioning organ in the maintenance of serum P homeostasis, wherein several upregulating or down regulating factors affect P reabsorption and retention, most importantly, parathyroid hormone (PTH), vitamin D, fibroblast growth factor-23 (FGF-23), secreted frizzled related protein-4 (sFRP-4) and Klotho [1, 11].

At the level of the intestine, dietary P absorption takes place in the duodenum and to a higher extent in the jejunum via a passive mechanism of ion diffusion and an

active sodium-dependent transport (SLC34A2 or NaPi-IIb). Interestingly, unlike other minerals, P absorption does not appear to increase when low P diet is consumed. As such, when investigating the effect of a low (0.1%), normal (0.6%) and high (1.2%) P diet on P absorption in rodents, it was demonstrated that dietary P did not affect jejunal absorption, nor the gene expression of the main phosphate transporter NAPi-IIb [12]. Nevertheless, several other factors affect the intestinal absorption of P. Vitamin D works opposite the phosphaturic compounds FGF-23 and sFRP-4 to enhance P transport across intestinal epithelial cells. Moreover, transport of P across the gastrointestinal track depends on several modulators like gastric acidity, compounds competition, P bioavailability, etc. [10].

Hence, the mechanism by which P metabolism is regulated is multifactorial, several of which alter P balance independently from ambient P concentration [13].

3. Food Sources and their Differential Bioavailability

Several studies have demonstrated that the amount of P in foods does not indicate the true P exposure due to the variability in P bioavailability [10, 14, 15]. Indeed, P bioavailability changes with the rate and efficiency of absorption of P from different food sources, as well as the availability of other nutrients that can bind to P and decrease its absorption, namely calcium [16].

There are basically two types of P in foods, natural and added, often referred to as organic and inorganic. Organic P depends on enzymatic digestion for hydrolysis to its inorganic form before absorption, thus it is slowly and less efficiently absorbed than

inorganic P. Animal protein sources such as meat and dairy, and plant foods like cereals, seeds, nuts and legumes, are major food sources of organic P (Table 1) [17].

Table 1: Phosphorus content of several common animal and plant foods

Animal Protein Sources	**Phosphorus (mg/100g)**	**Phosphorus content /g Protein**	**Plant foods**	**Phosphorus (mg/100g)**	**Phosphorus content /g Protein**
Ground beef	222	8.5	Wheat flour	135	10.8
Chicken breast	246	8.8	Whole wheat bread	202	15.5
Salmon	252	12.6	Brown rice, cooked	83	31.9
Tuna, canned in water	216	9.4	Pasta, cooked	58	9.6
Milk, whole	91	28	Soybeans	245	13.6
Yogurt, plain	95	27	Black beans, cooked	142	15.8
Whole egg	172	12.7	Chickpeas	168	18.7
Cheddar cheese	512	20.5	Lentils, cooked	180	20
Feta cheese	337	24	Peanuts	358	12.8
Cottage cheese	150	15	Broccoli, cooked	66	27.5

Adapted from USDA (U.S. Department of Agriculture) Agricultural Research Service. USDA Nutrient List from Standard Reference Legacy (2018). https://www.nal.usda.gov/sites/www.nal.usda.gov/files/phosphorus.pdf

However, there is a significant variation in bioavailability between these two groups. Organic P from animal protein is easily hydrolyzed and absorbed as compared to the P in plants, as the latter is in the form of phytates [18]. In mammals, due to the absence of phytase, the enzyme required for the degradation of phytates, the bioavailability of P from plant sources is relatively low, even though they contain high

amounts [19]. In effect, the bioavailability of P is nearly 40% from plant sources and 60% from animal protein sources [20]. As an example, estimated P bioavailability ranges from around 6% for sesame seeds and 35% in chickpeas and lentils, to 60% in meat, chicken, fish and dairy [21, 22]. Hence, when examining dietary P from natural food sources, a gram is not truly considered a gram [23].

On the other hand, depending on food choices, additives and preservatives comprise an important source of inorganic P, notably in individuals consuming a lot of processed foods. These inorganic forms, which are added to foods during processing or preparation, are rapidly hydrolyzed in the acid stomach requiring no enzymatic digestion. Thus, inorganic phosphate is quickly and easily absorbed with a high degree of bioavailability ranging from 80 to 100% [19]. Contrary to popular belief, carbonated beverages are not major contributors to dietary P, as each standard cola beverage contains between 10-15mg P/100g [22] which translates to around 24.1-37.5mg P/240ml [24]. When compared to a glass of milk which contains 222-247mg/240ml (approximately 1mg P/ml), these beverages contain considerably less P. In effect, P is mostly derived from proteins sources (Table 1).

In a recent study conducted on cats, it was revealed that diets with added inorganic P resulted in a dose-dependent temporary increase in plasma phosphate and P regulatory factors, whereas organic P derived from natural food ingredients, fed at the same levels, did not affect plasma P concentration [25]. Phosphate additives are also added in unknown amounts, which makes it more problematic in assessing dietary intake of P [23, 26]. Nonetheless, in certain dietary habits, food additives can contribute up to 30% of the adult daily P intake [27].

Although P digestibility is a major determinant of its bioavailability, their direct relation may be adjusted by factors that affect P absorption, namely Vitamin D status and the nutrient-nutrient interaction in the intestinal lumen [15]. This nutrient-nutrient interaction is mostly related to calcium. When calcium intake increases without a subsequent rise in dietary P, the absorption of P decreases and the risk of P insufficiency increases [28]. Hence, excess calcium binds to dietary P forming insoluble complexes and reducing its bioavailability. For this reason, calcium carbonate or citrate salts are used as phosphate binders in end-stage renal disease, yet the excessive use of non-phosphate calcium supplements can have a detrimental impact on P bioavailability and homeostasis [29].

Therefore, due to the inconsistency between digestibility of dietary P, its absorption and excretion, more balance studies are needed to determine the bioavailability of the different sources of P, and here comes the importance of adjusting for P bioavailability in future research.

4. *Daily Intake and Reference Recommendations*

The modernized dietary trends favor the consumption of refined cereals, potatoes, fat, sugar, fructose and other sweeteners, which contain negligible amount of P [30, 31], and these foods contribute to more than 50% of the food supply in most countries [32]. Conversely, P content in non-refined foods, like whole-grains, pulses and meat is about 1mgP/Kcal, which would equate to a daily intake of around 2500mg P if a person consumes 2500 Kcal/day. This kind of diet is believed to be the traditional diet trend that was consumed by our ancestors [33]. Consequently, it is implied that P

intake has decreased over time; and, with a lack of an adaptive mechanism that improves P absorption at low intake levels, the decrease in P consumption can affect its availability for overall cellular metabolism and function. In effect, a reduction in serum P and ATP synthesis rate were observed as a result of the consumption of a low P diet [34, 35].

On the other hand, despite the growing accessibility of processed food and the extensive use of phosphate additives, the daily P intake data (NHANES 2011-2012) has revealed an average P intake of 1754mg/day and 1276mg/day in adult men and women consecutively (Table 2). P intake was assessed by 24-hour dietary recall with its numerous limitations based on evidence of differences in bioavailability of the various forms of P compounds and effects of co-nutrient modification, thus, it reflects a poor assessment of true P exposure and bioavailability. Nevertheless, these values are above the current adult Recommended Dietary Allowance (RDA) of 700 mg/day for adults. Noteworthy is the fact that the establishment of the US reference intake Estimated Average Requirement (EAR) at 580mg/day, was not based on P balance studies, but on the lower end of the normal serum P concentration range (2.7mg/dl). If it were based on the middle value of that range (3.6mg/dl), the EAR would have been ~2100mg/day [36], and consequently the RDA at ~2520mg/d, hence, the current daily intake would have been considered lower than recommended.

Table 2: 1997 Dietary Reference Intake (DRIs) of phosphorus and daily intake by age and gender in the NHANES 2011-2012 survey.

Age (years)	**RDA (mg/day)**	**EAR (mg/day)**	**UL (mg/day)**	**Daily intake (mg/day)**	
				Men	Women
6-11	500-1250	380-1055	3000	1347	1225
12-19	1250	1055	4000	1700	1162
20-39	700	580	4000	1754	1276
40-59	700	580	4000	1670	1187
60-69	700	580	4000	1469	1100
≥70	700	580	3000	1469	1100

Source: DRIs (Institute of Medicine, 1997). RDA, Recommended Daily Allowance; EAR, Estimated Average Requirement; UL, Tolerable Upper Intake Level.
Daily intake data estimated from day 1 of individuals, from What We Eat in America, NHANES 2011–2012. Data available from: https://www.ars.usda.gov/ARSUserFiles/80400530/pdf/DBrief/15_Phosphorus_intake_1112.pdf

Another reason why the RDA is considered at the low end is further explained as follows. A gram of protein contains 15-17mg of P [37, 38]. Generally, the average protein requirement in humans is 0.8g/kg body weight [39], thus an average 70kg man requires 56g proteins/day, and consequently ingests 840mg of P from protein sources only, a value that is higher than the RDA, and which does not account for P content of whole grains, legumes and other plant sources.

Moreover, there exist a wide disparity in P requirements among animals and humans, as the average P requirements in adult animals span between 0.4 and 0.7% (4-7g/kg feed) [40], and with an energy density ranging from 2.8 to 4.55 Kcal/g, the

amount of P required by various species would be around 1-2.5mg/Kcal, a level that conforms with the nutrient requirements suggested by the National Research Council (NRC) (Table 3) [41-48]. However, in humans, an RDA of 700 mg P/day in individuals consuming 2500 Calories, equates to 0.3 mg P/Kcal which is approximately 1/3rd the amount of P required for animals. If the same computation of 1mg P/Kcal was used in humans, the daily recommendation for P would have been 2500mg/day for every 2500 Calories ingested.

Table 3: Phosphorus requirements for various animal species.

Species	P requirements (%) [40]*	Energy density (Kcal/g)**	P requirements per Calorie (mg/Kcal)***
Rodents	0.4	3.8-4.1 [41]	0.98-1.05
Guinea pigs	0.4-0.7	2.8-3.5 [41]	1.14-2.5
Non-human primates	0.4	3.87-4.55 [42]	0.88-1.03
Dogs	0.44	3.49-4.00 [43, 44]	1.00-1.26
Cats	0.6	4.00 [43, 44]	1.50
Swine	0.4-0.7	3.27 [45]	1.22-2.14

*Expressed as % per unit animal feed either on as-fed (10% moisture) or dry matter; Based on National Research Council NRC 1978-2001.

**Energy density of animal feed expressed in Calories per gram; based on NRC.

***Phosphorus intake per Calorie calculated in milligram phosphorus per Calorie.

B. Phosphorus Effects

1. Phosphorus Effect on Body Composition

Extensive research has highlighted the ability of P to alter body weight and composition. Recently, the analysis of 3 independent cohorts of 1979 healthy subjects has shown an inverse association between serum phosphate concentration and measures of adiposity in both men and women [49]. Furthermore, in a 12-week double-blind, randomized, placebo-controlled study, an improved dietary P intake has resulted in a significant decrease in body weight, body mass index (BMI) and waist circumference [50]. Additionally, in rats, the resulting effect of a high phosphate diet on a lower body fat mass accumulation was accompanied by a reduction in lipogenic and increase in lipolytic gene expression in visceral fat [51]. Similarly, lower visceral fat accumulation and decreased hepatic lipid synthesis were documented in rodents ingesting a high P diet over 8 weeks [52]. This can be partly explained by the capacity of a high dietary P to enhance insulin sensitivity and reduce fasting insulin levels [51, 52], resulting in suppression of lipid synthesis and fat accretion and stimulation of fat breakdown, thereby playing a role in reducing visceral fat mass [53].

Noteworthy is the result that upon ingestion of diets deficient in P, study animals exhibit reduced weight gain and body fat percent, whereas adding an adequate amount of P to a low-protein diet (10% of the calories) enhances these growth parameters and improves weight gain and body composition to a similar extent as rats ingesting a standard protein diet of 20%. In effect, as compared to low P diets, a standard dietary P content was associated with an increase in body protein content [54] and higher percent of body protein [55].

2. Phosphorus Effect on Energy Balance

Recent studies have demonstrated that P supplementation has an impact on both energy intake (EI) and energy expenditure (EE). Regarding EI, acute P ingestion with a carbohydrate preload has been shown to decrease subsequent EI by around 30% [56]. Additionally, P supplementation for 12 weeks has resulted in reduced appetite scores and number of snacks consumed [50]. Similarly, individuals supplemented with P exhibited a lower appetite described as a lower desire to eat a meal, indicating an improved postprandial satiety [57]. These effects may be explained by the involvement of hepatic ATP in the control of food intake and its dependency on P availability in the body [58]. Noting that P availability was reported to be directly influenced by dietary P intake and replenishment [35, 59].

On the other hand, animal studies have demonstrated that diets deficient in P are linked to lower EI [60]. Moreover, food intake improves when the diet provides a standard level of P (0.3%P) as compared to very low P (0.015%P-0.1%P) diets [54].

On the other side of the energy balance equation, being incorporated in ATP, P has been shown to enhance energy expenditure (EE). Ingestion of P was able to elevate both resting metabolic rate (RMR) upon consumption of a low energy diet [61], and postprandial thermogenesis in obese subjects [62, 63]. In line, P caused a significant increase in RMR in obese women during weight reduction [64]. Besides, it was recently demonstrated that the addition of 500mg of P to a high carbohydrate meal was able to improve postprandial EE of both lean and obese subjects [65], as well as enhance the postprandial thermogenesis in healthy lean individuals upon ingestion of diets of different nutrient compositions [66]. P ingestion in obese subjects has also been shown

to alter substrate oxidation and result in a 23% elevation in the diet-induced thermogenesis [57]. Such increases in different components of EE are largely dependent on ATP production, which may be related to P availability [58].

As reviewed by Obeid (2013), the rational for the importance of a regular supply of exogenous P for adequate P availability to support cellular metabolism is explained by two endogenous factors. First, only limited amounts of free phosphate are stored intracellular, and most tissues depend upon extracellular P for their metabolic phosphate requirements, and under low extracellular P levels as in cases of low P diet, this negatively impacts ATP production and cellular metabolism. Second, fractional P absorption is virtually constant across a broad range of consumption, suggesting a lack of an adaptive mechanism that enhances P absorption at low intake levels [33]. Thus, P availability in food becomes an essential factor that governs P levels in the circulation and in turn its availability for cellular ATP production and phosphorylation reactions.

C. Structured Exercise Impacts

1. Exercise Impact on Body Composition

There is strong evidence supporting the role of regular exercise (E) in body weight and fat reduction, maintenance of body weight and fat loss, metabolic fitness and maintenance or improvement of muscle mass. Both men and women exhibited lower body weight, fat mass and increased LBM after 3 months of physical training [67]. In addition to a lower fat mass, physical training lead to an increase in LBM, and fast-twitch, aerobic type muscle fibers in obese men [68]. In line, after 12 weeks of

structured physical E, slightly obese women showed a reduction of 2.6 kg of body fat and increased 1.9 kg of LBM, hence were able to improve their body composition [69]. Additionally, healthy normal-weight pre-menopausal women who habitually perform physical E maintained lower body fat levels, in which their mean percent body fat averaged 18.9% as compared to 28.8% in the sedentary controls [70]. Moreover, an epidemiologic study of adult individuals revealed that those who practiced vigorous physical activity on regular basis had lower central (measured as waist-to-hip ratio) and total adiposity (assessed by skinfold thicknesses) than individuals not engaging in physical E [71].

In experimental animals, an 8-week aerobic E regimen resulted in a significant reduction in body weight, fat percentage and adiposity index [72]. Similarly, endurance training decreased body weight, adipocyte size and visceral adiposity, wherein the exercised rats also displayed lower adipogenesis-related markers (SREBP1c and acetyl CoA carboxylase) after 8 weeks of structured training when compared to sedentary rats fed isoenergetic diets [73]. Moreover, daily running on the treadmill for 10 weeks resulted in lower weight gain, higher gastrocnemius muscle weight and a reduction in adipose tissue growth in rats [74].

2. *Exercise Impact on Energy Balance*

E effect on EI has long been controversial. Generally, short-term E does not lead to elevation in EI in men [75], but increases EI in women [76]. Acute E training can modify energy intake through the adjustment of the energy-regulating hormones [77]. Most studies have revealed that the subjective perceptions of appetite are temporarily

suppressed during E sessions performed at ≥60% peak oxygen uptake (VO_2 max), known as E-induced anorexia. These feelings are short-lived and return to resting normal values within 30-60 minutes of E termination, and do not result in changes in energy intake on the day of aerobic training [78]. In experimental animals, 8 days of training did not affect EI in either exercised male or female rats as compared to their sedentary controls [79]. Similar to human studies, appetite-regulating hormones seem to alter EI acutely, in which, an acute session of E resulted in a reduction in food intake in male rats only during the first hour after physical activity, independently of the E intensity [80].

Extensive research has also highlighted the ability of structured E, denoting supervised, prescribed and/or planned training sessions, to alter various components of EE. One way in which aerobic physical E can affect EE is through improving brown adipose tissue activity, and thus inducing adaptive thermogenesis [72, 81]. Additionally, it was demonstrated that E enhances RMR when measured by indirect calorimetry [70]. Generally, animal studies have shown that both single E sessions and long-term E training result in increases in RMR [81, 82]. Of note is that the greater the intensity of the E, the higher the post-exercise EE [83]. However, the energy cost of E was reported to be partially compensated over time [84], through alterations in energy intake and/or energy expenditure [85, 86].

Although there is a large inter-individual response to energy deficit induced by E, most studies reported an increase in food intake as a response to chronic E. As such, after 3 months of training, subjects portrayed an elevation in hunger, a reduction in satiety and an increase in EI [87]. On the long run, adaptation to E is evident as the drive to eat rises [84], fasting hunger amplifies [88], and average energy compensation

is approximately 30% [85, 89]. Additionally, in rodents, the exercised groups had markedly increased their food intake following structured training sessions [81].

The alternate part of energy compensation as a response to long-term E training is reflected in reducing energy output. The effect of structured E on total daily EE is apparent though its impact on normal daily activities or non-exercise activity thermogenesis (NEAT). NEAT is defined as the energy expenditure for all activities except structured E, such as activities of daily living, occupation, leisure, postural maintenance, spontaneous muscle contraction, talking and fidgeting [90]. As such, numerous studies found a reduction in EE from NEAT as a compensation for the energy spent during E sessions [88, 91]. More specifically, as volume and intensity of E increases, NEAT decreases [91]. Additionally, aerobic E results in a reduction in NEAT [84, 86], possibly due to increases in fatigue associated with aerobic training [92]. On the other hand, few studies have documented neither a compensatory reduction in NEAT [93], nor increases in sedentary behavior post-exercise [87]. Further, a study reported an elevated NEAT after E [94].

Therefore, the effect of supervised E on behavioral and/or physiological compensatory changes in energy balance are subject to differences in inter-individual responses to energy deficit incurred by training, E intensity and duration.

D. Protein Synthesis

1. Initiation of Protein Synthesis

Protein synthesis is essential for growth and maintenance of body tissues. Regulation of global rates of protein synthesis happens mainly at the level of translation

initiation, which is positively controlled by several stimuli, including nutrients (essential amino acids), circulating hormones (insulin), and mechanical stimuli (in muscle tissue) [95], and is facilitated by the availability of energy [96, 97]. These stimuli converge on mTOR (mammalian target of rapamycin), a serine-threonine protein kinase, which acts as the cell's nutrient and energy sensor and plays an essential role in regulating protein synthesis and cell growth [95]. Control of translation initiation by mTOR is mediated through phosphorylation of multiple downstream targets and highly organized protein-protein interactions between the initiation factors that modulate the binding of mRNA to the 40S ribosomal subunit, most importantly the eukaryotic initiation factor 4E-binding protein-1 (4E-BP1), and the ribosomal protein p70S6 kinase (S6K1) [95, 98].

Another essential translational initiation factor is eukaryotic initiation factor 4E (eIF4E), a part of the heterotrimeric complex eIF4F, which interacts first with the mRNA and facilitates the recruitment of the 40S ribosomal subunit, resulting in the activation of mRNA translation. More specifically, when eIF4E is bound to 4E-BP1, it cannot bind to eukaryotic initiation factor 4G (eIF4G) to form the mRNA cap-binding complex eIF4F (Figure 1). And the binding of eIF4E to 4E-BP1 is regulated by phosphorylation of 4E-BP1, with hyperphosphorylation resulting in the dissociation of 4E-BP1.eIF4E complex, thus permitting eIF4E to bind to eIF4G and initiate the cap dependent translation [99]. Indeed, protein synthesis was found to be linked with increased association of eIF4E.eIF4G. Further, phosphorylation of eIF4E was reported to facilitate the translational process [100] due to the formation of a more stable complex with eIF4G [101], and a greater binding affinity for the mRNA cap [102]. Moreover, in cardiocytes, increases in eIF4E activity selectively improves the efficiency of protein translation [103].

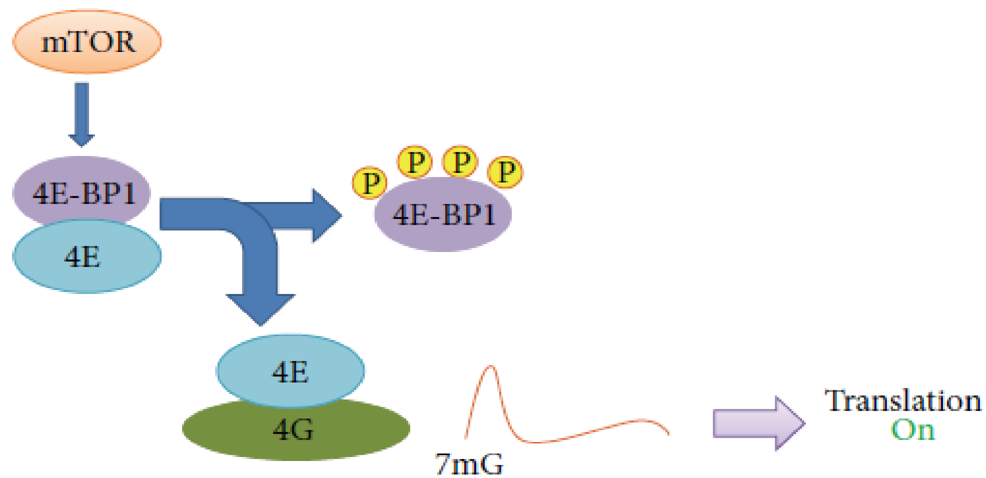

Figure 1: Regulation of cap-dependent translation.
7mG (7-methylguanosine cap) cap structure at the 5'end of mRNA; Protein complex: eIF4E (4E). eIF4G (4G) recruited to the cap to facilitate mRNA translation.
Source: Showkat et al. [104]

Protein synthesis is an energy-expensive process [105] where a constant supply of energy is necessary to maintain the transcription and translation reactions [106]. 4 ATP equivalents are required for the formation of 1 peptide bond for each amino acid residue added to the growing chain, or the equivalent of 0.67 kcal per 1g of protein synthesized [105, 106]. Numerous studies have demonstrated that hepatic ATP depletion, induced by ethionine, D-fructose or other compounds lead to the inhibition of protein synthesis [59, 107-109]. Acute ethionine intoxication produced a decline in the level of hepatic ATP which indicated impairment in the initiation of protein synthesis, and resulted in polysome disaggregation and accumulation of inactive ribosomal subunits [109]. Additionally, reducing the levels of hepatic ATP was reported to inhibit protein synthesis due to alterations in the phosphorylation states of translational initiation factors, where 4E-BP1, S6K1 and eIF4E were found to be hypo-phosphorylated. The administration of methionine and/or adenine rapidly restored ATP

levels which brought about a complete recovery of the phosphorylation states of these initiation factors and the resumption of protein synthesis [110]. Similar alterations in phosphorylation of translational elongation factors and mTOR signaling were observed in energy depleted cardiomyocytes, which resulted in the arrest of protein synthesis [111].

Therefore, protein synthesis initiation, the rate-limiting step of protein synthesis, is primarily mediated through the mTOR signaling pathway, and is enabled by the availability of cellular energy.

2. Phosphorus and Protein Synthesis

Could there be an association between P and Protein synthesis?

As previously illustrated, several observations suggest that the availability of cellular energy is highly dependent on P availability [35, 59, 112]. P produces and stores energy in phosphate bonds of ATP, the body's main energy currency, which is needed for numerous metabolic processes. Hence, P plays an important role in cellular metabolism (generation of ATP) and regulation of subcellular processes (phosphorylation of key enzymes) [5].

Depletion of inorganic phosphate causes a dose-dependent reduction of ATP levels in liver and kidney cortex as a result of a decrease in the rate at which ATP is regenerated [58]. Similarly, restriction of dietary P was reported to reduce gastrocnemius muscle tension; the muscles of phosphate-deficient mice fatigued more rapidly compared to control mice owing to a slow rate of ATP synthesis and diminished intracellular ATP, and this myopathy was reversed following P supplementation [35]. In a recent study, mice have shown a reduction in serum inorganic P concentration and

measured muscle ATP synthesis rates following a diet-induced hypophosphatemia. The reintroduction of P in the diet contributed to the normalization of serum P levels, as well as muscle ATP synthesis flux [34].

Correspondingly, in humans, patients with hypophosphatemia had reduced respiratory muscle function as compared to patients with normal P levels, and the respiratory muscle weakness was improved upon phosphate repletion. The mechanism responsible for the muscle fatigue was thought to be the reduction in the supply of "high-energy phosphate compounds" required for proper muscle contraction [113].

Dietary lifestyle is a major contributor to P availability for cellular ATP production. Current food habits, which are affected by modernization, globalization of food markets and industrialization, favor the consumption of refined high carbohydrates foods that are usually low in P. This type of diet can lead to a sharp increase in insulin secretion which stimulates the peripheral uptake of P and phosphorylation of macronutrients (namely carbohydrates). As a consequence of the increase in phosphorylation reactions, P becomes less available for ATP production and the regulation of energy metabolism [33].

Each ATP is regenerated 1000-1500 times/day, which means that in a resting adult, around 65kg of ATP are recycled daily [114]. As discussed, the rate of ATP synthesis is directly related to blood P concentration [34], which is ultimately related to P availability in food, its intake and replenishment [58]. And, protein synthesis is strongly associated with cellular ATP levels, as it was shown to be reduced with low intracellular ATP levels. Hence, a link may exist between P and protein synthesis.

Theoretically, a low P diet may result in lower cellular ATP content and regeneration rate, leading to a reduction in protein synthesis and enzyme function. To our knowledge, only one study measured protein fractional synthetic rate in rat liver upon P depletion. As concentration of P in the liver fell to around 50%, the concentration of ATP decreased to around 20% of control value. Subsequently, the rate of L-leucine incorporation into hepatic proteins was reduced to 35% of initial levels, which indicated a marked reduction in hepatic protein synthesis rate [112]. More recently, two studies correlated P intake with plasma urea nitrogen (PUN), a marker of protein metabolism. Where, a reduction in PUN was shown with increasing P levels in the diet from deficient to standard. This inverse relationship between dietary P content and PUN, supports the ability of P to alter protein metabolism [54, 55]. However, the mechanism involving P in protein metabolism was not elucidated.

3. *Exercise and Protein Synthesis*

Studies on the association between protein metabolism and E usually tackle either the effect of different types of E on skeletal muscle protein turnover and/or the effect of post-exercise feeding, especially repletion of amino acids/leucine on recovery of muscle.

Short-term changes in protein metabolism caused by E are mainly catabolic with net negative balance between the rates of protein synthesis and protein degradation, wherein the magnitudes of these catabolic processes are determined by the type of E (Figure 2). Nevertheless, these catabolic acute effects do not cause muscle wasting; instead, regular E is crucial to enhance muscle growth. Hence, a series of metabolic

adjustments are required to alternate from the catabolic period of E to the anabolic period of recovery [115].

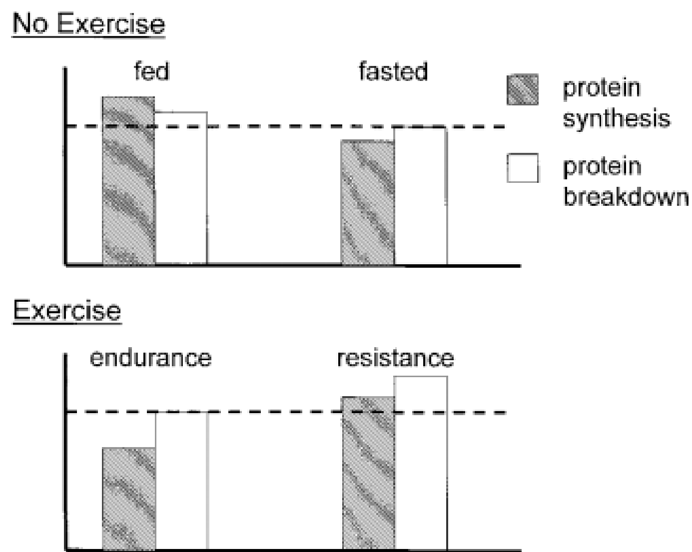

Figure 2: The effect of exercise or rest on the balance of protein synthesis (shaded parts) and protein breakdown (open bars). Upper: Portraying the anabolic conditions after a meal and catabolic period after an overnight fast. Lower: Depicting variances in protein turnover depending on type of exercise. Endurance exercise represents an exhaustive aerobic session, and resistance exercise, a prolonged bout of strength exercise.
Source: Norton and Layman[115]

During endurance E (aerobic training), the rate of muscle protein synthesis decreases (Figure 2), and the degree of reduction is linked to the duration and intensity of the activity [116]. The reduction in protein synthesis is associated with inhibition of mTOR pathway, which includes inhibition of the translation initiation factors eIF4E and eIF4G and ribosomal protein S6, under the regulatory control of insulin signaling and intracellular leucine concentrations. More specifically, eIF4E binding to the inhibitor

4E-BP1 is increased and binding with eIF4G is reduced, due to increased activity of AMP-activated protein kinase (AMPKα) [117]. A prolonged aerobic E session decreases ATP/AMP ratio and lowers glycogen stimulation. This in turn enhances AMPKα levels which leads to phosphorylation of TSC2, formation of TSC1/TSC2 complex, and inhibition of Rheb and mTOR [118].

Resistance E results in a different pattern of protein turnover as compared to endurance training (Figure 2). Although protein synthesis is stimulated simultaneously with protein breakdown, a single bout of resistance training remains catabolic [119]. Yet, it was shown that resistance training has a prolonged effect on muscle protein synthesis, which continues for up to 48 hours post-exercise [120]. This elevation in protein synthesis is likely mediated by PI3-kinase/ PKB/mTOR signaling, where experimental animals experience an elevation in PKB, resulting in hyperphosphorylation of TSC1 and decreased formation of the TSC1/TSC2 inhibitory complex, hence, permitting the binding of Rheb with mTOR and activation of ribosomal protein S6 and eIF4E [117]. Additionally, several studies have shown that the elevation of protein synthesis 16 hours following resistance E were mainly mediated by the activation of mTOR downstream initiation factor eIF2B [121, 122].

Therefore, a direct relationship does exist between the mTOR signaling pathway and short-term elevation of global protein synthesis post-resistance training, mediated through an increase in translation initiation. On the long run, the elevation of mTOR activity upon chronic resistance E results in increases in myofibrillar protein synthesis leading to increases in muscle mass and strength. Similarly, mTOR activation is necessary for the chronic endurance-induced myofibrillar protein synthesis, which mostly occurs after protein ingestion [123]. As such, 3 months of aerobic E training

resulted in increased global rate of protein synthesis in soleus muscles of mice attributed to stimulation of PKB/mTOR signaling pathway [124]. Nevertheless, endurance E was shown to primarily elevate mitochondrial protein synthesis independently of mTOR, thereby leading to improved muscle oxidative capacity and muscular endurance [125].

After both endurance and resistance exercises, a net positive nitrogen balance in skeletal muscle is only achieved when adequate protein and energy are provided for recovery. Hereafter, the combination of amino acids and carbohydrates stimulate the PI3-Kinase and mTOR pathways, resulting in reduced AMPKα and TSC2 activity and activation of translational initiation factors, most importantly, 4E-BP1, eIF4E and eIF4G, consequently bringing about maximal stimulation of protein synthesis rates post-exercise [126-128].

Over and above that, E was shown to activate mTOR in diverse tissues [123]. In the heart, the activation of mTOR in cardiac muscle during endurance E leads to physiological hypertrophy [129]. Moreover, increases in mTOR pathways signaling could contribute to the benefits of E on mental health and cognition [130]. On the other hand, some studies have shown that prolonged endurance E can result in metabolic stress, and metabolic stress stimulates AMPKα, which can lead to inhibition of mTOR in non-muscular tissues, such as liver and fat tissues. As mTOR plays a role in lipid metabolism, then decreasing mTOR through AMPKα activation may reduce adiposity in liver and fat tissues [131], and lower protein synthesis in the liver [132].

CHAPTER II
AIMS

As elaborated on in the previous section, both P and E individually affect body composition measures and energy balance parameters. However, the combination of P and structured E has never been studied before. It is also not clear whether the capacity of P to stimulate EE would impact energy compensation following E. Accordingly, the 2 inter-related prominent objectives of the project were to:

1. Examine the combined effect of dietary P and moderate-intensity running E routine on body composition and energy balance.
2. Assess whether the capacity of P to stimulate EE would outweigh energy compensation following E.

On the molecular level, most of the EE in the body is related to LBM, and it is known that protein synthesis is a major regulator of lean tissue accretion. This leads to an important research question: Is there an individual or joint effect of P and structured E on liver and muscle protein synthesis?

As formerly discussed, an association exists between P and the availability of energy which is known to facilitate protein synthesis. Additionally, chronic endurance E is a well-known activator of protein synthesis in skeletal muscle, but may not be so in other tissues. However, there are no studies investigating the combined effect of P and moderate-intensity aerobic physical E on the mTOR pathway and markers of protein

synthesis initiation in different tissues. Accordingly, a further important objective of this research was to:

3. Investigate the individual and joint P + E impact on liver and muscle protein synthesis initiation factors, and propose the potential signaling pathway involved in protein anabolism.

CHAPTER III

MATERIALS AND METHODS

A. Experimental Procedure

Approval of the experimental protocol was obtained from the Institutional Animal Care and Use Committee (IACUC) of the American University of Beirut (AUB), Lebanon.

1. Animal Housing and Adaptation

Seven-week old male Sprague-Dawley rats weighing around 250 grams were housed individually in wire-bottom cages that enable the collection of food spillage, under 12:12h dark/light cycle (inverse light cycle, light off at 13:00 h) in controlled environmental conditions (22 ± 1°C and 70% humidity). Rats had free access to water and semisynthetic powder control diet for 1-week adaptation period in order to familiarize with the environment before being randomly assigned to their experimental groups.

2. Experimental Design

Two sequential experiments were run. Following the 1-week adaptation period, 48 rats in Experiment 1 (Low Phosphorus- LP diets) and 48 rats in Experiment 2 (High Phosphorus- HP diets), were randomly divided into different experimental groups (8

rats per group), each presented with their corresponding experimental diet containing various levels of P, and following either a sedentary (S) or an exercise (E) routine.

Experiment 1 (LP diets):

- Group 1 (Low P level): 0.1%P and Sedentary (0.1S)
- Group 2 (Low P level): 0.1%P and Exercise (0.1E)
- Group 3 (Marginally low P level): 0.2%P and Sedentary (0.2S)
- Group 4 (Marginally low P level): 0.2%P and Exercise (0.2E)
- Group 5 (Standard P level): 0.3%P and Sedentary (0.3S)
- Group 6 (Standard P level): 0.3%P and Exercise (0.3E)

Experiment 2 (HP diets):

- Group 1 (Standard P level): 0.3%P and Sedentary (0.3S)
- Group 2 (Standard P level): 0.3%P and Exercise (0.3E)
- Group 3 (Moderately high P level): 0.6%P and Sedentary (0.6S)
- Group 4 (Moderately high P level): 0.6%P and Exercise (0.6E)
- Group 5 (High P level): 1.2%P and Sedentary (1.2S)
- Group 6 (High P level): 1.2%P and Exercise (1.2E)

Rats were maintained on their respective diet *ad libitum* for the whole experimental period of 6 weeks, during which their body weight and body composition were measured once per week. Food intake was measured twice weekly, and averaged in order to calculate weekly changes. More specifically, food intake calculation was

done by subtracting the weight of the food at the current time point from its weight recorded on the previous time point, taking into account any possible food spillage.

Upon termination of the experiment, overnight-fasted rats were anesthetized with isoflurane (Forane; Abbott, Berks, UK) and blood was drawn from the superior vena cava and collected in EDTA tubes that were directly placed on ice. The rats were then sacrificed by severing their hearts. Immediately afterwards, tissue samples (liver, gastrocnemius muscle, epididymal fat pad) were excised, weighed, frozen in liquid nitrogen, and then stored at -80°C pending analysis. Blood samples were centrifuged at 2200xg for 15 minutes at 3°C, and aliquots of plasma were collected and stored at -80°C until further analysis.

3. *Experimental Diets*

The semisynthetic powder experimental diets (Table 3) were isocaloric, in which protein content accounted for 20% of the calories. The diets were all prepared using the same ingredients. Dried egg white was used as the main source of protein, because it supplies all essential amino acids and contains negligible amounts of P (mean±SD: 1.5±0.013 g/kg) (EPA reference). To control the level of P in the various diets, P-free mineral mix (AIN-93G mix without P) was used, and different proportions of potassium phosphate from Dyets Inc. (Pennsylvania, USA) were added accordingly. Potassium phosphate was used as the P source because potassium does not affect the growth of laboratory rodents [133, 134].

Under standard conditions, the recommended P content of rats' diet is 0.3% based on the AIN-93 recommendation for optimal growth of laboratory rats [135]. The

0.1%P is equivalent to 0.263mgP/Kcal, 0.2%P~0.526mgP/Kcal,

0.3%P~0.789mgP/Kcal, 0.6%P~1.579mgP/Kcal and 1.2%P~3.158mgP/Kcal (Table 3).

Table 4: Dietary composition of different semi-synthetic experimental diets following the AIN-93G recommendations, consumed by the different groups of rats in experiments 1 and 2.

The composition of the diet (g/kg)	**0.1P**	**0.2P**	**0.3P**	**0.6P**	**1.2P**
Egg white[1*]	225	225	225	225	225
Cornstarch	314.4	310	305.6	300	273
Sucrose	307.5	307.5	307.5	307.5	307.5
Corn oil	50	50	50	50	50
Cellulose[*]	55	55	55	55	55
Mineral mix[2*]	35	35	35	35	35
Vitamin mix[3*]	10	10	10	10	10
Potassium phosphate[4*]	3.1	7.5	11.9	18.2	44.5
Calculated phosphorus content of the diets					
Phosphorus (%)	0.1	0.2	0.3	0.6	1.2
Phosphorus (mg/kcal)	0.263	0.526	0.789	1.579	3.158

0.1P: 0.1% phosphorus, 0.2P: 0.2% phosphorus, 0.3P: 0.3% phosphorus, 0.6P: 0.6% phosphorus, 1.2P: 1.2% phosphorus

[1*]The diets contained 20% protein coming exclusively from egg white

[2*]Phosphorus-free" mineral mix (AIN-93G mineral mix phosphorus-free, used as 35 g/kg of diet)

[3*]Vitamin mix (AIN-93VX vitamin mix, used as 10 g/kg of diet)

[4*]Potassium phosphate KH_2PO_4, molecular weight 136 g/mol of which phosphorus 31 g/mol

*Obtained from Dyets Inc., Bethlehem, Pennsylvania, USA

4. Structured Exercise

For the exercising groups, a motor-driven rodent treadmill apparatus (4-lane Rat Model locally manufactured for experimental purposes) was used for the implementation of structured E training sessions. Initially, during a 3-day acclimation period, the physically exercised groups underwent a low-intensity running protocol for 10 min/day at 10m/min. Thereafter, during the experimental period, 30 minutes of moderate-intensity E protocol (equivalent to 60% VO_2 max) was implemented 5 days a week for a total of 6 weeks. Wherein, exercising rats were started at 10m/min for 5 minutes, and speed was increased up to 14m/min for another 5 minutes, then kept constant at 18m/min [136] to reach a total of 30 minutes of aerobic E per session. To encourage the rats to run forward during the whole training session, a mild electrical stimulus (0.1mA at 90 volts and 25 Hz) was delivered [137]. Correspondingly, sedentary rats were placed on the treadmill in static mode for the same period of time.

B. Measurement of Body Weight and Composition

Body weight and body composition analysis were measured once per week till the end of the experiment. For non-invasive measurement of body composition, rats were placed in a whole-body composition analyzer (Minispec LF110; Brucker, MA, USA) based on nuclear magnetic resonance (NMR) technology, yielding measurements for fat tissue (fat mass-FM), lean tissue (Lean Body Mass-LBM) and free fluid in living rats. The NMR technique offers a fast in vivo method of body composition analysis which is well-correlated to but with greater overall precision than whole-body chemical carcass composition analysis [138].

C. Assessment of Energy Balance Parameters

As previously explained, food intake was monitored twice weekly and averaged in order to calculate weekly food intake in grams. Then, weekly energy intake was calculated by multiplying food intake by 3.8 Kcal/g, and subsequently summed up to obtain total energy intake (EI). Total energy expenditure (TEEx) was estimated using an energy balance technique, which is highly correlated with indirect calorimetry. It was specifically calculated as the difference between total EI over the whole experimental period and the change in energy stores (ΔE_{st}) (energy accumulated in fat and lean mass gain) [139]. More specifically, body composition parametes (FM and LBM) were recorded at the start and the end of the experiment (in grams), and the mass gains were computed in terms of accumulated energy stores (in Kilocalories-Kcal) i.e the sum of the change in fat stores (ΔFat_{st}) and the change in LBM stores (ΔLBM_{st}). The caloric equivalent of FM was assigned 9.4 Kcal/g and LBM 1.0 Kcal/g [140, 141]. Below are the detailed calculations of TEEx:

$TEEx = EI - (\Delta Fat_{st} + \Delta LBM_{st})$

Where, $\Delta Fat_{st} = (FM_{Final} - FM_{Initial}) \times 9.4$ Kcal/g

And, $\Delta LBM_{st} = (LBM_{Final} - LBM_{Initial}) \times 1.0$ Kcal/g

Consequently, the accumulated energy stores aka the change in energy stores, i.e. ΔE_{st}, is the sum of ΔFat_{st} and ΔLBM_{st}, which are equivalent to the total Kcal gain in body tissues over the whole 6-week experimental period. Percent Fat Stores ($\%Fat_{st}$) represents the proportion of the gain in body stores attributed to fat accumulation, and

the Percent LBM Stores ($\%LBM_{st}$) denotes the proportion of the gain in body stores attributed to LBM accumulation. The specific calculations are as follows:

$$\Delta E_{st} = \Delta Fat_{st} + \Delta LBM_{st}$$

$$\%Fat_{st} = (\Delta Fat_{st} \div \Delta E_{st}) \times 100$$

$$\%LBM_{st} = (\Delta LBM_{st} \div \Delta E_{st}) \times 100$$

Finally, Energy efficiency (EEf) is determined as the amount energy stored per 100 kcal consumed.

$$EEf = \text{proportion of } \Delta E_{st} / 100 \text{ kcal ingested} = (\Delta E_{st} \times 100 \text{ Kcal}) \div EI$$

D. Determination of Plasma Metabolites

In order to obtain a complete understanding of the effect of P and/or E on plasma specific parameters, the following were analyzed. Fasting plasma glucose, total cholesterol (TC), high density lipoprotein (HDL) cholesterol, triglycerides (TG), total P, plasma urea nitrogen (PUN) and plasma creatinine were measured with an enzymatic colorimetric method on the Vitros 350 Chemistry System (Ortho-Clinical Diagnostics; Raritan, NJ, USA).

The plasma insulin concentration was determined by an enzyme immunoassay using the Rat/Mouse Insulin ELISA Kit (EZRMI-13K; EMD Millipore Corporation, Billerica, MA, USA). The plasma parathyroid hormone (PTH) and Fibroblast Growth Factor-23 (FGF-23) were quantitatively measured by a sandwich enzyme immunoassay technique using the Rat PTH and Rat FGF23 ELISA Kits respectively (My BioSource, Thermo Fisher Scientific, California, USA).

E. Hepatic Fat Extraction

Hepatic fat extraction is performed for the purpose of assessing the proportion of fat in the livers of the experimental animals. Liver samples were freeze-dried for 48 hours (2.5 Liter Bench top Freeze-Dry System, LABCONCO). Approximately 1g of the dried liver samples were placed in moisture free sealable filter bags and accurately weighed prior to fat extraction. Fat extraction was implemented for 40 minutes per run using petroleum ether solvent (BP 400-600C) in Ankom XT10 (ANKOM Technology, Macedon, NY, USA).

Fat weight in liver was calculated as the weight difference of the samples before and after fat extraction; and the percentage of fat in liver and defatted weight were inferred.

F. Analysis of Protein Expression Levels of Translational Initiation Factors

To check whether the level of dietary P, E or their combination affect the mechanism of protein synthesis at the molecular level, protein expression of signaling proteins that affect translation initiation in gastrocnemius muscle and liver was determined by Western Blot. Namely, total and phosphorylated levels of mTOR and AMPKα, and downstream translation initiation factors S6K1 and 4E-BP1, and eIF4E were assessed. Briefly, the procedure starts with measurement of protein concentration in tissues, gel electrophoresis, immunoblotting, detection and imaging, and finally analyzing the amount of protein expression. The detailed methodology is described below.

Rat liver and gastrocnemius muscle tissues (n=8) were lysed using RIPA buffer containing 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 300 mM sodium chloride, 100 mM EDTA, 100 mM Tris-hydrochloride, 1% Tergitol (NP40), 1% of protease and phosphatase inhibitors and 1mM phenylmethylsulfonyl fluoride. The lysates were centrifuged at 13,600 rpm for 30 minutes at 4°C. The total protein concentration in the supernatants was measured by the Lowry Protein Assay [142].

For immunoblotting, 20-40 μg of proteins were separated by electrophoresis (Bio-Rad Laboratory, CA, USA) on either a 10%, 12% or 15% polyacrylamide gel (depending on MW of protein of interest), and subsequently transferred to nitrocellulose membranes (Bio-Rad Laboratory, CA, USA). The blots were then blocked with 5% BSA in Tris-buffered saline for 1 hour at room temperature, and then incubated overnight at 4°C at a dilution of 1:1000 with their respective primary polyclonal antibodies. Antibodies against total mTOR and phosphorylated mTOR (Ser 2448), total S6K1 and phosphorylated S6K1 (Thr 389), total 4E-BP1 and phosphorylated 4E-BP1 (Thr 37/46), total AMPKα and phosphorylated AMPKα (Thr 172) and total eIF4E and phosphorylated eIF4E (Ser 209) were purchased from Cell Signaling (Danvers, MA, USA). The primary antibodies were detected using a secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG; Cell Signaling; 1:1000), in which membranes were incubated for 1 hour at room temperature. The membranes were rinsed with TBST before and after the incubation with the secondary antibody. Finally, chemiluminescent reagents were used to facilitate the detection of protein bands, which were then visualized by enhanced chemiluminescence. Densitometric analysis was performed using Image J software. Phosphorylation levels were determined by the expression of phosphorylated protein divided by the expression of total protein.

G. Statistical Analysis

The required number of rats (n=8) was based on previously determined weight gain data (6.0 ±0.95 g/d) and assuming a 25% difference in the mean with 90% statistical power and 5% significance.

Results were expressed as mean ± SD for all values. Except for the western blot data, the statistical analysis was performed using the SPSS Statistics 25.0 software (IBM Corp.). In both experiments, multiple-way analysis of variance ANOVA (general linear model, GLM), with time, P and E as well as their interactions was used to analyse the results throughout the 6-week experimental period. For the variables that were not measured on weekly basis, GLM, with P and E as well as their interactions was applied.

The western blot statistical analysis was done using GraphPad Prism 8. Statistical significance was determined by two-way ANOVA Fisher's post-test for multiple comparisons. P-value < 0.05 is considered significant and is indicated by (*), and a trend towards significance at P-value < 0.10.

CHAPTER IV

RESULTS

A. Body Weight Gain and Composition Variations

In the LP diets experiment (Experiment 1), body weight increased gradually with time, though differences were observed between the various groups. In which, body weight was increased with P content of the diet ($p<0.001$), while a significantly lower body weight was displayed in the exercising groups ($p=0.001$) (Figure 3A). At the body composition level, significant differences were found according to P and E. % body fat in the 0.3%P sedentary group was higher than that of the 0.1%P and 0.2%P sedentary groups ($p<0.001$) (Figure 3B). Additionally, the groups who were regularly exercising accumulated a lower % body fat throughout the experimental period ($p=0.005$). Epidydimal adipose tissue weight was also found to be significantly lower in the exercising groups as compared to their sedentary counterparts ($p=0.045$) (Table 5).

As for LBM accretion, an increasing trend was observed in all the groups over the 6-week experimental period ($p<0.001$). Additionally, the percentage of LBM was respondent to P level ($p<0.001$) and E ($p=0.013$) (Figure 3C). However, there was no change in gastrocnemius muscle weight among the 6 groups (Table 5).

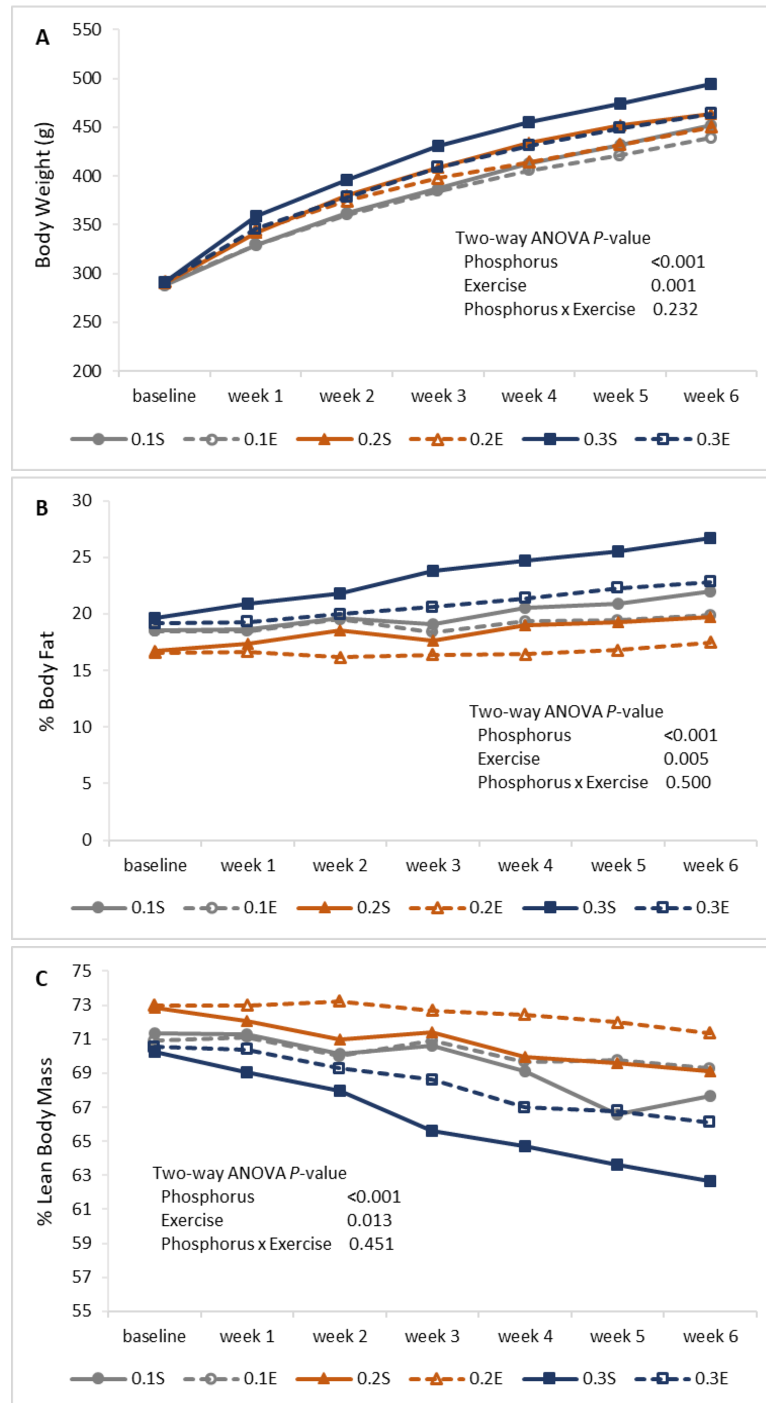

Figure 3. Experiment 1- Effect of phosphorus and exercise on body weight and composition measures. Weekly body weight gain in grams (A), percent body fat (B) and percent lean body mass (C), of the six groups of rats over the 6-week experimental period.

Group 0.1S: 0.1% P and sedentary; Group 0.1E: 0.1% P and exercise; Group 0.2S: 0.2% P and sedentary; Group 0.2E: 0.2% P and exercise; Group 0.3S: 0.3% P and sedentary; Group 0.3E: 0.3% P and exercise.

Data are expressed as the mean ± SD of all values. A multiple-way ANOVA (general linear model) was performed with time, phosphorus and exercise as factors. Significance was set at *P*-value<0.05.

Table 5. Experiment 1- Effect of phosphorus and exercise on weights of epididymal adipose tissue and gastrocnemius muscle in the six group of rats.

	0.1S	0.1E	0.2S	0.2E	0.3S	0.3E	*P*-value		
							P	E	PxE
Epididymal adipose tissue weight (g/100gBW)	2.25±1.05	1.46±0.40	2.28±1.14	1.72±0.32	2.05±0.93	1.87±0.91	0.882	0.045	0.603
Gastrocnemius muscle weight (g/100gBW)	0.62±0.08	0.62±0.04	0.62±0.15	0.68±0.07	0.61±0.06	0.61±0.07	0.387	0.471	0.659

P, phosphorus; E, exercise
Group 0.1S: 0.1% P and sedentary; Group 0.1E: 0.1% P and exercise; Group 0.2S: 0.2% P and sedentary; Group 0.2E: 0.2% P and exercise; Group 0.3S: 0.3% P and sedentary; Group 0.3E: 0.3% P and exercise.
Data are expressed as the mean ± SD of all values. A two-way ANOVA was performed with phosphorus and exercise as factors. Significance was set at P-value<0.05.

Looking at the quantity and type of energy stores accumulated throughout the study period (Table 6), it is clear that the ΔE_{st} of the 0.3%P sedentary group was the highest, differing by around 300Kcal as compared to the other groups. While ΔE_{st} in the exercising groups was always lower than the sedentary groups, though failed to reach statistical significance ($p=0.063$). The ΔFat_{st} was significantly altered by P level ($p=0.043$) and trended towards a lower level with E ($p=0.074$), yet the ΔLBM_{st} was similar according to both P ($p=0.159$) and E ($p=0.544$) (Table 5).

Hence, the differences in ΔE_{st} between the groups was mainly related to changes in body fat content, as the amount of LBM gained was similar between the groups. Subsequently, the lower ΔFat_{st} observed in the exercising groups resulted in a higher proportion of gain in body stores coming from LBM accumulation (% LBM_{st}) among these groups.

Table 6. Experiment 1- Effect of phosphorus and exercise on body energy storage in the six groups of rats over the 6-week experimental period.

	0.1S	0.1E	0.2S	0.2E	0.3S	0.3E	*P*-value		
							P	E	PxE
Est Initial (Kcal)	714.40±172.80	710.23±127.86	668.61±111.15	671.78±107.69	744.02±149.37	732.64±117.86	0.353	0.915	0.988
Est Final (Kcal)	1265.34±429.21	1144.02±398.26	1190.15±286.73	1072.70±292.03	1569.87±502.68	1322.14±399.13	0.071	0.159	0.867
ΔEst (Kcal)	550.94±261.42	433.79±290.83	521.54±215.75	400.91±230.79	825.85±392.85	589.51±294.03	0.039	0.063	0.800
Fatst Initial (Kcal)	509.35±171.32	504.56±131.06	457.94±104.81	458.87±103.55	540.06±150.20	527.71±113.21	0.269	0.887	0.990
Fatst Final (Kcal)	961.77±430.02	841.98±404.37	870.49±290.00	752.80±291.66	1262.23±528.86	1017.48±404.42	0.068	0.171	0.877
ΔFatst (Kcal)	452.42±263.76	337.42±289.51	412.55±222.31	293.94±230.70	722.16±414.61	489.76±300.80	0.043	0.074	0.814
Fatst %	78.66±9.48	71.88±11.76	75.74±9.91	66.09±15.94	83.48±10.49	79.85±8.75	0.034	0.047	0.755
LBMst Initial (Kcal)	205.05±15.26	205.67±20.90	210.67±12.12	212.92±8.72	203.96±10.75	204.93±9.17	0.255	0.744	0.984
LBMst Final (Kcal)	303.57±18.66	302.04±13.33	319.66±21.62	319.89±14.14	307.6±29.0	304.67±9.05	0.034	0.794	0.971
ΔLBMst (Kcal)	98.52±11.40	96.37±12.23	108.99±14.81	106.98±8.70	103.69±23.60	99.74±12.67	0.159	0.544	0.980
LBMst %	21.34±9.48	28.12±11.76	24.26±9.91	33.91±15.94	16.51±10.49	20.15±8.75	0.034	0.047	0.755

P, phosphorus; E, exercise; E_{st}, Energy Stores; Fat_{st}, Fat Stores; LBM_{st}, Lean Body Mass Stores
Group 0.1S: 0.1% P and sedentary; Group 0.1E: 0.1% P and exercise; Group 0.2S: 0.2% P and sedentary; Group 0.2E: 0.2% P and exercise; Group 0.3S: 0.3% P and sedentary; Group 0.3E: 0.3% P and exercise. Data are expressed as the mean ± SD of all values. A two-way ANOVA was performed with phosphorus and exercise as factors. Significance was set at P-value<0.05.

In the HP experiment (Experiment 2), body weight was significantly different according to P, E and their interaction. In the sedentary groups, the 0.3%P showed a higher body weight than that of the other groups. As expected, the groups who were consistently exercising had a significantly lower body weight than the non-exercising groups, and the magnitude of the difference in weight being greatest among the 0.3%P group (E vs. S) ($p<0.001$) (Figure 4A). % body fat was also significantly influenced by P, E and the combined P x E factors, and the pattern of change was very similar to that of body weight, noting a lowest % body fat in the 1.2%P exercising group ($p=0.01$) (Figure 4B). The epidydimal adipose tissue weight showed a clear tendency towards a lower level in the exercising groups, however it didn't reach significance ($p=0.150$) (Table 6).

With respect to LBM, although all groups increased their LBM with time ($p<0.001$), the sedentary groups displayed a lower %LBM than their exercising counterparts and the extent of this difference was greatest among the 0.3% and 1.2%P groups (S vs. E) ($p<0.001$) (Figure 4C). A combined P and E factors significantly influenced mean %LBM ($p=0.009$). Notably, the 1.2%P exercising group best maintained their %LBM throughout the 6-week experimental period (71.02% initial %LBM vs. 70.68% final %LBM). However, the weight of gastrocnemius muscle was shown to be lower among the exercising groups as compared to the sedentary ones (Table 7).

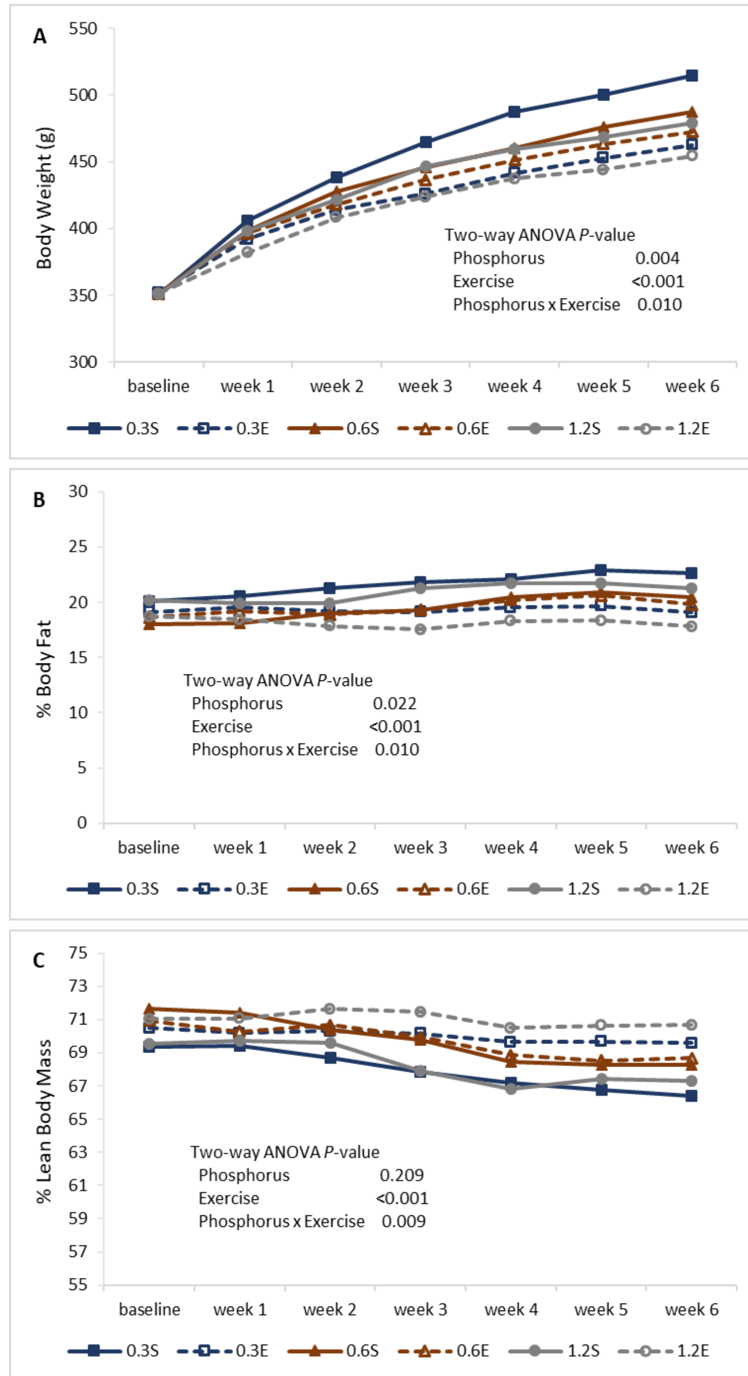

Figure 4. Experiment 2- Effect of phosphorus and exercise on body weight and composition measures. Weekly body weight gain in grams (A), percent body fat (B) and percent lean body mass (C), of the six groups of rats over the 6-week experimental period.
Group 0.3S: 0.3% P and sedentary; Group 0.3E: 0.3% P and exercise; Group 0.6S: 0.6% P and sedentary; Group 0.6E: 0.6% P and exercise; Group 1.2S: 1.2% P and sedentary; Group 1.2E: 1.2% P and exercise.
Data are expressed as the mean ± SD of all values. A multiple-way ANOVA (general linear model) was performed with time, phosphorus and exercise as factors. Significance was set at *P*-value<0.05.

Table 7. Experiment 2- Effect of phosphorus and exercise on weights of epididymal adipose tissue and gastrocnemius muscle in the six group of rats.

	0.3S	0.3E	0.6S	0.6E	1.2S	1.2E	*P*-value		
							P	E	PxE
Epididymal adipose tissue weight (g/100gBW)	1.88±0.75	1.65±0.38	1.79±0.43	1.61±0.31	1.75±0.28	1.44±0.43	0.738	0.150	0.433
Gastrocnemius muscle weight (g/100gBW)	0.74±0.13	0.69±0.04	0.70±0.07	0.68±0.06	0.69±0.04	0.60±0.13	0.098	0.035	0.533

P, phosphorus; E, exercise
Group 0.3S: 0.3% P and sedentary; Group 0.3E: 0.3% P and exercise; Group 0.6S: 0.6% P and sedentary; Group 0.6E: 0.6% P and exercise; Group 1.2S: 1.2% P and sedentary; Group 1.2E: 1.2% P and exercise.
Data are expressed as the mean ± SD of all values. A two-way ANOVA was performed with phosphorus and exercise as factors. Significance was set at P-value<0.05.

At the level of energy stores (Table 8), it was evident that the exercising groups had significantly lower ΔE_{st} as compared to the sedentary groups ($p<0.001$). The differences in the accumulation of energy stores were mainly attributed to lower gains in Fat_{st}, though LBM_{st} gains was also reduced but to a lesser extent. There was also a pronounced trend towards a reduction in ΔE_{st} gain as the level of P increased in the diet ($p=0.091$). Noteworthy is the result that the proportion of accumulated energy balance coming for LBM ($\%LBM_{st}$) were significantly higher in all the exercising groups ($p=0.004$).

Table 8. Experiment 2- Effect of phosphorus and exercise on body energy storage in the six groups of rats over the 6-week experimental period.

	0.3S	0.3E	0.6S	0.6E	1.2S	1.2E	*P*-value		
							P	E	PxE
Est Initial (Kcal)	917.53±223.35	886.75±162.82	847.73±87.22	866.33±119.03	911.52±114.32	869.36±132.55	0.667	0.671	0.824
Est Final (Kcal)	1456.41±417.74	1155.04±196.98	1271.59±178.06	1205.89±158.59	1285.17±144.31	1090.08±217.55	0.378	0.009	0.381
ΔEst (Kcal)	465.00±120.80	268.29±79.84	423.86±103.50	339.56±136.71	373.65±110.08	220.72±118.50	0.091	<0.001	0.384
Fatst Initial (Kcal)	675.04±212.65	639.20±159.76	595.95±78.37	617.16±121.38	667.57±115.16	620.09±137.76	0.591	0.620	0.771
Fatst Final (Kcal)	1116.61±420.31	833.27±206.09	939.46±162.11	881.69±163.29	962.94±113.19	769.57±215.96	0.434	0.013	0.407
ΔFatst (Kcal)	366.10±132.70	194.07±84.76	343.51±96.31	264.52±138.04	295.37±97.39	149.48±110.68	0.116	<0.001	0.489
Fatst %	77.34±7.53	68.72±16.26	80.63±3.34	74.76±10.81	78.28±3.86	60.77±20.17	0.175	0.004	0.376
LBMst Initial (Kcal)	242.49±17.64	247.56±11.01	251.78±10.76	249.16±10.54	243.94±11.04	249.27±10.37	0.435	0.465	0.581
LBMst Final (Kcal)	339.80±25.69	321.77±16.70	332.13±22.58	324.20±11.18	322.23±19.25	320.51±14.95	0.360	0.100	0.479
ΔLBMst (Kcal)	98.82±22.37	74.21±20.92	80.35±13.72	75.04±13.55	78.28±15.09	71.24±16.16	0.156	0.018	0.240
LBMst %	22.66±7.53	31.28±16.26	19.37±3.34	25.24±10.81	21.71±3.86	39.23±20.17	0.175	0.004	0.376

P, phosphorus; E, exercise; Est, Energy Stores; Fatst, Fat Stores; LBMst, Lean Body Mass Stores
Group 0.3S: 0.3% P and sedentary; Group 0.3E: 0.3% P and exercise; Group 0.6S: 0.6% P and sedentary; Group 0.6E: 0.6% P and exercise; Group 1.2S: 1.2% P and sedentary; Group 1.2E: 1.2% P and exercise.
Data are expressed as the mean ± SD of all values. A two-way ANOVA was performed with phosphorus and exercise as factors. Significance was set at P-value<0.05.

B. Energy Balance Outcomes

In the LP diets experiment (Experiment 1), EI was significantly different according to P ($p<0.001$), in which the 0.3%P groups had the highest intake. While, EI of the exercised rats was significantly lower than that of the sedentary rats ($p=0.009$) (Figure 5A). Surprisingly, the TEEx was not found to be affected by neither P nor E.

Changes in EEf were similar to that of EI, in which EEf was increased as the P content of the diet increased, and this was highly pronounced in the 0.3%P groups ($p<0.001$), whereas EEf decreased among the exercising groups ($p=0.001$) (Figure 5B). In line with the trajectory of EEf, the ΔE_{st} were altered between groups with different P levels, demonstrating reduced accumulation of body stores with lower dietary P intake as compared to the standard 0.3%P group ($p=0.039$).

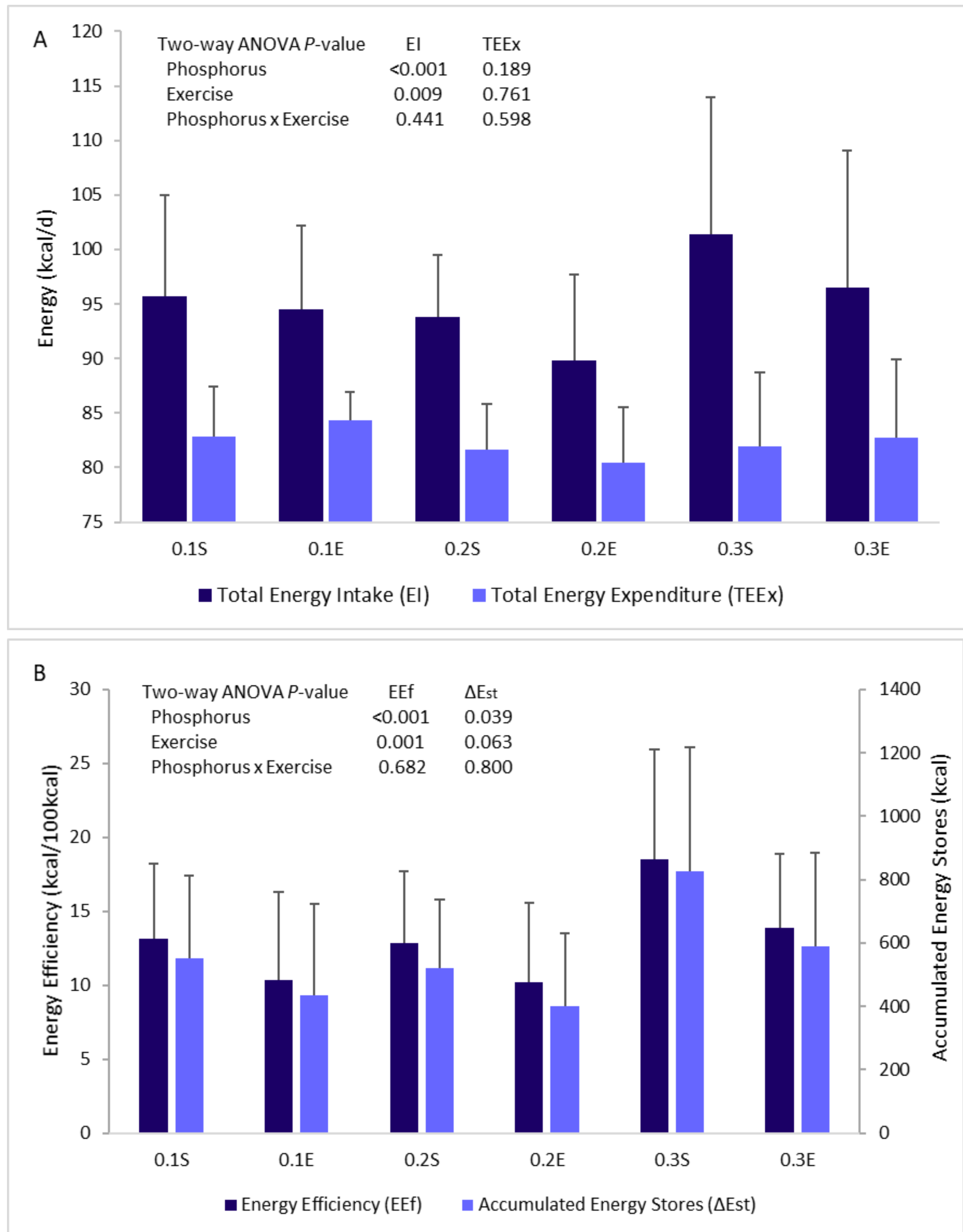

Figure 5. Experiment 1- Effect of phosphorus and exercise on total energy intake (EI) (A), total energy expenditure (TEEx) (A), energy efficiency (EEf) (B), and accumulated energy stores (ΔEst) (B) in the six group of rats over the 6-week experimental period. Group 0.1S: 0.1% P and sedentary; Group 0.1E: 0.1% P and exercise; Group 0.2S: 0.2% P and sedentary; Group 0.2E: 0.2% P and exercise; Group 0.3S: 0.3% P and sedentary; Group 0.3E: 0.3% P and exercise.
Data are expressed as the mean ± SD of all values. A multiple-way ANOVA (general linear model) was performed with time, phosphorus and exercise as factors. Significance was set at P-value<0.05.

In the HP diets experiment (Experiment 2), EI significantly varied between the various P levels ($p=0.001$) (Figure 6A). Additionally, P x E interaction was significantly different between all of the 6 groups ($p=0.002$), demonstrating an incremental increase in intake in the exercising groups of both 0.6%P and 1.2%P as compared to their sedentary counterparts. In contrast to LP diets experiment, TEEx significantly increased with increasing P levels ($p<0.001$) and supervised E routine ($p<0.001$). Additionally, TEEx responded to the combined effect of these factors, showing a greater EE in the 1.2%P sedentary group (81.17 Kcal/d) as compared to the 0.3%P (74.34 Kcal/d) ($p=0.05$) and 0.6%P (76.24 Kcal/d) ($p=0.034$) sedentary groups.

EEf was also significantly influenced by P ($p=0.010$) and E ($p<0.001$) (Figure 6B). Notably, the EEf was altered by the joint P x E factors, where it was significantly lower in the 1.2%P groups as compared to the 0.3%P groups ($p=0.047$). A similar course of change in ΔE_{st} was documented, where incremental levels of dietary P resulted in a decreasing trend in ΔE_{st}, which were significantly reduced in response to physical E.

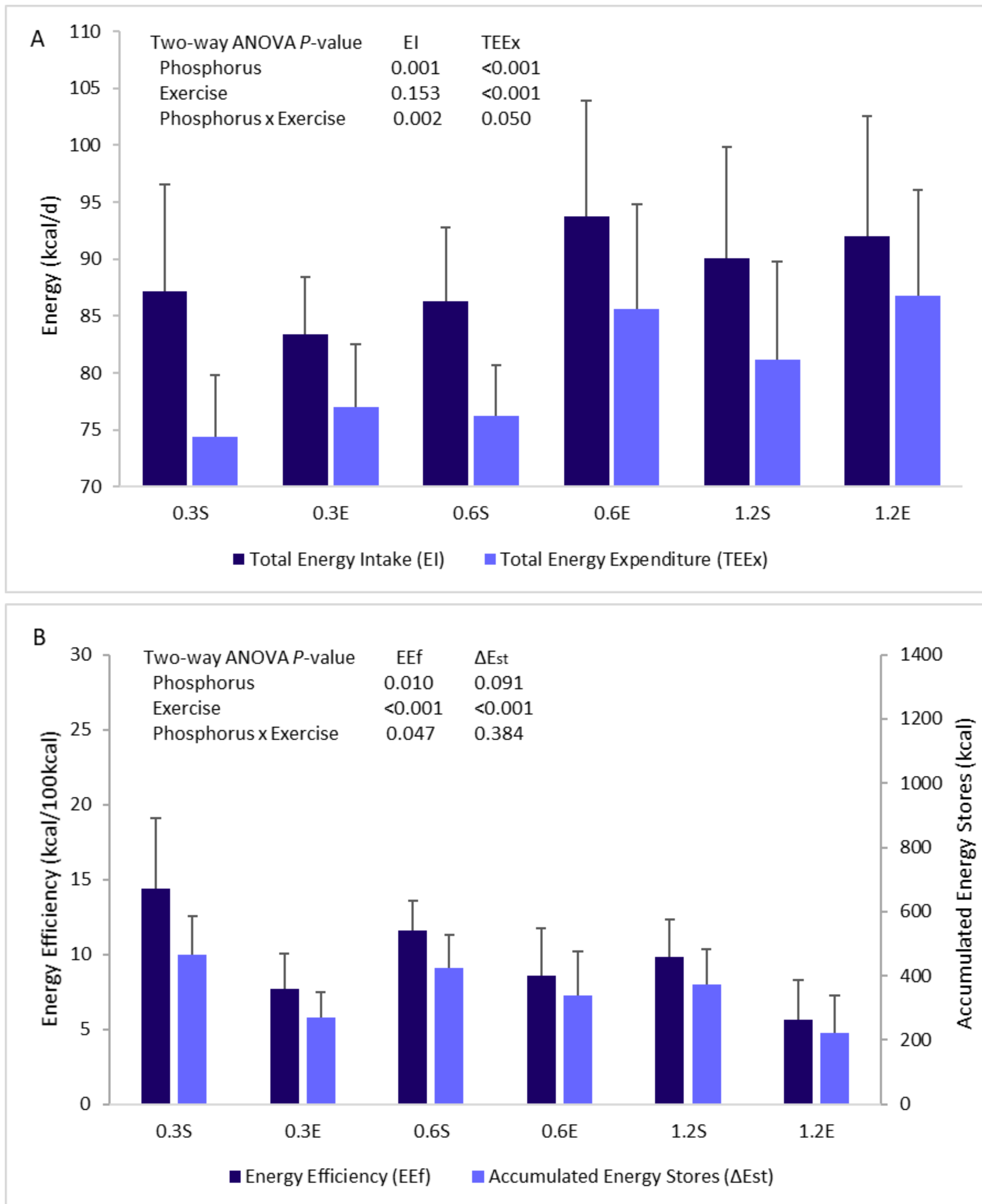

Figure 6. Experiment 2- Effect of phosphorus and exercise on total energy intake (EI) (A), total energy expenditure (TEEx) (A), energy efficiency (EEf) (B), and accumulated energy stores (ΔEst) (B) in the six group of rats over the 6-week experimental period. Group 0.3S: 0.3% P and sedentary; Group 0.3E: 0.3% P and exercise; Group 0.6S: 0.6% P and sedentary; Group 0.6E: 0.6% P and exercise; Group 1.2S: 1.2% P and sedentary; Group 1.2E: 1.2% P and exercise.
Data are expressed as the mean ± SD of all values. A multiple-way ANOVA (general linear model) was performed with time, phosphorus and exercise as factors. Significance was set at P-value<0.05.

C. Plasma Analysis

In the LP diets experiment (Experiment 1), plasma glucose and insulin levels were found to be similar among the groups (Table 9). Total cholesterol concentrations was significantly lower in the exercising groups as compared to their sedentary counterparts ($p=0.044$). Plasma TG showed a similar trend though failed to reach significance ($p=0.072$). Plasma P was not affected by P content of the diet, while PUN was reduced with increased P content of the diet ($p=0.004$). No changes in plasma PTH or FGF-23 were detected between the different groups.

In the HP experiment (Experiment 2), plasma glucose and insulin were also consistent between the various groups (Table 10). Both plasma total cholesterol ($p=0.001$) and TG ($p=0.001$) were significantly reduced in the groups who exercised regularly. Increased P content of the diet was associated with a reduction in plasma P ($p<0.001$) and an increase in PUN ($p=0.001$), which was also significantly affected by the joint P x E factors ($p=0.016$). Serum PTH and FGF-23 levels remain unchanged in response to increasing dietary P level.

Table 9. Experiment 1- Effect of phosphorus and exercise on blood metabolites in the six groups of rats.

	0.1S	0.1E	0.2S	0.2E	0.3S	0.3E	*P*-value		
							P	E	PxE
Glucose (mg/dL)	156.13±20.18	141.13±18.13	150.63±10.06	145.13±22.49	159.75±22.59	153.38±21.78	0.393	0.123	0.754
Insulin (ng/mL)	1.84±0.93	1.74±1.53	2.08±0.69	1.55±0.81	3.34±2.46	1.95±0.51	0.178	0.113	0.444
Total-cholesterol (mg/dL)	96.38±20.91	83.13±18.19	96.3±43.5	75.88±12.91	102.40±30.7	88.38±21.11	0.609	0.044	0.916
HDL-cholesterol (mg/dL)	54.75±9.13	52.13±9.89	50.25±6.50	50.50±8.62	53.25±7.83	52.25±12.21	0.617	0.674	0.907
Triglycerides (mg/dL)	93.30±54.30	76.40±55.40	63.25±12.49	42.75±22.15	82.4±38.2	57.30±32.30	0.082	0.072	0.956
Phosphorus (mg/dL)	8.64±0.83	9.25±1.21	8.69±0.96	8.65±0.98	8.35±0.84	8.22±1.15	0.215	0.568	0.557
Plasma urea nitrogen (mmol/L)	16.88±3.09	18.13±2.95	15.50±2.33	15.38±2.39	15.25±1.67	13.75±2.05	0.004	0.861	0.298
Creatinine (µmol/L)	0.34±0.05	0.36±0.05	0.34±0.09	0.28±0.04	0.32±0.04	0.30±0.07	0.268	0.385	0.368
PTH (pg/ml)	534.7±100.2	573.5±84.4	483.5±108.7	493.1±39.2	510.7±71.8	552.1±56.1	0.075	0.204	0.826
FGF-23 (pg/ml)	16.95±3.43	8.54±4.49	11.41±3.78	12.04±8.00	13.23±5.79	11.11±6.88	0.902	0.085	0.135

P, phosphorus; E, Exercise; HDL, High Density Lipoprotein; PTH, Parathyroid Hormone; FGF-23, Fibroblast Growth Factor-23
Group 0.1S: 0.1% P and sedentary; Group 0.1E: 0.1% P and exercise; Group 0.2S: 0.2% P and sedentary; Group 0.2E: 0.2% P and exercise; Group 0.3S: 0.3% P and sedentary; Group 0.3E: 0.3% P and exercise.
Data are expressed as the mean ± SD of all values. A two-way ANOVA was performed with phosphorus and exercise as factors.
Significance was set at P-value<0.05.

Table 10. Experiment 2- Effect of phosphorus and exercise on blood metabolites in the six groups of rats.

	0.3S	0.3E	0.6S	0.6E	1.2S	1.2E	*P*-value		
							P	E	PxE
Glucose (mg/dL)	167.00±11.39	160.50±8.94	162.00±7.17	165.63±12.64	179.75±19.67	165.38±16.60	0.115	0.146	0.177
Insulin (ng/mL)	1.97±0.58	1.71±0.65	1.84±0.67	1.99±0.75	2.20±1.13	1.40±0.61	0.899	0.132	0.156
Total-cholesterol (mg/dL)	94.75±19.23	76.13±11.05	86.38±12.86	77.50±7.07	89.25±11.51	76.13±12.51	0.723	0.001	0.567
HDL-cholesterol (mg/dL)	49.63±13.85	52.38±5.71	58.00±6.39	53.25±5.09	57.88±6.75	51.88±8.01	0.240	0.265	0.272
Triglycerides (mg/dL)	99.00±44.90	62.50±21.18	104.1±36.8	74.50±31.90	90.75±26.56	60.50±29.40	0.494	0.001	0.947
Phosphorus (mg/dL)	8.54±0.48	8.36±0.90	7.84±1.13	6.86±0.52	6.15±0.54	6.10±0.49	<0.001	0.061	0.156
Plasma urea nitrogen (mmol/L)	14.50±1.51	15.50±0.93	16.00±2.00	14.75±1.91	15.75±1.98	18.50±2.67	0.001	0.369	0.016
Creatinine (µmol/L)	0.41±0.06	0.39±0.06	0.41±0.06	0.36±0.07	0.35±0.05	0.35±0.05	0.075	0.174	0.534
PTH (pg/ml)	505.1±72.5	482.2±99.8	460.3±47.9	495.3±67.7	446.9±39.3	503.7±56.7	0.704	0.241	0.232
FGF-23 (pg/ml)	10.70±9.15	9.89±4.03	8.55±2.53	12.93±8.97	12.47±3.08	6.99±2.61	0.899	0.726	0.095

P, phosphorus; E, Exercise; HDL, High Density Lipoprotein; PTH, Parathyroid Hormone; FGF-23, Fibroblast Growth Factor-23
Group 0.3S: 0.3% P and sedentary; Group 0.3E: 0.3% P and exercise; Group 0.6S: 0.6% P and sedentary; Group 0.6E: 0.6% P and exercise; Group 1.2S: 1.2% P and sedentary; Group 1.2E: 1.2% P and exercise.
Data are expressed as the mean ± SD of all values. A two-way ANOVA was performed with phosphorus and exercise as factors. Significance was set at P-value<0.05.

D. Hepatic Composition Evaluation

In the LP diets experiment (Experiment 1), liver weight was unchanged among the groups, as was the percentage of fat in the liver (Table 11). Similarly, the defatted liver, which represents the protein as mineral mass and considered fairly constant, showed no difference.

In the HP diets experiment (Experiment 2), liver weight was significantly lower in the exercising groups as compared to the sedentary groups (p=0.007) (Table 12). Liver fat % was also markedly reduced in response to E (p=0.007). The defatted liver weight was significantly affected by dietary P levels, showing the lowest values at the 1.2%P (p=0.013).

Table 11. Experiment 1- Effect of phosphorus and exercise on liver weight, liver fat and defatted percentages in the six group of rats.

	0.1S	0.1E	0.2S	0.2E	0.3S	0.3E	*P*-value		
							P	E	PxE
Liver weight (g/100gBW)	3.43±0.75	3.28±0.81	3.40±0.37	3.28±0.48	3.84±0.64	3.48±0.56	0.266	0.251	0.831
Liver water weight (%)	68.11±0.47	68.11±0.46	68.81±0.77	68.63±0.48	68.88±0.28	68.64±0.96	0.345	0.044	0.409
Liver dry weight (%)	31.89±0.47	31.19±0.46	31.37±0.77	31.1 ±0.48	31.36±0.28	31.21±0.96	0.345	0.044	0.409
Liver fat in dry weight (%)	9.13±4.33	8.53±2.23	9.99±4.72	8.61±2.78	11.04±2.51	11.42±2.68	0.111	0.583	0.758
Liver fat in wet weight (%)	2.92±1.39	2.66±0.68	3.16±1.53	2.67±0.84	3.47±0.81	3.58±0.92	0.134	0.502	0.737
Liver defatted (%)	22.77±4.16	22.67±2.49	21.38±4.23	22.52±3.07	20.32±2.38	19.79±2.26	0.064	0.857	0.748

P, phosphorus; E, exercise
Group 0.1S: 0.1% P and sedentary; Group 0.1E: 0.1% P and exercise; Group 0.2S: 0.2% P and sedentary; Group 0.2E: 0.2% P and exercise; Group 0.3S: 0.3% P and sedentary; Group 0.3E: 0.3% P and exercise.
Data are expressed as the mean ± SD of all values. A two-way ANOVA was performed with phosphorus and exercise as factors.
Significance was set at P-value<0.05.

Table 12. Experiment 2- Effect of phosphorus and exercise on liver weight, liver fat and defatted percentages in the six group of rats.

	0.3S	0.3E	0.6S	0.6E	1.2S	1.2E	*P*-value		
							P	E	PxE
Liver weight (g/100gBW)	3.01±0.57	2.63±0.27	2.96±0.34	2.72±0.19	2.96±0.34	2.67±0.41	0.978	0.007	0.876
Liver water weight (%)	65.29±1.78	67.53±0.68	67.88±0.37	68.72±1.00	67.87±0.86	68.59±1.08	<0.001	<0.001	0.090
Liver dry weight (%)	34.71±1.78	32.47±0.68	32.12±0.37	31.28±1.00	32.13±0.86	31.41±1.08	<0.001	<0.001	0.090
Liver fat in dry weight (%)	10.07± 2.46	6.78±2.53	7.15±2.97	6.97±2.20	10.66±3.84	7.82±2.89	0.107	0.015	0.263
Liver fat in wet weight (%)	3.53±1.01	2.21±0.87	2.30±0.96	2.19±0.73	3.45±1.33	2.46±0.94	0.096	0.007	0.214
Liver defatted (%)	24.64±1.27	25.69±2.17	24.97±3.04	24.31±2.05	21.47±3.23	23.60±2.83	0.013	0.257	0.299

P, phosphorus; E, exercise
Group 0.3S: 0.3% P and sedentary; Group 0.3E: 0.3% P and exercise; Group 0.6S: 0.6% P and sedentary; Group 0.6E: 0.6% P and exercise; Group 1.2S: 1.2% P and sedentary; Group 1.2E: 1.2% P and exercise.
Data are expressed as the mean ± SD of all values. A two-way ANOVA was performed with phosphorus and exercise as factors. Significance was set at P-value<0.05.

E. Protein Expression Levels of Translational Initiation Factors

In the LP diets experiment (Experiment 1), there was no difference in the phosphorylation of mTOR on its Ser_{2448} residue in liver among any of the experimental groups (Figure 7A). Similarly, there was no effect of P nor E on the phosphorylation of 4E-BP1 on its $Thr_{37/46}$ residue among the study groups (Figure 7B). Further, western blot results of p-S6K1 (Thr_{389}) on a limited number of liver samples have shown no trend of change between groups, hence, protein signaling pathway assessment was focused on the HP experimental groups (Experiment 2).

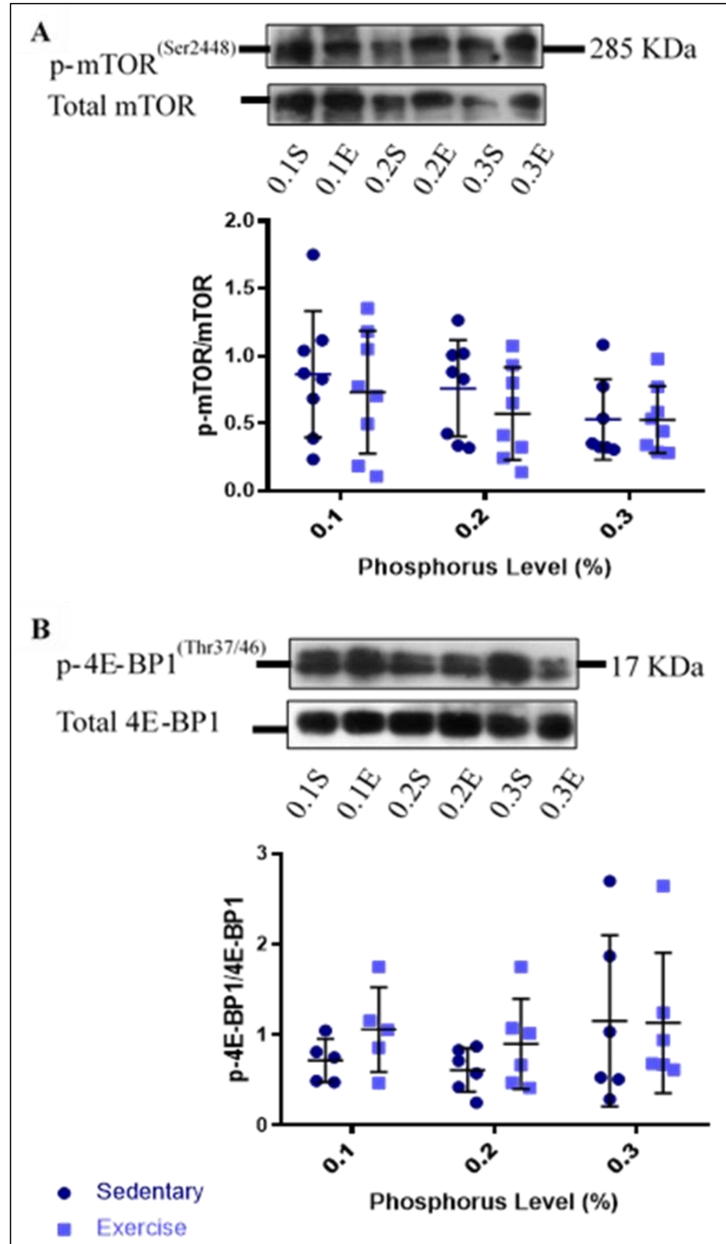

Figure 7. Experiment 1- Protein expression levels of p-mTOR (A) and p-4E-BP1 (B) in liver of rats fed various P levels and were either sedentary or exercising. Upper: Representative figure for Western blot reflecting the protein expression of p-mTOR (A) and p-4E-BP1 (B) normalized by the levels of corresponding total protein for all study animals. Lower: Scatter plot reflecting the quantification of protein expression of p-mTOR against total mTOR (A), and p-4E-BP1 against total 4E-BP1 (B). Data is expressed as mean ± SD. Statistical significance was determined by two-way ANOVA Fisher's post-test for multiple comparisons. P-value < 0.05 is considered significant and is indicated by (*).

As shown in Figure 8A, protein level of p-mTOR (Ser_{2448}) in liver increased as P levels in the diet increased. In which, the 1.2% sedentary group displayed a significantly higher level of p-mTOR as compared to the 0.3%P sedentary group (p=0.004) and a prominent trend as compared to the 0.6%P sedentary groups (p=0.077). Additionally, p-mTOR in the 0.6%P exercising group (p=0.041) and 1.2%P exercising group (p=0.002) were significantly enhanced as compared to the 0.3% exercising group.

The values of p-4E-BP1 ($Thr_{37/46}$) and p-S6K1 (Thr_{389}) in liver were not altered by either P level or E (Figure 8B, 8C). Yet, as shown in Figure 8D, differences in phosphorylation of the initiation factor eIF4E on its Ser_{209} residue were observed. As P levels increased, the exercising groups displayed significant differences in eIF4E activation as compared to their sedentary counterparts. Notably, the p-eIF4E was significantly higher in the 1.2%P exercising group as compared to the 1.2%P sedentary group (p=0.002), and the 0.6%P exercising group demonstrated a higher trend as compared to its sedentary group (p=0.080). Additionally, PxE effect was revealed as the p-eIF4E levels were shown to be significantly higher in the 1.2%P exercising group as compared to the 0.3%P exercising group (p=0.031).

Lastly, in liver, there was no change in the phosphorylation of AMPKα on its Thr_{172} residue among the different experimental groups, denoting no effect of P nor E (Figure 8E).

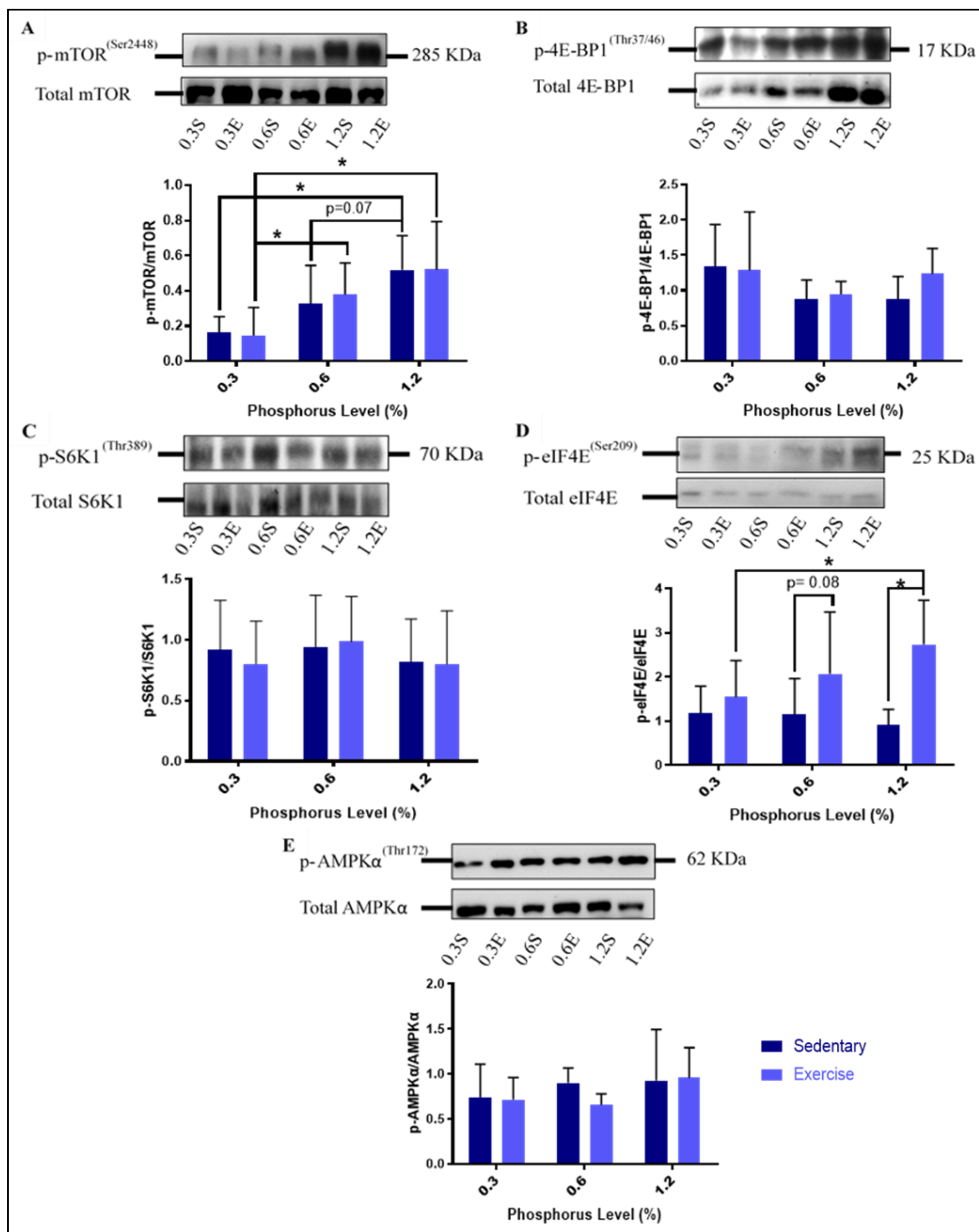

Figure 8. Experiment 2- Phosphorylated protein expression levels of key signaling proteins involved in protein synthesis initiation in liver of rats fed various P levels and were either sedentary or exercising. Upper: Representative figure for Western blot reflecting the protein expression of p-mTOR (A) and p-4E-BP1 (B), p-S6K1 (C), p-eIF4E (D) and p-AMPKα (E) normalized by the levels of corresponding total protein for all study animals. Lower: Bar graph reflecting the quantification of protein expression of p-mTOR against total mTOR (A), and p-4E-BP1 against total 4E-BP1 (B), p-S6K1 against total S6K1 (C), p-eIF4E against total eIF4E (D), and p-AMPKα against total AMPKα (E). Data is expressed as mean ± SD. Statistical significance was determined by two-way ANOVA Fisher's post-test for multiple comparisons. P-value < 0.05 is considered significant and is indicated by (*).

In gastrocnemius muscle tissue of the HP experimental groups (Experiment 2), phosphorylation of mTOR on its Ser_{2448} residue was significantly increased in the 0.6%P exercising group as compared to its 0.6%P sedentary counterpart (p=0.050) (Figure 9A). Moreover, PxE effect is observed among the exercising groups with higher P intake. As such, p-mTOR level in the 0.6%P exercising group was significantly elevated relative to the 1.2%P exercising group (p=0.026).

Similar to their unchanged expression in liver, there was no significant difference in the phosphorylation of 4E-BP1 on its $Thr_{37/46}$ residue, nor S6K1 on its Thr_{389} residue in response to P level or E in gastrocnemius muscle (Figure 9B, 9C). However, p-eIF4E (Ser_{209}) was respondent to P level displaying a significant elevation in its expression in the 1.2%P sedentary group as compared to both the 0.6% P (p=0.044) and 0.3%P sedentary groups (p=0.035) (Figure 9D). Additionally, p-eIF4E was markedly reduced in the 1.2% exercising group as compared to the 1.2%P sedentary group (p=0.017).

In contrast to liver expression values, the phosphorylation of AMPKα on its Thr_{172} residue varied in response to the joint effect of PxE (Figure 9E). In which, the 1.2%P exercising groups had significantly higher p-AMPKα level as compared to the 0.6%P exercising group (p=0.046), and an elevated trend as compared to the 0.3%P exercising group (p=0.08). No significant changes were recorded due to the individual effects of P and E.

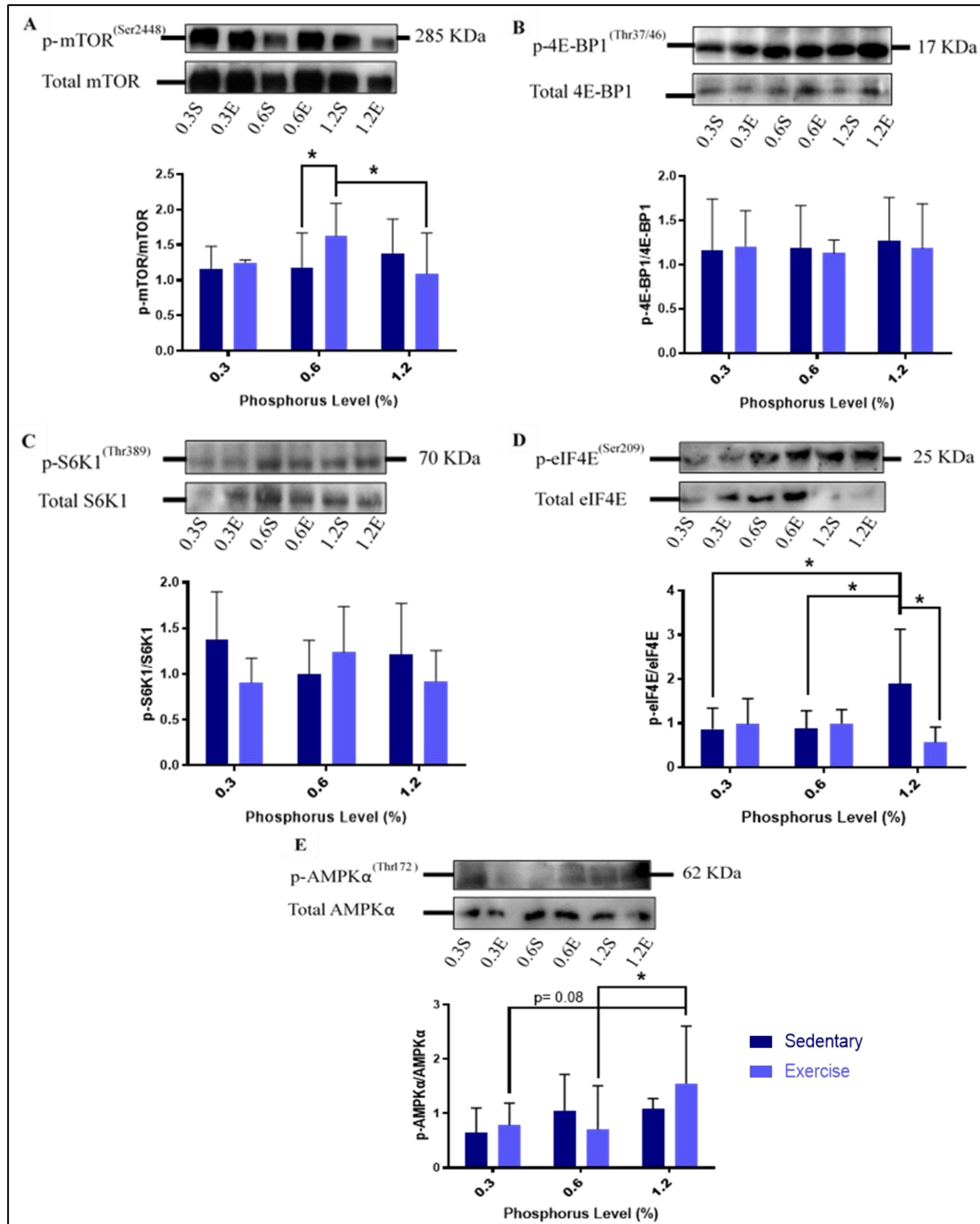

Figure 9. Experiment 2- Phosphorylated protein expression levels of key signaling proteins involved in protein synthesis initiation in gastrocnemius muscle of rats fed various P levels and were either sedentary or exercising. Upper: Representative figure for Western blot reflecting the protein expression of p-mTOR (A) and p-4E-BP1 (B), p-S6K1 (C), p-eIF4E (D) and p-AMPKα (E) normalized by the levels of corresponding total protein for all study animals. Lower: Bar graph reflecting the quantification of protein expression of p-mTOR against total mTOR (A), and p-4E-BP1 against total 4E-BP1 (B), p-S6K1 against total S6K1 (C), p-eIF4E against total eIF4E (D), and p-AMPKα against total AMPKα (E). Data is expressed as mean ± SD. Statistical significance was determined by two-way ANOVA Fisher's post-test for multiple comparisons. P-value < 0.05 is considered significant and is indicated by (*).

CHAPTER V

DISCUSSION

Our data shows that the relation between dietary P content and body weight or energy stores is not linear. Highest body weight and ΔE_{st} were at 0.3%P and decreased with lower or higher levels. EI did not follow the same pattern, it was the lowest in the 0.1 and 0.2%P groups and the highest in the 0.6 and 1.2% groups. Yet, the lower body weight gain and ΔE_{st} of the 0.1 and 0.2%P groups were the result of a combination of reduced EI as well as EEf. While, lower body weight gain and ΔE_{st} of the 0.6 and 1.2% groups were attributed to an increase in TEEx that resulted in a decrease in EEf.

In our experiments, ΔLBM_{st} were similar between the groups (0.1%P to 1.2%P). Hence, the change in body weight and ΔE_{st} were due to differences in FM, as LBM was preserved.

Our results are in line with others, as studies in animals have shown that diets deficient in P are associated with a lower food intake and weight gain [60]. Besides, a comparable rising trend in food intake and EEf was previously demonstrated when P was increased gradually from very low to standard P level of 0.3%, which can be related to the capability of young animals to regulate their food intake in order to support their nutritional requirements for growth and maintenance [54]. However, at higher intakes of P (>0.3%P), food intake was shown to be inconsistent among different studies. No difference in food intake was noted in rats when P level spanned from 0.2-1.2% [52]. Whereas in humans, lower intakes were reported on the long-term when main meals were supplemented with 375mg P [50], and on the short-term when subsequent ad libitum food intake was reduced following a 500mg P supplement [56]. In our

experiment, food intake continued to rise as the P level increased in the diet. Nonetheless, the higher EI was accompanied by a significant elevation in TEEx and a reduction in EEf which explains the lower weight and fat gains. In agreement, rats fed a high P diet displayed an elevated thermogenesis, which was manifested by an increase in the expression of UCP-1 in brown adipose tissue [52]. Bassil et al. has also demonstrated an implication of P in energy metabolism, altering substrate oxidation and increasing thermogenesis in healthy individuals [57]. In effect, P ingestion with high carbohydrate meal elevated post-prandial EE in both lean and obese subjects [65]. Similarly, P supplementation lead to an increase in RMR and post-prandial EE in overweight [61] and obese women [62, 63]. Moreover, in both animal and human studies, a reduction in respiratory quotient upon P supplementation indicates preferential utilization of fat for energy production resulting in enhanced energy expenditure and reduced body weight gain and lower fat accumulation [52, 65].

In support of our results demonstrating lower weight gain and % body fat in the HP groups, numerous studies have shown that P supplementation affects fat metabolism. Imi et al. has demonstrated that a high P diet (1.5%P) results in less fat accretion in rats and a reduction in white adipose tissue activity by increasing lipolytic gene expression and decreasing lipogenic gene expression in visceral fat tissue [51]. Similarly, rats ingesting 1.2%P diet for 8 weeks had lower visceral fat accumulation and lower hepatic lipid synthesis due to altered m-RNA expression of lipogenesis-related genes [52]. In addition, P supplementation for 12 weeks in obese and overweight subjects has significantly improved body weight, body mass index and waist circumference indicating a reduction in abdominal obesity [50].

Two plasma parameters responded to dietary P levels. The first was plasma urea nitrogen (PUN), a marker for protein breakdown. In the LP diet experiment, PUN decreased significantly as P intake increased to 0.3%P, which reflects an enhanced protein anabolism as P intake increases from deficient to standard level. This is in accordance with Ragi et al., who revealed that P-containing diets were associated with a reduced PUN and an increase in body protein %, hence implying that protein metabolism was affected by the P level in the diet [55]. Correspondingly, P supplementation of low-protein diets was able to improve total body protein content, and reduce urinary nitrogen excretion and thus improved protein anabolism [54]. At higher P intake, although the PUN was significantly different, the pattern of values was inconsistent. As ΔLBM_{st} were found to be similar among different levels of P intake, there is a possibility that the higher PUN observed in the 0.6%P and 1.2%P may be attributed to both a higher protein breakdown and subsequent protein anabolism, which may suggest a higher protein turnover in these groups. Though not directly measured in this study, we speculate that a probable higher protein turnover, which is known to be energy expensive, may have contributed to the elevation of TEEx observed in the HP diets experiment.

The second plasma parameter to examine is plasma P. Our study found that at low levels of P intake, plasma P levels were not affected. Yet, the higher P levels in the diet resulted in lower serum P levels, and showed no difference in the phosphaturic hormones PTH and FGF-23, knowing that an elevated serum P generally results in an elevation of these two parameters [1]. In agreement, Kremsdorf et al. have shown that a 33% increase in dietary P does not result in significant changes in plasma P level and its regulatory hormones (PTH and FGF-23) [143]. Another study has shown no difference

in fasting plasma P concentration upon ingestion of different P levels in the diet, however the random plasma P level was significantly higher in the high dietary P group as compared to the control [51]. This confirms that an elevated P intake is not associated with an elevated fasting serum P, which is in line with findings in humans where serum P was found to be a poor indicator of dietary P intake [144, 145]. Yet, dietary P can affect post-prandial P levels [65], where P is required for metabolic processes and is involved in the elevation of post-prandial EE [57, 65].

Starting with the effect of E on the various parameters, the data presented in the current study demonstrated a significant reduction in plasma measurements of total cholesterol and/or TG in the exercising groups. Essentially, the decrease in total cholesterol and maintenance of HDL-cholesterol implies that the reduction was mainly from LDL-cholesterol. This is consistent with the therapeutic and preventive effects of structured E on improving cardio-metabolic parameters [146]. Structured E denotes weekly prescribed or programmed E intervention, which in the current study consisted of 30 minutes of moderate-intensity supervised aerobic training (equivalent to 60% VO_2 max) 5 times per week for 6 weeks.

Our research also demonstrates that structured E results in a lower fat accumulation. In the LP diets experiment, the lower body weight gain, % body fat and epidydimal fat weight in the exercising groups were mainly related to the reduction in EI rather than the effect of physical E on EE. Conversely, in the HP exercising groups, the lower % body fat and body weight gain (including ΔE_{st} and ΔFat_{st}), were highly attributed to an increase in TEEx and a decrease in EEf. The lower fat and weight gains

upon E are in line with previous observations, where dynamic aerobic E training was found to significantly reduce body weight, fat percent and adiposity index [72] and adipogenesis-related markers [73]. Moreover, in the HP experiment, structured E resulted in a lower liver weight and % fat in liver. In agreement, numerous studies have shown that physical E, at different intensities and frequencies, reduces hepatic fat content by decreasing fatty acid synthesis and increasing fatty acid oxidation [147].

Regarding the second aspect of body composition, our findings indicate that the exercising groups always maintained a higher %LBM. As previously noted, in experiment 1, even though the exercising groups had a lower EI, they had a similar ΔLBM_{st} to their sedentary counterparts. Hence, the prominent trend towards lower fat storage in these groups resulted in an apparently higher %LBM and $\%LBM_{st}$.

On the other hand, in experiment 2, the lower level of accumulated LBM stores in response to E was mostly related to the lower overall gain in accumulated body stores, i.e ΔE_{st}. Nevertheless, the major contributor to the lower gain in ΔE_{st} was ΔFat_{st}. Hence, although the ΔLBM_{st} was lower in the exercising groups, these best maintained their %LBM from the start till the end of the experiment. In accordance, structured physical E is well-known to maintain or improve LBM in human studies [69], and treadmill training was shown to improve muscle mass and strength in study animals [148].

Despite the 6-week maintenance of %LBM with moderate aerobic E training, the weight of gastrocnemius muscle was shown to be lower in the exercising groups. This can mean that the current E intensity and duration of the E session, and overall period of the study were not sufficient enough to result in muscle hypertrophy. Yet, to

provide a clearer picture of the weights of rat hind legs and their response to E, in addition to the recorded weight of gastrocnemius muscle, the weights of both soleus and plantaris muscles should have been measured. In effect, in rodents, longer duration endurance training can result in muscle growth after 4-12 weeks of training. However, it was revealed that hind limb muscle mass either remains unchanged or increases, with discernible differences in weights of gastrocnemius, soleus and plantaris muscles [149]. Similarly, clinical studies have shown that regular aerobic E training initiates muscle hypertrophy comparable to resistance training only when performed at higher intensity (80% heart rate reserve), longer session duration (30-45 minutes) and over an extended period of time (12 weeks onwards) [150].

Another important finding of this research is related to the effect of a moderate-intensity E routine on TEEx and energy compensation either through changes in EE and/or EI. Our data shows that in the LP experiment, the increase in EE as a result of structures E did not result in an overall increase in TEEx as would be expected, which was probably due to a reduction in spontaneous activity during the day as a means of compensation for the energy expended during the E session. Essentially, a decrease in NEAT and an increase in sedentary behavior was reported following E [91]. Additionally, King et al., has shown a reduction in NEAT following routine E sessions [88], and as a result of this compensation, TEEx remains unchanged in response to physical E [151].

On the other hand, in the HP experiment, the energy expended during E was partially compensated for by an increase in EI which was shown in the 0.6% and 1.2%P exercising groups. In line, Pinto et al. has shown that exercised rats had significant elevation in EI following E training [81]. Moreover, in human studies, individuals have

experienced an increase in EI accompanied by an increase in hunger and a decrease in fullness after meals after a 12 week E intervention [87]. Subjects experienced an elevated drive to eat as an adaptive response to long-term E [84], and on average, individuals compensated for around 30% of the energy deficit induced by E through increasing their EI [85, 89]. Nonetheless, in contrast to the LP experiment results, the exercising groups of the HP experiment demonstrated a significantly higher TEEx which may be attributed to a higher NEAT as rats remained active post-exercise. This implies that there was no compensation for the energy cost of E through less daily activity. This is in agreement with research reporting no reduction in NEAT in response to prescribed E training [93], and no increase in sedentary behavior following supervised aerobic E [87]. Moreover, Alahmadi et al. has shown that walking E even had a delayed effect on increasing NEAT [94].

It is the first time that a study combines the dual effect of P and regular E training on energy balance, energy compensation and body composition. We can infer from the above discussion that, with the ingestion of low P levels, NEAT seems to be lowered, as the TEEx in exercising rats was shown to be similar to the sedentary ones. This can mean that the low P availability affects the way in which rats respond to daily movement, thereby increasing their sedentary behavior and reducing their spontaneous activity. On the other side, at higher P intakes (0.6 and 1.2%), although there was a partial compensation evident in an elevated EI in the exercising group, they displayed a higher TEEx. This means that the energy deficit induced by E was not compensated for by reduced spontaneous everyday activities or increased sedentary behavior. This implies that an increased P intake reduced the bodily ability to compensate for the E-

induced energy deficit. This, in turn, increased TEEx which further enhanced body composition measures. The cost of elevated TEEx appears to have been derived from fat stores and not LBM as the data showed that the 1.2%P exercising group had the lowest fat accretion and the best maintenance of %LBM throughout the experimental period.

Moving on to the second part of our research related to the effect of P and/or structured E on markers of protein synthesis, our data shows that protein synthesis translation initiation responded to both the individual and combined P x E effect, with distinct differences between liver and gastrocnemius muscle, namely observed in the degree and pattern of activation of various signaling proteins.

However in the LP experiment, no differences between the groups were noted in mTOR activation, nor its downstream initiation factors in rat liver. This may be due to the fact that increasing the level of dietary P from deficient to standard may have diverted the availability of energy to bodily processes other than protein synthesis, such as phosphorylation of compounds, nutrients or enzymes that are essential for survival. If we pursued this further, we may have gotten alternate results in muscle tissue, however, due to time constraints and the overall situation with COVID-19, we concentrated our efforts in the Western Blot analysis of tissues pertaining to the HP experiment, as will be discussed hereafter.

In the liver, our data indicated that mTOR activation increased markedly with increasing P levels in the diet from 0.3 to 1.2%. Numerous studies have shown that as

dietary P increases, P availability in the body increases, which enhances the availability of cellular energy [35, 59, 112]. Indeed, an adequate supply of dietary P is required for hepatic ATP production [33], and making ATP available to facilitate protein synthesis initiation [96]. Additionally, being incorporated within ATP, P becomes essential for the regulation of subcellular processes, namely the phosphorylation of key compounds [5], in this case mTOR (Ser_{2448}). This serine-threonine protein kinase is known to be a nutrient and energy sensor of the cell, and plays an essential role in the regulation of protein synthesis [96]. To our knowledge, this is the first study to assess the effect of dietary P on the protein expression of phosphorylated mTOR (Ser_{2448}), leading to an important association between P, mTOR activation and potential enhancement of protein synthesis in rat liver.

mTOR starts a cascade of downstream phosphorylation of several initiation factors and promotes protein-protein interactions that favor the binding of mRNA to the 40S ribosomal subunit, thus controlling protein synthesis translation [95]. Generally, mTOR activation results in the phosphorylation of 4E-BP1 ($Thr_{37/46}$), and S6K1 (Thr_{389}) [95, 98]. Phosphorylation of 4E-BP1 ($Thr_{37/46}$), results in it dissociating from the initiation factor eIF4E and permitting it to bind to eIF4G to form the active mRNA cap-binding complex [95, 110]. Additionally, activated S6K1 (Thr_{389}) phosphorylates ribosomal protein S6, among other functions, leading to activation of mRNA translation [98]. However, our results have shown that there were no differences between the groups in activation of the aforementioned proteins, neither responding to P nor to E. In line, some studies reported enhanced protein synthesis rates in tissues without any observable changes in the phosphorylation levels of these 2 initiation factors. As such, Bolster et al., have shown that the rate of skeletal muscle protein synthesis can be

increased in the absence of phosphorylation changes in 4E-BP1 (Thr$_{37/46}$) and S6K1 (Thr$_{389}$) [152]. Instead, other considerations, such as the activation of eIF4E (Ser$_{209}$), eIF4G (Ser$_{1108}$) and the formation of the active complex eIF4E.eIF4G enable mRNA binding to the 40S initiation complex, thereby resulting in increased translation initiation and consequently protein synthesis rates [153].

Additionally, it is likely that mTOR exhibited its enhanced effect on protein synthesis through encoding for protein components of the translation machinery independently of 4E-BP1 and S6K1. In agreement, as reviewed by Laplante and Sabatini, mTOR can upregulate protein synthesis through activation of the regulatory protein TIF-1A, which promotes its interaction with RNA polymerase I and the expression of rRNA, and inhibition of Maf 1 (Pol III repressor) which leads to the induction of 5S rRNA and tRNA transcription, hence improving ribosome biogenesis [154].

Another possible explanation for the unchanged expression in 4E-BP1 and S6K1 could be the timing of sacrifice, which in our study, was after an overnight fast. In effect, Reynolds et al., has observed no change in the phosphorylation status of S6K1 (Thr$_{389}$) although phosphorylated mTOR (Ser$_{2448}$) expression was elevated, which was attributed to the fasted state of the mice. Alternatively, if the measurements were performed in the post-prandial state, they speculated an upward shift in S6K1 (Thr$_{389}$) phosphorylated level [124].

A prominent finding in our study was the combined P x E effect on the activation of eIF4E (Ser$_{209}$) in the exercising group of 1.2%P as compared to the 0.3%P group. Additionally, at high P intakes (0.6 and 1.2%), the exercising groups had higher

phosphorylated levels of eIF4E (Ser_{209}) than sedentary groups, indicating an E effect on the activation of eIF4E and probable enhancement of protein synthesis initiation. In fact, eIF4E comprises the cap-binding subunit of the eIF4F translation initiation complex and facilitates the recruitment of mRNA to the ribosome [155]. In its phosphorylated form, it forms a more stable complex with eIF4G [101], and binds with more affinity to the mRNA cap [102], facilitating the translational process of protein synthesis [100]. In particular to the liver, preceding animal studies have shown that stimulation of hepatic protein synthesis involves the association of eIF4E.eIF4G to form the eIF4F translation initiation complex [156]. Remarkably, eIF4E (Ser_{209}) gets phosphorylated after its association to eIF4G [100], hence the hyperphosphorylation of eIF4E in response to the joint effect of P and E in our study may be indicative of an increased association of eIF4E to eIF4G, allowing the cap-dependent translation to begin, successively leading to an increased rate of protein synthesis in rat liver.

It's noteworthy to mention that liver mTOR expression was not affected by structured E, as both sedentary and exercising groups at each level of P intake had similar level of phosphorylated mTOR (Ser_{2448}). Similarly, AMPKα expression was not respondent to neither P, nor E. In contrast, Reiter et al. has shown that prolonged endurance E can result in metabolic stress which is known to stimulate AMPKα. This, in turn, can lead to mTOR inhibition, and subsequently, can result in reduced protein synthesis in liver [132]. Taken together, it is likely that the higher availability of P may have prevented hepatic AMPKα (Thr_{172}) phosphorylation in the exercising groups, which could have resulted in the maintenance of mTOR activation and its stimulatory effect on protein synthesis. Therefore, P may play a role in mitigating the effect of E on hepatic protein synthesis.

In the gastrocnemius muscle, the activation of mTOR and other signaling proteins were somewhat different than those observed in liver. Wherein, the individual effect of P on phosphorylated mTOR (Ser_{2448}) level was not demonstrated. Instead, there was a combined P and E effect only at high P intakes. In which, the 0.6%P exercising group had a higher level of phosphorylated mTOR (Ser_{2448}) than the 1.2%P exercising group. This is unexpected as the high P availability associated with higher P intake was supposed to initiate higher cellular ATP levels, activate mTOR and positively impact protein synthesis. Indeed, ATP is known to enhance the synthesis of proteins, nucleic acids and all other bodily building blocks [157]. However, this was not demonstrated in rat muscle and may be explained by the marked activation of AMPKα in the 1.2%P exercising groups which may have prevented the activation of mTOR in that group. It is widely known that AMPKα (Thr_{172}) phosphorylation is associated with decreased activation of mTOR signal transduction pathway and inhibition of anabolic processes, including protein synthesis [158].

Another interesting explanation of how the abundance of ATP could reduce protein synthesis may be related to the depletion of Mg^{2+} pools [159]. Essentially, given that ATP has a high affinity for Mg^{2+} ions, a high cellular ATP level would chelate free cytosolic Mg^{2+} pools. As Mg^{2+} is required for all the steps of protein synthesis, especially initiation [160], then its depletion can disrupt Mg^{2+} -dependent cellular processes including protein synthesis [161, 162].

In contrast to the non-existent E effect on mTOR activation in liver, in the gastrocnemius muscle, regular structured E resulted in higher mTOR (Ser_{2448})

phosphorylation in the exercising group ingesting 0.6%P. In agreement, aerobic E has been shown to increase mTOR (Ser$_{2448}$) phosphorylation with corresponding effects on protein synthesis [163]. Additionally, numerous studies have shown that on the long run, endurance/aerobic E results in muscle anabolism [123-125, 164], despite reductions in muscle protein synthesis experienced during individual aerobic E sessions [117, 118]. In effect, both types of muscle protein synthesis are enhanced with regular E, the mTOR-independent sarcoplasmic and mitochondrial protein synthesis [125, 164], and the mTOR dependent myofibrillar protein synthesis [123]. Essentially, in animal studies, Reynolds et al. has demonstrated elevated global rate of protein synthesis in soleus muscles of mice following 3 months of aerobic E training, and this was attributed to stimulation of PKB/mTOR signaling pathway [124]. Yet, concerning the expression of downstream initiation factors in muscle, all the groups displayed similar phosphorylated levels of 4E-BP1 (Thr$_{37/46}$) and S6K1 (Thr$_{389}$), as was previously noted in the liver. In parallel to the discussion provided previously, this could be attributed to the fasted state when the rats were sacrificed in which plasma insulin and amino acids levels were low. Whereas, in the postprandial state, studies have shown prominent activation of 4E-BP1 and S6K1 in skeletal muscles after training [126, 165] . In line with our findings, Li et al., has demonstrated an activation of mTOR in response to nutrient manipulation, however, both 4E-BP1 (Thr$_{37/46}$) and S6K1(Thr$_{389}$) phosphorylated levels remained unchanged in the psoas major muscle of experimental pigs [166]. Additionally, mice undergoing long term aerobic E training demonstrated no changes in level of S6K1 in gastrocnemius muscle [124].

Hence, it is likely that activation of mTOR in the 0.6% exercising group due to E and joint P x E may have exerted its effect on enhancing protein synthesis through

other signaling molecules such as eIF4G or the eukaryotic elongation factor 2 (eEF2). As an example, the stimulatory effect of leucine on mTOR activation and consequent protein synthesis in rat skeletal muscle was not observed through further activation of S6K1 and 4E-BP1, but through hyperphosphorylation of eIF4G (Ser_{1108}), which increased its availability for assembly with eIF4E [152]. Another key component of the mTOR-dependent mRNA translational machinery is eEF2, which was shown to be activated in skeletal muscle following anabolic stimuli [167]. Accordingly, the effect of mTOR on skeletal muscle protein synthesis is revealed through numerous downstream effectors, with variations in the degree of phosphorylation of the different signaling proteins.

Protein synthesis is also associated with the activation of eIF4E [168]. Another important finding in our study, was the direct association of dietary P with the elevated expression of eIF4E. In which the 1.2%P sedentary group had significantly higher phosphorylated levels of eIF4E (Ser_{209}) as compared to the 0.3% and 0.6% sedentary groups, a result that reflects an enhanced translation initiation rate at higher P intakes. As the phosphorylation trend of eIF4E was different than that of mTOR, we speculate that the effect of eIF4E on muscle protein synthesis was independent of the effect of mTOR. In accord, Watson et al., has revealed that skeletal muscle protein synthesis can be induced by an mTOR-independent pathway, particularly the MAPK-Mnk1/2 which was shown to directly activate eIF4E [123]. Besides, phosphorylated eIF4E (Ser_{209}) modulates the function of eIF4F complex, thereby increasing mRNA cap binding affinity resulting in increased translation rates [102]. In effect, Jayapalan et al., has demonstrated nutrient-induced increases in muscle protein synthesis attributed to

phosphorylated eIF4E and active eIF4E.eIF4G complex, without changes in expression of 4E-BP1 and S6K1, hence emphasizing that muscle protein synthesis can be controlled independently of mTOR-mediated signaling [153].

On the other hand, in the 1.2%P exercising group, a marked reduction in phosphorylation of eIF4E in muscle was noted, similar to the one observed with mTOR expression. As highlighted previously, this can be attributed to the activation of AMPKα in that group, preventing both eIF4E and mTOR activation. In contrast, other studies have shown that long-term E activates the PI3-Kinase/mTOR pathways among others, resulting in reduced AMPKα activity and activation of multiple initiation factors most importantly, 4E-BP1, eIF4E and eIF4G, consequently leading to maximal stimulation of protein synthesis rates post-exercise [126-128].

Then, how can it be explained that a high dietary P intake (1.2%) combined with E resulted in AMPKα activation and subsequent reductions in phosphorylation of mTOR (Ser_{2448}) and eIF4E (Ser_{209}) in gastrocnemius muscle? We have 2 hypothetical explanations:

1- The higher P availability and cellular ATP level in exercising muscle of the 1.2%P group may have led to a negative feedback mechanism. Wherein, cells regulate their metabolism and modify the activity of the main ATP-generating processes in order to lower ATP synthesis [159]. Consequently, the level of ATP drops, initiating the activation of AMPKα, which in turn reduces mTOR expression and eIF4E activation.

2- The 1.2%P exercising group displayed the highest TEEx. The energy expended in this group through structured E and an elevated NEAT may have resulted in lower intracellular levels of ATP relative to AMP. This kind of stress is known to activate AMPKα which promotes processes that increase ATP generation such as enhanced glucose transport and fatty acid oxidation and decrease those that consume ATP such as lipid and protein synthesis [169]. Consequently, with activated AMPKα comes a decline in the levels of phosphorylated mTOR (Ser_{2448}) and eIF4E (Ser_{209}). However, some studies have shown that despite persistent increases in AMPKα, protein synthesis increases post-exercise [170], which in this case casts a doubt about the capacity of AMPKα to reduce protein synthesis in muscle tissue. In effect, after E, metabolic pathways respond with more sensitivity to insulin, including protein synthesis, amino acid transport and glycogen synthesis, which may compensate for the increase in AMPKα activity [171].

Here comes the link in our 2 sets of results; we have demonstrated that the 1.2%P exercising group had the lowest % body fat and ΔE_{st}, and significant reductions in plasma TG/TC levels. Knowing that AMPKα activation decreases TG synthesis in liver and adipose tissue [172, 173], it can be inferred that AMPKα activation may be one factor contributing to these effects. Additionally, although accumulation of LBM was lower in the 1.2%P exercising group, this group maintained the highest %LBM. Hence, the elevated AMPKα may have promoted the beneficial effects on reducing adiposity, without negatively affecting protein synthesis in rat skeletal muscle of rat.

In our study, the effect of E on initiation of muscle protein synthesis was observed in the moderately high P (0.6%) group as they exhibited an elevated mTOR expression. This can be indicative of enhanced protein synthesis in skeletal muscle, which may ultimately lead to higher LBM accretion. Yet, to establish that relationship, measuring protein fractional synthesis rate in several muscle groups (gastrocnemius, soleus and plantaris) is recommended. Then, further analysis of translational initiation factors can further highlight the pathways involved in protein synthesis activation, ultimately leading to LBM maintenance or accumulation.

What we found intriguing were the elevated PUN values at higher P intake, which do not seem to reflect lower protein anabolism, but a higher protein turnover. In effect, in the liver, mTOR was activated with increasing P intake, and phosphorylated eIF4E (Ser_{209}) level was higher in the exercising groups ingesting a high P diet, both of which are indicative of enhanced protein synthesis rates. Additionally, in gastrocnemius muscle, eIF4E was significantly more activated in the high P (1.2%) sedentary group. Thus, our combined findings suggest that P impacts protein metabolism and turnover, hence, playing a role in LBM accumulation and/or maintenance. Moreover, as this process is known to consume a high percentage of cellular ATP, it may also have contributed to the increase in TEEx observed with high P intakes.

CHAPTER VI

CONCLUSION

Our study exclusively assessed the impact of P and E on body composition, energy balance, and protein synthesis initiation. The data revealed that P intakes above standard level combined with structured E result in lower gains in body weight and fat accumulation, while maintaining %LBM. As for energy balance parameters, at low P intakes, TEEx was not affected by E, a finding which may associate low P availability to energy compensation through lowering NEAT and increasing sedentary behaviour. In contrast, at higher P intakes (0.6 and 1.2%), an elevated TEEx indicated an absence of energy compensation. Hence, an increased P intake reduced the bodily ability to compensate for the E-induced energy deficit. This, in turn, maintained an elevated TEEx, which further enhanced body composition measures.

Regarding protein synthesis, tendencies of significance in expression of signaling proteins involved in protein synthesis initiation were varied in liver and gastrocnemius muscle. Most prominently, hepatic protein synthesis seems to be activated as P level increases, through both mTOR and eIF4E activation. Most importantly, phosphorylated AMPKα (Thr_{172}) levels were unchanged with E, which implies that P availability may have prevented the activation of AMPKα in liver, hence maintained phosphorylated mTOR (Ser_{2448}) levels and resultant protein synthesis activation.

In gastrocnemius muscle, AMPKα activation may have played a part in lowering the levels of phosphorylated mTOR (Ser_{2448}) and eIF4E (Ser_{209}) in the high P exercising group, yet may not have affected protein synthesis to a pronounced degree. Notably, moderately high P intake (0.6%P) combined with E resulted in enhanced mTOR phosphorylated levels, which may signify greater stimulation of muscle protein synthesis through novel signaling proteins. Moreover, an mTOR-independent pathway may be responsible for the effect of high dietary P level (1.2%) on eIF4E activation. Hence, at the level of skeletal muscle, both an mTOR-dependent and mTOR-independent pathway may play a role in protein synthesis in response to P and/or E.

Finally, taken together, the results of the study reveal that P ingestion above standard level of 0.3%P combined with regular structured moderate-intensity E result in improvements in body composition measures and energy balance outcomes, and may lead to enhancement in protein synthesis through activation of various initiation factors in liver and muscle.

The strength of this research lies in its objective of combining P and E. The range of P intakes was wide (0.1-1.2%) and the number of rats (n=8) was statistically powerful. Yet, there were no direct measurement of TEEx nor protein fractional synthesis rate, both of which would have provided a more accurate picture. Besides, a noted shortcoming was the starting weight of the rats which was different in the 2 experiments. Future research can investigate the combined effect of P and E in obese and/or diabetic rats, or the joint effect of P and other forms of structured E (resistance E or vigorous aerobic E) on body composition, energy balance and protein synthesis.

Additionally, it would be interesting to assess the activation of translation initiation factors in the post-prandial state, and explore other pathways involved in protein synthesis, such as MAPK-Mnk1/2 pathway. On a clinical perspective, conducting a trial assessing the combined effect of P and E on body composition and energy expenditure measures in lean and obese subjects would be worthwhile.

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