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Effet d'un supplément de fructose, protéines et lipides sur le rétablissement des stocks musculaires de triglycérides et de glycogène après exercice chez des athlètes entraînés : effets sur la performance d'endurance

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Contents

Résumé

Un problème récurrent pour les sportifs réguliers concerne la récupération de leurs réserves de substrats entre leurs séances, et notamment ceux en glycogène musculaire. Celui-ci est un facteur limitant la performance d'endurance, et il a été postulé qu'il pourrait être économisé à l'effort en augmentant le métabolisme des lipides via leurs réserves locales sous forme de lipides intramyocellulaires (IMCL). Nos 5 athlètes volontaires ont suivi à tour de rôle les séquences suivantes : après un exercice déplétif de 2h30, consommation d'une diète isocalorique riche en graisses et fructose (FRU : 45% glucides dont 35% fructose, 47% lipides et 8% protéines) ou riche en graisses et glucose (GLU : 45% glucides dont 40% glucose, 47% lipides et 8% protéines) sous forme de repas liquides répétés durant 36 heures, avant un second exercice de 3h effectué le lendemain et suivi d'un effort mesurant la performance d'endurance.

Notre hypothèse était que FRU augmenterait plus les IMCL que GLU tout en replétant le glycogène. La seconde hypothèse était que cela épargnerait le glycogène musculaire lors d'un exercice subséquent, aboutissant à une amélioration de la performance d'endurance.

Les niveaux musculaires en glycogène et IMCL ont été mesurés par spectroscopie à résonance magnétique (^{1}H-MRS) après l'effort initial, et suite au dernier repas liquide. Les échanges gazeux respiratoires, la glycémie, la lactatémie, les niveaux de triglycérides, acides gras libres et acide urique ont été mesurés lors de l'ingestion de 3 repas liquides, et le lendemain lors du deuxième effort sur ergomètre.

Si FRU n'a pas augmenté le remplissage des IMCL par rapport à GLU ($P=0.922$), FRU a augmenté le glycogène musculaire 2.6 fois plus ($P<0.05$). Si FRU a effectivement augmenté lors de son ingestion les oxydations totales de glucides ($P<0.05$), tandis que la glycémie était mieux maîtrisée ($P<0.01$) et que la lactatémie était augmentée ($P<0.001$), les concentrations plasmatiques en triglycérides n'ont pas été modifiées par FRU comparé à GLU ($P=0.166$). Le lendemain, FRU a favorisé l'oxydation plus précoce des lipides lors de l'exercice prolongé ($P<0.01$) tandis que les oxydations glucidiques n'étaient pas modifiées ($P=0.098$) dans la durée. Une baisse de glycémie plus précoce a également été observée, expliquant peut-être l'absence d'amélioration de la performance d'endurance ($P=0.134$).

En conclusion, les stocks en glycogène musculaire peuvent être augmentés en consommant une diète riche en fructose durant les 36 heures précédant un effort, mais cela n'aboutit pas à une amélioration de performance, peut-être car le contrôle hépatique de la glycémie est perturbé et ce, même chez des athlètes protégés des autres effets négatifs du fructose.

Introduction

Glycogen as a performance limiting factor

During prolonged (ie >5 min) exercise, skeletal muscle energy provision rely on oxidation of endogenous (i.e. intracellular stores) and exogenous (i.e. blood borne) substrate sources, mainly glucose and fatty acids. Other substrates, such as amino acids, lactate or ketone bodies may also be used for ATP production. There is a constant trade-off between glucose and fat as well as the proportion arising from endogenous and exogenous stores (Romijn et al., 1993) to ensure energy provision. Therefore, skeletal muscle endogenous stores, glycogen and intramyocellular triglycerides –or intramyocellular lipids (IMCL) –, are in competition in-between and with blood-borne glucose and free fatty acids (FFA). Exercise intensity and

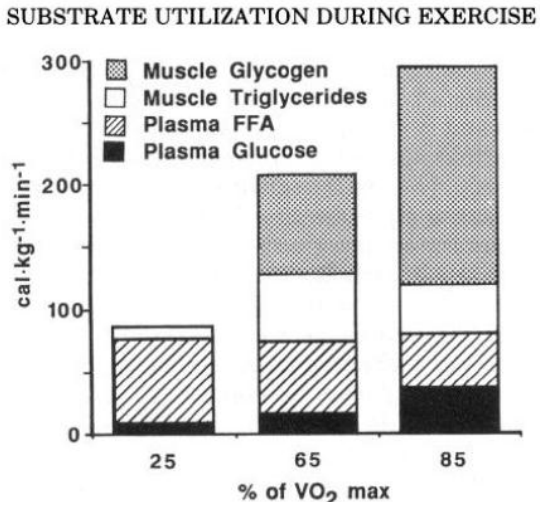

Figure 1: Substrate utilization depending on exercise intensity (Romijn et al., 1993)

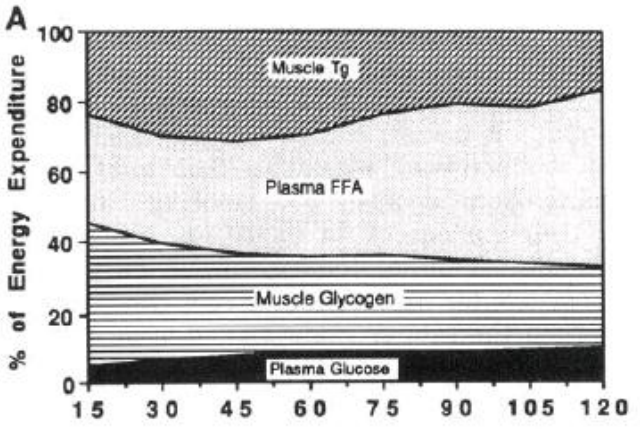

Figure 2: Substrate utilization at 60% VO_2max depending on exercise duration (Romijn et al., 1993)

duration are the main factors influencing substrate use (Figures 1 and 2), but other environmental factors, such as baseline levels of energy stores, gender, training status, temperature, etc., play a significant role.

With increasing exercise intensity the proportions of fat and carbohydrate (CHO) used for contraction declines and increases, respectively. With the increased contribution of carbohydrates, and the small contribution of circulating glucose to skeletal muscle glucose oxidation (Figures 1 and 2) during exercise performed at 60% or more of maximal aerobic power (MAP), muscle glycogen (carbohydrate's storage form: a branched polysaccharide) availability is considered to be a limiting factor. Typically, when glycogen levels become low, circulating glucose cannot match the demand, and exercise intensity cannot be maintained. For example, the resulting fatigue is well known among marathoners as the "hitting the wall"

phenomenon. This also occurs in many other aerobic sports, in which it is a limiting factor for endurance exercise performance. As a consequence, much research in the field of sport science has focused on developing strategies to delay glycogen depletion and hence exhaustion. First, several protocols aiming at raising total glycogen content over normal physiological levels have been explored, combining diet interventions with or without exercise training modifications. With success, as some protocols led to substantial improvements in aerobic performance (for review, see Ivy, 2001).

Pre-competitive protocols could not only aim at maximizing glycogen levels before exercise, but also at diminishing glycogen use: increasing the contribution of any other substrate would lead to a sparing of glycogen in myocytes. Fat could potentially fulfill that purpose, because it contributes substantially to energy provision at exercise intensities up to 85% VO_2max[1] (Fig.1) and this is even increased by exercise duration (Fig.2). Precisely, conditions in which total fat use is increased coincide with that in which glycogen use declines. Then, it was hypothesized that increasing the availability of fatty acids to the mitochondria would allow decreasing the reliance on glycogen.

Cellular fat molecular metabolism

Blood-derived lipids are used by the cells in various manners depending on conditions (Figure 3). They can cross the plasma membrane, then can be stored as IMCL for subsequent use or be directly oxidized for energy purposes. Circulating fat –in form of lipoproteins-bound particles- is processed by the lipoprotein lipase (LPL) complex at the blood-cell membrane interface. LPL catalyzes the hydrolysis of the triacylglycerol component of circulating fat particles, liberating free fatty acids (FFA). Importantly, LPL is regulated at transcriptional, posttranscriptional, and posttranslational levels in a tissue-specific manner (for review, see Wang and Eckel, 2009). For instance, its activity is increased in muscle cells by contraction but decreased by insulin. In the adipose tissue, insulin affects LPL activity in an opposite manner. Released FFA can then cross the sarcolemma membrane by the fatty acid transporter fat/CD36 (McFarlan et al., 2012). Holloway (et al., 2006) showed that CD36-mediated FA import is limited. Because of that, high circulating plasma FFA levels may not coincide with high intracellular FA pool.

[1] For writing simplification, oxygen and carbon dioxide consumptions will be referred as VO_2 and VCO_2 respectively, even if they represent gas flows and not volumes.

Once inside the cell, FFA are believed to form a common cytosolic pool from which some may be used or stored as lipid droplets named, in the case of skeletal muscle, intramyocellular lipids (IMCL) depending on cellular needs (Langfort et al., 2003).

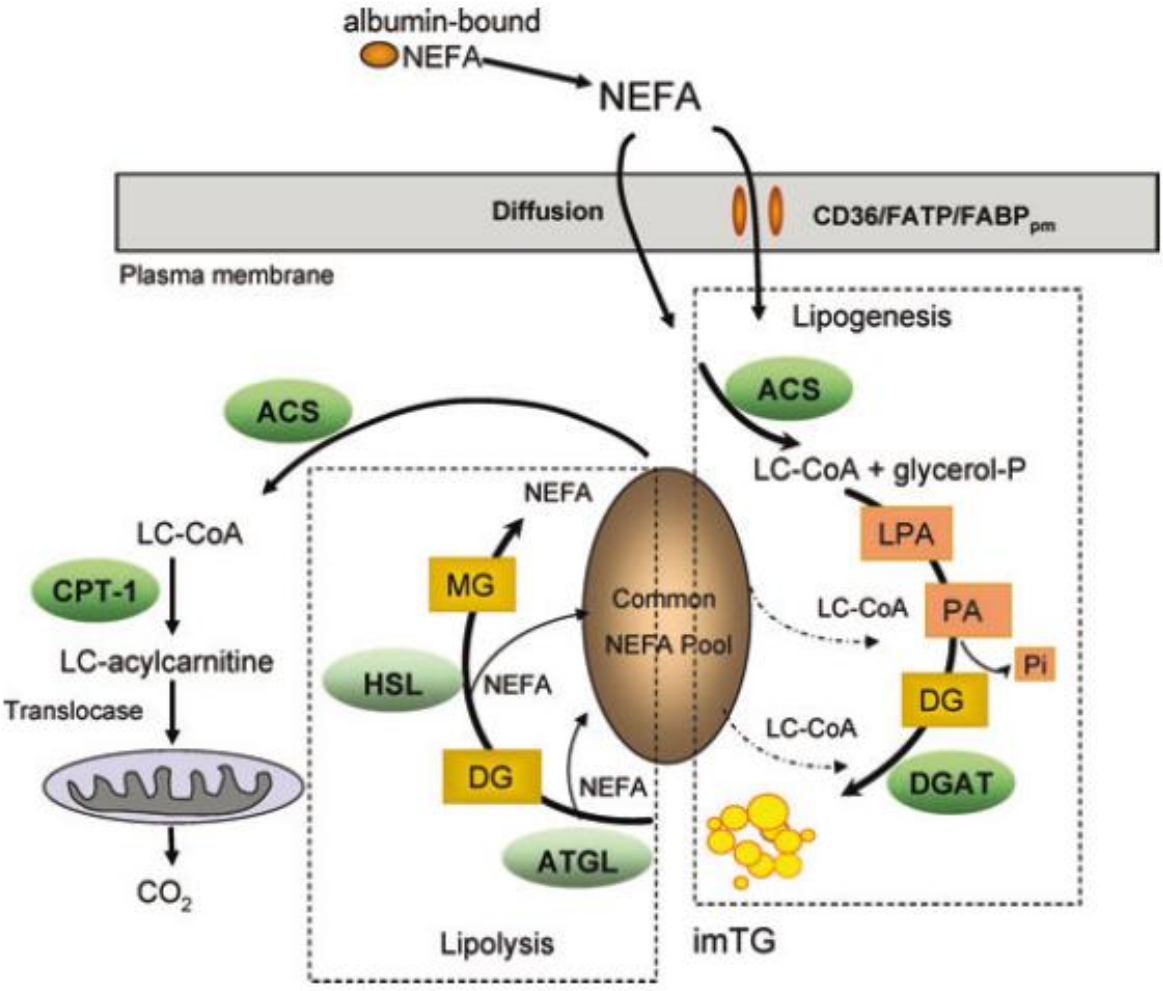

Figure 3: Cellular fat metabolism keypoints (Kanaley et al., 2009)

Fatty acids can be processed to acetyl-CoA that is able to enter the mitochondria where aerobic oxidation occurs to produce cellular energy. Importantly, the fat-derived ac-CoA mitochondrial entry by the carnitin-palmitoyl transferases (CPT) system is tightly regulated. Actually, it has been demonstrated that at rest, there is a constant competition between fatty acids and carbohydrates (CHO), with fat modulating CHO oxidation (Randle et al., 1963). During exercise however, as energy need raises, glycolytic flux appear to limit fat oxidation: with increasing intensity, it is proposed that the increasing glycolytic flux produces acetyl-CoA in excess to the Krebs cycle capacity (Jeppesen and Kiens 2012). The proposed mechanism is that within mitochondria, acetyl-CoA binds to free carnitine, which limits

carnitine availability that can be bound to fatty-acyl-CoA. This limits fatty-acyl-CoA movement across the mitochondrial membrane, and hence fat oxidation (Figure 4, Jeppesen and Kiens 2012).

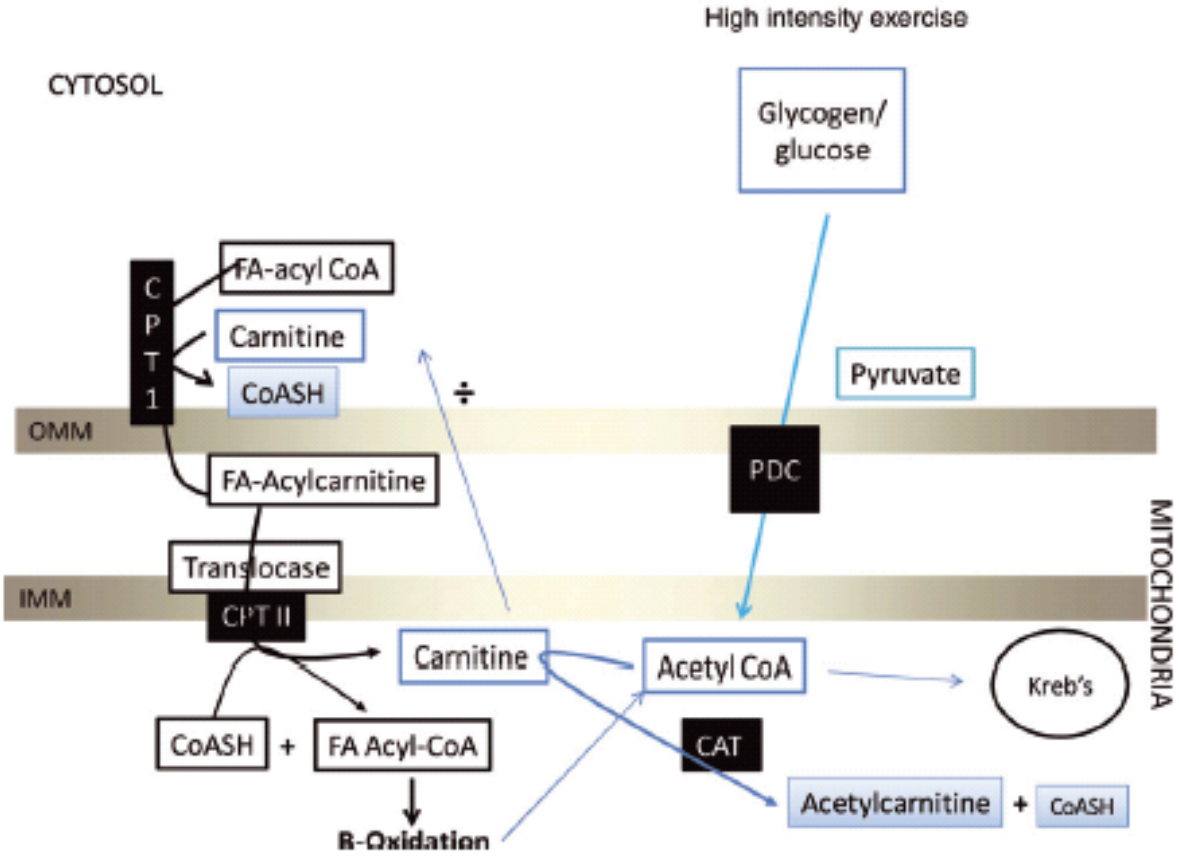

Figure 4: Schematic overview of a proposed interaction between fatty acids metabolism and glycolysis in skeletal muscle (Jeppesen and Kiens, 2012)

Carbohydrates can then be viewed as leading on an energy switch in high energy needs conditions, while fat is mainly used at rest.

At any time, IMCL content is dependent on the balance between synthesis (lipogenesis) and hydrolysis (lipolysis). Briefly, lipids esterification, or lipogenesis, is performed by adding FAs to a glycerol molecule, GPAT and DGAT believed to be intracellular regulatory enzymes in the process. GPAT and DGAT expression were shown being themselves regulated by hormones, insulin favoring it and glucagon diminishing it.

The breakdown, or lipolysis, however, is believed to be regulated by HSL (hormone-sensitive lipase) that was found in skeletal muscle (Langfort et al., 1998). This enzyme is believed to be

the key regulator of lipolysis and its activation has been reported to be activated by catecholamines and/or muscle contraction (Langfort et al., 2003).

Mechanisms implicated in particular intracellular FA fate are still not very clear, but several regulatory proteins, the lipid droplets physico-chemical properties and the particular properties of the FA themselves seem implicated into decision whether each FA subtype is stored in IMCL or recycled in the cytosolic pool.

Because limitations on IMCL metabolism concern all mentioned regulating endpoints, as well as possible additional unidentified ones, the question whether IMCL form an important substrate source during exercise and how its utilization is regulated is still not fully elucidated.

Various strategies were tried to adapt metabolic pathways toward an increase in IMCL oxidation, combining both endurance training and a high-fat diet. Such studies effectively resulted in an increased fatty acids oxidation (for review, see Burke et al., 2000). However, chronic high-fat diets had no effective improvement in subsequent endurance performance, comparing to high-carbohydrate diets (Erlenbusch et al., 2005). There were also side effects to chronic high-fat intake such as: reduction of glycogen content, difficulties to train hard, increased sympatho-adrenal activation, down regulation of glycogenesis and decreased mood state (Zehnder et al., 2006). All of these side effects may be linked to the lack of performance improvement.

To benefit from the effects of augmented fat oxidation without the negative chronic effects, pre-competitive dietary protocols were proposed, and included a glycogen loading phase over a few days, followed by a specific high-fat intake the last day. Indeed, it was shown that depleted IMCL levels can be replenished within a day (Boesch et al., 1999, Décombaz et al., 2000) and that IMCL are used during a subsequent exercise in proportion to their initial content (Zehnder et al., 2006). In this study, IMCL lipolysis increased while total carbohydrate and net fat oxidation rates were maintained, thereby resulting in a sparing of plasma FFA, but not of glycogen. Moreover, Costill et al. (1979) showed that pre-exercise elevated FFA decreased the carbohydrate oxidation during exercise and Zehnder et al. (2006) arrived to the same conclusion concerning elevated IMCL content. However, in both cases, no effect on performance was noticed. The conditions in which glycogen is the performance limiting factor and the mechanisms controlling its use are not fully elucidated.

Still, because FFA availability is somewhat low at the onset of exercise and increases with time, and because exercise, even in competitive settings such as cycling, may be performed at low to moderate intensity before high-intensity bursts where glycogen becomes a limiting factor, increasing IMCL content, and thereby FA availability for mitochondria may spare glycogen and improve exercise capacity/performance.

Together, it means that, in fasted, low-insulin conditions, post-prandial lipids should be directed from whole body to muscle cells. Moreover, preceding contraction accelerates lipids intake into skeletal muscle cell. This would suggest that a specific short-term (24-36h) high-fat protocol could aim at repleting selectively IMCL in skeletal muscle by limiting circulating insulin levels, together with a sufficient carbohydrate intake in order to increase endurance performance.

A particular sugar

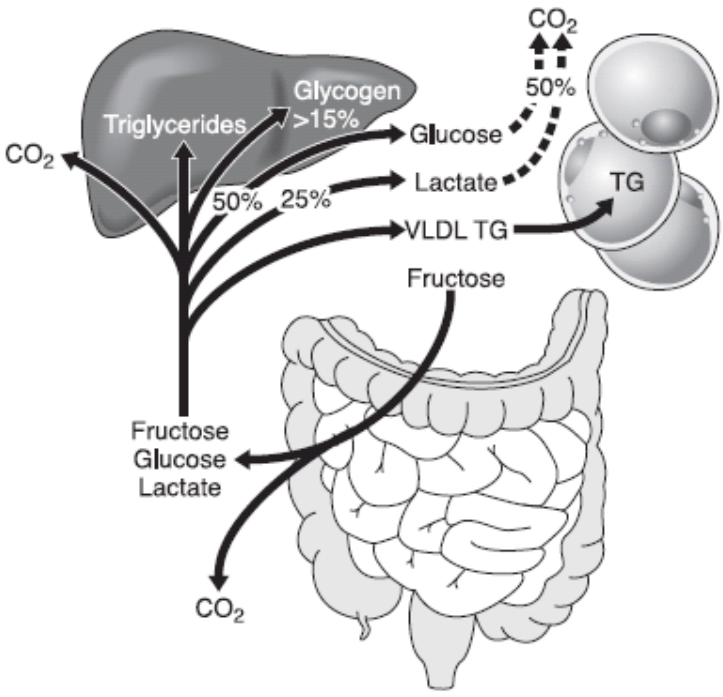

Figure 5: Metabolic fate of an oral fructose load (Tappy and Lê, 2010)

Fructose, a simple sugar, has been intensively studied due to its particular metabolism. It is absorbed from the intestine via specific transporters (GLUT 5), before entering the bloodstream. Fructose metabolism markedly differs from that of glucose in that it is not metabolized in non-hepatic tissues. Therefore, it is almost completely processed in the liver and plasma concentrations are very low. Whilst dietary glucose can be processed by almost all tissues, fructose can only enter hepatocytes cytosol. Here, both glucose and fructose can be converted to pyruvate. However, while glucose follows the glycolysis pathway that is regulated by insulin and cell energy status, fructose bypasses the main regulatory step (phosphofructokinase) and is rapidly converted to pyruvate (Cortez-Pinto et al., 1999). This quick metabolism coming from a rapid

entry, the lack of negative feedback loops and independence on insulin, results in high pyruvate levels in hepatocytes following a fructose load. Pyruvate can then be used in various manners. Fructose-derived extra pyruvate carbons can be discharged to bloodstream by lactate conversion. These carbons can also newly synthetize glucose (neoglucogenesis). However, it was shown that the extra glucose produced by neoglucogenesis wasn't delivered to bloodstream contrarily to lactate. This was shown to be due to compensatory hepatic glycogenolysis down regulation at rest (Tounian et al., 1994). This coupling, however, is lost during exercise due to hyperglucagonemia (Surmely et al., 1999).

Pyruvate can also be oxidized through import in the hepatocytes mitochondria and Krebs cycle. It can as well be stored locally, either as hepatic glycogen, or following *de novo* lipogenesis pathway. Resulting intrahepatic cellular lipids pool forms the basis of VLDL-bound triglycerides (Figure 5).

As a result, fructose intake was reported to raise acutely blood lactate and lipoproteins-bound triglycerides levels, and to diminish the increase in glycaemia comparing to glucose. Tracer studies aiming at defining the different pathways proportions concluded that the vast majority of the fructose carbons were converted to glucose (50%), lactate (25%) and glycogen (>15%) (Figure 5). *De novo* lipogenesis seemed of quantitatively low importance (<1%), however this pathway seems responsible for suspected deleterious effects of fructose and such a percentage may underestimate the delayed in time conversion (for review, see Sun, 2012).

Fructose is known to be less insulinemic than comparable amounts of glucose. Lower post-prandial insulin levels result in a lower lipoprotein lipase activity in adipose tissue, diminishing blood lipids clearance. Any blood metabolite levels are determined by input (production) and output (clearance). As fructose raises hepatic lipid production while diminishing clearance, it was related to induce hyperlipidemia, even if its underlying mechanisms remain controversial. Sun (2012) reviewed not only *de novo* lipogenesis stimulation, but also possibly energy source shifting and lipid sparing effect. In any case, fructose-induced hyperlipidemia was implicated in the occurrence of several cardiovascular or metabolic diseases (for review, see Tappy and Lê, 2010).

However, fructose has been commonly used by the sport beverages industry as combined fructose-glucose ingestion during exercise was found to enhance total carbohydrate oxidation

(Jentjens et al., 2004). Because of its fat metabolism enhancing role, fructose may also favor fat metabolism in myocytes. However, Lê et al. (2006) found no significant augmentation of IMCL content following two weeks of fructose overfeeding. As both the influences of high fat diets and physical activity were shown to influence IMCL and muscle glycogen stores (Zehnder et al., 2005), it is hypothesized in this study that preceding physical activity in combination with fructose may somehow interfere on IMCL metabolism.

Aim of the study

This work focused on the effects of fructose versus glucose on IMCL and glycogen replenishment between two exercise bouts. It was hypothesized that, after a prolonged exercise bout, a 36-hour high-fructose/high-fat diet would enhance IMCL content more than a high-glucose/high-fat diet. As a consequence, it was hypothesized that during the subsequent aerobic exercise, IMCL oxidation would rise, leading to a decrease in glycogen breakdown, possibly allowing better exercise performance.

Furthermore, the aim of this study was to explore the feasibility of labeling IMCL stores with stable isotopes and to measure their oxidation non-invasively during exercise. MRS has been well documented as a non-invasive, patient friendly, reproductive method with a good correlation with other techniques for assessing skeletal muscle glycogen and IMCL contents (Schrauwen-Hinderling et al., 2006). Therefore, this study is a proof-of-concept study aiming at validating the use of stable isotopes to measure IMCL breakdown kinetics during exercise.

Techniques

Indirect calorimetry

The term calorimetry refers to the science of measuring the heat of chemical or physical reactions. While direct calorimetry measures energy exchanges, they can also indirectly be measured by monitoring gas exchanges. Short, to be oxidized a substrate needs oxygen (O_2) and produces carbon dioxide (CO_2), water (H_2O) and energy in various amounts. The indirect calorimetry is based on known stoichiometry of macronutrients' oxidation, i.e. when a specific macronutrient is oxidized, a fixed amount of O_2 is consumed and a fixed amount of CO_2 is produced. Other assumptions include that consumption of O_2 that is not linked to respiration is negligible, and that oxidative phosphorylation is not uncoupled. Considering

this, indirect calorimetry allows measurement of net substrate oxidation (Tappy and Schneiter, 1997) both at rest and during exercise.

In our study, at rest, briefly, the volunteer's head was placed in an open-circuit canopy, which was constantly ventilated. As the indirect calorimeter measured the air flows with a turbine and the gas compositions (in/out of the canopy), it was possible to assess the gas flows:

O_2 consumption: VO_2 [ml/min] = V (F_{in} O_2 – F_{out} O_2) [2]
CO_2 production: VCO_2 [ml/min] = V (F_{out} CO_2 – F_{in} CO_2)
Respiratory quotient: RQ = VCO_2 / VO_2

The VO_2 and VCO_2 values were then interpolated per time intervals to calculate macronutrients net oxidation rate. These rates were calculated using total O_2 consumption, CO_2 production and urinary nitrogen excretion (corresponding to proteins oxidation) using Weir's equations (1949) corrected by Livesey and Elia (1988). Respiratory quotient for glucose, lipids and proteins were assumed as 1.0, 0.7 and 0.8 respectively. Net substrate oxidations were calculated as (Frayn 1983):

Total lipid oxidation (LOX) [mg/min] = 1.67 VO_2 – 1.67 VCO_2 – 1.92 N
Total carbohydrate oxidation (CHOX) [mg/min] = 4.55 VCO_2 – 3.21 VO_2 – 2.87 N
Total protein oxidation (POX) [mg/min] = 6.25 N

N: Nitrogen excretion [mg/min] was measured by urine collection throughout the tests. Energy expenditure was calculated using each macronutrient's oxidation rates:

EE [kcal/min] = 3.73 CHOX + 9.3 LOX + 4.1 POX

During exercise, the measurement was slightly different as gas exchanges were measured with a mask coupled to a flow meter. Flow meter determined the ventilation rate and air flow direction, while a tube collected expired air to measure gas composition. Together, it was

[2] With V being the ventilation rate (ml/min), Fin and Fout O_2 and CO_2 the inspired and expired fractions of oxygen and carbon dioxide, respectively.

possible to measure VCO_2 and VO_2 breath-by-breath. This allowed the assessment of the oxidation rates of carbohydrate and fat.

Because of the normal physiological variation of gas exchanges and urine was collected throughout the test, obtained data were averaged over 30min intervals and summarized to obtain total oxidation values.

Body impedance analysis (BIA)

This technique is commonly used to measure body composition. Four electrodes were placed on the volunteer's wrist (styloïd processus of ulna bone), hand (distal part of third metacarpal), ankle (medial part between inner and external malleolus) and foot (distal part of third metatarsus). A small electrical current circulated between electrodes as the volunteer had to rest in supine position. The electrical impedance was measured and it was used to estimate total body water. As the quantity of water is not the same between fat and fat-free masses, this technique is able to assess body fat-free mass and body fat mass. This is performed by measuring a single electric arc, and then using algorithms depending on age, height and weight for whole body distributions.

Tracers- stable isotopes tracers

Isotopes are defined as variations of neutrons number of an element (sharing same proton number), resulting in different atomic weights. With the example of carbon, depending on the number of neutrons, some are stable (carbon-12, carbon-13) and others are unstable (radioactive carbon-11, carbon-14). Although natural abundance of isotopes species is varying, it is assumed to be almost invariant in given conditions (Nier and Gulbransen, 1939). Particularly, if the molecule comprising the specific atom is modified, (f.eg. during metabolic reactions) it is still possible to trace atom's fate. This is based on the assumption that the isotopic species of a same element are metabolized in the same fashion. The manipulated rare isotope specie is called the tracer, whereas the molecule comprising the most abundant isotope is called the tracee. The ratio tracer to tracee is called isotopic enrichment and can be measured. Particularly, as the isotopic enrichment of the ingested food is known, the isotopic enrichment of expired carbon dioxide allows calculating which part was oxidized while the remaining stayed in body.

Concerning lipid metabolism, tracers comprising stable isotopes can be used in diverse manners that are safe for subjects. For instance, we used stable isotopes for estimation of oxidation of exogenous fatty acids at rest and during exercise. Volunteers ingested ^{13}carbon-containing fatty acids, and expired $^{13}CO_2$ in breath air was measured by isotopes-ratio mass spectrometry (IRMS).

Briefly, IRMS is a specialization of mass spectrometry that measures the relative isotopic abundances in a given sample. The latter is processed before entering the mass spectrometer, separating atom of interest (carbon here) by chemical methods from similar weighting atoms. Then, the atomic specie of interest is ionized and resulting ions are accelerated. A magnetic field diverts them depending on atomic weight and a screen detects collisions, reflecting isotopic species proportions (isotopic enrichment).

Magnetic resonance spectrometry (MRS)

Also known as nuclear magnetic resonance (NMR) spectroscopy, this method can be used to characterize tissues. This technique is highly associated to magnetic resonance imaging. Both use a standard whole-body scanner. Using a magnetic field and varying frequency, MRS is able to measure resonance frequencies of various body molecules. In skeletal muscles, proton MRS (^{1}H-MRS) can be used to measure IMCL (demonstrated by Boesch et al., 1997) and glycogen. In ^{1}H-MRS, because water includes hydrogen atoms, a process called water suppression has to be performed by measuring the water signal, then removing it to assess lipids-associated protons signals in methylene (CH_2) groups. Thus, this method quantifies the abundance of lipids in a tissue area, but not the type of lipids. Particularly, ^{1}H-MRS is not able to distinguish between intra- and extra myocellular lipids (peaks of IMCL and EMCL partially overlap). Thus, in volunteers with a high EMCL content, IMCL value may be overestimated. To avoid this, the voxel can be moved in an area with less EMCL and, as this protocol included athletes, measurements should not have been enormously overestimated.

Comparing to other IMCL measuring methods (electron microscopy, histochemistry, computed tomography, biochemical analysis or magnetic resonance imaging), MRS has numerous advantages: it is non-invasive, patient friendly and reproducible (coefficients of variation about 6% versus more than 12% in biopsies according to Boesch et al., 1997). MRS allows repeated measurements on identical muscle volumes, and is therefore especially interesting to monitor the effect of an intervention.

Glycogen quantification can be performed in a similar manner by ^{1}H-MRS. Both isotopes allow relative measurements and, by comparison to assumed invariables peaks (phosphocreatine f.eg.), absolute quantification is possible, though laborious.

Blood analyses

Blood was collected on EDTA-coated tubes for measurement of free fatty acids, triglycerides, glucose, lactate, uric acid, total cholesterol, cortisol and insulin, as EDTA-trasylol-coated tubes were used concerning glucagon. After collection, blood was immediately centrifuged (10min, 3600g at 4°C) and plasma was aliquoted, then stored at -20°C until analyzes. Lab technicians performed the following analyzes. Briefly, lactate concentration was measured enzymatically by using kits from Boehringer (Boehringer Mannheim, Mannheim, Germany). Plasma glucose concentration was measured by the glucose oxidase method with a Beckman glucose analyzer II (Beckman Instruments, Fullerton, USA). Colorimetric methods were used to assess plasma concentrations of FFAs (kit from Wako Chemicals, Freiburg, Germany). Commercial radioimmunoassay kits were used for the determination of plasma cortisol, insulin and glucagon (Linco Research, St Charles, USA).

Subjects and study conception

Whole study design

This was a randomized cross-over trial in which subjects were their own controls. Treatments order was randomly chosen and neither the subjects, nor the investigators were aware of allocation. The study was performed in Lausanne University and Hospital and in Bern Hospital. As illustrated in Figure 6, volunteers first came to a screening visit before being enrolled in the study.

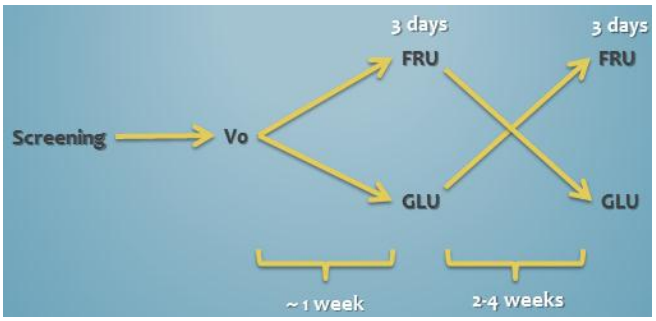

Figure 6: Whole study design

The trial began with a preliminary visit (V0), during which maximal aerobic capacity and anthropometric measurements were performed. To avoid training status variations, soon after that the volunteers began with experiment itself. They faced the two conditions in a row, respecting a 2-4 weeks' washout interval in-between. Each condition consisted in two

successive three-day periods of tests (first condition: V1, V2 and V3, and second condition: V4, V5 and V6).

A food-controlled period lasting 2 days was set before each condition. Diet was weighted and provided to the volunteers for the two forthcoming days, and the morning of the first day of tests (V1 and V4). Menus included 55% carbohydrate (40% polysaccharides, 15% sugars), 30% fat (10% saturated, 10% monounsaturated, 10% polyunsaturated) and 15% protein in isocaloric amounts, trying to avoid ^{13}C and fructose-rich foods. Total calories amounts were calculated using anthropometric measurements of V0 (age, height, weight) with the Harris-Benedict equation (Harris and Benedict, 1918). A correcting factor of 1.7 was then applied, considering this population's usual high physical activity levels.

In order to complete standardization of their metabolic status before the tests, volunteers were instructed to perform a two-hour, low intensity cycling training two days before V1 and V4, and then to refrain from physical exercise the day before tests periods.

Three-day tests periods (Figure 7) were similar, except for the diet composition submitted to the volunteers on the first two days (V1-2 and V4-5): high-fructose versus high-glucose. On

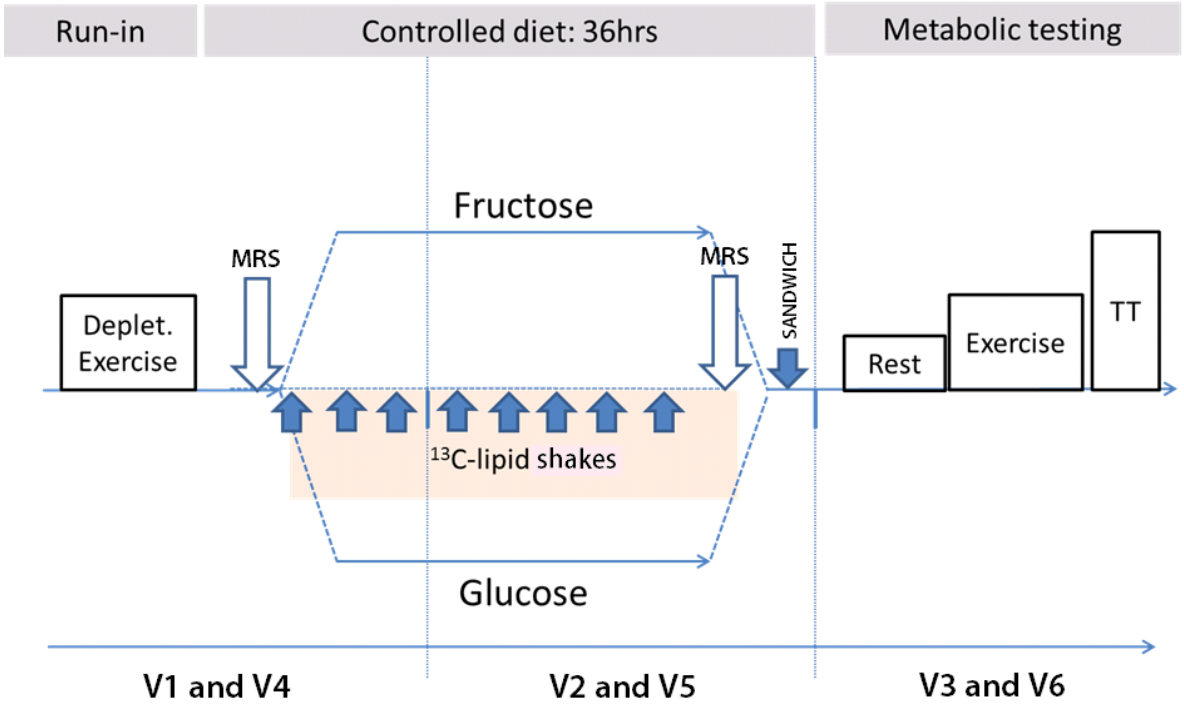

Figure 7: Three-day tests periods

first testing day (V1 and V4), subjects reported to the lab at 1300, were instructed to void and were weighted. They performed a depleting exercise (2.5 hour at 50% MAP) on a cycle ergometer in order to lower their glycogen and IMCL stores. Décombaz et al. (2001) and

Vermathen et al. (2012) showed decreases in IMCL and glycogen contents of 25-30% and 40-45% respectively after comparable exercises in duration and intensity. Within 3h post-exercise, IMCL and glycogen stores were measured by ^{1}H-MRS in medium vastus of quadriceps muscle. Time interval between end of exercise and MRS was shortened maximally, trying to lower refilling by other organs' stores. During that interval, calories intake was null and expenditure minimized in order to get depleted values of skeletal muscle stores.

Following MRS measurement, a repletion period started using liquid shakes of known composition. That period lasted 24h, but covered 36h EE by adding the depletion plus MRS measurement time. Shakes were prepared in order to achieve 80% of theoretical EE, achieving a slight hypocaloric status of subjects: Energy intake was set as 0.8 multiplied by: 36h-energy expenditures (Harris-Benedict with factor 1.0) plus energy expenditure of the depleting exercise (2.5 hours at 50% MAP assuming a mechanic yield of 22.5%).

In order to direct energy to depleted muscle cells, shakes were absorbed in equal amounts at eight different time points, the first three taking place on the first testing day (V1, V4) immediately after MRS in proximate time points. The last five shakes were ingested the day after (V2, V5). The two conditions differed in the sugar composition of the shakes: containing either a fructose/glucose 7:1 proportion[3], or glucose alone. The macronutrients proportions in the shakes were: 45% carbohydrates (40% of known industrially purified pure glucose or fructose), 47% fat (17% saturated, 14% monounsaturated, 16% polyunsaturated) and 8% proteins. Labeled ^{13}C-palmitate will be incorporated in tracer amounts only in next volunteers, and this is why it is not presented in our results.

Repletion continued on second testing day (V2 and V5), and metabolic response to feeding was assessed (see below post-prandial testing section for details). IMCL and glycogen stores of vastus medialis of quadriceps muscle were again measured by MRS after completing the 24h refilling repletion period. For compliance reasons, subjects were then provided a low-lipid, small sandwich comprising white bread and jerked/dried meat (achieving a slight hypocaloric status: 90% of 36h-EE covered since run-in).

[3] For writing simplification, high fructose-containing condition is referred as FRU, being opposed to GLU, even if 5% of the total calories consisted of glucose as well in FRU condition. Glucose adjunction was performed to enhance intestinal absorption of fructose (for review, see Latulippe and Skoog, 2011).

The last day of test periods (V3 and V6), subjects reported to the laboratory at 0700, being overnight fasted. Body composition was firstly measured, following what they underwent a metabolic testing (see below the so named section for details) to measure IMCL oxidation rates and blood metabolites kinetics both at rest and during exercise. Finally, endurance performance was assessed the same day by a simulated 20km time-trial protocol (see below time-trial section for details).

Subjects

A total of twelve endurance cycling trained males (cyclists and triathletes) with competitive experience will be enlisted in the study. The characteristics of the first five subjects having completed the study are presented in Table 1. All subjects had a cycling training history of at least 5many years including competitive status. Subjects were selected after a first informal meeting. All were aged 18-45 years old, having a body mass index (BMI) between 18 and 25kg/m^2 and in general good health, as assessed by a general health questionnaire and rest electrocardiogram examination. None of them was taking any medications, smoked, drank over 10g/day alcohol, had a history of cardiovascular, nor type 2 diabetes events, or was anemic. Before participation, all of the volunteers were fully informed on the risks associated with the study and its purpose, and they fulfilled a written consent. This protocol was effectuated with respect to Helsinki's Declaration and was approved by the Ethics Committee of the Faculty of Biology and Medicine, University of Lausanne as an addendum to protocol 451/11.

Table 1: Study participants characteristics

N = 5	Mean	SD
Age [y]	30	10
Height [cm]	180	7
Weight [kg]	72	5.2
Body fat [%]	16.9	1.5
MAP [W]	376	28
VO_2max [l/min]	4.809	0.349
VO_2max/kg [ml*min^{-1}*kg^{-1}]	67.1	4.5

Preliminary visit (V0)

Within two weeks of the beginning of the first testing period, subjects were weighted, measured for height and their peak oxygen consumption (VO_2max, [ml*min^{-1}*kg^{-1}]) and its associated power were measured. Briefly, weight was obtained to the nearest 0.1 kg with a Seca 708 electronic weighing scale, and height was measured to the nearest 0.1 cm with a Seca height scale, allowing calculating their body mass index (BMI).

To measure maximal oxygen consumption (VO_2max) and maximal aerobic power (MAP), an incremental test to exhaustion was performed on a cycle ergometer (Lode Excalibur Sport, Lode B.V., the Netherlands). Subjects started cycling at 60 W for 5 min, followed by incremental power steps of 35W every 3 min until exhaustion. Heart rate (HR) was continuously recorded by a Polar heart rate monitor (Polar Vantage NV, Kempele, Finland). At the end of each power steps, several parameters were measured: Subjects' rate of perceived exhaustion was monitored using Borg's scale. Drops of blood from the earlobe were collected after each step to measure lactate concentrations (Lactate Pro, Arkray, Kyoto, Japan). MAP was calculated from the last completed step, plus the fraction of time cycled in the final non-completed work rate multiplied by 35W. The obtained MAP was used to determine exercise intensity percentages used in further tests.

Gas exchanges were monitored breath-by-breath by indirect calorimetry (Vmax, SensorMedics srl, Italy) throughout the VO_2max testing with a mouthpiece. The volume sensor and gas analyzers were calibrated using a three liters syringe and variations of known gas compositions respectively. Oxygen uptake (VO_2) was considered to be maximal (VO_2max) when at least two of the three following criteria were met: 1) a leveling off of VO_2 with increasing workload (increase of no more than 2 ml*min^{-1}*kg^{-1}), 2) a HR within 10 beats/min of predicted maximum (HR 220 minus age), and 3) a respiratory quotient exchange ratio (RQ=VCO_2 / VO_2) over 1.05 (Jentjens, 2005). VO_2max was calculated as the average VO_2 (ml*min^{-1}*kg^{-1}) over the last 30 seconds of the test. The volunteer's VO_2max and MAP are also presented in Table 1.

After a period of rest, subjects were familiarized to the time-trial (see the so-called section). It was reported that exercise reproducibility is accurate enough after a single familiarization time-trial (Laursen et al., 2003).

Post-prandial testing (V2 and V5)

After fasting overnight, subjects reported at 0700 to the laboratory where they were asked to void and weighted. A catheter was then inserted into an antecubital vein (right arm) by which blood samples were collected. Trying to avoid homeostasis perturbation due to the catheter positioning, a period of 30min was respected before first values were collected. To obtain most relaxed resting values, subjects watched TV, laid in a bed during the whole 6h-test. Baseline expiratory air samples were collected in quadruplicate to assess expired air $^{13}CO_2$ isotopic enrichment and blood was drawn to measure plasma metabolites basal concentrations.

Subjects were then asked to drink first shake, after what indirect calorimetry measurement was started for two hours. Every 30min, breath and blood samples were collected throughout the test as, to assess post-prandial response, volunteers were provided other shakes at 120 and 240min to repeat three times a 2h-procedure shown in Figure 8. Indirect calorimetry was performed to assess resting EE, LOX and CHOX. POX was assessed by collecting total urine production during the test and measuring urea concentration.

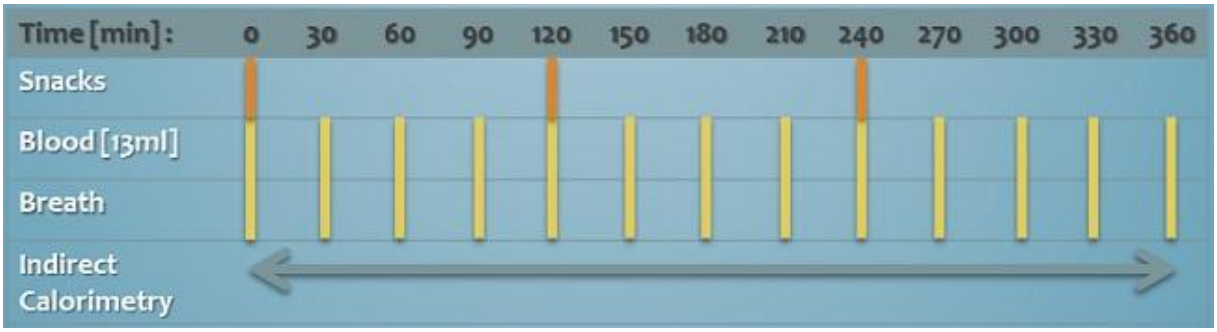

Figure 8: Post-prandial testing (V2 and V5)

Metabolic testing (V3 and V6)

The day after, being overnight fasted, volunteers reported at 0700 to the laboratory where they were again voided and weighted. Blood and air samples were collected in the same manner as the day before. First, resting metabolism was measured by indirect calorimetry. During this time, blood and air were collected respectively three and five times to standardize baseline resting values.

After a 20min transition period, still fasted volunteers were asked to start pedaling constantly at 50% MAP during three hours as measurements were made every twenty minutes. That data collection comprised 5min of indirect calorimetry, blood and air samples. Hearth rate was

monitored during the whole exercise. After a 3h cycling period, the volunteers were provided a 65g carbohydrate-rich snack as they were allowed to recover during 20min before the time-trial.

Time-trial (V3 and V6)

In order to assess performance, a 'time-trial' testing was organized. The volunteers had to complete as fast as possible an individualized work on ergometer. This work was calculated as the mechanical energy corresponding to 20km cycled at 85% of personal MAP at preferential pedaling frequency. Volunteers were isolated from distractions and no information of time, power nor pedaling rate was given. Time-trial duration, exercise intensity (as a percentage of MAP) and absolute power were collected as endurance performance indexes.

Randomization

Condition's order were randomized for each volunteer and kept under key. Neither the volunteers, nor the investigator (until data analysis) were aware of it. Precisely, simple sugars, glucose (GLU) or Fructose to Glucose in a 7:1 proportion (FRU) were added in the shakes preparation by a different person than the investigators. Thus, it was a double-blind trial.

Statistics

Before statistical analysis, all variables were tested for normality using the Kolmogorov-Smirnov test. Then, Student's two-tailed paired t-tests were applied on single parameters. Time-evolving variables were analyzed for treatment (GLU compared with FRU) and time effects using a 2-way (time x condition) repeated-measures analysis of variance (ANOVA), followed, if applicable by Bonferroni post hoc test. Differences beyond 95% of confidence interval (P values lower than 0.05) were considered significant. All statistical operations were performed using Prism 5.0 (GraphPad Software, San Diego, USA). All data are presented as means ± SD.

Results

1) Depleting exercise (V1 and V4)

On run-in of the three-days testing period, the volunteers exercised during 150 min at 50% MAP to deplete quadriceps muscle glycogen and IMCL stores. Monitored mean heart rates were similar between GLU and FRU conditions (135 ± 8 vs. 134 ± 8 bpm, paired t-test: P=0.791).

2) Medium vastus energy stores (V1-2 and V4-5)

Quadriceps muscle IMCL contents were comparable between GLU and FRU conditions in depleted (4.85 ± 1.47 vs. 4.63 ± 1.78 mmol/kg ww, P=0.832), as well as in repleted state (8.27 ± 2.76 vs. 8.13 ± 2.96 mmol/kg ww, P=0.884). IMCL 24h repletion was effective in both conditions, although no treatment effect was detected between GLU and FRU respectively (Fig.8A: 3.42 ± 1.60 vs. 3.5 ± 1.49, 2-way ANOVA: treatment, P=0.922; time, P<0.01; interaction, P=0.950). Glycogen 24h recovery was higher in FRU as compared to GLU (Fig.8B: 0.12 ± 0.13 vs. 0.32 ± 0.04 a.u.[4], 2-way ANOVA: treatment, P<0.05; time, P<0.01; interaction, P<0.05), although this difference may be due to initial glycogen content (1.33 ± 0.13 vs. 0.94 ± 0.11 a.u., P<0.05) rather than final glycogen value (1.45 ± 0.23 vs. 1.26 ± 0.10 a.u., P=0.201) in GLU and FRU respectively. See Table 2 for summary and Figure 9 for illustration.

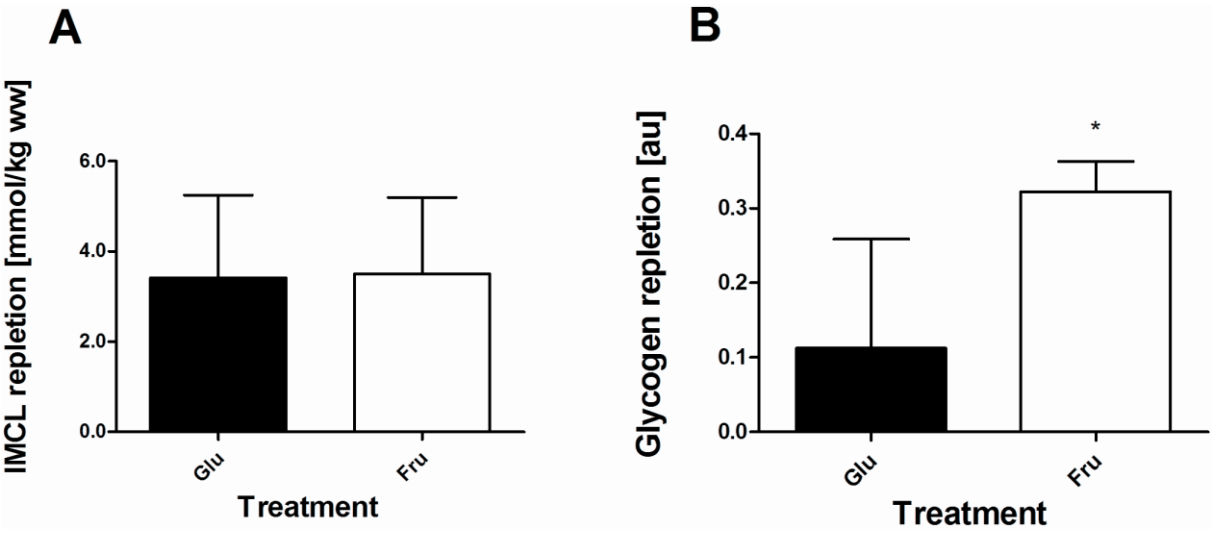

Figure 9: Mean augmentation of quadriceps muscle energy stores contents in response to ingestion of glucose (GLU) or fructose (FRU) containing shakes. For all variables, n=5. Analyses were performed by paired Student's t-tests in all panels (A-B). A: P=0.984. B: P<0.05. Significant differences between GLU and FRU treatments: *P<0.05, **P<0.01 and ***P<0.001 (Bonferroni post hoc test). Data are shown as means ± SD.

[4] a.u. : Arbitrary Unit to creatine content, considered as stable.

Table 2: Repletion of myocellular stores (V1-2 & V4-5)

Symbols: GLU, glucose alone condition; FRU, fructose-containing condition; SD, standard deviation; IMCL, intramyocellular lipids; Δ IMCL, differences between depleted and repleted IMCL contents; Δ Glycogen, differences between depleted and repleted glycogen contents T x T, Treatment x Time interaction. P values were obtained by paired Student's t-tests for not evolving values and 2-way repeated-measures ANOVA for 24h repletion. Data are shown as means ± SD.

n = 4	GLU		FRU		P values		
	Mean	**SD**	**Mean**	**SD**	**Treatment**	**Time**	**T x T**
Depleted IMCL [mmol/kg ww]	4.85	1.47	4.63	1.78	0.832	-	-
Repleted IMCL [mmol/kg ww]	8.27	2.76	8.13	2.96	0.884	-	-
Δ IMCL [mmol/day/kg ww]	3.42	1.60	3.50	1.49	0.922	<0.01	0.950
Depleted Glycogen [a.u.]	1.33	0.13	0.94	0.11	<0.05	-	-
Repleted Glycogen [a.u.]	1.45	0.23	1.26	0.10	0.201	-	-
Δ Glycogen [a.u.]	0.12	0.13	0.32	0.04	<0.05	<0.01	<0.05

3) Post-prandial response to the diet (V2 and V5)

At baseline, none of the fasting indirect calorimetry parameters differed between conditions (Student's paired t-tests). Overall, whole-body lipid oxidation was 103.0 ± 17.6 vs. 87.0 ± 29.0 mg/min (P=0.199) and protein oxidation 18.0 ± 2.8 vs. 17.3 ± 3.2 mg/min (P=0.750) were not different between GLU and FRU. However, carbohydrate oxidation was lower in GLU, as compared to FRU (101.4 ± 39.4 vs. 156.8 ± 70.9 mg/min; P<0.05), even if that difference was mostly imputed to first testing hour. Overall, this resulted in a comparable energy expenditure (EE: 1.42 ± 0.12 vs. 1.48 ± 0.20 kcal/min; P=0.607). Because of that increased CHOX, the mean RQ was augmented as well in FRU (0.78 ± 0.03 vs. 0.82 ± 0.05; P<0.05). Taken together, whole body oxidations were 67.9% lipids, 26.8% carbohydrates, 5.2% proteins vs. 55.2% lipids, 39.9% carbohydrates, 4.8% proteins in GLU and FRU respectively.

Interestingly, QR peaks following shakes ingestion did not appear at the same time between treatments (2-way ANOVA: T x T component: P<0.001). They seemed to happen earlier in FRU condition (Fig.10) and this may be due to differences in both LOX (P<0.01) and CHOX (P<0.001) in T x T interaction effect. See Figure 10 for LOX and CHOX kinetics illustration.

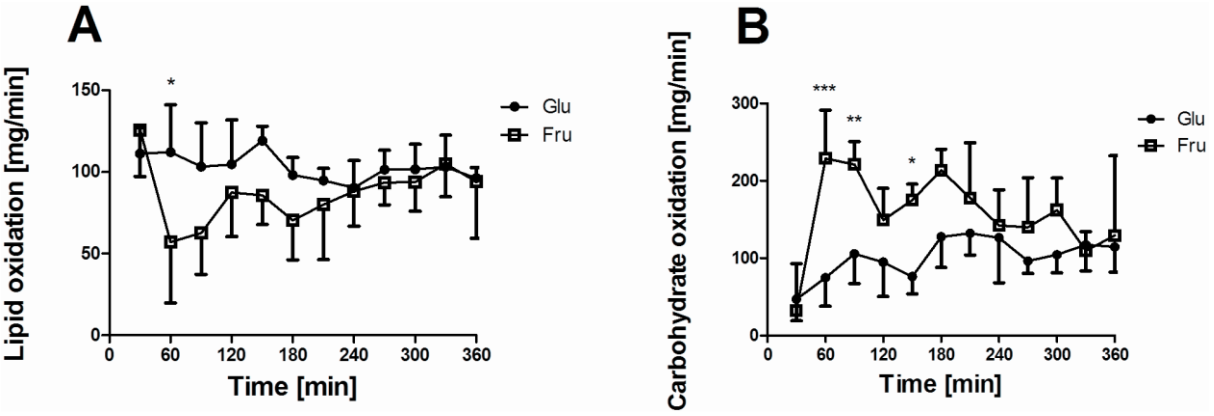

Figure 10: Mean postprandial 30min-averaged macronutrient oxidation rates in response to ingestion of glucose (GLU) or fructose (FRU) containing shakes at three successive time occurrences (T=0;120;240). For all variables, n=5. Analyses were performed by 2-way repeated ANOVA in both panels. A: P=0.199 for treatment, P<0.01 for time, P<0.01 for interaction. B: P<0.05 for treatment, P<0.001 for time, P<0.001 for interaction. Significant differences between GLU and FRU treatments at certain time points: *P<0.05, **P<0.01 and ***P<0.001 (Bonferroni post hoc test). Data are shown as means ± SD.

Concerning plasma metabolites, at baseline, triglycerides (0.39 ± 0.08 vs. 0.41 ± 0.07, P=0.145), glucose (4.61 ± 0.13 vs. 4.80 ± 0.25, P=0.060) and uric acid (0.40 ± 0.05 vs. 0.39 ± 0.04, P=0.121) were similar between GLU and FRU. Free fatty acids (0.50 ± 0.16 vs. 0.34 ± 0.09, P<0.05) and lactate (0.68 ± 0.10 vs. 0.55 ± 0.09, P=0.051) were higher in GLU as compared to FRU.

In both conditions plasma concentrations of free fatty acids (P<0.001), triglycerides (P<0.001), glucose (P<0.001), and lactate (P<0.001) were significantly increased with time. However, no significant effect of time was found for uric acid concentrations (P=0.077).

As compared to GLU, changes over time of plasma triglycerides (Fig.11A) and lactate (Fig.11D) were increased in FRU. On the other hand, plasma concentrations of free fatty acids (Fig.11B), glucose (Fig.11C) and uric acid (Fig.11E) were not modified as compared to GLU.

Combined effect of fructose and time (2-way ANOVA: Treatment x Time parameter) resulted in statistical differences between GLU and FRU conditions for free fatty acids (P<0.01), triglycerides (P<0.01), glucose (P<0.01) and lactate (P<0.001), while uric acid response was similar (P=0.122). See Figure 11 for illustration and Table 3 for summary of the postprandial test.

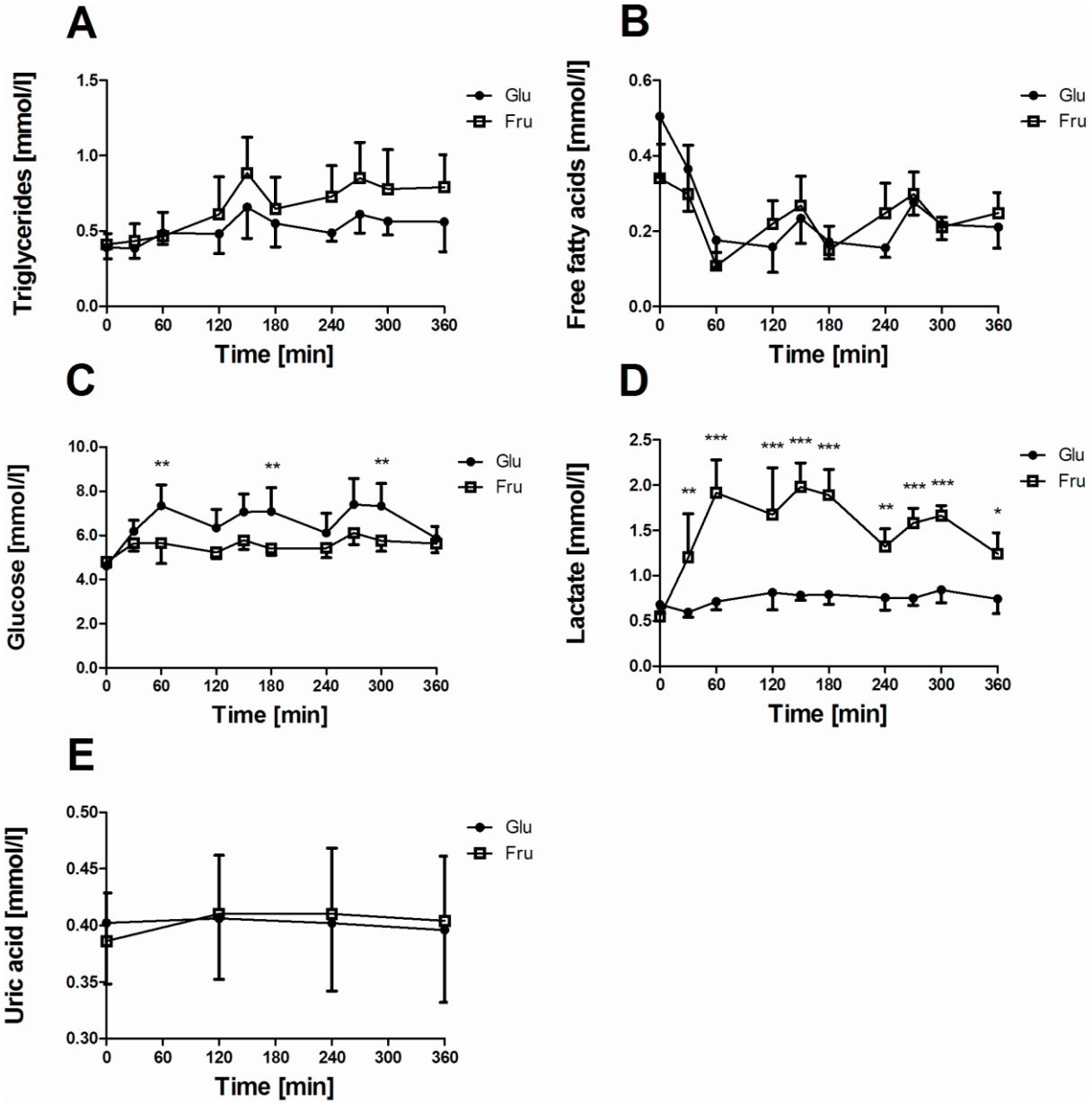

Figure 11: Mean plasma metabolites concentrations in response to ingestion of glucose (GLU) or fructose (FRU) containing shakes. For all variables, n=5. Analyses were performed by 2-way repeated ANOVA in all panels (A-E). A: P<0.01 for treatment, P<0.001 for time, P<0.01 for interaction. B: P=0.769 for treatment, P<0.001 for time, P<0.01 for interaction. C: P<0.01 for treatment, P<0.001 for time, P<0.01 for interaction. D: P<0.001 for treatment, P<0.001 for time, P<0.001 for interaction. D: P=0.978 for treatment, P=0.077 for time, P=0.122 for interaction. Significant differences between GLU and FRU treatments at certain time points: *P<0.05, **P<0.01 and ***P<0.001 (Bonferroni post hoc test). Data are shown as means ± SDs.

Table 3: Postprandial metabolic characteristics (V2 & V5)

Symbols: GLU, glucose alone condition; FRU, fructose-containing condition; SD, standard deviation; VO_2, oxygen consumption; VCO_2, carbon dioxide production; T x T, Treatment x Time interaction. P values were obtained by Student's t-tests for baseline values and 2-way repeated-measures ANOVA for time evolving variables. Data are shown as means ± SD.

n = 5	GLU		FRU		P values		
Duration [min]	360	-	360	-	-	-	-
Meals time [min]	0 ; 120 ; 240		0 ; 120 ; 240		-	-	-
Indirect calorimetry	**Mean**	**SD**	**Mean**	**SD**	**Treatment**	**Time**	**T x T**
VO_2 [l/min]	0.297	0.025	0.305	0.033	0.701	<0.01	0.794
VCO_2 [l/min]	0.233	0.021	0.251	0.03	0.295	<0.001	0.087
Total lipid oxidation [mg/min]	103.0	17.6	87.0	29.0	0.199	<0.01	<0.01
Total carbohydrate oxidation [mg/min]	101.4	39.4	156.8	70.9	<0.05	<0.001	<0.001
Total protein oxidation [mg/min]	18.0	2.8	17.3	3.2	0.750	0.453	0.453
Energy expenditure [kcal/min]	1.42	0.12	1.48	0.20	0.607	<0.001	0.790
Lipids (% energy)	67.9	-	55.2	-	-	-	-
Carbohydrates (% energy)	26.8	-	39.9	-	-	-	-
Proteins (% energy)	5.2	-	4.8	-	-	-	-
Respiratory quotient	0.78	0.03	0.82	0.05	<0.05	<0.01	<0.001
Plasma metabolites	**Mean**	**SD**	**Mean**	**SD**	**Treatment**	**Time**	**T x T**
Triglycerides: baseline [mmol/l]	0.39	0.08	0.41	0.07	0.290	-	-
Triglycerides : evolution [mmol/l]	0.51	0.15	0.66	0.25	0.166	<0.001	<0.01
Free fatty acids: baseline [mmol/l]	0.50	0.16	0.34	0.09	0.090	-	-
Free fatty acids: evolution [mmol/l]	0.24	0.13	0.24	0.10	0.769	<0.001	<0.01
Glucose: baseline [mmol/l]	4.61	0.13	4.80	0.25	0.119	-	-
Glucose: evolution [mmol/l]	6.48	1.13	5.54	0.55	<0.01	<0.001	<0.01
Lactate: baseline [mmol/l]	0.68	0.10	0.55	0.09	0.102	-	-
Lactate: evolution [mmol/l]	0.74	0.13	1.50	0.50	<0.001	<0.001	<0.001
Uric acid [mmol/l]	0.40	0.05	0.39	0.04	0.242	-	-
Uric acid: evolution [mmol/l]	0.40	0.05	0.40	0.05	0.978	0.077	0.122

4) Metabolic testing (V3 and V6)

At rest, VO_2, VCO_2, RQ, and net substrate oxidation were comparable between GLU and FRU (Table 4). Fasting concentrations of plasma free fatty acids (0.526 ± 0.143 vs. 0.594 ± 0.269 mmol/l, P=0.303), triglycerides (0.565 ± 0.116 vs. 0.569 ± 0.156 mmol/l, P=0.549), glucose (4.996 ± 0.553 vs. 5.028 ± 0.299 mmol/l, P=0.276) and uric acid (0.398 ± 0.061 vs. 0.390 ± 0.046 mmol/l, P=0.488) did not differ between GLU and FRU conditions. Resting plasma lactate concentration was however higher in GLU as compared to FRU (0.681 ± 0.065 vs. 0.579 ± 0.072, P<0.01) (Table 4).

As expected, EE as well as total substrate oxidation rates increased significantly (p<0.001) with exercise, but did not differ between conditions (p for treatment effect =NS, Table 4). Mean heart rate did also not statistically differ between treatments (P=0.273) although it was slightly higher (146 ± 6 vs. 142 ± 6 bpm) in GLU condition. Interestingly, there was a significant interaction between treatment and time for net lipid oxidation, suggesting different kinetics of lipid oxidation rates between conditions (Fig.12). During the first, second and third

exercising hours, lipid oxidation represented 44.2 vs. 46.6%, 47.4 vs. 50.5% and 51.1 vs. 50.6 % of total energy expenditure in GLU and FRU, respectively, while carbohydrate oxidation decreased with time and represented 55.6 vs. 53.2%, 52.4 vs 49.3% and 48.7 vs. 49.2% of total energy expenditure in GLU and FRU respectively. Because POX was obtained through whole day urine collection, intra-exercise time effect was not analyzable. Overall, lipids, carbohydrates and proteins provided respectively 47.5%, 52.3% and 0.2% of total energy expenditure during the exercise bout, corresponding to 2673 kcal in GLU condition. In FRU 49.2% of EE was accounted for lipid, 50.6% for carbohydrate and 0.2% for protein oxidation, averaging 2666 kcal. See Figure 12 for LOX and CHOX kinetics and Table 4 for summary of other parameters.

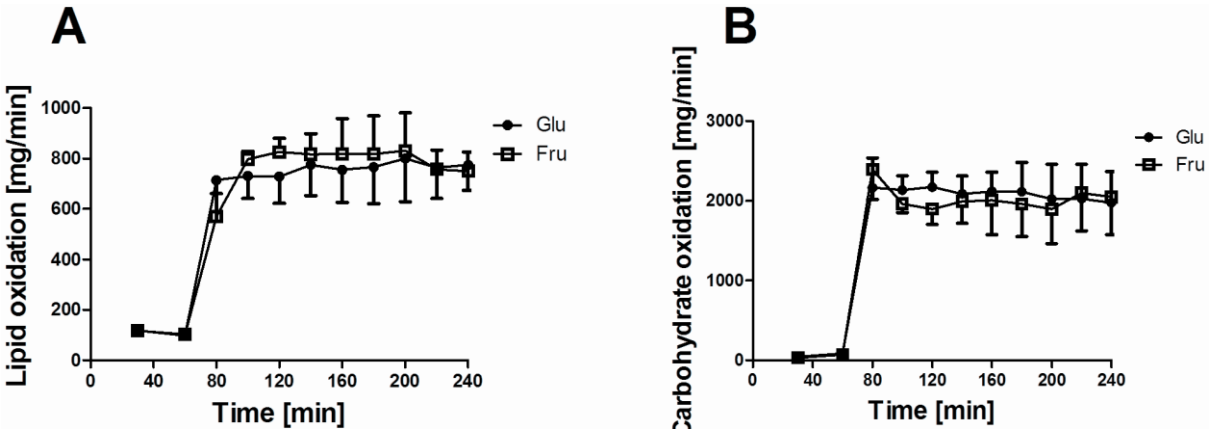

Figure 12: Mean 20min-averaged fasted macronutrient oxidation rates one day after ingestion of glucose (GLU) or fructose (FRU). For both variables, n=5. Analyses were performed by 2-way repeated ANOVA in both panels. A: P=0.670 for treatment, P<0.01 for time, P<0.01 for interaction. B: P=0.756 for treatment, P<0.05 for time, P=0.092 for interaction. Data are shown as means ± SD.

All plasma metabolites were significantly ($p<0.05$) changed by exercise. Interestingly, even if no significant change was found between GLU and FRU with regard to the integrated response of plasma metabolites (Table 4), plasma lactate concentrations exhibited a different pattern (p for interaction <0.001), as shown in Fig 13D).

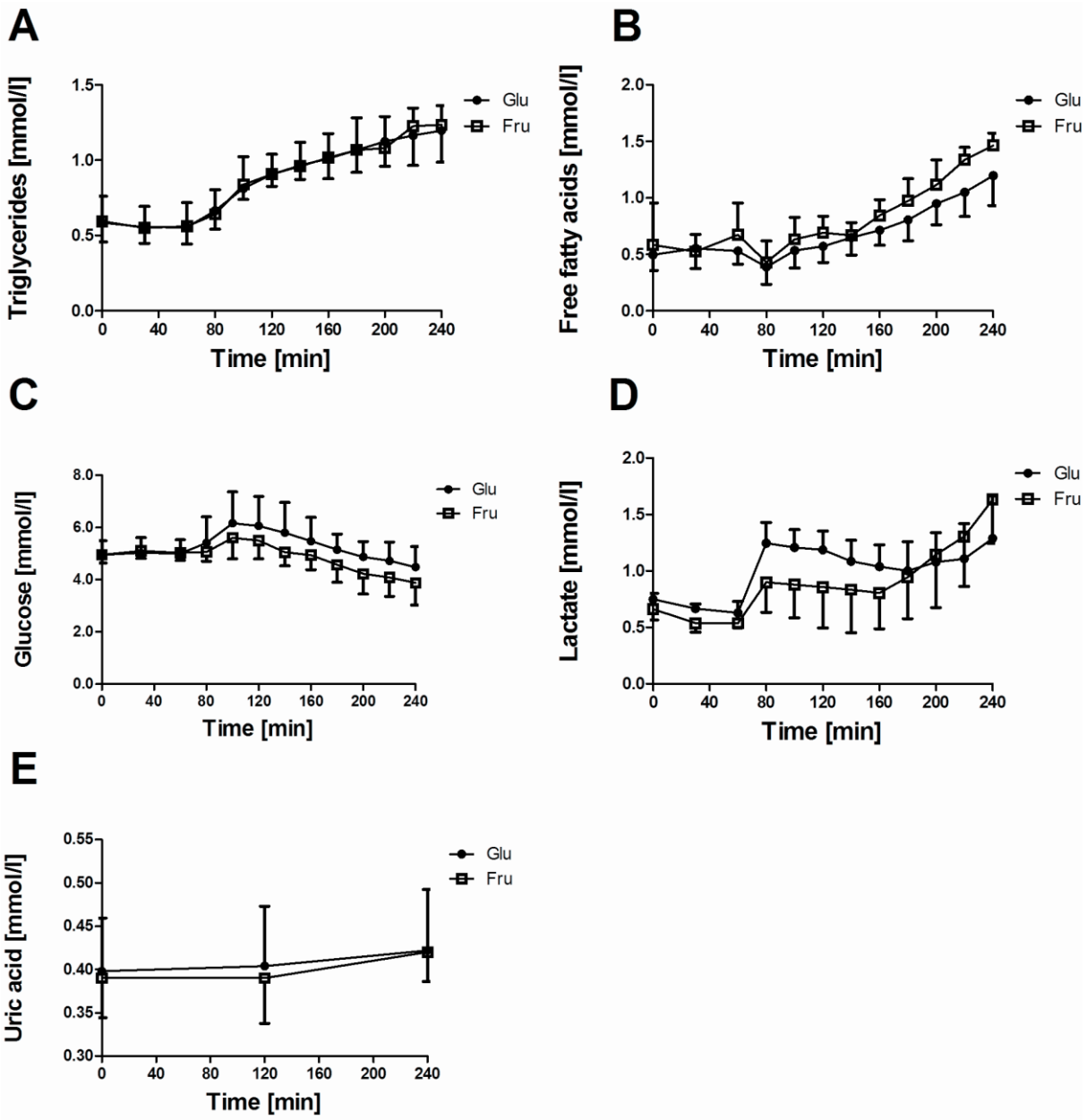

Figure 13: Mean plasma metabolites concentrations, the day after ingestion of glucose (GLU) or fructose (FRU). Subjects firstly rested during one our (T≤60min), then exercised (50% MAP) for 3h (60<T≤240min). For all variables, n=5. Analyses were performed by 2-way repeated ANOVA in all panels (A-E), considering mean resting values (0<T≤60min) as baseline. A: P=0.941 for treatment, P<0.001 for time, P=0.984 for interaction. B: P=0.147 for treatment, P<0.001 for time, P=0.183 for interaction. C: P=0.256 for treatment, P<0.001 for time, P=0.729 for interaction. D: P=0.524 for treatment, P<0.001 for time, P<0.001 for interaction. E: P=0.824 for treatment, P<0.01 for time, P=0.763 for interaction. Significant differences between GLU and FRU treatments: *P<0.05, **P<0.01 and ***P<0.001 (Bonferroni post hoc test). Data are shown as means ± SD.

With respect to kinetics, plasmatic concentrations of triglycerides and free fatty acids were continuously raised (TG: T=240; 1.198 ± 0.210 vs. 1.237 ± 0.127; FFA: T=240; 1.198 ± 0.268 vs. 1.465 ± 0.109) in GLU and FRU respectively.

Glucose concentration however, firstly peaked soon after the onset of exercise (T=80; 6.164 ± 1.200 vs. 5.602 ± 0.797) above resting mean values (+1.168 vs. +0.574), then constantly diminished until the end of exercise (T=240; 4.484 ± 0.784 vs. 3.875 ± 0.849) in GLU and FRU respectively. Maximal values were correlated (R^2 =0.92) to minimal, end-exercise, values.

Plasma lactate also peaked soon after the onset of exercise (T=80; 1.246 ± 0.184 vs. 0.900 ± 0.265) above resting values (+0.565 vs. +0.321) in both GLU and FRU. Exercise effect then decreased lactate concentration reaching minimal values (1.038 ± 0.193 vs. 0.804 ± 0.316) at different time points (180 vs. 160) for GLU and FRU respectively. Lactate concentration then rose again in both conditions until the end of exercise (T=240; 1.288 ± 0.377 vs. 1.633 ± 0.390). See Table 4 for summary of whole metabolic testing. Results of insulin, glucagon and cortisol dosages are not known yet.

Table 4: Day after metabolic characteristics at rest and during moderate exercise (V3 & V6) Symbols: GLU, glucose alone condition; FRU, fructose-containing condition; VO_2, oxygen consumption; VCO_2, carbon dioxide production; MAP, maximal aerobic power; SD, standard deviation; T x T, Treatment x Time interaction. P values were obtained by Student's t-tests for baseline values and 2-way repeated-measures ANOVA for time evolving variables. Data are shown as means ± SD.

n = 5	GLU		FRU		P values		
Rest	**Mean**	**SD**	**Mean**	**SD**	**Treatment**	**Time**	**T x T**
Duration [min]	60	-	60	-	-	-	-
Indirect calorimetry	**Mean**	**SD**	**Mean**	**SD**	**Treatment**	**Time**	**T x T**
VO_2 [l/min]	0.274	0.020	0.268	0.022	0.437	-	-
VCO_2 [l/min]	0.209	0.016	0.201	0.017	0.261	-	-
Total lipid oxidation [mg/min]	108.5	16.6	110.0	16.7	0.745	-	-
Total carbohydrate oxidation [mg/min]	66.0	34.0	52.8	32.7	0.116	-	-
Total protein oxidation [mg/min]	6.9	2.3	7.3	2.0	0.812	-	-
Energy expenditure [kcal/min]	1.31	0.10	1.28	0.10	0.399	-	-
Lipids (% energy)	78.6	-	81.8	-	-	-	-
Carbohydrates (% energy)	19.2	-	15.8	-	-	-	-
Proteins (% energy)	2.2	-	2.4	-	-	-	-
Respiratory quotient	0.76	0.03	0.75	0.03	0.205	-	
Plasma metabolites	**Mean**	**SD**	**Mean**	**SD**	**Treatment**	**Time**	**T x T**
Free fatty acids [mmol/l]	0.54	0.14	0.56	0.27	0.303	-	-
Triglycerides [mmol/l]	0.61	0.11	0.59	0.15	0.549	-	-
Glucose [mmol/l]	4.98	0.51	4.93	0.28	0.276	-	-
Lactate [mmol/l]	0.67	0.08	0.56	0.09	<0.01	-	-
Uric acid [mmol/l]	0.39	0.06	0.37	0.05	0.512	-	-
Exercise	**Mean**	**SD**	**Mean**	**SD**	**Treatment**	**Time**	**T x T**
Duration [min]	180	-	180	-	-	-	-
Set power (50% MAP) [W]	188	13	188	13	-	-	-
Mean heart rate [bpm]	146	6	142	6	0.215	<0.001	0.949
Indirect calorimetry	**Mean**	**SD**	**Mean**	**SD**	**Treatment**	**Time**	**T x T**
VO_2 [l/min]	3.135	0.110	3.099	0.177	0.659	<0.01	0.798
VCO_2 [l/min]	2.658	0.107	2.627	0.178	0.737	0.865	0.997
Total lipid oxidation [mg/min]	758.2	117.9	783.2	120.4	0.670	<0.001	<0.01
Total carbohydrate oxidation [mg/min]	2083.4	297.8	2009.1	363.1	0.756	<0.05	0.092
Total protein oxidation [mg/min]	6.9	2.3	7.3	2.0	0.812	1.000	1.000
Energy expenditure [kcal/min]	14.85	0.48	14.81	0.90	0.902	0.075	0.779
Lipids (% energy)	47.5	-	49.2	-	-	-	-
Carbohydrates (% energy)	52.3	-	50.6	-	-	-	-
Proteins (% energy)	0.2	-	0.2	-	-	-	-
Respiratory quotient	0.85	0.02	0.85	0.02	0.919	<0.001	0.184
Plasma metabolites	**Mean**	**SD**	**Mean**	**SD**	**Treatment**	**Time**	**T x T**
Free fatty acids [mmol/l]	0.70	0.29	0.81	0.35	0.147	<0.001	0.183
Triglycerides [mmol/l]	0.88	0.27	0.88	0.28	0.941	<0.001	0.984
Glucose [mmol/l]	5.32	0.87	4.86	0.75	0.256	<0.001	0.729
Lactate [mmol/l]	1.02	0.29	0.90	0.41	0.524	<0.001	<0.001
Uric acid [mmol/l]	0.41	0.06	0.40	0.05	0.824	<0.01	0.763

4.3) Endurance performance (V3 and V6)

Finally, endurance exercise performance was assessed by a time-trial protocol in which subjects had to complete an individualized work on ergocycle as fast as possible. No significant difference was observed with regard to work completion time (36:38 ± 2:35 vs. 37:19 ± 1:28 min; P=0.157) that can also be expressed as average power (267 ± 21 vs. 257 ± 20 W; P=0.150) or %MAP intensity (71.1 ± 4.8 vs. 68.4 ± 2.6) between GLU and FRU respectively. Time-trial mean heart rate difference (169 ± 4 vs. 166 ± 7; P=0.141) was also not different between conditions. See Table 5 for summary.

Table 5: Performance assessment (V3 & V6)
Symbols: GLU, glucose alone condition; FRU, fructose-containing condition; SD, standard deviation; P values were obtained by paired Student's t-tests. Data are shown as means ± SD.

n = 4	GLU		FRU		P values
	Mean	**SD**	**Mean**	**SD**	**Treatment**
Mechanical work [kJ]	574	38	574	38	-
Time [min]	36:38	02:35	37:19	01:28	0.157
Average power [W]	267	21	257	20	0.150
Intensity (% MAP)	71.1	4.8	68.4	2.6	0.134
Mean heart rate [bpm]	169	4	166	7	0.141

Discussion

This study indicates that a post-exercise, high-fructose high-fat diet does not replenish more IMCL levels than does an iso-energetic high-glucose high-fat diet. Although not enhancing pre-exercise IMCL levels, and opposite to our expectations, our study indicates that a high-fructose diet may increase skeletal muscle glycogen more than does a high-fructose diet.

The effects of the diet composition in exercise recovery and next bout preparation have been intensively studied (for review, see Hawley et al., 2007 or Burke et al., 2011). Previous studies focussed on high glucose intakes and their ability to increase skeletal muscle glycogen within a few hours (Ivy et al, 1998, Jentjens et al., 2003). To our knowledge, if such diets (high-simple carbohydrates, high-fat, low protein) have been already tested on skeletal muscle energy stores dynamics, it is the first time that the particular role of fructose is tested. On the opposite, if the particular effects of fructose during or immediately before exercise are well known, few data exist on influence of fructose ingested the day before exercise. Therefore,

this study represents one of the first work in which fructose is used toward a fat adaptation strategy.

Myocellular energy stores

Our feeding protocol resulted in no change in 24h repletion rates of IMCL after fructose, but an enhanced repletion of glycogen comparing to glucose alone as directly measured by ^{1}H-MRS.

It was assumed that the lipogenic effects of fructose feeding and the low insulin levels associated to fructose intake would drive to an increase in circulating TG (Lê et al, 2006; 2009; Lê and Tappy, 2010) and a higher activity of skeletal muscle LPL, thereby leading to an increase in IMCL. Because fructose intake was spread over 36 hours only, it may be that fructose-induced *de novo* lipogenesis was not stimulated enough to induce a significant increase in circulating VLDL-TG, thereby preventing a further increase in IMCL levels as compared to the GLU condition. Our diminished TG response might be responsible for that low IMCL repletion, and is consistent with observations that physical activity prevents fructose-induced dyslipidemia (Egli et al., in revision, 2013). These results may indicate that in addition to an acute effect of exercise, chronic exercise may also prevent ectopic fat deposition, as shown earlier in our laboratory (Egli et al., in revision, 2013). Actually fructose is highly consumed among exercising athletes, partly because of its ability to raise total carbohydrate oxidation and intestinal absorption during exercise. As this highly exposed population doesn't develop on the long term suspected health-concerning effects of fructose, Johnson et al. (2010) concluded that they may be protected. Then, as Burstein et al. (1984) showed that insulin receptors number is modulated by physical training, this could possibly explain that "protection". Our measures on highly aerobic trained individuals are in line to such considerations, and therefore the lack of fructose-induced hypertriglyceridemia could be explained by a protective mechanism due to chronic physical activity. This is further supported by the fact that numerous studies performed with less important fructose doses than ours (about 3g/kg per day) resulted in hypertriglyceridemia (f.eg. Bantle et al., 2000).

IMCL appeared to be higher the first day subjects were tested, irrespective of treatment order (5.51 ± 1.87 vs. 3.97 ± 1.40 mmol/kg ww; $P<0.05$). It may be that for some reason the conditions' facing order was of importance. The reason of this effect is unknown, and may still be related to the fact that the randomization was unbalanced in the five first subjects (i.e.

four faced GLU and then FRU vs. only one faced FRU and then GLU). Moreover, IMCL absolute contents after repletion on first faced condition were still higher than on second condition, although this did not reach significance (8.87 ± 3.52 vs. 7.53 ± 2.81 mmol/kg ww; P=0.056) while treatments still resulted in no difference. Considering that the depleting exercise was performed within the same mean heart rates ranges independently on order or treatment, it is possible that, for some reason, volunteers presented themselves on second testing period with lower basal IMCL levels.

Concerning glycogen, its repletion was most effective in FRU. This was quite unexpected and in contradiction to previous work. Ivy et al. (1998) determined that glucose or glucose polymers were the most effective for replenishment of muscle glycogen. They also noticed the importance of calories intake partitioning, concluding that continuous high insulin levels induced by glucose allowed a higher glycogen synthesis. Fructose, being less insulin stimulating, was mostly beneficial for the replenishment of liver glycogen (Conlee, Lawler and Ross, 1987). Glycogen synthase, the key-regulating enzyme controlling glycogen synthesis, has been showed being stimulated by insulin or previous contraction (Nielsen et al., 2004). Jentjens and Jeukendrup (2003), however, hypothesized that carbohydrate availability was the main limiting factor for glycogen synthesis. Because in our results glycaemia remained lower with fructose in the postprandial state, it could be expected that glycogen synthesis would be lowered in fructose condition. Because fructose increases less glycaemia than glucose, it also prevents insulin stimulation of glycolysis and oxidation. Then, glycogen breakdown may have been lowered in the postprandial state in the same time as synthesis was diminished. As glycogen content can be assumed being in constant balance between synthesis and breakdown, a diminution of both components induced by low insulin levels could still result in the overall higher net gain observed in fructose condition.

However, Jentjens and Jeukendrup (2003) showed that low muscle glycogen concentration can be a potent regulator of glycogen synthase, while breakdown can be stimulated by high contents by direct binding to glycogen phosphorylase (Johnson et al., 1992). Because our subjects faced glycogen repletion with lower initial glycogen contents in FRU, synthesis was locally enhanced while breakdown was diminished and may also explain this discrepancy.

Fructose postprandial metabolism

Our study also gave the opportunity to verify several traditional effects of fructose feeding (for review, see Sun 2012). Soon after ingestion, it effectively induced hyperlactatemia, diminished the glycemic peaks, and increased total carbohydrate oxidation that occurred earlier following the shakes.

Uric acid is produced during the breakdown of purines, and fructose-induced raised plasmatic uric acid concentrations (hyperuricemia) were associated to cardiometabolic diseases (Johnson et al., 2007). The proposed mechanism was a drop in liver cellular ATP due to fructose metabolism, which in turn could induces AMP degradation and raises plasma and urine uric acid concentrations. Our study, however, didn't result in any change in plasma uric acid concentrations. Wang et al. (2012) performed a meta-analysis on current knowledge concerning fructose-induced hyperuricemia. He concluded that while fructose overfeeding induced hyperuricemia, isocaloric fructose amounts did not increase plasma concentration of uric acid. He also concluded that non-diabetic patients were less exposed, moreover if the duration of fructose feeding was short. Our experiment, performed with a short time isocaloric fructose feeding on non-diabetic athletes, corroborate to these conclusions.

Day after fasting resting consequences

To our knowledge, only one study focused on the 24h effect impact of fructose on several metabolites following an acute fructose load. In that study, Teff (et al., 2009) also reported day after fasting metabolites values. Interestingly, the only fasting metabolite modified with fructose was plasma lactate that was decreased in their study as observed in ours. To explain this, the authors referred to Carmona and Freedland (1989) who associated lactate concentration to liver lipogenic activity. These authors propose that fructose depletes ATP in hepatocytes, resulting in SIRT-1 mediated activation of pyruvate dehydrogenase. In turn, acetyl-CoA levels rise, leading to an increase in lipogenic precursor availability. Therefore, it is possible that in our study the lower day after lactate fasting concentration could be explained by some compensatory mechanism occurring during the night following fructose ingestion, and that this mechanism involved an overnight decrease in liver lipogenesis.

Contrarily to previous results (Bantle et al., 2000), we didn't observe higher fasting triglycerides levels in fructose condition. As subjects were measured initially the day before, being fasted after 3/8 shakes, we can compare their fasted concentrations after 3/8 shakes and after 8/8 shakes on day of metabolic testing. In that order, both diets, however, increased

fasting triglycerides concentrations with increasing number of shakes. This has to be explained by our high fat diet composition (47%) in both conditions, by a possible protective effect of chronic physical activity or by the low duration of fructose exposure.

Exercise metabolism

Exercise allows the specific study of skeletal muscle metabolism, which represents the vast majority of energy expenditure in such conditions. Because opposite to glucose, fructose does not inhibit fat metabolism and oxidation, it is probable that much of its effect observable the day after should be similar to those observed after a high fat protocol and be especially visible during exercise. Therefore, much of our results can be compared to the study of Zehnder et al. (2006), which investigated the effects of a 36h high fat hypercaloric diet and their effects the day after on a subsequent 3h, 50% MAP continuous exercise performed by trained athletes, while they measured IMCL contents before and after exercise.

Our results show no differences in total macronutrients oxidations, or whole body energy expenditure. This may signify that such a short-term change in the diet does not result in a change in substrate selection system. However, accordingly to Zehnder et al. (2006) our data indicate a trend toward higher total lipid oxidation during the first part of exercise in fructose condition that was no more present in the last hour. Zehnder et al. (2006) and Sacchetti et al. (2002) postulated that this initial higher lipid oxidation was due to IMCL utilization, which was proportional to initial content and decreased with exercise time.

Interestingly, while Zehnder et al. (2006) observed that total fat utilization was enhanced only during the first exercising hour, they also noticed that it was correlated with participants' aerobic capacity. Then, this confirms previous work on fat oxidation that is raised in athletes comparing to sedentary subjects (for review, see Kelley, 2005). As a result, highly trained athletes would more benefit from such fat adaptation protocols by increasing their lipid oxidation percentages during a longer exercising time.

At that point, our fructose loading diet may seem interesting because of increased fat oxidation during the initial two exercising hours. However, it was also marked by the negative counterpart: lower glycemic values. Traditionally, at the onset of exercise, catecholamines are secreted via sympatho-adrenal stimulation via the fight-or-flight response (Bracha, 2004), resulting in an increased and decreased glucagon and insulin secretion, respectively. Then,

these hormones are responsible for increased liver glucose output while the exercising muscles increase their glucose uptake due to contraction. Depending on exercise intensity, muscle uptake overpasses limited liver glucose production, and glycaemia falls with time (for review, see Suh, Paik and Jacobs, 2007).

Interestingly, our results show a trend to a lower glycaemia at the onset of exercise in fructose condition. Therefore, liver glucose output function may have been impaired by our high fructose, high fat diet, despite the documented effects of fructose enhancing liver glycogen (Décombaz et al., 2011). It is possible that this was due to decreased catecholamines in response to fructose, as this was already reported by Da Silva-Grigoletto et al. (2010).

Our results further seem to confirm previous studies showing that fructose decreases exercising mean heart rate. Because heart rate is modulated by the autonomous nervous system (Glick and Braunwald, 1965), including its sympathetic component, it is possible that the possible diminution in catecholamines was responsible for heart rate diminution in fructose condition.

Finally, volunteers reported hypoglycemia symptoms in both conditions. We can then suppose that muscular blood glucose uptake remained stable during exercise between conditions (Jeukendrup et al., 1999), and that our 3h/50% MAP exercise tested low glycemic response during the last hour.

Interestingly, because lactate concentrations remained steady during the first two exercising hours and then raised dramatically (Fig.13D), it can be hypothesized that gluconeogenesis from lactate may have been increased by fructose ingestion the day before, as it was already reported in exercising subjects ingesting fructose (Lecoultre et al., 2010). In our study, it is possible that fructose caused an increase in liver gluconeogenesis that was still enhanced the day after. Supposing this, it would explain our diminution in resting lactatemia in fructose condition. As expected, during exercise, lactate concentration arose in both conditions because of the muscles increased production. The liver gluconeogenesis was again supposed more active in fructose condition, as lactate concentrations remained lower. However, at the end of the exercise the marked difference in curves may be explained by the lowered glycemic values in FRU more than GLU, as low blood glucose was reported to raise lactate concentration (Medalle et al., 1971).

In fact, probably both lowered glycemic values and already maximized gluconeogenesis could explain the higher lactatemia in last exercising hour of fructose condition. In absence of direct flux measurements, this remains highly speculative, but interestingly all volunteers reported effectively more hypoglycemia symptoms during the last exercising hour of fructose condition and one volunteer had to drop out at that time.

Plasma FFA were raised during exercise in both conditions, but there was a trend toward higher levels in fructose condition, especially marked during the last hour (Figure 13B). As plasma free fatty acids concentration represents a balance between adipose tissue lipolysis and exercising skeletal muscle uptake, one or both components can be responsible for these differences. While Romijn et al. (1995) showed that the muscle free fatty acids relay is constant or increased with exercise duration, lipolysis was shown to be effectively less repressed by low insulin (Yen and Steinmetz, 1972) or blood glucose (Frizzell et al., 1988) levels. As written before, fructose is known to induce lower insulin concentration. Then, persistent lower insulin levels resulting from FRU may have result in less repressed lipolysis during the whole exercise, while lower blood glucose may have been responsible for the last exercise hour differential lipolysis stimulation between treatments. Both fructose-induced lower insulinemia and lower glycaemia may have been responsible for the tendency to increased FFA concentration throughout the test.

Sondergaard et al. (2011) showed that total triglycerides concentration falls during exercise in parallel to VLDL-TG production, assuming this subtype being responsible for the overall fall. On the other side, Magkos et al. (2008) showed that acute endurance exercise did not result in modifications of VLDL-TG kinetics. In both cases, acute exercise resulted in hypotriglyceridemia. However, in our data, plasma triglycerides levels rise similarly in both conditions. Because simple sugars were shown to increase day after triglyceridemia, it is not surprising that both diets induced the same profile the day after, furthermore because of the high fat content (47%) of both diets.

As a summary, fructose may have favored lipid oxidation during the first two hours of exercise. This time was also marked by possible increased hepatic lactate clearance for gluconeogenetic purpose. However, on the counterpart, fructose seemed to diminish hepatic flexibility to exercise, and this may have induced an earlier glycemic fall. At that point our

data, however, do not provide any further information concerning the muscle glycogen and IMCL particular use during exercise. It is probable that at the times in which glycemic concentrations were low, volunteers had highly depleted muscle glycogen and IMCL as well. To test that, subjects were provided carbohydrate-rich food to raise blood glucose, as the following time-trial aimed to test muscle glycogen stores state according to their limitative role for performance in such conditions.

Performance

It is widely accepted that under most conditions glycogen appears to be limiting performance. Even if fructose repleted more stores, feeding values were maximized in glucose-only condition. Tentative to correlate maximal glycogen values to time-trial performance failed, despite normalization of data that were subtracted of familiarization intensity. Thereby, it is possible that in our study design glycogen was not the limiting factor for performance, or that the number of participants was too restrictive. A power analysis was run with actual time-trial intensity mean difference and SD and estimated that 12 subjects should be enough to reach significance (power 0.80). Therefore, any firm conclusion cannot be drawn yet.

Conclusion

Whereas both of our high-simple sugars/high-fat effectively enhanced both IMCL and glycogen contents, fructose did not increase IMCL content but surprisingly enhanced glycogen repletion, although this may be due to lower initial glycogen contents or a possible carry over effect. Much of the reported health-concerning fructose effects on postprandial metabolism were attenuated by the highly trained status of our volunteers.

The following day, fructose did not result in significant modifications of fuel selection during exercise, although fat relay tended to increase. These possible advantaging effects during exercise were however counterbalanced by lower blood glucose concentrations, possibly limiting performance.

Importantly, one major limiting factor of this study for now consists in the limited number of subjects. The insufficient number of observations may be responsible for both Type I and II errors, and this also induced a dysbalance in conditions facing order. To avoid carry over

effect, trials traditionally are randomized to assume an independency from faced conditions order. Because of the low actual number of subjects, our randomization resulted in 80% of the subjects facing GLU condition before FRU (75% concerning MRS measurements). The independence of observations then could not be ensured, resulting in a possible important bias. However, as this represents a time-scheduled MSc work, and because our study will continue and include further volunteers, a first insight of the future results, even if statistically incorrect, was presented and discussed above.

For future perspective, it would be interesting to test a combined diet enriched in fructose and amino acids, as these latter have been reported to increase glucagon secretion (Assan et al., 1977). Increased glucagon in turn was shown to enhance muscle LPL activity while diminishing adipose tissue lipoprotein lipase activity in the post-absorptive state (Wang and Eckel, 2009). Then, our diet would have replete more selectively IMCL than adipocytes. Furthermore, supposing that its concentration stayed elevated the day after, glucagon could have possibly helped liver glucose output during exercise in fructose condition, in turn limiting the observed glycemic fall, possibly responsible for our volunteers' hypoglycemia during the last exercising hour.

In any case, with our current protocol, the future use of ^{13}C labeled palmitate will allow quantification of the oxidized fraction of dietary shakes and, thereby an estimation of IMCL oxidation. Therefore it will be possible to estimate IMCL and FFA contributions to fat fuel and compare the obtained data to Zehnder et al. (2006) values after high-fat feeding.

From a personal point of view, this work represented my Masters of Science thesis. This study might seem little productive considering that my master lasted almost one year. To me, it is worth to be mentioned that I was first involved in another study protocol (effects of fructose on lactate kinetics) and second, that I have been involved in designing the study, submitting it to the Institutional Review Board, established the current protocol, scheduled the agenda and recruited the volunteers. All these procedures are time consuming but may not appear as a result in this manuscript.

As I took much pleasure in Luc Tappy's laboratory, and as I learned a lot during the whole year, I want to take the opportunity to thank every lab team member.

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I also take the opportunity to render thanks to the volunteers that kindly accepted to give blood and sweat for this study.

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