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A Test between Plant and Fish Oil Sources: The Potential Benefits of Diet Enhanced with Omega-3 Fatty Acids

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Acronyms:

AA: Arachidonic Acid

Akt (1): Protein Kinase B

ALA: Alpha-linolenic acid

AMPK: Adenosine Monophosphate Kinase

AS-160: Akt Substrate-160

ATF6: Activating Transcription Factor 6

BiP: Binding Immunoglobulin Protein/ 78kDa Glucose Response Protein (GRP 78)

C: Control

CD36: Scavenger Receptor A

CHOP: CCAAT/Enhancer Binding Protein (C/EBP) Homologous Protein

COX 1/2: Cyclooxygenases 1/2

DAG: Diacylglycerol

DHA: Docosahexaenoic Acid

eEF2K: Eukaryotic Elongation Factor 2 Kinase

eIF4E: Eukaryotic Initiation Factor 4E

EO: *Echium* Oil

EPA: Eicosapentaenoic Acid

ER: Endoplasmic Reticulum

FO: Fish Oil

FOXO1: Forkhead Box-Protein O1

GLUT (2/4): Glucose Transporter

GRP78: Binding Immunoglobulin Protein/78kDa Glucose Response Protein (BiP)

GSK-3β: Glycogen Synthase Kinase-3β

HF: High Fat Diet

iNOS: Inducible Nitric Oxide Synthase

IL-6: Interleukin-6

ipGTT: Intraperitoneal Glucose Tolerance Test

ipITT: Intraperitoneal Insulin Tolerance Test

IP3: Inositol 1,4,5-triphosphate

IP3R: Inositol 1,4,5-triphosphate receptor

IRE-1α: Serine/Threonine-Protein Kinase/Endoribonuclease

IRS 1/2: Insulin Receptor Substrates 1/2

K+: Potassium ion(s)

$K+_{ATP}$: ATP-targeted Potassium ion

LBD: Ligand Binding Domain

LDL: Low Density Lipoprotein

LF: Low Fat Diet

LPL: Lipoprotein Lipase

LPO: Lipoxygenase

mTORC1: Mammalian Target of Rapamycin Complex 1

mTORC2: Mammalian Target of Rapamycin Complex 2

MUFA: Monounsaturated Fatty Acids

NAFLD: Non-alcoholic Fatty Liver Disease

NCoR: Nuclear Receptor Corepressor

NFκB: Nuclear Factor Kappa Light Chain Enhancer of Activated B

n-3: Omega 3 fatty acid

n-6: Omega 6 fatty acid

PGC-1α: Peroxisome Proliferator Activated gamma Coactivator- 1 alpha

PERK: Eukaryotic Translation Initiation Factor 2-Alpha Kinase 3/PRKR-like Endoplasmic Reticulum Kinase or Protein Kinase R (PKR)-like Endoplasmic Reticulum Kinase

PI3K: Phosphatidyl inositol 3 Kinase

PPAR α: Peroxisome Proliferator Activated Receptor alpha

PPAR β/δ: Peroxisome Proliferator Activated beta/delta

PPAR γ: Peroxisome Proliferator Activated gamma

PPAR γ1: Peroxisome Proliferator Activated gamma 1

PPAR γ2: Peroxisome Proliferator Activated gamma 2

PUFA: Polyunsaturated Fatty Acid

PIP2: Phosphatidylinositol 4,5-bisphosphate

p-Akt (1): Phosphorylated/Activated Protein Kinase B

p-FOXO1: Phosphorylated/Inhibited Forkhead Box-Protein O1

p-GSK-3β: Phosphorylated/Inhibited Glycogen Synthase Kinase-3β

p-Stat(-3): Phosphorylated/Active Signal Transducer and Activator of Transcription

RXR: Retinoic Acid/Vitamin D/Calciferol Receptor

SFA: Saturated Fatty Acid

SCFA: Short Chain Fatty Acid

Stat(-3): Signal Transducer and Activator of Transcription

TNF-α: Tumor Necrosis Factor-alpha

TSC 1/2: Tumor Suppressor Complexes 1/2

T2DM: Type 2 Diabetes Mellitus

U: Units

XBP1: X-box Binding Protein 1

XBP1s: X-box Binding Protein 1-spliced

4E-BP1: Eukaryotic Initiation Factor 4E- binding protein 1

Abstract:

In a world where the epidemic of type 2 diabetes mellitus (T2DM) is spreading like wildfire, it is absolutely critical to develop more effective and globally available options to combat the host of metabolic problems that can lead to T2DM and its other affiliated diseases. Beyond lifestyle interventions, drugs such as insulin sensitizers and β-cell insulin secretion enhancers have been used to treat T2DM. However, novel mechanisms are being explored in both pharmaceutical and dietary strategies to improve insulin sensitivity, counter inflammation, and improve glucose tolerance and lipid metabolism in "insulin-sensitive tissues". Recently, it has been found that specific dietary polyunsaturated fats (PUFAs), such as those found in high concentrations in fish oil, are able to bind to mediate many of these effects through multiple biological mechanisms. However, PUFA supplementation, known to heighten insulin sensitivity and curtail inflammation, is controversial due to obscure guidelines of what concentrations are therapeutic, and which are toxic. Regardless, there is a need to examine new sustainable sources of omega 3 fatty acids (n-3s) effective in supplying docosahexaenoic acid (DHA) that can compare to levels detected in fish oil, as DHA is one of the most critical n-3 fatty acids is very difficult to synthesize in the body from precursors.

Skeletal muscle mediates 70-80% of glucose uptake [25, 67]. When the skeletal muscle's capability to absorb glucose is reduced, the individual becomes insulin resistant and develops, if

unabated, diabetes. Here we performed a high fat feeding study on inbred diet-induced obesity in C57BL/6 (B6) mice over the course of 12 weeks, in which we compared the well-established effects of n-3 rich fish oil (FO) versus the poorly understood effects of balanced n-3 and omega-6 fatty acid (n-6) rich plant derived *Echium* oil (EO). Although there were little differences in HFEO or HFFO supplemented groups compared to the high fat control group with respect to blood glucose or plasma insulin concentrations, the *Echium* oil-enriched diet improved glucose tolerance by 12 weeks. Skeletal muscle was examined for diet-specific changes in insulin sensitivity, growth and survival signaling, and anti-inflammatory activity. We found that the n-3 supplemented high fat diet-fed mice, and especially the HFEO group, showed enhanced Akt1 activity, as well as increased GSK-3β phosphorylation and inactivation in the HFEO group, suggesting increased insulin signaling and glycogen synthesis. Although skeletal muscle PPARγ levels were not different among the high fat diet groups, BiP, a marker of ER stress, had a trend of being reduced in the HFEO group. Thus, the improved glucose tolerance of *Echium* oil-supplemented high fat diet-fed mice were due to changes in skeletal muscle physiology resulting in enhanced insulin sensitivity and reduced ER stress.

Background:

A brief overview of the global epidemiology of dysmetabolism:

Currently, 285 million patients have been estimated to have type II diabetes mellitus (T2DM) and the epidemic growing rapidly [82]. The epidemics of obesity, metabolic syndrome/pre-T2DM (which is characterized by increased adiposity, poor fatty acid and lipoprotein metabolism, hepatic steatosis, elevated fasting glycaemia, abnormal protein/haemoglobin glycation, and insulin resistance/glucose intolerance [10, 21]), and T2DM

are spreading quickly throughout other densely populated countries [82]. The diseases of metabolic syndrome and the latter T2DM (the more obvious dysfunction pertaining to insulin resistance and glucose intolerance) have become quite a burden on health, with the cost of health expenditure estimated to be $9800 per patient in the U.S. during 2013 [104]. These three conditions can lead to cardiovascular events, carcinogenesis, and hepatosteatosis/non-alcoholic fatty liver disease (NAFLD)- further burdening the cost of health care [10, 21].

The nature of insulin resistance

Obesity is highly correlated to the incidence of metabolic syndrome and T2DM [53, 83], but this association is often very misleading. Insulin resistance is considered as a reduced capability of "insulin-sensitive" tissues to recognize insulin to stimulate glucose uptake [25, 48, 67]. To understand the onset of insulin resistance, it is critical to comprehend the physiology behind insulin signal transduction. Normally, insulin-sensitive tissues respond to insulin through receptor tyrosine kinases that amplify and *transduce* signaling through Insulin Receptor Substrates 1/2 (IRS1/2) [37, 102]. These proteins then interact with "downstream" intermediates to yield an amplified response: phosphoinositide 3-kinase (PI3K) triggers 3-phosphoinositide-dependent kinase (PDK1), through inositol 1,4,5-triphosphate (IP3) synthesis, to phosphorylate protein kinase B (Akt) at its threonine 308 residue, and stimulate mammalian target of rapamycin complex 2 (mTORC2/PDK2) which further phosphorylates Akt at its serine 473 and threonine 308 residues. In skeletal muscle, especially upon stimulus of Akt, Akt phosphorylates Akt substrate 160 (AS 160), which would otherwise inhibit the recruitment of glucose transporting proteins to the membrane (glucose transporter 4 (GLUT4)), and also phosphorylates and inhibits glycogen synthase-3β (GSK-3β) which inhibits glycogen synthase when unphosphorylated [37, 102]. In addition, in skeletal muscle, active Akt is able to phosphorylate transcription factor

forkhead box protein O1 (FOXO1) and prevent it from entering the nucleus to promote transcription of genes that lead to muscular atrophy/degradation, known as "atrogenes" [41]. It is these atrogenes that are able to produce ubiquitinating/degradative enzymes, MAFbx and MuRF1 to reduce skeletal muscle structure volume and integrity [41]. Finally, a predominant response to Akt activation is the indirect activation of the mammalian target of rapamycin complex 1 (mTORC1) [55]. The MAPKKK pathway is also stimulated in a separate cascade to activate ERK1/2 and c-Jun transcription factors. Both of these pathways influence growth and survival through proteogenesis, lipogenesis, as well as stimulate glucose uptake and glycogenesis [37, 102]. In this study, these pathways intertwined with Akt are critical to the understanding of n-3 behavior upon regulating insulin sensitivity and glucose tolerance.

With insulin resistance and the onset of pre-T2DM, insulin signal transduction is reduced [103]. With this, the pancreatic β-cells continue to be forced to secrete even more insulin to keep up with the demand. The secretion of insulin involves glucose entry into the β-cells of the islets of Langerhans through glucose transporter 2 (GLUT 2) [18, 50, 66]. Upon intake, glucose is aerobically degraded to generate ATP, and ATP then gets shuttled from the mitochondria [74, 85] where it is then able to bind to and antagonize the Kir6.2 subunits of ATP-sensitive potassium ion ($K+_{ATP}$) channels- channels that would normally pump out potassium ions to maintain the resting potential across the β-cell plasma membrane [18, 50, 66]. After inhibition of $K+_{ATP}$ channels, the resting potential across the cell membrane becomes diminished [18, 50, 66]. The depolarization of the membrane activates and opens L-type voltage sensitive calcium ion channels to allow the influx of extracellular calcium ions into the β-cell, and trigger the endoplasmic reticulum (ER) (both directly and indirectly) to release calcium- overall in a pulsatile/oscillatory manner [18, 20, 50, 66]. Upon elevations in calcium, calcium-modulated

proteins become stimulated to trigger the synthesis and secretion of insulin [18, 39, 50, 63, 66]. As the insulin resistant state progresses, though, this pathway is overstimulated, and unfortunately, the β-cells become unable to produce and secrete insulin in a pulsatile matter, and instead become damaged and undergo "ER stress", that may lead to β-cell apoptosis [31, 39]. Whereas insulin resistant individuals have elevated insulin levels for several years, this may eventually develop into T2DM with β-cell failure, hypoisulinaemia, and hyperglycaemia [18, 74].

However, this canonical linear sequence (or cascade) is not fully supported by literature [25, 48]. Rather, the relationship between fat gain and insulin resistance is a network, in which one condition can cause the other, and vice versa [25, 48]. Adipose tissue is able to expand and proliferate when other insulin-dependent tissues become resistant, as fat can remain more sensitive to insulin in contrast, and thus fat tissue in patients that are highly insulin resistant in skeletal muscle and the liver is still able to absorb glucose [48]. Lean individuals with normal insulin sensitivity, on the other hand, dispose 70-80% of blood glucose to skeletal muscle, followed by most of the remainder being funneled to the liver [25, 67]. Yet, a similar scheme can be seen with individuals with good insulin sensitivity, in an acute overfeeding *(think-pizza and ice cream... good stuff)*, individuals can secrete too much insulin, and the surplus can then trigger adipose tissue expansion. In chronic hyperinsulinaemia, it is feasible for skeletal muscle to become resistant to insulin, in comparison to fat tissue, as chronic expression of mTORC1 and its downstream protein, p70S6K, are able to inhibit the activity of IRS1/2, and provide a means to reduce insulin signaling [103]. In terms of overfeeding and lethargy as well, the skeletal muscle, liver, fat tissue, and the pancreas can be introduced to a harmful environment, in which their ERs can become abnormally stressed due to a response from an aggregation of unfolded

proteins as well as from oxidative stress/radical oxygen species, an excess intake of fats (palmitic acid, in particular) through lipoprotein lipase (LPL), and stimuli from inflammatory proteins/cytokines resulting in the unfolded protein response (UPR) [28, 77]. UPR can then trigger a multitude of mechanisms that hinder insulin sensitivity, and trigger inflammatory secretion and apoptotic mechanisms [28, 77]. Should this happen to the liver, pancreas, and skeletal muscle, adipose would be the only tissue to be able to take up nutrients for growth and survival. In this sense, it is insulin resistance, or an excess of insulin, that leads to adiposity. But, the old dogma generally remains true, as tissues become resistant to other highly expressed fat derived protein hormones (leptin). Leptin would promote insulin sensitivity, but fat tissue loses its insulin sensitivity and modulates the production of critical peptide hormones to disrupt systemic insulin sensitivity and satiety. Adipose can decrease its adiponectin generation that would normally heighten insulin sensitivity, and secrete resistins that reduce insulin and leptin sensitivity, for example [48].

Lifestyle factors impacting insulin sensitivity

As described, the story of the relation of insulin resistance and obesity is not as easy to explain as the previously stated canonical picture would have made to appear, rather, it includes the influences of a variety of factors, including intake of key/essential omega-3 fatty acids (n-3s). A lifestyle encouraging obesity (lethargy and poor "over-nutrition"/diet) does promote resistance to insulin [53, 83]. But the broad picture of the onset of insulin resistance varies from factors that range from influence of the synthesis/expression, delivery, proper conversion, and activity of steroid and thyroid hormones, as well as protein and signaling expression upon (intensity, frequency, and volume of) exercise, epigenetic factors, (corepressor/coactivator recruitment in DNA expression, and methylation), (chronic vs. acute) inflammation, nutrition, sensitivity to key

specific key peptide hormones (excluding insulin), and metabolism. It is all these factors that influence the ability for cells to respond insulin and other signaling hormones. My study is focused on the proteins regulating anti-inflammatory drive, insulin sensitivity, and glucose transport in skeletal muscle after chronic n-3 PUFA supplementation, comparing fish oil (FO) and *Echium* oil (EO), in the context of a low fat diet (LF) vs. high fat diet.

Proposed molecular mechanisms affecting insulin sensitivity:

In prior studies, there is abundant evidence supporting the agonistic activity of fats, in particular, short chain fatty acids (SCFAs), medium chain fatty acids (MCFAs), omega 6 fatty acids (n-6s), and n-3s on insulin sensitivity, anti-inflammation, and improved lipid (lipoprotein) metabolism. Here, we focused on and tested the potential therapeutic effects of the n-3 source, FO, and the plant n-3/n-6 source, EO, in testing the potential benefits of these fats. However, much of the information is supportive of the n-3s in having beneficial effects. N-3 sources can be metabolized effectively into advanced forms of n-3s (especially into DHA) in contrast to their flax seed and walnut counterparts (DHA synthesis *in vivo* from precursors, especially the most abstract alpha-linolenic acid, ALA, which conversion is very inefficient) [99]. In addition, it is imperative, in this world where marine life is becoming increasingly impacted by overfishing, to take a pharmacognosic approach, in which research seeks to find lead drugs in nature, or in this case, find new promising sources of n-3s. Should we continue to rely on marine life in our oceans, they will continue to be threatened as endangered species, as observed recently, with illegal whale shark slaughter [57].

One of the most fundamental mechanisms to set the stage of PUFA biological activity in cells is how n-3s and n-6s bind to and stimulate the nuclear receptor and transcription factor, peroxisome-proliferator activated receptor γ (PPAR γ), and its other affiliated fatty

acid/eicosanoid/thiazolidinedione ligand-binding family (PPARα and PPARδ/PPARβ), which are individually bound to retinoic acid/vitamin D/cholecalciferol transcription factor (RXR) [v, 3, 15, 24, 29, 88]. Out of the PUFAs, n-3s eicosapentaenoic acid (EPA), eicosatrienoic acid, DHA, and ALA, and n-6s linoleic acid, arachidonic acid (AA), γ-linolenic acid, and dihomo-γ-linolenic acid will bind to PPARs' ligand binding domains (LBDs), and effectively change PPAR structure, allowing PPARs to influence complex patterns of gene expression [v, 3, 15, 24, 52, 99]. In addition, it has been shown that the oxygenated/oxidized products of PUFAs act as more potent activators of PPARs [29]. This includes n-6 products produced by lipoxygenases (LPOs), dehydrogenases, and cyclooxygenases (COX-1/2) to form (hydoxylated or "(-)oxo-" octadecanoates, and the (oxygenated) prostaglandins (which covalently bind PPARγ) produced from AA [29]. In addition, DHA can be modified into more potent agonists forming hydroxylated DHA isomers, and these isomers can be further modified into other PPARγ agonists (such as oxo-DHA) [29]. Finally, these PUFAs can also be converted into nitrates, upon reaction with nitric oxide, which also prove to be PPAR agonists [29]. In adipocytes and skeletal muscle, this tends to eject corepressors from PPARs and allows recruitment of coactivators to achieve a conformation where DNA transcription can effectively initiate, and promote target gene expression that leads to growth, differentiation, and proliferation [15, 29, 32].

PPARγ, though highly expressed in adipose (predominantly as PPARγ2, rather than PPARγ1), is also expressed in skeletal muscle (which solely expresses the PPARγ1 isoform) [32]. As a transcriptional factor, it is responsible for the transcription of gene encoding proteins involved in mediating insulin and mTOR signal transduction and glucose tolerance, such as GLUT4, IRS 1/2, and PI3K, along with those encoding lipogenic enzymes lipoprotein lipase (LPL), acyl-CoA-binding protein (ACBP), and fatty acid binding protein 2 (aP2), and glycogenic

enzymes [v, 3, 5, 15, 29, 32]. Regardless this transcriptional activity, it has also been suggested that PPARγ agonism has been directly responsible for the phosphorylation and activity of insulin transduction proteins in skeletal muscle and other tissues, which has been suggested through reduction of inducible nitric oxide synthase (iNOS) activity [2, 23, 32, 43, 60, 86, 87, 91]). In addition, PPARγ activity also triggers the production of anti-inflammatory proteins and insulin-sensitizing peptide hormones thought to be specifically synthesized from fat tissue, known as adipokines [3, 11, 15, 32]. Recently, it had been found that skeletal muscle is even able to secrete the adipokine, adiponectin, from PPARγ activation [5]. PPARγ's augmenting transcriptional effects on lipogenesis in fat and liver tissue would necessarily promote further fattening of an obese patient with T2DM (which is quite concerning as you would be only removing the damaging symptoms acutely, and not treating the condition) by giving them a PPARγ agonist (such as a thiazolidinedione) [3, 15]. However, by stimulating fat uptake from the blood via PPARγ, serum triacylglycerides are lowered, and lipoprotein metabolism concerning chylomicron and very low density lipoprotein (VLDL) become amplified via LPL and signal transducer and activator of transcription (STAT) expression [3, 15]. Finally, in macrophages and foam cells (cells responsible for forming fatty streaks of endothelial walls of the vasculature, which then rupture to form atherosclerotic plaques), upon binding of PPARγ agonists, PPARγ ceases transcription of scavenger receptor A (CD36), which recognizes (per)oxidized low density lipoprotein that abnormally enters the endothelium (through lesions of blood vessels), probably through a "tethering" process [32]. After recognition, the low density lipoprotein (LDL) becomes engulfed by the macrophages (also known as monocytes in circulating blood), forming foam cells- which are characterized as monocytes filled with triacylglycerides from devouring oxidized LDL proteins. These cells, as mentioned earlier, eventually rupture upon engulfing too

many oxidized lipoproteins, forming the fatty streaks in the endothelium. Hence, by preventing the ability for these cells to uptake as much of these oxidized lipoproteins, atherosclerotic plaques may decrease [7].

Given how the "-oxo-" products have yielded the greatest potency in activating PPARs and how these products have been reported to develop in cells with elevated amounts of oxidative reactions, it has been hypothesized in previous experiments that PPARs can be sensors of oxidative stress, and can then dictate whether the cell should survive and grow (or undergo apoptosis in some tissues), though this is controversial as mainstream belief is, and many studies show, that chronic oxidative stress stimulates chronic ER stress and thereby reduces insulin senstivity [29]. Though, perhaps this is not such a cellular activity with a "one way path behavior", as acute ER stress is beneficial for skeletal muscle, as well as defense from infection [75, 95, 96, 98, 100]. It also depends on the sort of tissue in which oxidative stress occurs that dictates the effects on physiology. This is quite relatable to James Watson's latest editorial concerning T2DM, in which he claims that the reason insulin resistance is not the effect of excessive oxidation and presence of reactive oxygen species, but that there is a lack of these (in active skeletal muscle, which would arise upon exercise) [94]. Comparing to the likelihood of insulin resistance originating in a "couch-potato" in contrast to an athlete, this would seem to make sense. There is evidence from other works supports this claim, as it would seem that oxidative mitochondrial stress during (intense) exercise could indeed trigger a cellular environment in which PUFAs become oxidized into their potent agonist structures and facilitate PPAR influence over transcription, as insulin sensitivity and the speed at which proteins involved insulin transduction interact with each other does become elevated upon exercise [33, 91, 94, 97, 103].

The precise mechanism principally responsible for heightening glucose disposal and insulin sensitivity is dubious, as both have been shown to be mediated through adenosine monophosphate kinase's (AMPK's) aid in promoting glucose tolerance by triggering GLUT4 membrane recruitment for glucose uptake [103], as well as hormonal and environmental influence over upstream insulin signaling. Environmental stimuli such as high amounts of dietary amino acids (in particular- leucine and branched chain amino acids which by binding to hVPS34 and being tRNA substrate) can trigger glucose disposal. Other potential stimuli include exercise-induced secretion of growth hormone (GH) followed by Insulin-like Growth Factor-1), secretion of muscular growth factor, and mechanotransduction through mTORC1 [41, 65, 68, 73, 89, 103]. These prevent muscular atrophy by inhibiting the key insulin cascade transcription factor FOXO1 [41, 65, 68, 73, 89, 103]. However, the physiological impact of these pathways during exercise are a contentious subject, being claimed to be negligible during exercise- with the primary mechanism not an enhancement of insulin sensitivity, but an enhancement of glucose disposal, which is through AMPK in recruiting GLUT4 [97]. This response does depend on the phosphorylation/inhibition of Akt substrate 160 (AS160), to promote GLUT4 recruitment to the sarcoplasmic membrane, but there is another path AMPK takes. Ironically, GLUT4 synthesis also becomes triggered by AMPK's activation by the same coactivator that all PPARs use to promote transcriptional activity, known as peroxisome proliferator- activated receptor coactivator 1 alpha (PGC-1α). It performs a metabolic activity contradictory to what the name would claim, rather the primary transcriptional protein that mediates this effect is myocyte enhancer factor 2 (MEF2), which regulates a wide diversity of genes to promote skeletal muscle hypertrophy [12]. In light of how MEF2's principal functions to promote hypertrophy involve analogous coactivators to that of PPARs, it would perhaps make sense that PPARs and MEF2

work together- MEF2 has the ability to elicit hypertrophy, whereas PPAR(γ) can augment that response by triggering the supply of nutrients and using them via PPARα and β/δ [12, 79, 91].

Regardless of these disagreements in the literature on the relative importance of these stimulatory pathways, the exercise-facilitated mechanisms that use analogous cascade signaling to the insulin pathway, described earlier, are able to activate mTORC1, and inhibit AS160 and GSK-3β. Upon doing so, they upregulate the translation of insulin-sensitizing proteins from (PPARγ and MEF2) transcription and promote improved glucose disposal as result, along with activating GLUT4 and glycogen synthase. mTORC1 is able to perform this by phosphorylating/inhibiting eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) from binding/inhibiting eIF4E [103]. This allows eIF4E to employ translation factor eIF4G to the 5' end of mRNA strands [103]. Furthermore, mTORC1 phosphorylates p70S6K1 to additionally promote translation by activating "eukaryotic elongation factor 2 kinase (eEF2K), S6K1 Aly/REF-like target, 80 kDa nuclear cap-binding protein, and eIF4B" [103]. mTORC1 activation of S6K1 Kinase also amplifies ribosomal synthesis by stimulating RNA Polymerase I- further enhancing the DNA expression mediated by PPARγ [103] through translation- promoting proteogenesis and glycogenesis, respectively. All of these factors, through enhancing translation and potentially heightening PPAR activity by PUFA oxidation, lead to heightened expression of PPAR mRNA by translation, and elevate insulin sensitivity [89].

Upon recovery from exercise, insulin has been found to promote hypertrophy of skeletal muscle [65, 89]. However, upon prolonged inactivity, it has been found that insulin-facilitated anabolism becomes reduced to instead prevent muscle protein breakdown, rather than grow skeletal muscle [89]. While this diminished influence from insulin is due to how skeletal muscle protein synthesis is influenced by other various stimuli (including those mentioned before,

energy balance, the need to recover from exercise-induced muscle fiber damage, and steroid activity), it could be reasoned that PPARγ activity (upon oxidative environment) is a critical player in muscle growth, especially with this odd behavior in which insulin, and presumably IGF-1 (considering IGF-1 mediates analogous pathways in comparison to insulin) mediate skeletal muscle hypertrophy upon an acute oxidative environment, as well as promoting enhanced glucose disposal activity upon exercise. This has been supported by a variety of studies- mTOR knockout (KO) or rapamycin (inhibitor of mTOR) administration had been found to silence PPARγ expression, and as a result, had yielded insulin resistant mice (thus promoting muscle atrophy), and the same has been observed upon selectively knocking out the *PPARγ* gene in skeletal muscle [5]. Furthermore, genetic constitutive activity of *PPARγ* and ligand-induced activity (from thiazolidinediones) have displayed comparable increases in insulin sensitivity and hypertrophy of skeletal muscle, emphasizing the importance of PPARγ in skeletal muscle metabolic homeostasis [5, 91]. As a suggestion for future study, further analysis of PPAR activity involved in insulin (and IGF-1) transduction should be performed to elucidate the hypertrophic vs. solely protein sparing behavior, as it would seem that PPARγ plays a critical role in skeletal muscle hypertrophy and may somehow be influenced by acute intense activity. A possible reason for why oxidation of PUFAs may be absolutely critical in insulin/IGF-1 triggering growth of skeletal muscle is that perhaps not enough mRNA can be produced from PPARγ transcription without effective agonism.

To further understand the extent of how PPARs perform insulin sensitizing and anti-inflammatory activity, it is also important to know biological role beyond the regulation of gene expression by comprehending the roles in the ER stress response. As mentioned before, a western diet (high fat and "carby" diet), oxidative stress, abnormal calcium signaling,

hyperglycaemia, chronic signaling of cytokines, and a high sugar intake can cause chronic ER stress in skeletal muscle [19, 26, 28, 51], while it is suggested that elongated bouts of lethargy and extreme strenuous activity (such as a "200 km run") can lead to the incidence of chronic ER stress more distinct to the skeletal muscle, in comparison to favored/normal acute ER stress during short bouts of intense activity (yet, exercise is suggested to prevent chronic ER stress) [26, 28, 75, 95, 96, 98]. This initiates an adaptive response in the rough ER to permit proper peptide folding and modification [26, 28]. This response involves glucose-regulated protein 78, also known as Binding Immunoglobulin Protein (GRP 78/BiP), which is normally bound to endoplasmic (or sarcoplasmic, for the sake of this thesis) reticulum bound proteins, Inositol-Requiring Enzyme 1 α (IRE1α, a Serine/threonine-protein kinase/endoribonuclease), Activating Transcription Factor 6 (ATF6), and protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK) to become displaced and bind to the aggregation of unfolded proteins that accumulates in the ER [26, 28].

With IRE1α and PERK free from BiP/GRP 78, these proteins are able to homodimerize and then autophosphorylate, similar to receptor tyrosine kinases and some G-protein coupled receptors (GPCRs/GPRs) [26, 28]. Then, these proteins, along with ATF6, promote autophagy, apoptosis, and systemic inflammation. PERK acts by phosphorylating eukaryotic initiation factor 2 α (eIF2α) to attenuate further translation of proteins that would otherwise be synthesized should stress not occur, and also promote atrophy/autophagy [26, 28]. Furthermore, ATF6 and IRE1α act to transcribe X-box Binding Protein 1 (XBP1) mRNA and splice it into Spliced X-box Binding Protein 1 (XBP1s) mRNA, respectively [26, 28]. XBP1(s) can then be selectively translated in the UPR process, and once produced into functional proteins, after processing, can act to stimulate CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) [26,

28]. CHOP can then continue to indirectly promote atrophy/autophagy and apoptosis, as well as amplify Tribbles 3 (TRIB 3) action in silencing Akt activity, and thus suppressing insulin sensitivity [26, 28, 54]. Finally, a last critical point to address about UPR/ER stress is how it silences insulin sensitivity as IRE1α leads to the activation of c-Jun N-terminal Kinase (JNK) to indirectly reduce IRS1 activity (thus, insulin sensitivity) through degradation [28, 86], but it can also promote transcription of inflammatory proteins through activating protein 1 (AP-1) [28]. This mechanism works in synchrony with other signaling proteins triggered in UPR- ATF6 and PERK act to indirectly stimulate Nuclear Factor kappa light chain enhancer of activated B (NFκB) to also transcribe mRNA encoding for inflammatory proteins [28]. In the acute stimulation of UPR during intense exercise, this process can be beneficial in directing nutrients to the active skeletal muscle and fuel continued exercise, such as promoting the transcription/translation and secretion interleukin-6 (IL-6) to trigger gluconeogenesis in the liver, and lipolysis in fat tissue [75, 95, 96, 98]. However, the overall response from chronic stimulation of these mechanisms promotes the onset of insulin resistance both specific to the cell, and in a systemic manner [19, 26, 28, 51, 54, 75, 95, 98].

So how can n-3s potentially manage this condition? To override the transcription of inflammatory mediators, these molecules are associated with cytokine signaling and stimulated by UPR, PPARs can modulate these by "squelching" or directly binding to other transcriptional coactivator proteins, as well as "tethering" to transcriptional cofactors, and transrepression activity (in which PPARs will bind to promoters and inhibit their transcription) [32]. PPARγ can inhibit iNOS production, but the mechanism is still very equivocal. PPARγ, on one hand, is thought to inhibit transcription of iNOS by a squelching mechanism upon the iNOS promoters that have AP-1, NFκB, and STAT-1 as transcriptional factors by binding onto and displacing

their CBP coactivators, which would normally become stimulated upon ER stress/UPR and cytokine signaling [28, 32, 100]. On the other hand, PPARγ has been proposed to interact with corepressor NCoR upon sumoylation and bind to the iNOS promoter to promote its deacetylation and thereby inhibit transcription through DNA condensation [32].

iNOS, uninhibited, does promote inflammatory activity, permits recovery from acute damage, and fights infection [80, 100]. But, its uglier side, when it is chronically expressed, is how it promotes the degradation of IRS 1 through JNK activation by nitric oxide production, and acts through other vague mechanisms, altering PPARγ activity to reduce insulin sensitivity [8, 19, 23, 28, 86, 87, 95, 98, 100]. So, by inhibiting iNOS activity by preventing NFκB , AP-1, and STAT-1 transcriptional activity, there is greater potential to promote enhanced insulin sensitivity, making PPARγ an even more influential target to prevent insulin resistance through non-transcriptional means, but through tethering [8, 23, 32, 87]. PPARs can also prevent UPR and external cytokine influence through other means, and prevent the expression of interleukins. Critical to pro-inflammatory IL-6 production, as mentioned before, PPARγ can tether to phosphorylated and unphosphorylated STAT-3 and PPARα can tether to NFκB and AP-1 to silence IL-6 transcription [32]. In preventing IL-6 synthesis, this is a very promising approach to therapy to ameliorate insulin resistance, as chronic IL-6 expression is significantly involved in the mechanism of T2DM and the metabolic syndrome [75, 95, 98].

In lieu of these activities of PPARγ, it is seems likely that a lack of PPARγ agonism in skeletal muscle can lead to insulin resistance in the skeletal muscle and systemically, directly and indirectly (via lack of cytokine inhibition). This has been confirmed *in vivo* by selectively knocking out PPARγ in skeletal muscle of mice. Hevener, A. L., et al.'s (2003) study displayed this in detail, in which the mice had reduced glucose disposal by "~80%", and after 3 weeks of

thiazolidinedione treatment, the mice did not recover and suffered from T2DM and fat gain [43]. Furthermore, it had been found that IRS-1 (tyrosine phosphorylation) and Akt phosphorylation/activation had been heavily reduced in skeletal to promote an insulin resistant state [43]. However, a separate study performed by Norris, A.W., et. al. (2003) which knocked out PPARγ selectively in skeletal muscle tissue had described continued glucose disposal upon thiazolidinedione treatment and claimed that skeletal muscle KO had not interfered with thiazolidinedione activity [64]. This claim was indeed supported by how glucose disposal had been elevated to near normal levels in KO mice, yet, it would appear from their data that rather the KO mice were still glucose tolerant from thiazolidinedione treatment because rather than the nutrients ingested going to skeletal muscle, as they normally should be, they failed to be absorbed by the muscle and were instead diverted to other tissues, especially adipose, which had been observable by the significant fat gain (Norris, A. W., et al. [64], Hevener, A. L., et al. [43]).Further support of these findings of insulin resistance arising in skeletal muscle upon lack of PPARγ activity has been provided by studies whereby PPARγ inactivated in human skeletal muscle (C2C12 cells) and further corroborates these findings [91]. Upon PPARγ KO, the rhabdomyocytes had demonstrated significant insulin resistance, whereas constitutive PPARγ expression had demonstrated enhanced insulin sensitivity comparable to the effects of thiazolidinedione treatment that are observed when administrated to the skeletal muscle cells, directly in tissue culture [91].

Excluding PPAR mechanisms specific to skeletal muscle, the big picture of n-3s' and n-6s' influences on insulin sensitivity and chronic inflammation amongst skeletal muscle and other tissues includes distinct tissue responses due to PPAR activity, as well as through influencing activity of other receptors. N-3s have been reported to increase PPAR mRNA levels in tissues,

allowing higher potential for PPAR activity, systemically [22, 93]. Upon this increase in the levels of PPAR protein, as well as the activation of PPARs, adipokine secretion from adipocytes is known to be increased by n-3 intake, as active PPARs are a necessary step in the differentiation of preadipocytes [29, 30, 58, 69, 90, 92]. This activity allows the production of adiponectin upon differentiation to occur, in concert with PPAR activity in macrophages described above, can non-transcriptionally interfere with cytokine production, thereby lowering the incidence of (chronic low grade) inflammation upon ER stress and promoting systemic insulin sensitivity [19, 32, 42, 51]. Indirect influences from macrophage PPARγ activity has been found to be highly contributory to enhancing the insulin sensitivity of skeletal muscle [42]. Adiponectin promotes insulin sensitivity and fat metabolism in skeletal muscle several ways. By binding to adiponectin receptor 1 GPCRs (AdipoR1), calcium channels of the sarcolemma/cellular membranes can be triggered to open and transport calcium ions into the cytosol. Calcium signaling then paves the way for adiponectin signaling, indirectly activating/phosphorylating AMPK and activating transcription mediated by coactivator PGC-1α and SIRT-1 to promote mitochondrial biogenesis [49]. Furthermore, adiponectin can interact with AdipoR1/2 receptors to promote the activity of (APPL) to promote fatty acid oxidation through AMPK, PPARα, and p38MAPK (and Rab5), thereby indirectly improving insulin sensitivity by promoting GLUT4 translocation and insulin sensitivity [14, 59]. AdipoR1/2 has also been shown to activate IRS1 and PI3K and stimulate the mTOR pathway, suggesting that adiponectin is highly involved in skeletal muscle hypertrophy as an indication of energy status, as well as preventing proteolysis [14]. However, an interesting paradoxical finding had been found, in which iNOS production had been upregulated in skeletal muscle upon adiponectin activity, alongside upregulation of manganese superoxide dismutase, suggesting that adiponectin

can reduce oxidative stress, and thus decrease ER stress and reduce its negative effects on insulin signaling [14].

Insulin sensitivity, by reducing inflammatory signaling and ER stress in tissues, can be indirectly enhanced in skeletal muscle in other ways through n-3 (and n-6) influence on tissues of the body. PUFA intake has been found to manipulate the plasma membrane structure of tissues. For instance, n-3 intake promotes the incorporation of such fatty acids into the skeletal muscle membranes [9]. This has been found to influence the formation of "lipid rafts" (hot-spots for membrane signaling), influence the activity and incorporation of receptors, and allow for a pool of substrate for cells to use in intracellular signaling (such as the PPAR agonism and COX interactions), especially upon influence by weight loss and exercise [6, 29, 38]. Finally, it has also been suggested that polyunsaturation of phospholipids (with DHA) permit optimal mitochondrial/oxidative phosphorylation [47]. Incorporation of n-3s into the cellular and mitochondrial membranes has been correlated with increased insulin sensitivity and fatty acid oxidation in skeletal muscle directly through PPAR agonism, and indirectly through adipokines and decreased inflammation, or restoring mitochondrial metabolism (in reducing oxidation) [13, 29, 32, 34, 47].

Aside from their affinity for membrane incorporation and ability to interact with PPARs, n-3s are able to impact skeletal muscle physiology in an indirect way to reduce low grade chronic inflammation as well as enhance insulin sensitivity. The G-protein Coupled Receptor, GPR 120, binds to free omega 3 fatty acids EPA and DHA [67] (and perhaps other n-3's, such as ALA[16], as well as dairy fat-derived palmitoleic acid [45] acting as agonists, while palmitate acts as an antagonist [17, 76]) and directly promotes anti-inflammatory actions in adipose tissue macrophages and increases insulin sensitivity. To mediate anti-inflammatory activity in

macrophages, β-arrestin protein 2 reacts to GPR 120 binding to an n-3 and so binds to the receptor, inducing endocytosis. The β-arrestin 2/GPR 120 complex then interacts with protein TAK 1, which would normally stimulate TAB 1 without the presence of n-3s, mediating an inflammatory response from cytokines IKKB and TNF-α. But in this case, β-arrestin 2's association with TAK 1 prevents TAK 1's interactions with TAB 1 and prevents cytokine-induced inflammation [67]. In addition, GPR 120, upon activation, hampers the expression of cytokine/myokine (M1) inflammatory expression and stimulates M2 anti-inflammatory expression by transcription, suggesting again that PPAR mechanisms are at hand [67]. Finally, to directly facilitate insulin sensitivity in adipocytes through GPCR 120, phosphorylation of G protein α/q11 occurs upon GPR 120 activation that leads to GLUT 4 translocation [67]. This pathway significantly improves glycaemia and energy metabolism in mice suggesting that n-3 activity through GPR 120 had a highly significant effect on skeletal muscle insulin sensitivity, however, indirectly [67], although the precise mechanisms are not yet known.

Pitfalls with n-3 supplementation in humans

Unfortunately, regardless the information I have provided, n-3s have been supplemented in very heavy doses by many lean and healthy individuals, as there has been a misconception spread by media in the health and fitness industry that these fats will provide equivalent insulin sensitivity and anti-inflammatory-boosting effects as those observed in studies that use patients or animals that are already diagnosed with a pathophysiological condition (commonly, T2DM). The idea many of these people have is that n-3s will amplify their insulin sensitivities, reduce inflammation, and promote skeletal muscle hypertrophy beyond normal levels. However, the effects of dietary n-3s are going to be much more significant in those already suffering from chronic low grade inflammation and/or insulin resistance. Lean individuals should realize that n-

3 supplementation should be used to prevent pathogenesis rather than enhance insulin sensitivity beyond normal levels, being that they are likely metabolically healthy. FO supplementation has been more commonly demonstrated to promote insulin sensitivity, anti-inflammatory, and eulipidemic effects with a desirable efficacy upon heavy dosing (>3 grams added, plus diet) for diabetic patients [67, 78]. Yet, this is not as well supported in lower doses, and may not be as applicable for individuals who are already healthy aside from providing the benefit of preventing insulin resistance and chronic low grade inflammation, rather than boosting insulin sensitivity, further. The capacity to improve insulin sensitivity have been reported to be less significant in a "*moderate-dosing*", which is consists of 1-2 grams of n-3s/day, where rather the anti-inflammatory effect and eulipidemic effects have been shown to persist [4, 56]. This difference in potencies in therapeutic effects is of some concern, though, as it is currently thought that an excess of PUFA (especially n-6s) intake by the "western diet" can lead to carcinogenesis [29], impaired glucose metabolism [61, 84], thrombogenesis/atherosclerosis, and chronic inflammation (and then leading to insulin resistance with n-6s) through peroxidation. It has been suggested that raising the amount of n-3s to the higher amounts that have benefited T2DM patients in ameliorating insulin resistance may also bring about toxic effects, as the therapeutic dosage of n-3s is not yet well distinguished from their toxic dosage. In this instance, a small increase in bioavailability of n-3s upon heavy supplementation (to treat insulin resistance) could elicit toxic effects. The peroxidation of n-6s have been reported to alter lipoprotein/protein structure by making LDLs denser and more thrombogenic, and peroxidation of both n-3s and n-6s have been reported to form DNA adducts bound to deoxyguanosine residues from formation of aldehydes [62, 70]. In addition, by acting as PPAR agonists, it has been revealed how modifying gene expression can bring about increased adiposity (in patients that were already

overweight), though probably "healthy" due to improved glucose metabolism [29, 35]. Furthermore, the potential for PPAR agonism can lead to development of hepatocellular carcinoma via PPARα, and colon cancers through AA stimulation of PPARβ/δ [29, 35]. This leads to an upregulation of COX-2, which allows an overproduction of prostaglandins to induce colon carcinogenesis [29, 35]. As the toxic dosage is approached, FO has been shown to elicit toxic effects on glucose metabolism, as shown in one study performed on subjects with T2DM whereby that heavy amounts of FO (5.8 grams worth of n-3s) have negative effects on glucose disposal, similar to that of n-6 rich corn oil [61]. Another similar study demonstrated that >6 grams of n-3 supplementation, an imbalance of n-3:n-6 (analogous to the western diet) can elicit negative effects on glucose disposal [84].

However, the opposite claim has been shown in some cases. An incongruity has been reported in which researchers had compared 67 individuals on either a diet consisting of saturated fatty acids (SFAs) versus a diet of sunflower-derived fats [11]. It was concluded that the 28 individuals on a diet of sunflower fats had lower serum levels of inflammatory proteins Tumor Necrosis Factor-R2 and Interleukin-1Rα, as well as less serum palmitate and (ironically) highly pro-inflammatory AA [11]. Additionally, *in vitro,* PPARγ activity has been identified to attenuate cancer growth in cells from distinct tissues [29]. However, studies on *in vivo* PPARγ stimulation have displayed a murkier view, in which PPARγ can stimulate carcinogenesis, showing dual effects [29]. Alongside this, heavy supplementation of n-3s has been suggested to enhance tissue n-3 levels which could promote the inhibition of carcinogenesis, as a high correlation exists between these n-3 rich diets/tissue levels with low incidence of cancer and the ability to ameliorate cancer [29]. Regardless of the hype about the potential benefits of excess PUFA supplementation, more research should be conducted before encouraging recreational

high dose supplementation of n-3s, as the field displays a very obscure view about the potential benefits vs. risks of these lipids' metabolic effects. As one of the fathers of toxicology, Paracelsus, had explained, "the dose makes the poison" [71]. Elucidating the toxicology of PUFAs is critical in assessing the safety of elevated intake- more studies must be performed to assess better ways to optimize PUFA use.

Study Rationale:

With a need to expand basic research on testing effective and safe n-3 supplements to improve metabolic health, as well as to rely less on marine sources of n-3s, we chose to test the effectiveness of plant derived *Echium* oil compared with well-studied fish oil with respect to improving glucose homeostasis, energy partitioning, and skeletal muscle physiology in normal mice subjected to both low and high fat diets.

Materials and Methods:

Animals, Feeding, and Husbandry:

Sixty 6-week old adolescent C57BL/6NTac mice (B6; Taconic) were originally weaned onto a control chow diet (Lab Diet 5001; 13.5% fat) in UVM's Animal Facility. They were then subjected to either low fat diet (LF) (Research Diets D12450B; 10% kcal from fat) or high fat diet formulae (HF) (Research Diets D14292; 60% kcal from fat), each supplemented with lard or n-3 rich oils. Mice were randomized into groups of 10 (n=10, six distinct groups) and were either subjected to control diets (C) consisting of lard, or diets supplemented with 20% of the total fat as menhaden fish oil (FO, n-3 control) or *Echium* oil (EO). Each of these diets had an equivalent amount of soybean oil added (~2.5%) to provide essential fatty acids. The protocols approved by

the UVM Institutional Animal Care and Use Committee (IACUC) had been followed throughout these studies.

Biometric and Physiological Parameters:

Mice were weighed, tested for fed glycaemia levels (FreeStyle monitor; Abbott), and had blood drawn to test plasma insulin levels (ELISA; Alpco), weekly. Intraperitoneal glucose tolerance tests (ipGTTs) were performed on 5 mice from each group after a 12 hour fast, on weeks 4, 8, and 12. In this procedure, glucose and plasma insulin levels were measured over the course of two hours following IP injection of dextrose (2g/kg body weight). Fasting glycaemia was measured immediately prior the test (time= 0 minutes), and at 15, 30, 60, 90, and 120 minutes, and blood was drawn immediately prior the test (time= 0 minutes), and at 15, 30, and 120 minutes of the test for subsequent plasma insulin measurements. Similarly, IP insulin tolerance tests (ipITTs) were performed on separate mice cohorts on weeks 8 and 12, following a 12 hour fast and glycaemia tested 0, 15, 30, 60, 90, and 120 minutes after injecting insulin (0.75U insulin/kg body weight).

Tissue Analyses:

After twelve weeks, the mice had been administrated a lethal dose of pentobarbital euthanasia solution. Upon death, the pancreas, liver, epididymal fat, quadriceps (skeletal muscle), spleen, hypothalamus, and blood had been extracted from the mice, and either placed immediately in a cryogenic container (of liquid nitrogen at -170°C with subsequent -80°C storage) or in histological fixative, pending an array of analytical techniques to investigate mRNA and protein expression, and measure specific activated intermediates of key proteins in these tissues. Whole blood was separated, cells from plasma, and then both stored at -20°C.

Skeletal Muscle Processing for Whole Tissue Proteins and Protein Estimation:

Skeletal muscle samples were quickly removed from the -80°C storage and kept on wet ice while homogenizing the tissue (with Tissue-Mizer) in lysis buffer, described in Table #1. After homogenization, the samples were centrifuged at 10,000 RPM for ten minutes. 50 µL aliquots of the supernatants were stored at -80°C freezer for later analysis. The Bradford reagent-based BioRad protein assay was used to estimate protein concentration using the microplate assay and read with a BioTek 800ELx plate reader. Microsoft Excel was used to generate a standard curve using bovine serum albumin as a reference and calculate the unknown protein values of diluted protein homogenates.

Western Blotting:

SDS-PAGE Gel Electrophoresis and Electroblotting:

10% acrylamide SDS-PAGE gels were cast with a 5% acrylamide stacking gel (Tables #2 and #3). The homogenates were removed from the -80°C freezer and placed on ice. Twenty-five µg of protein were loaded per lane with β-mercaptoethanol and "4x sample buffer" (Invitrogen). Molecular weight protein standards were aliquots from Invitrogen Novex® Benchmark™ (10748-010). The reduced homogenates were electrophoresed using a BioRad Mini PROTEAN® 3 cell with TRIS/glycine/SDS running buffer. The gels were electroblotted onto PVDF membranes (Millipore) using TRIS/glycine/SDS/methanol (see Table #4) buffer using a BioRad Mini blotting apparatus run at 100 volts for two hours.

Immunoblotting and Chemiluminescence Detection:

Following transfer, the membranes were equilibrated and blocked in phosphate buffered saline +0.05% Tween 20 (PBST) + 5% nonfat dry milk. Primary antibodies were diluted 1:1000-1:2000 in this same buffer solution and then vacuum sealed in separate pouches. The pouches were set on a rotator, overnight, at 4°C. The next day, the membranes were washed with PBST,

then incubated with goat anti-rabbit-horseradish peroxidase (BioRad) at 1:7500 for 90 minutes at room temperature with agitation. Following washes in PBST 3 times, the membranes were then subjected to Western Lighting® Plus-ECL, Enhanced Chemiluminescence Substrate (PerkinElmer) for 2 minutes before being wrapped in Saran wrap and blotted. The membranes were then exposed to KODAK XAR film that was subsequently processed by an automated film developer. Antibodies used can be observed on Table #9.

Immunoreagent Stripping of Membrane:

After ECL detection, the membranes were subjected to stripping buffer (as described in Table #5) for 35 minutes at 70°C. Afterwards, the membranes were immediately washed twice for 10 minutes with PBST, then reblocked and immunostained for another protein.

Densitometry/Statistical Analysis:

The exposed films were digitally scanned using EPSON Scantastic software. The images were converted to TIF files, and then densitometrically analyzed via NIH ImageJ. Band intensities were normalized the β-actin signal for each lane and entered into Microsoft Excel for comparative analyses. Finally, the data from each marker and each animal group (n=6) were compared using 2-tailed T-Tests (Microsoft Excel). Probability (P) values of <0.05 were considered statistically significant.

Results:

Analysis of Diet

Analysis of diets verified that the low fat diets had 10% of their caloric intake coming from fat, while high fat diets had 60% of their calories come from fat (Table #6) [9]. Accounting for these intakes, it was found that the LFC diet caloric (kcal%) content consisted of 4.44% lard and 5.56% soybean oil, and the LFFO and LFEO diets' caloric content were 3.56% lard, 5.56%

soybean oil, and 0.09% fish oil and *Echium* oil, respectively. The HFC diet consisted of 54.44% of calories coming from lard, and 5.56% of calories coming from soybean oil. The HFFO and HFEO diets' caloric contents consisted of 43.56% lard, 10.88% fish oil and *Echium* oil, respectively, and 5.56% soybean oil. Overall, the LFC diet had ~0.4% of calories consisting of n-3s, and the LFFO and LFEO diets had ~0.6% of their calories coming from n-3s, while ~3.9% of calories came from n-6s for each of the diets (Table #7) [9]. As result, the LFC diet had a fatty acid ratio of ~9 n-6:n-3, whereas the LFFO diet had a ~6 n-6:n-3, and the LFEO diet had a ~5 n-6:n-3 ratio. The HFC diet consisted of 1.1% of calories attributed to n-3s, and 15.19% coming from n-6s. The HFFO diet had 4.16% of calories come from n-3s and 13.32% from n-6s, and the HFEO diet had 5.63% come from n-3s and 15.52% come from n-6s. The n-6:n-3 ratios for the HFC diet was ~14.6, whereas the ratio for HFFO and HFEO diets was ~3. It was noted that the addition of fish oil supplied DHA, which was not a constituent in either control or *Echium* oil diets, whereas the *Echium* oil had supplied a significantly higher concentration of stearidonic acid (an intermediate n-3).

High fat diet influenced glucose and insulin metabolism, and feeding efficiency, after long exposure.

Glycaemia and insulinaemia were measured every week for 11 weeks. Glycaemia in the high fat diet groups was increased in contrast to the low fat diet groups (n=10) (*Fig.* 1A, 1B). AUC 2-tailed T-test analyses were performed for the groups via Graphpad Prism, and the high fat diet groups exhibited a mild fed hyperglycaemia,(*Fig.* 1B). It is also noted, though not statistically significant, that the HFEO group appeared to exhibit an elevated fed glycaemia in comparison to the HFFO (AUC, P=0.069). All of the diet groups had elevated glycaemias during the start of the 11 week glycaemia period. The low fat diet groups' glycaemias had dropped after

2 weeks, whereas the high fat diet groups' glycaemias remained elevated around ~150 mg/dL through 12 weeks (Figure 1A). Similarly, plasma insulin measurements were performed each week with blood draws (*Fig.* 2A). We were then able to determine the AUC for weekly insulinaemia measurements (n=10) (*Fig.* 2B), which show that the high fat diet groups all had significantly elevated insulinaemia levels compared to each of the low fat diet groups, though it was noted that the HFEO group had higher plasma insulin levels during weeks 2 and 5 (*Fig.* 2A).

The mice were also weighed weekly (n=10) (*Fig.* 3A) and were compared by compiling the weight values, chronologically to determine AUC over the treatment period (*Fig.* 3B). The high fat diet groups were observed to gain more body weight than the low fat diet groups over 12 weeks. It was also found that the LFFO group was heavier than the LFC group throughout the 12 weeks. LFEO mice also tended to be a bit heavier than the LFC mice during the 12 week period, too though not statistically different (P=0.1215). However, the LFEO did exhibit a trend in being lighter than that of the LFFO group (P=0.0736). During the last week, the only notable difference between the mice was that the high fat diet groups were heavier than the low fat diet groups by an average of 15 more grams per individual than the low fat groups.

Every week, caloric (food) intake was determined by calculating the difference between the weight of food supplied and the food remaining when replacing the feed, weekly (n=2, cages) (*Fig.* 4A). Weekly values were used to calculate an AUC for caloric intake (*Fig.* 4B), in which the high fat diet groups had consumed more kcal/week than the low fat groups, and the LFFO group consumed more calories than the LFC group. In addition, two trends were also observed, though not statistically significant, in which the LFEO group had a trend of consuming less than that of the LFFO group (P=0.0566), and the HFEO group had a trend of consuming more than the HFFO group (P=0.0926). After comparing the weekly food intake values and the weekly

weights, we calculated the weekly feeding efficiencies overall feeding efficiency from the 12-week period (n=2, done by cage) (*Fig.* 4C and 4D). The LFFO and LFEO groups were found to have decreased feeding efficiency in contrast to the LFC group. The HFEO group had a significantly higher feeding efficiency than that of the LFC, LFFO, and LFEO groups, whereas the HFFO and HFC groups had significantly higher feeding efficiency compared to the LFFO and LFEO groups. Though not statistically significant, the HFFO group had also exhibited a trend in having a higher feeding efficiency than the LFC group (P=0.0583) over the 12 week care period. Therefore, we find that the high fat diet groups had decreased satiety and impaired metabolic function. We also find that the LFFO group had gained more weight and had decreased satiety, and fish oil supplementation during high fat feeding exhibited a trend of having better glucose metabolism than with *Echium* oil supplementation (P=0.069). In general, the addition of proportionally more n-3s in the low fat diets may yield increases in body weight, as the LFEO group also had a trend of being heavier than the LFC group (P=0.1215), but lighter than the LFFO group (P=0.0736).

High fat diets impact glucose homeostasis and insulin sensitivity

ipGTTs were performed on mice after 4, 8, and 12 weeks, in which glycaemia had been measured before, and 2 hours after intraperitoneal injection of dextrose, and the glycaemia per time interval was used to formulate AUC, for each. The ipGTT (n=10) performed on week 4 of the trial resulted in the high fat diet groups having a significantly higher AUC glycaemia than that of the low fat diet groups, and the HFEO group having a higher AUC glycaemia than the HFFO group (*Fig.* 5A and 5B). In addition, though not statistically significant, the HFEO group did show a trend of having a higher glycaemia than the HFC group (P=0.105), and the HFFO group having a lower glycaemia than the HFC group (P=0.113). The ipGTT during week 8

(n=5) had demonstrated that, again, the high fat diet groups had an elevated glycaemia (thus reduced tolerance) in comparison to the low fat diet groups (*Fig.* 5C and 5D). Comparing glycaemia measured at distinct time intervals (not AUC) did show that the LFEO had a decreased glycaemia compared to the LFC and LFFO groups at the 120 minute endpoint. Though not significant, the HFEO group also showed a trend of having increased glycaemia in comparison to the HFC (P= 0.112) and HFFO (P=0.119) groups at 60 minutes, and increased in comparison to the HFFO group at 120 minutes (P=0.121). Finally, the week 12 ipGTTs (n=4) AUC analysis revealed that the HFC and HFFO having an elevated glycaemia in comparison to the low fat diet groups (Fig 5E and 5F). In addition, the HFEO group was found to have a higher glycaemia compared to the LFEO group, and the LFEO group was found to have a decreased glycaemia compared to the LFC group. Significantly, the HFEO was found to have a decreased glycaemia compared the HFC group. Though not statistically significant, it was also found that the HFEO group had a trend of having a better glucose tolerance than the HFFO group (P=0.0752).

ipITTs were also performed during weeks 8 and 12 in order to measure insulin sensitivity through acute response to an insulin bolus (0.75mU/g body weight). Mice were injected with insulin, and glycaemia was measured at intervals. Glycaemia per increment of time was used to calculate AUC of blood glucose levels. At both 8 (n=5) and 12 weeks, although the high fat diet groups exhibited worsened insulin tolerance compared to the low fat diet groups, there were no differences within the low fat and high fat diet groups, though during the ipITT from week 12, it was noted that the LFEO group exhibited a trend of having decreased glycaemia compared to the LFC group (P=0.107) (n=10) (*Fig.* 6A to 6D).

High Fat Diet Increases Epididymal Body Fat Percentage, Pancreatic Mass, and Hepatic Mass

After euthanasia, cardiac blood was immediately drawn for analysis. Furthermore, pancreata, epididymal fat pads, and the liver lobes were dissected and weighed (as well as the quadriceps skeletal muscle, spleen, and hypothalamus), to assess where energy was being distributed. We found that the high fat diet groups had significantly higher amounts of epididymal fat tissue, as well as a heightened visceral fat proportion, compared to the low fat diet groups (n=10) (*Fig.* 7A and 7B). In addition, we had found that the HFEO group was not statistically different compared to the LFC group in visceral fat (percentage of total body mass). Interestingly, we also found that the LFEO group carried a lower percentage of epididymal fat mass than the LFC group, while the LFFO group followed in a non-significant, but noticeable trend (P=0.084). The pancreatic weight was found to be elevated in all the high fat diet groups compared to the low fat diet groups, however, the HFEO group only exhibited this increase compared to the LFC group (n=6) (*Fig.* 7C). We also observed that the HFC and HFEO groups had a decreased percentage for how much pancreatic mass contributed to total body mass (n=6) (*Fig.* 7D). Finally, we also observed that the livers of the high fat diet groups had increased masses in comparison to the low fat diet groups, and the HFEO group's livers were even heavier than the HFC group's livers (n=10) (*Fig.* 7E). The HFEO group was also observed to have higher proportional liver weight than the HFC group. It would appear as if more energy was diverted to the HFEO group's livers (n=10) (*Fig.* 7F). From this, we find the high fat diets had significantly increased visceral fat, pancreatic, and liver weights, that the high fat diet groups had more visceral fat contributing to body weight (percentage). The HFEO group gained less visceral fat but acquired more liver mass through the 12-week period.

N-3 Supplementation Prevents High Fat Diet-Induced Reduction of Insulin Signaling in Skeletal Muscle

Endogenous n-3s and their metabolites have been revered for their broad ligand-binding ability to mediate insulin-sensitizing effects directly and indirectly [4, 29, 32, 35, 52, 67, 69, 78, 90, 93, 99]. Since the HFEO group exhibited improved glucose disposal at the end of the trial, based on the ipGTT performed on week 12, and the HFFO had non-significant but noticeable trends in improved glucose metabolism as observed in the week 4 ipGTT (P=0.113) and week 8 ipITT (P=0.104), we sought to determine to observe if these observations were the result of enhanced skeletal muscle insulin signaling. Using western blotting, we examined the activity and prevalence of proteins involved in insulin receptor transduction, inflammation, and ER stress. As demonstrated (*Fig.* 8B), we found that the HFC group's skeletal muscle a 51% reduction in insulin signaling as judged by the percentage of activated (phosphorylated) Akt1 versus the total amount of Akt1, compared to the LFC group (n=5). When solely comparing the high fat diet groups against each other (*Fig.* 8D), no statistical differences were observed. However, it was noted that the HFFO group exhibited a trend of an increased proportion of p-Akt1 relative to total amount of Akt1 (n=6) (P=0.1092). Since there were considerable differences in total Akt1 (*Fig* 8A: the HFFO group did have 1.91-fold higher levels of Akt1 in comparison to the LFC group) among the groups, we also compared amounts of p-Akt1 normalized to β-Actin. A comparison between the high fat diet groups (*Fig.* 8C) showed that the HFEO displayed heightened skeletal muscle activated Akt1 in comparison to the HFC group by 2.56-fold (n=6). Although not statistically significant, the HFFO group exhibited a well-defined trend of having higher levels of activated Akt1 in comparison to the HFC group (P=0.089).

Insulin transduction is also known to trigger glycogen storage in skeletal muscle by inhibiting GSK-3β through phosphorylation via Akt, otherwise GSK-3β would normally inhibit glycogen synthase from producing glycogen. Being that GSK-3β is a critical target for regulation for glycogen synthesis, we also probed for GSK-3β levels for comparison in expression (n=6) to see if the supplementation of n-3s had improved glycogen storage, aside from insulin transduction alone. The, high fat diet groups showed variability, in which the HFEO showed increased amounts of p-GSK-3β (inactive) compared to the HFC by 2.37-fold (*Fig.* 9). This observation was in accordance with the increase in p-Akt1 in the HFEO group observed in comparison to the HFC group (*Fig.* 8C), as well as the decrease in p-Akt1 activity observed in the HFC group (*Fig.* 8B), implicating that insulin transduction-induced glycogen storing activity had been enhanced with n-3 supplementation. Though not statistically significant, the HFFO showed a similar trend in higher amounts of p-GSK-3β in contrast to the HFC (P=0.0577), which is also analogous to the trend observed with the HFFO group having an increased amount of Akt1 compared to the HFC group (*Fig.* 8C), and analogous to the decrease in %Akt1 activity observed in the HFC group (*Fig.* 8B).

Considering PUFAs have been reported elicit much of their insulin-sensitizing and anti-inflammatory activity through PPARγ [15, 24, 29, 52, 69, 90, 99], and high fat diets and the n-3 fatty acid EPA have been shown to increase mRNA encoding PPARγ [22, 69, 72], we tested whether the n-3 supplemented diets had an augmenting effect on PPARγ levels. We found that PPARγ levels had been increased in the HFFO group in comparison to the low fat diet groups by 2-3-fold, and its levels were elevated in the HFEO group in comparison to the LFC group by 3.1-fold (n=6) (*Fig.* 10). The HFEO group also exhibited a (non-statistically significant) trend in having higher amounts of PPARγ in comparison to LFFO (P=0.0578) and LFEO (P=0.09271)

groups. Although there was no statistically significant difference between the HFC and low fat diet groups, the HFC group did display a trend of having higher amounts of PPARγ compared to LFC (P=0.0785), LFFO (P=0.104), and LFEO (P=0.1326) groups. There were no statistical differences among the high fat diet groups. Phospho-p70 S6 Kinase, IRS1/2, and p-FOXO1 of the canonical insulin signaling cascade were also examined. However, no reproducible differences were observed among the groups. IRS-1/2 and p-FOXO1 were undetectable upon western blotting. From the data observed, it is concluded that the n-3-supplemented groups, especially the HFEO group, have a higher insulin transduction activity in skeletal muscle by enhancing Akt1 and GSK-3β activity.

High Fat Diets Lead to Inflammation and UPR, but What About *Echium* Oil's Effects?

Considering the vast amount of data there is on n-3s acting as anti-diabetic and anti-inflammatory agents, as well as their toxicity at certain high concentrations, and how iNOS inhibits insulin transduction in skeletal muscle [1, 2, 8, 23, 86, 87], we decided to examine iNOS expression. However, iNOS exhibited no observable differences in expression. We then investigated if dietary n-3s may protect skeletal muscle from high fat diet induced UPR, and tested for levels of p-Stat3, Xbp1s, and BiP which are critical markers in UPR activity. BiP recognizes misfolded proteins in the ER, which would normally be due to an energy deficit. But, this process has previously been found to be susceptible to the influence of a high fat diet [19, 26, 27, 51, 54, 82]. Xbp1s mediates secondary messenger activity in the cell to eventually lead to transcriptional activity by Stat3. Because BiP and Xbp-1s become increased by activation of UPR, and Stat3 is activated (phosphorylated) during UPR, we examined the expression of these intermediates. Whereas p-Stat3 and Xbp1s were undetectable, the HFFO group showed significantly increased BiP levels when compared to the LFC and LFFO groups by 4.3-fold

(n=6) (*Fig.* 11A). The HFFO group also showed a trend in increased levels of BiP in comparison to the LFEO group (P=0.0546). In addition, though not statistically different, the HFC showed a trend in having increased BiP in comparison to the LFC (P=0.087), LFFO (P=0.102), and LFEO (P=0.113) groups. HFEO was not statistically different to the low fat diet groups. Finally, the high fat diets were compared to one another, and though no statistical differences had been observed, one trend had certainly piqued our interest: the HFEO BiP expression had a tendency to be greatly decreased in comparison to the HFC (P=0.05614) by 69.5%and the HFFO (P=0.0659) by 68.9% (*Fig.* 11B). From these observations, we find that high fat diets do induce BiP expression through UPR, but it would appear that supplementation of *Echium* oil can either ameliorate chronic UPR induced by high fat dieting or maybe interfere with the synthesis of BiP, directly- either through translation, which is highly regulated [36], or transcription [98].

Previous related studies: Fatty acid composition:

Skeletal muscle samples were also used to determine sarcolemmal membrane fatty acid composition, by Pamela Bay and Jana Kraft [9]. Regardless the amount of stearidonic acid the *Echium* oil, or any other n-3 carrying fats, thereof, there was no detectable trace of stearidonic acid within the membraneous tissue of skeletal muscle (Table #8). The *Echium* oil-supplemented groups had a higher incorporation of n-6s into the muscle, including a high amount of ALA, contributing to the trend of having higher PUFA content, in comparison to the fish oil and control groups' muscles. However, this behavior mimicked how many n-6s the control diet groups had also incorporated into skeletal muscle. Intriguingly, though, the supplementation of fish oil had decreased the total percentage of n-6s, including ALA, contributing to skeletal muscle tissue fatty acid composition in both the low fat and high fat dieting scenarios. Consequently, fish oil-supplemented groups' muscles did carry more n-3s than *Echium* oil-

supplemented and control diet groups, yet, also contributed more saturated fatty acids (including palmitic acid) to skeletal muscle membranes. The saturated fatty acid contents (including palmitic acid), though lower than the fish oil diet groups, were indistinguishable between the control and *Echium* oil supplemented diets. The fish oil groups had a higher EPA (20:5, n-3) and DHA (22:6, n-3) content in their skeletal muscle than the *Echium* oil-supplemented and control groups. There was no significant difference in DHA and EPA levels between the *Echium* oil-supplemented and control groups, though the LFEO did appear to have a trend of having increased DHA incorporated into skeletal muscle than the LFC group. An interesting observation was that, although EPA content increased in the high fat diets (vs. low fat diets), the DHA content, though not significant, did appear to have a trend in being decreased in the high fat diet groups in comparison to the low fat diet groups. Finally, it was noted that an increase in n-3 intake and total fat intake was directly associated with increasing levels of docosapentaenoic acid (22:5, n-3) levels in skeletal muscle, which can be interconverted into EPA or DHA. Aside from ability to interconvert docosapentaenoic acid to other metabolites, the intermediate's influence on metabolism are not well known, though docosapentaenoic acid can also stimulate PPARγ activation after conversion by COX-2 [35]. Fish oil and *Echium* oil provided higher levels of docosapentaenoic acid in comparison to the control groups. Finally, it was also observed that the low fat diet groups had significantly higher levels of monounsaturated palmitoleic acid in their membranes in comparison to the high fat diet groups. However, not much is known about palmitoleic acid in skeletal muscle, though it is suggested that it can ameliorate insulin resistance [45, 101].

These observations do indicate that fish oil and *Echium* oil modulate fatty acid composition in skeletal muscle. However, the most significant distribution between n-3s, n-6s

(including ALA), and saturated acids had been established in the fish oil-supplemented diets, which carry fewer n-6s (and ALA) in exchange for more n-3s and saturated fats. In addition, it would be reasonable to claim that the incorporation of DHA is dependent on DHA's bioavailablity and has a trend of being associated with body composition (higher in low fat diet groups supplemented with n-3s, in comparison to high fat diet groups supplemented with n-3s, being lean vs. fat), and the incorporation of DHA may reduce the incorporation of n-6s and increase saturated fatty acid levels in the skeletal muscle membrane. Perhaps the increased availability of DHA decreases the need for skeletal muscle to carry as many n-6s. Furthermore, further experimentation should be done to elucidate why there is a decrease in palmitoleic acid that occurs along with a high fat diet. Finally, it is noted that the distribution of polyunsaturated fatty acids is increased by supplementation of *Echium* oil, while not increasing saturated fatty acid content from the control. It would seem that prevention of palmitate-induced dysfunction in metabolism is not due to reduction of palmitic acid, as it was in fact observed that palmitic acid increased with application of fish oil.

Discussion

The extent to which PUFAs, or more specifically, n-3s, affect skeletal muscle physiology to impact whole-body insulin sensitivity and anti-inflammatory drive has been difficult to assess. Furthermore, of n-3 supplementation may have toxic consequences should levels exceed a specific concentration [29, 61, 84]. In skeletal muscle, n-3s have been suggested to improve insulin sensitivity through an array of mechanisms: direct PPARγ agonism and membrane incorporation/enhanced mitochondrial function, and indirectly through inhibition of inflammatory cytokine secretion from distinct tissues, and improved adipokine signaling [4, 5, 13, 16, 29, 32, 34, 38, 40, 45, 52, 67, 69, 78, 90, 91, 93, 99]. Glucose metabolism had been

impaired in the high fat diet mice over the 12-week trial, yet, there were several n-3-specific effects within the HF group.

First of all, the HFEO group exhibited a marked improvement in glucose tolerance by 12 weeks. This improvement may be the result of increased insulin sensitivity, especially in skeletal muscle. Although the ipITT assays failed to show differences in insulin tolerance among the HF groups, and by inference, no detectable differences in systemic insulin sensitivity, the skeletal muscle immunoblot analyses demonstrated a significant increase in insulin signaling in the HFEO and HFFO groups, as demonstrated by the enhanced Akt1 activation, and the downstream inhibition/phosphorylation of GSK-3β. Perhaps if the study were continued beyond 12 weeks, differences in insulin tolerance among the high fat diet groups would become apparent.

The n-3 enriched high fat diets differentially affected tissue-specific energy partitioning. Body mass was found to be significantly increased in the high fat diet groups, but there were no differences among the high fat diet groups. *Echium* oil supplementation led to augmented liver growth in the high fat diet groups, suggesting that PPARγ activity in the liver may be enhanced to promote triglyceride uptake and lipogenesis (*Fig.* 7E and 7F) [32]. This possibility warrants investigation in future studies. Interestingly, the HFEO group displayed a significant decrease in (epididymal) fat mass compared to the HFC group (*Fig.* 7A and 7B). Since body weight in the HFEO group was unchanged after 12 weeks compared to the HFC group, this might reflect an increase in lean muscle mass.

As expected, there were distinct differences in the fatty acids incorporated into skeletal muscle tissue between fish oil- and *Echium* oil- supplemented high fat diets. The HFFO group had elevated concentrations of EPA and DHA, known to stimulate PPARγ. HFEO group displayed increased concentrations of total PUFAs (as a trend) and reduced levels of saturated

fatty acids (including palmitic acid) compared to the HFFO group. However, this observation resembles the saturated fatty acid and n-6 level in the HFC group, whereas the HFFO group had significantly decreased levels of n-6s, and elevated n-3s and saturated fatty acids (including palmitic acid). The behavior is difficult to explain, though it would appear to be influenced by the bioavailability of DHA. The ratio of saturated fatty acids and PUFAs could render the HFEO less prone to mitochondrial dysfunction and ER stress due to saturated fat (and especially palmitic acid) metabolism in skeletal muscle in a high feeding model, regardless of inflammatory influence from NFκB [44, 46]. However, this behavior needs to be studied further, as it is well established that HFFO ameliorates mitochondrial dysfunction [47]: perhaps the dysfunction can be ameliorated with distinct members of the n-3 family, in this case DHA, and so the rhabomyocytes would need to rely less on other n-6s/PUFAs to satisfy metabolic function. Regardless, the fact that we detected a trend in reduced ER stress in the HFEO group using the BiP marker seems to reinforce this notion, though the mechanism facilitated by *Echium* oil, if this trend can be reconfirmed, must be elucidated to see if it has an effect on UPR, or a separate effect on BiP's translation, as BiP is highly regulated by translation [36] and transcription [98]. Finally, it was found that palmitoleic acid was sharply declined in the high fat diet groups compared to the low fat diet groups. The effects of this FA on skeletal muscle insulin sensitivity and inflammation warrants further studies.

Skeletal muscle glycogen synthesis potential appears to be enhanced in the n-3 supplement high fat diet groups, and especially the HFEO group, as suggested by increased GSK-3β inactivation, which in turn, activates glycogen synthase activity. The finding of increased levels of p-GSK-3β correlate well with *Hirabara, S. M. et al. 's* observations on how higher saturated fatty acid concentrations can dull glycogen synthesis [44]. PPARγ was found to

be elevated in the HFFO and HFEO groups (and the HFC group, non-significantly) when compared to the low fat diet groups, which was anticipated, since obese and hyperinsulinaemic subjects exhibit increased PPARγ levels [72].

Collectively, these studies suggest that skeletal muscle's insulin sensitivity is protected when n-3s are supplemented in a high fat diet. Furthermore *Echium* oil appears to be just as effective as fish oil as judged by (1) improved glucose tolerance, (2) improved skeletal muscle insulin signaling, as determined by Akt1 activation and GSK-3β inactivation, (3) possible ability to reduce ER stress, (4) possible incorporation of more PUFAs into the sarcolemmal membrane, and (5) reduced visceral fat accumulation. Future studies are necessary to establish how DHA bioavailability appears to allow the sarcolemma to incorporate more saturated fatty acids (including palmitic acid), In addition, future analysis must establish the biochemical changes in other insulin sensitive tissues (e.g., liver, adipose, and pancreatic islets) in response to *Echium* oil and fish oil supplemented diets that impact glucose tolerance and energy distribution.

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Table #1: Preparation of tissue homogenization (lysis buffer)

Lysis Buffer Recipe: Start with 2x Extract Buffer	
Preprepared and frozen: **2x Extract buffer (10 mL)**	
Ingredient	Volume (mL) per 10 mL
1 M Tris-HCl pH 7.4	0.2
5 M NaCl	0.2
0.5 M EDTA	0.04
0.1 M EGTA	0.2
10% Triton X-100	1
50% Glycerol	2
10% SDS	0.1
10% Deoxycholate	0.5
Double Distilled Water	5.76
Prepared fresh with pre-prepared Extract Buffer: **Basic Lysis Buffer (Final Homogenization Buffer) (10 mL)**	
Ingredient	Volume (mL) per 10 mL
2x Extract Buffer	5
0.2 M Na-orthovanadate	0.1
0.2 M Na-pyrophosphate	1
0.1 M Na-fluoride	0.1
10x Protease Inhibitor	1
Double Distilled Water	2.8

Table #2: Preparation of Western blotting resolving gel

10% Acrylamide Resolving Gel Recipe	
Ingredient	Volume (mL)
Double distilled water	9.9
30% Acrylamide	8.3
1.5 M TRIS (pH 8.8)	6.3
10% SDS	0.25
10% Ammonium Persulfate	0.25
TEMED	0.01

Table #3: Preparation of Western Blotting Stacking Gel

5% Acrylamide Stacking Gel Recipe	
Ingredient	Volume (mL)
Double distilled water	5.5
30% Acrylamide	1.3
1.0 M TRIS (pH 6.8)	1
10% SDS	0.08
10% Ammonium Persulfate	0.08
TEMED	0.008

Table #4: Preparation of Western Blotting Transfer Buffer

Transfer Buffer Preparation	
Ingredient	Amount
TRIS Base	5.8 g
Glycine	2.9 g
SDS	0.37 g
Double Distilled Water	800 mL
Methanol	200 mL

Table #5: Preparation of Stripping Buffer

Stripping Buffer Preparation	
Ingredient	Volume (mL)
2M Glycine pH 2.8	20
1M NaCl	15
Tween 20	0.5
Double Distilled Water	64.5

Table #6: Bay, P., et. al. (2014). Plant-Derived Bioactive Lipids Impacts Glucose Homeostasis and Energy Metabolism in Mice. *Department of Animal Science, and Department of Medicine, University of Vermont.*

	Treatment					
	LF			HF		
% of kcal	CON	FO	EO	CON	FO	EO
Protein	20	20	20	20	20	20
Carbohydrate	70	70	70	20	20	20
Fat	10	10	10	60	60	60
Total	100	100	100	100	100	100
Ingredient						
Sucrose	275	275	275	275	275	275
Maltodextrin 10	500	500	500	500	500	500
Corn Starch	2025	2025	2025	0	0	0
Lard	180	144	144	2205	1764	1764
Menhaden Oil	0	36	0	0	441	0
Echium oil	0	0	36	0	0	441
Soybean Oil	225	225	225	225	225	225

Table #7: Bay, P., et. al. (2014). Plant-Derived Bioactive Lipids Impacts Glucose Homeostasis and Energy Metabolism in Mice. *Department of Animal Science, and Department of Medicine, University of Vermont.*

Feed	Treatment					
	LF			HF		
Fatty Acids	CON	FO	EO	CON	FO	EO
C16:0	16.15	15.87	14.95	20.29	19.75	17.76
C18:0	7.10	6.77	6.56	10.46	9.28	9.21
C18:1 n-7	26.91	24.61	25.04	34.57	29.08	30.48
C18:1 n-9	1.63	1.70	1.54	1.98	2.17	1.71
C18:2 n-6	38.80	37.75	38.62	24.16	21.50	23.26
C18:3 n-6	ND	ND	0.76	ND	0.08	1.68
C18:3 n-3	4.08	4.25	6.63	1.65	1.81	7.02
C18:4 n-3	ND	0.17	1.00	ND	0.47	2.22
C20:5 n-3	ND	1.02	ND	ND	2.39	ND
C22:5 n-3	ND	0.19	ND	0.05	0.42	0.05
C22:6 n-3	ND	0.64	ND	ND	1.84	ND
Saturated fatty acids	25.37	25.07	23.15	32.84	32.80	28.71
Mono-unsaturated fatty acids	30.57	29.36	28.40	39.74	36.87	35.09
Poly-unsaturated fatty acids	43.60	44.66	47.63	27.33	29.24	35.42
n-3	4.12	6.26	7.63	1.79	6.93	9.39
n-6	39.33	38.32	39.83	25.31	22.20	25.86
n6/n3	9.55	6.12	5.22	14.12	3.20	2.75

Table #8: Bay, P., et. al. (2014). Plant-Derived Bioactive Lipids Impacts Glucose Homeostasis and Energy Metabolism in Mice. *Department of Animal Science, and Department of Medicine, University of Vermont.*

Muscle Tissue	Treatment							Significance		
	LF			HF						
Fatty Acid	CON	FO	EO	CON	FO	EO	SEM	Fat Level (F)	Treatment (T)	F x T
C14:0	1.67^{A}	1.55^{AB}	1.39^{B}	1.02	1.66^{A}	0.97	0.20	***	***	***
C16:0	22.47^{BC}	23.87^{A}	22.68^{ABC}	21.70^{C}	23.66^{AB}	21.70^{C}	1.34	NS	**	NS
C16:1 n-7	10.97^{A}	9.65^{AB}	8.48^{B}	3.99	3.62	3.02	2.41	***	NS	NS
C18:0	6.89	7.41	7.40	7.42	7.52	7.85	1.78	NS	NS	NS
C18:1 n-9	19.79^{BC}	17.61^{C}	19.13^{BC}	26.35^{A}	23.14^{AB}	24.44^{AB}	5.53	**	NS	NS
C18:1 n-7	0.03	0.04^{A}	0.02^{A}	0.03^{B}	0.04^{BC}	0.02^{C}	0.30	***	**	NS
C18:2 n-6	12.88^{AB}	11.14^{A}	13.07^{B}	17.25^{CD}	15.81^{C}	17.86^{D}	1.97	***	*	NS
C18:3 n-6	0.05	0.05	0.06	0.04	0.04	0.27^{A}	0.10	*	**	**
C18:3 n-3	0.55^{A}	0.50^{A}	0.74^{AB}	0.66^{AB}	0.85^{B}	2.75^{C}	0.30	***	***	***
C20:5 n-3	0.12^{A}	0.36^{B}	0.15^{A}	0.11^{A}	0.71^{C}	0.24^{AB}	0.20	*	***	*
C22:5 n-3	0.90^{A}	1.72^{BC}	1.50^{BD}	1.20^{AD}	1.95^{CE}	2.24^{E}	0.47	**	***	NS
C22:6 n-3	8.06^{BC}	13.22^{A}	10.94^{AB}	7.17^{C}	11.77^{A}	7.68^{BC}	3.65	NS	**	NS
Saturated Fatty Acids	31.44	33.29	31.92	30.64^{A}	33.51^{B}	31.01^{A}	2.47	NS	*	NS
Mono-unsaturated fatty acids	36.64	32.70	33.04	34.66	31.10	31.42	6.47	NS	NS	NS
Poly-unsaturated fatty acids	30.89^{A}	32.89	34.04	33.96	34.40	36.49^{B}	4.48	NS	NS	NS
n-3	9.62^{A}	15.81^{B}	13.32^{B}	9.13^{A}	15.28^{B}	12.91^{AB}	3.99	NS	***	NS
n-6	21.22^{A}	16.98^{B}	20.68^{AC}	24.80^{D}	19.07^{C}	23.54^{D}	1.81	***	***	NS

*P < 0.05; **P < 0.01; ***P < 0.001

^{1} Values are means for n = 10/ group. Means in row with superscripts without a common letter differ (Tukey's *post hoc* test).

Table #9: Antibodies used in protocol

Used Antibodies	
Cell Signaling	
Antibodies	Catalog #
Phospho-Akt1 (S473) (Rabbit mAb)	9018S
Akt1 (Rabbit mAb)	2938S
Phospho-GSK-3beta (S9) (Rabbit mAb)	9322S
GSK-3beta (Rabbit mAb)	9351S
BiP (Rabbit mAb)	3183S
beta-Actin (Rabbit mAb)	4967S
Phospho-FOXO1 (S256) (Rabbit mAb)	9461P
FOXO1 (Rabbit mAb)	9454S
Phospho-p70 S6 Kinase (Rabbit mAb)	9234S
Phospho-IRS-1 (S307) (Rabbit mAb)	2381S
IRS-2 (Rabbit mAb)	4502S
Phospho-Stat3 (Y705) (Rabbit mAb)	9145S
Xbp1s (Rabbit mAb)	12782S
Millipore	
Anti-iNOS/NOS II (Rabbit polyclonal IgG)	06-573
Affinity BioReagents	
Anti-PPAR Gamma (Rabbit Polyclonal IgG)	PA3-821A

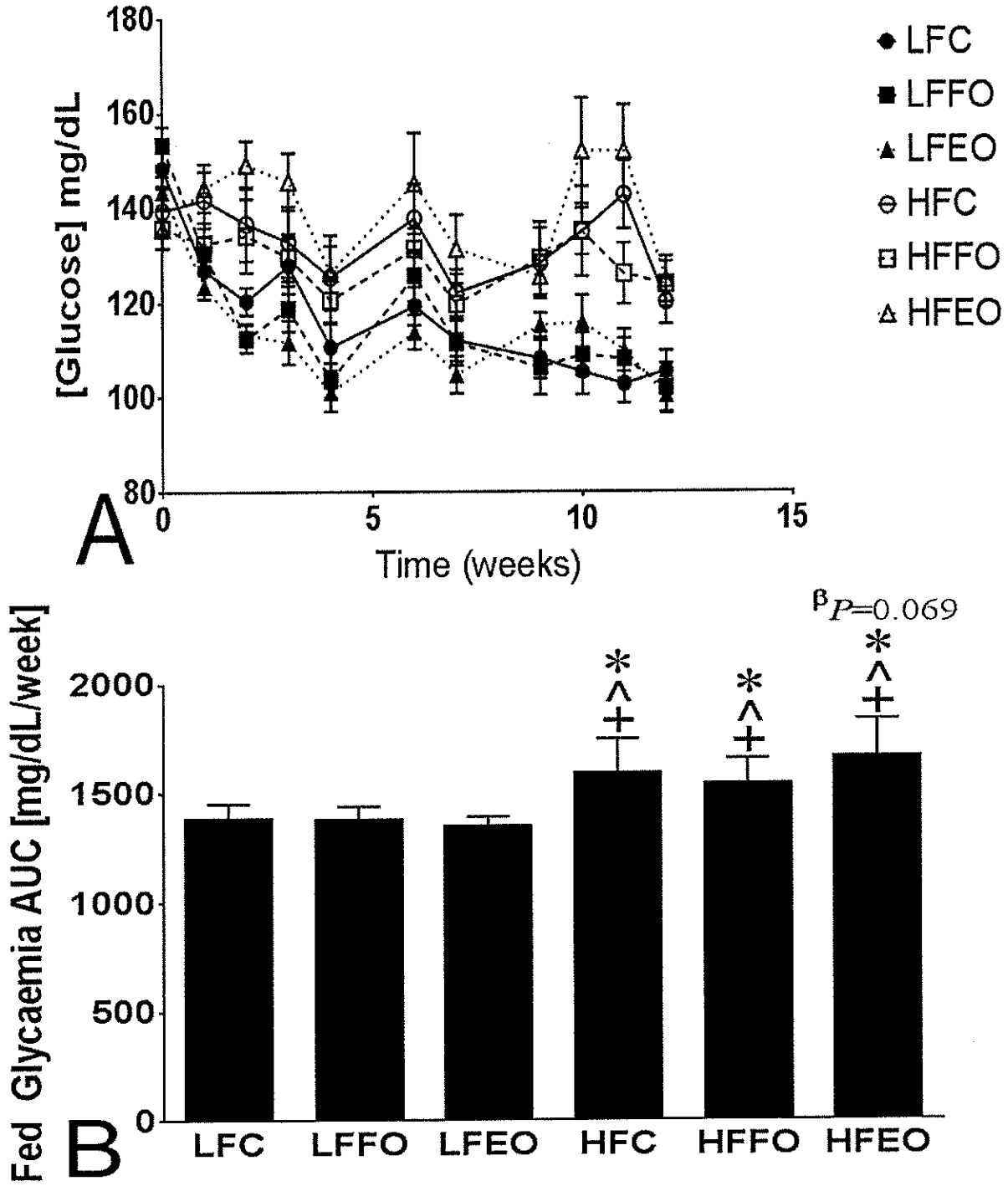

Figure 1: High fat diet disrupts glucose tolerance, chronically.
(**A** to **B**) Baseline fed glycaemia levels had been measured intermittently, every week, excluding weeks when ipGTTs and ipITTs were being performed. (A) The weekly glycaemia measurements had been analyzed to derive (A) the AUC for glycaemia of each group. Data from (A) to (D) signify means ± SEM. *P<0.05 to LFC, ^P<0.05 to LFFO, +P<0.05 to LFEO, #P<0.05 to HFC, and βP<0.05 to HFFO. Significance determined by individual T-tests.

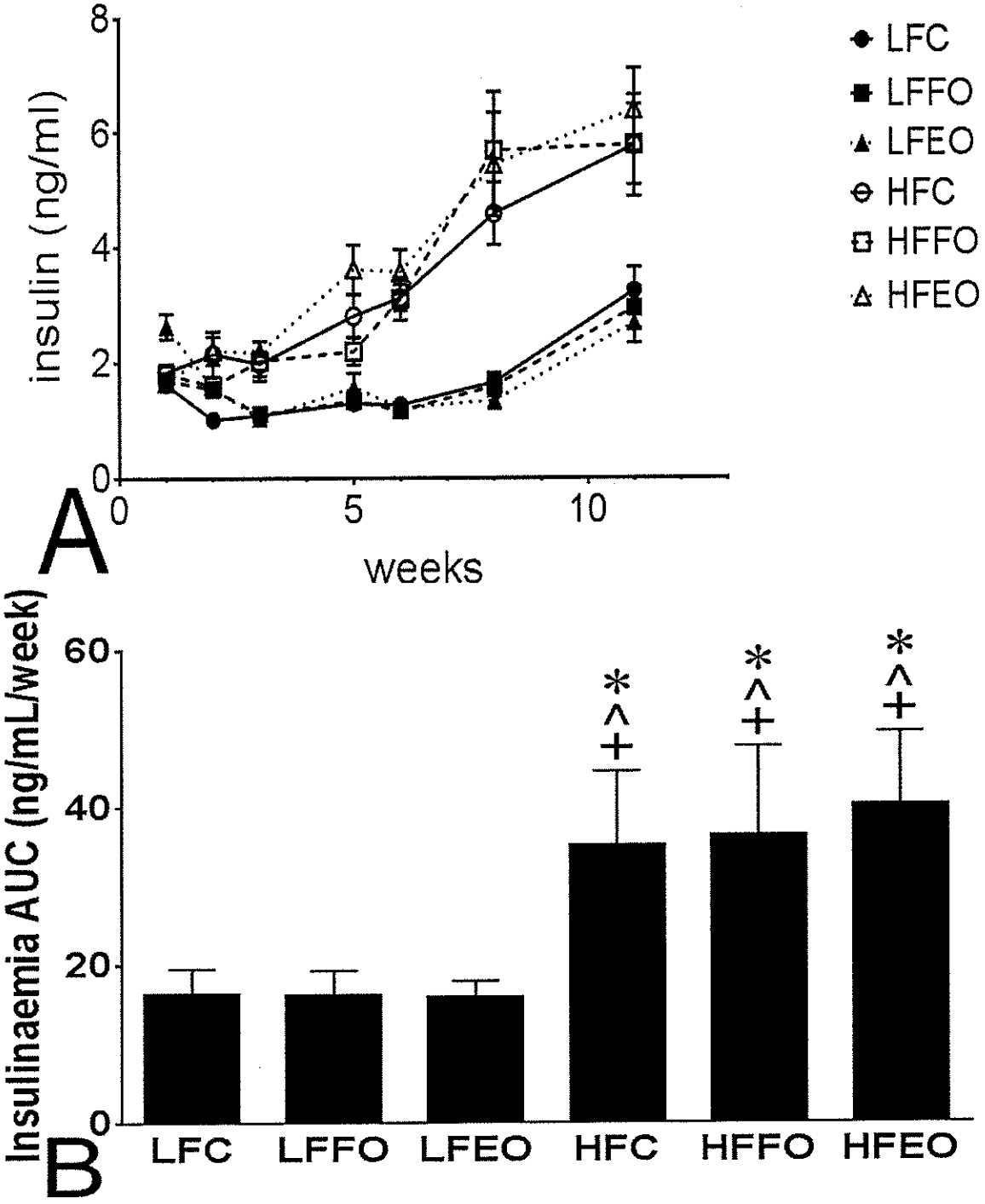

Figure 2: High fat diet disrupts insulin sensitivity, chronically.
(**A** to **B**) Baseline fed plasma insulin levels had been measured intermittently every week, excluding weeks when ipGTTs and ipITTs were performed. (A) Weekly plasma insulin levels had been analyzed to derive (B) the AUC for insulinaemia of each group. Data from (A) to (B) signify means ± SEM. *P<0.05 to LFC, ^P<0.05 to LFFO, +P<0.05 to LFEO, #P<0.05 to HFC, and βP<0.05 to HFFO. Significance determined by individual T-tests.

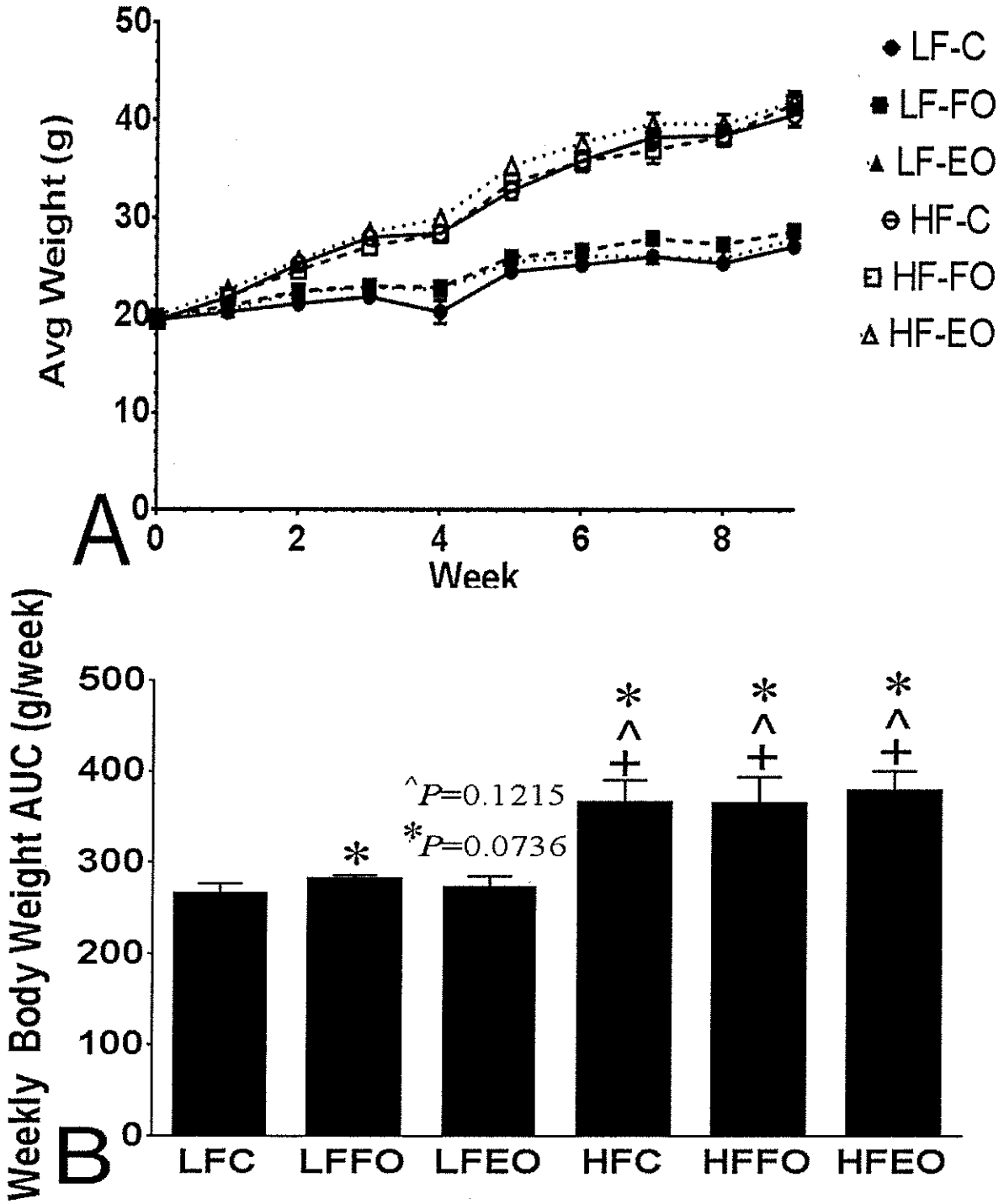

Figure 3: High fat diet promotes weight gain.
(A) Mice were weighed weekly, and the data was compiled in order to derive (B) AUC and compared for weight gain. Data from (A) to (B) signify means ± SEM. *P<0.05 to LFC, ^P<0.05 to LFFO, +P<0.05 to LFEO, #P<0.05 to HFC, and βP<0.05 to HFFO. Significance determined by individual T-tests.

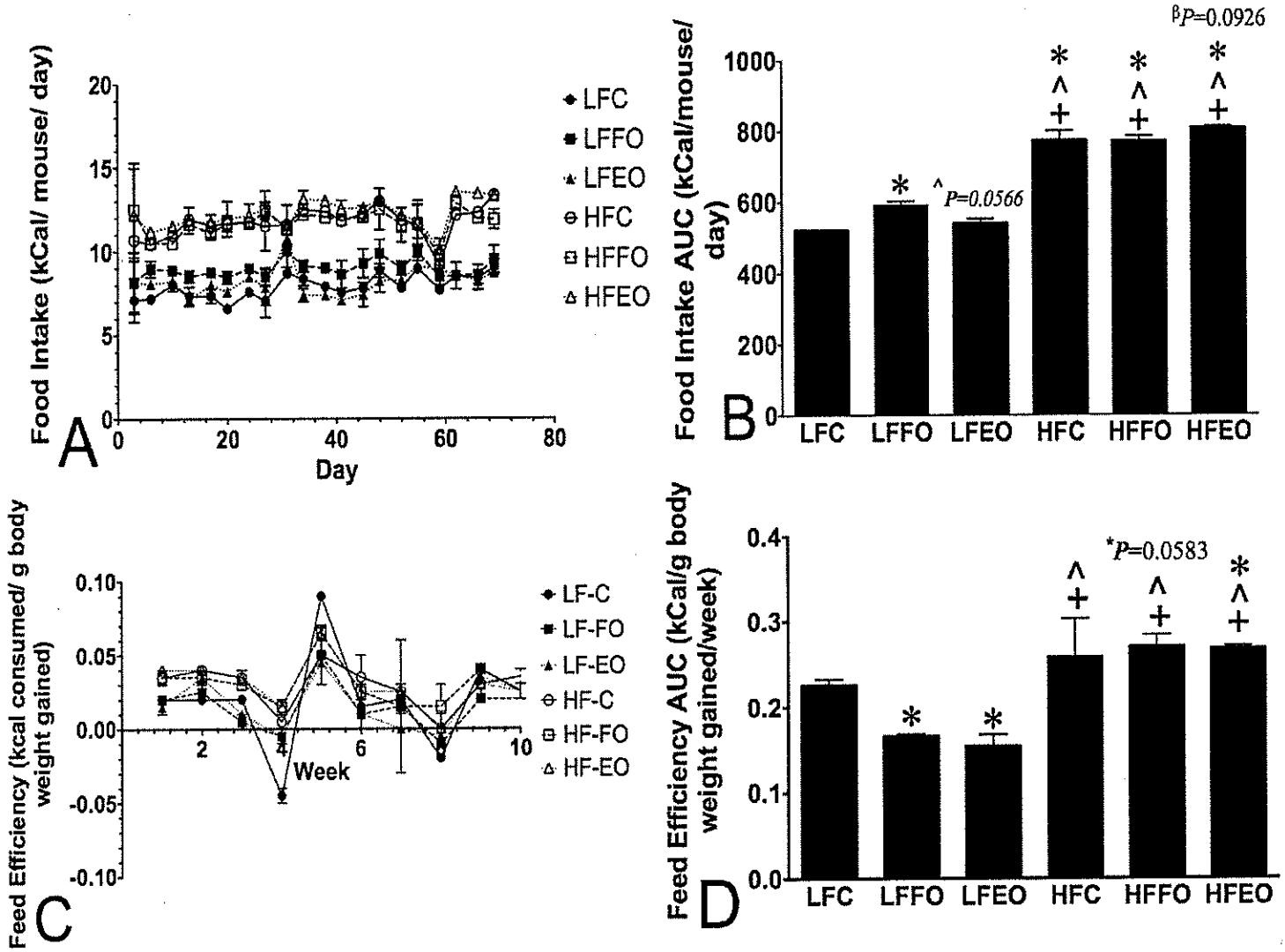

Figure 4: High fat diet decreases satiety and increases feeding efficiency. N-3s increase feeding efficiency. (A) Food intake was measured weekly by weighing the remaining food from each cage, and subtracting the value from the food's weight when it had initially been placed in the cage, prior replacement. The food intake weight had been converted to caloric values, and displayed. (B) The weekly values were then compiled to determine the AUC. Afterwards, (C) weekly and (D) AUC feeding efficiencies had been determined by dividing weekly and AUC food intakes (kcal) by body weights. Data from (A) to (D) signify means ± SEM. *P<0.05 to LFC, ^P<0.05 to LFFO, +P<0.05 to LFEO, #P<0.05 to HFC, and βP<0.05 to HFFO. Significance determined by individual T-tests.

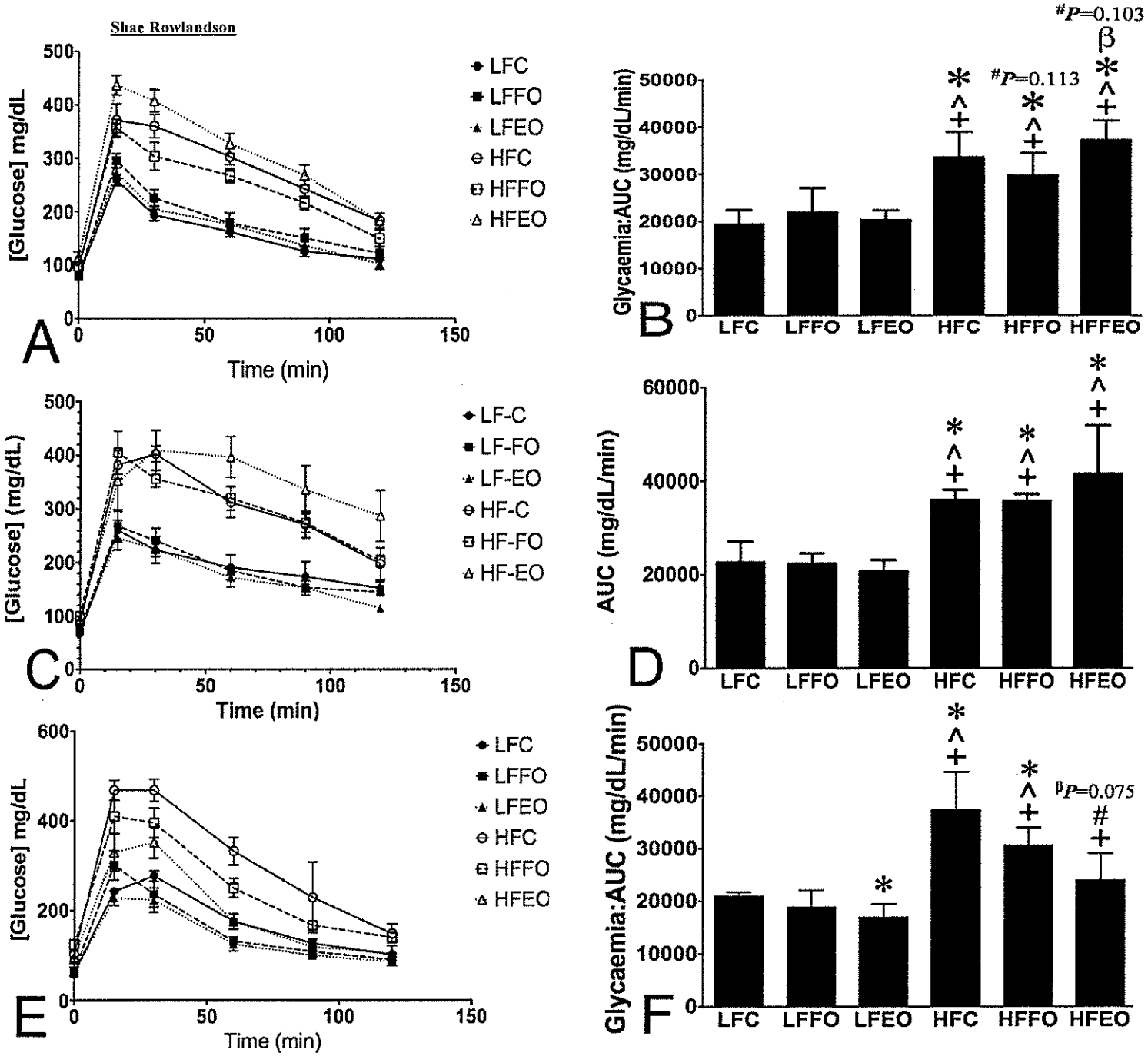

Figure 5: High fat diet afflicts response to acute hyperglycaemia, with unclear phenotypic influences from n-3s.

(**A** to **F**) 3, 2-hour ipGTTs were performed. Area under the curve (AUC) for blood glucose levels had been determined from (B) week 4 (n=10), (D) week 8 (n=5), and (F) week 12 (n=4) ipGTTs by measuring glycaemia for 2 hours after injection of dextrose, as represented in (A), (C), and (E), respectively. (Data from (A) to (F) signify means ± SEM. **P*<0.05 to LFC, ^*P*<0.05 to LFFO, +*P*<0.05 to LFEO, #*P*<0.05 to HFC, and β*P*<0.05 to HFFO. Significance determined by individual T-tests.

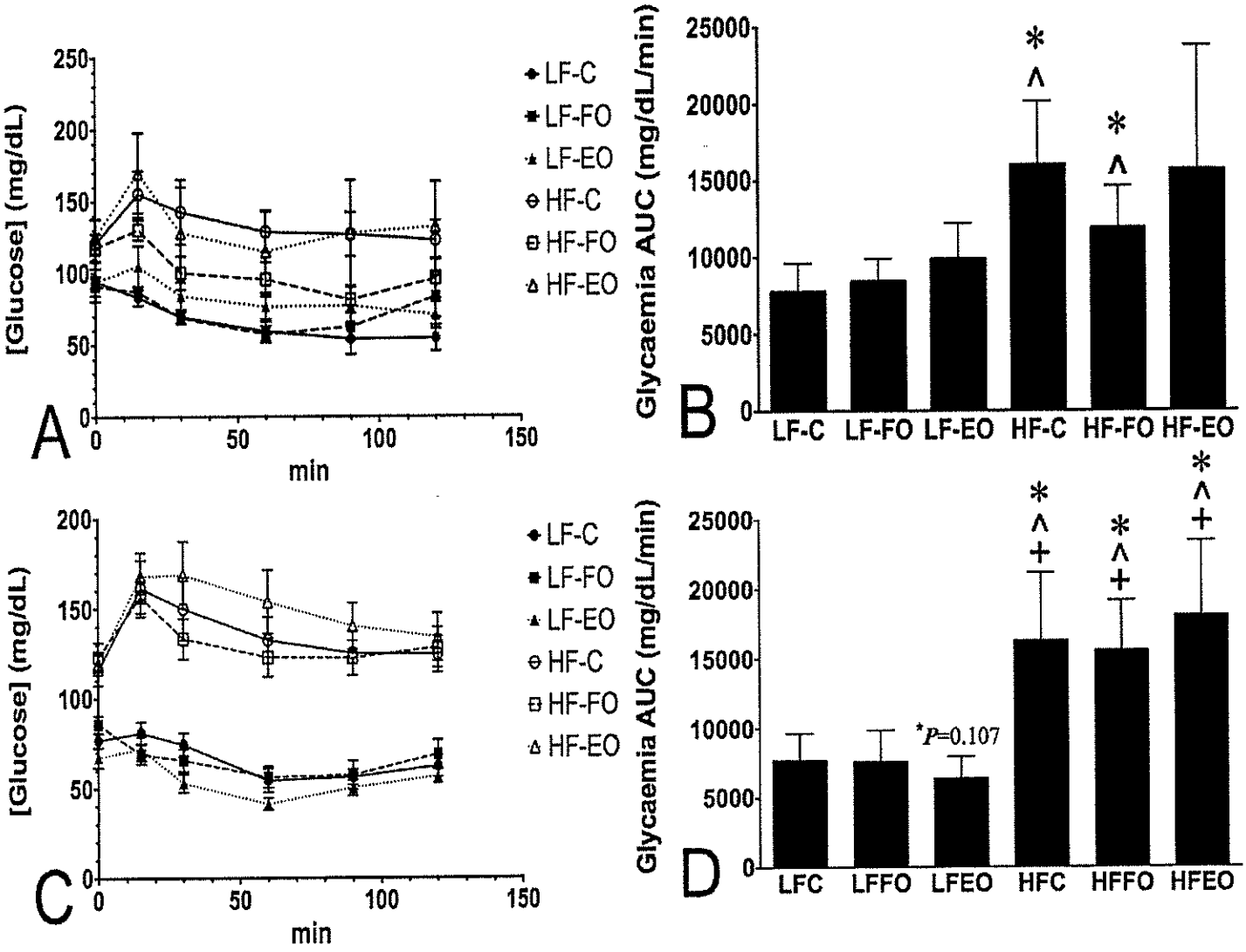

Figure 6: High fat diet afflicts response to acute hyperinsulinaemia
(**A** to **D**) 2, 2-hour ipITTs were performed. AUC for blood glucose levels had been determined from (B) week 8 and (D) week 12 ipITTs by measuring glycaemia for 2 hours after injection of insulin, as represented in (A) and (C). Data from (A) to (D) signify means ± SEM. *P<0.05 to LFC, ^P<0.05 to LFFO, +P<0.05 to LFEO, #P<0.05 to HFC, and βP<0.05 to HFFO. Significance determined by individual T-tests.

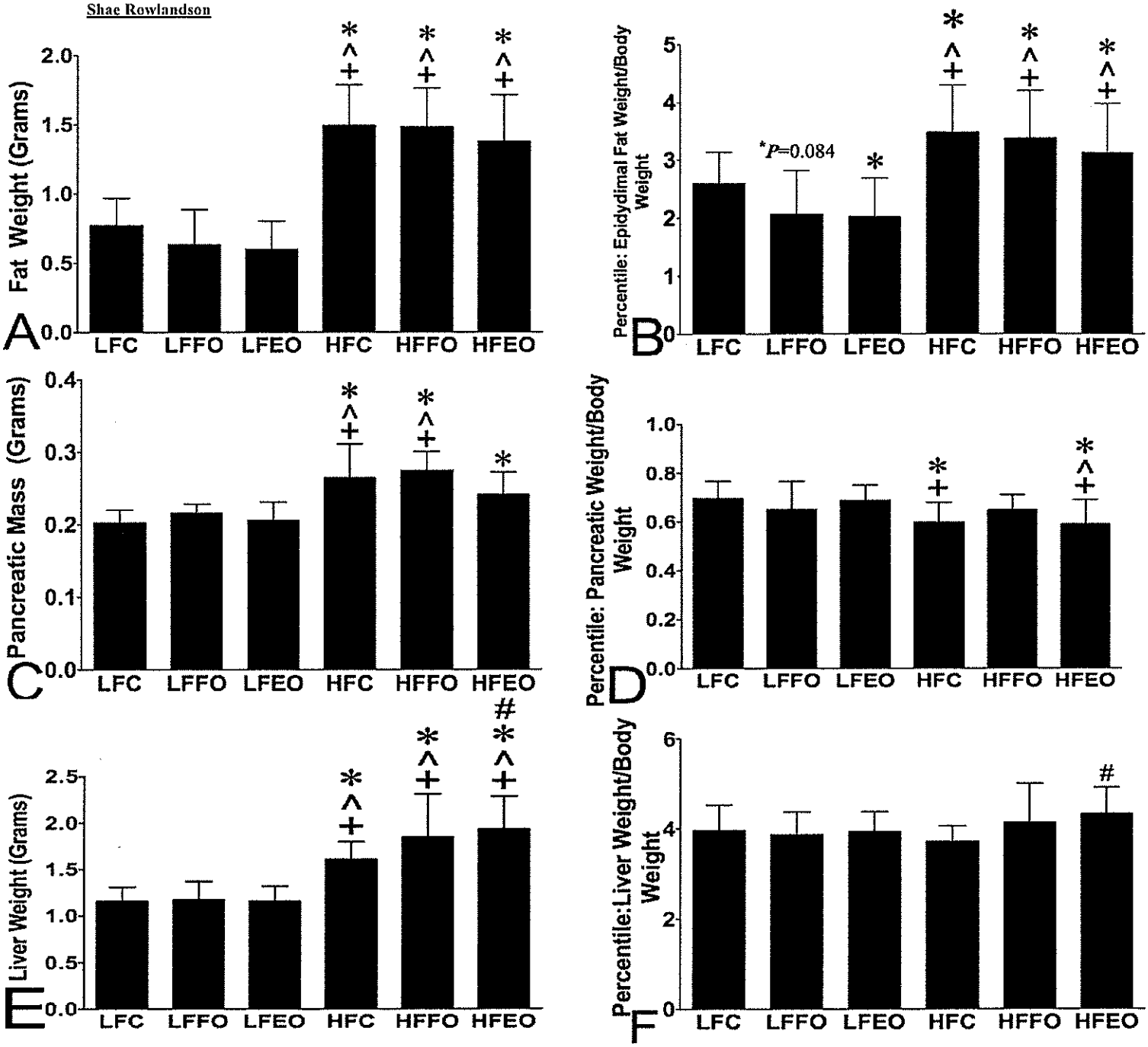

Figure 7: High fat diet increases fat mass, pancreatic mass, and hepatic steatosis.
(**A**, **C**, and **E**) The epididymal fat pads, pancreas, and liver, respectively, had been dissected and weighed. (**B, D,** and **F**) The weight from the epididymal fat pads, pancreas, and liver were then compared to body weight to derive how much weight each respective organ tissue contributed to total body weight. Data from (A) to (F) signify means ± SEM. **P*<0.05 to LFC, ^*P*<0.05 to LFFO, +*P*<0.05 to LFEO, #*P*<0.05 to HFC, and β*P*<0.05 to HFFO. Significance determined by individual T-tests.

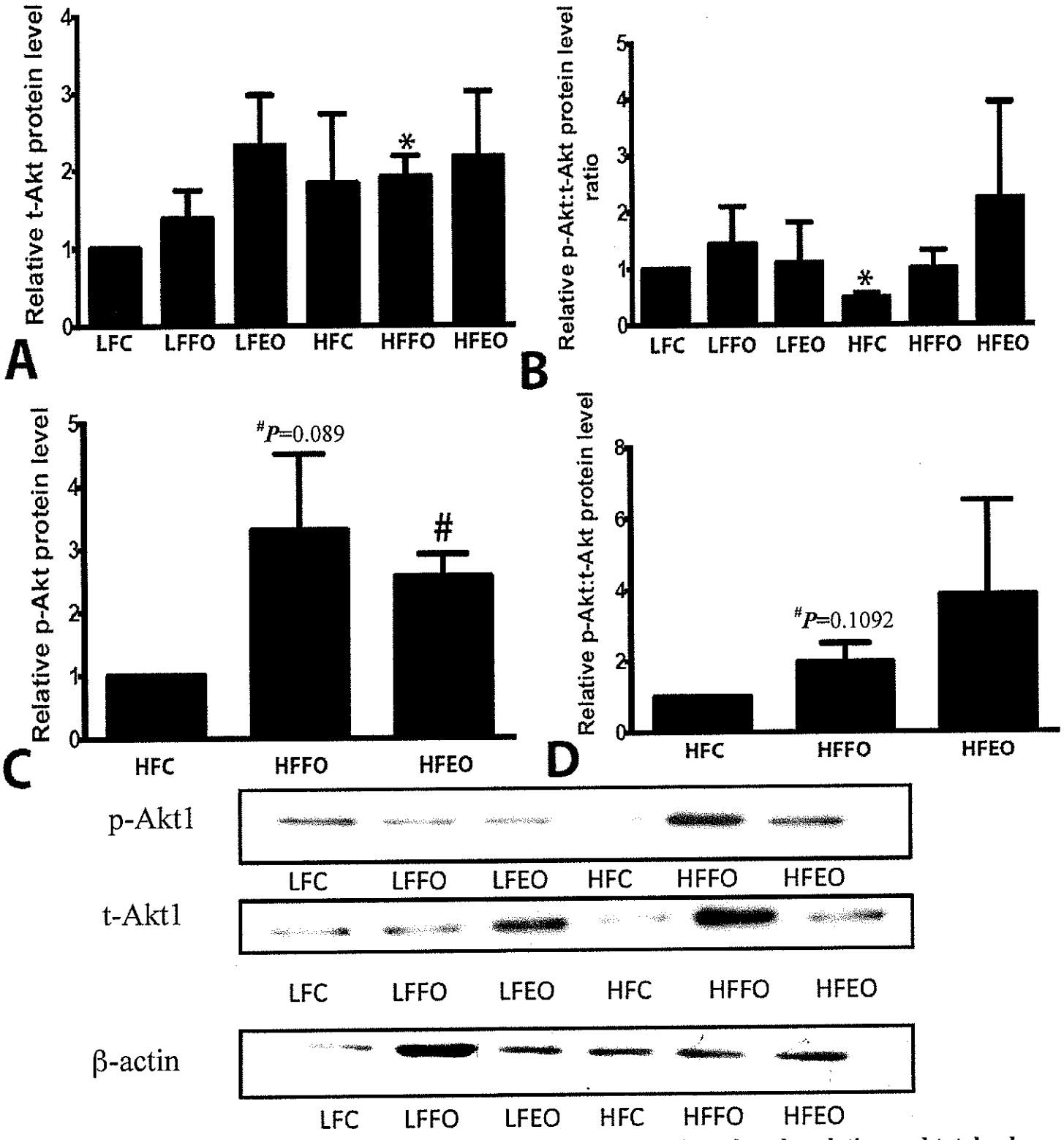

Figure 8: N-3s elevate levels of Akt1 and improve insulin sensitivity: phosphorylation and total values. (**A** to **D**) Western blotting analyses of Akt1, after normalization with respective β-Actin levels. Values presented were relative to protein expression in LFC or HFC. Data from (A) to (F) signify means ± SEM. **P*<0.05 to LFC, ^*P*<0.05 to LFFO, +*P*<0.05 to LFEO, #*P*<0.05 to HFC, and β*P*<0.05 to HFFO. Significance determined by individual T-tests comparing protein values to the LFC and HFC.

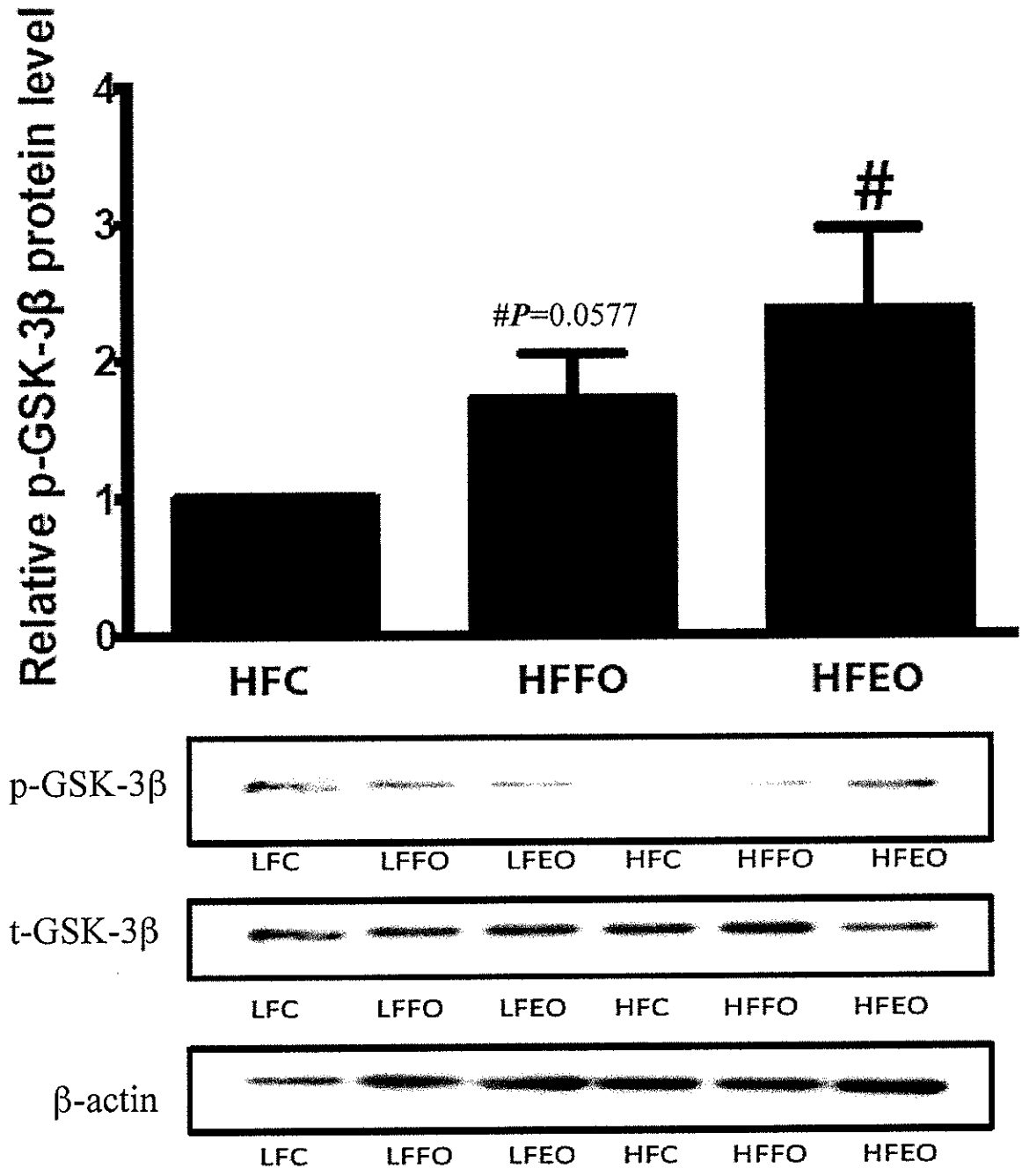

Figure 9: N-3s elevate levels of GSK-3β expression and promote glycogen synthesis: phosphorylation and total values.

Western blotting analysis of GSK-3β, after normalization with respective β-Actin levels. Values presented were relative to protein expression in LFC or HFC. Data from (A) to (F) signify means ± SEM. *$P<0.05$ to LFC, ^$P<0.05$ to LFFO, +$P<0.05$ to LFEO, #$P<0.05$ to HFC, and β$P<0.05$ to HFFO. Significance determined by individual T-tests comparing protein values to the LFC and HFC.

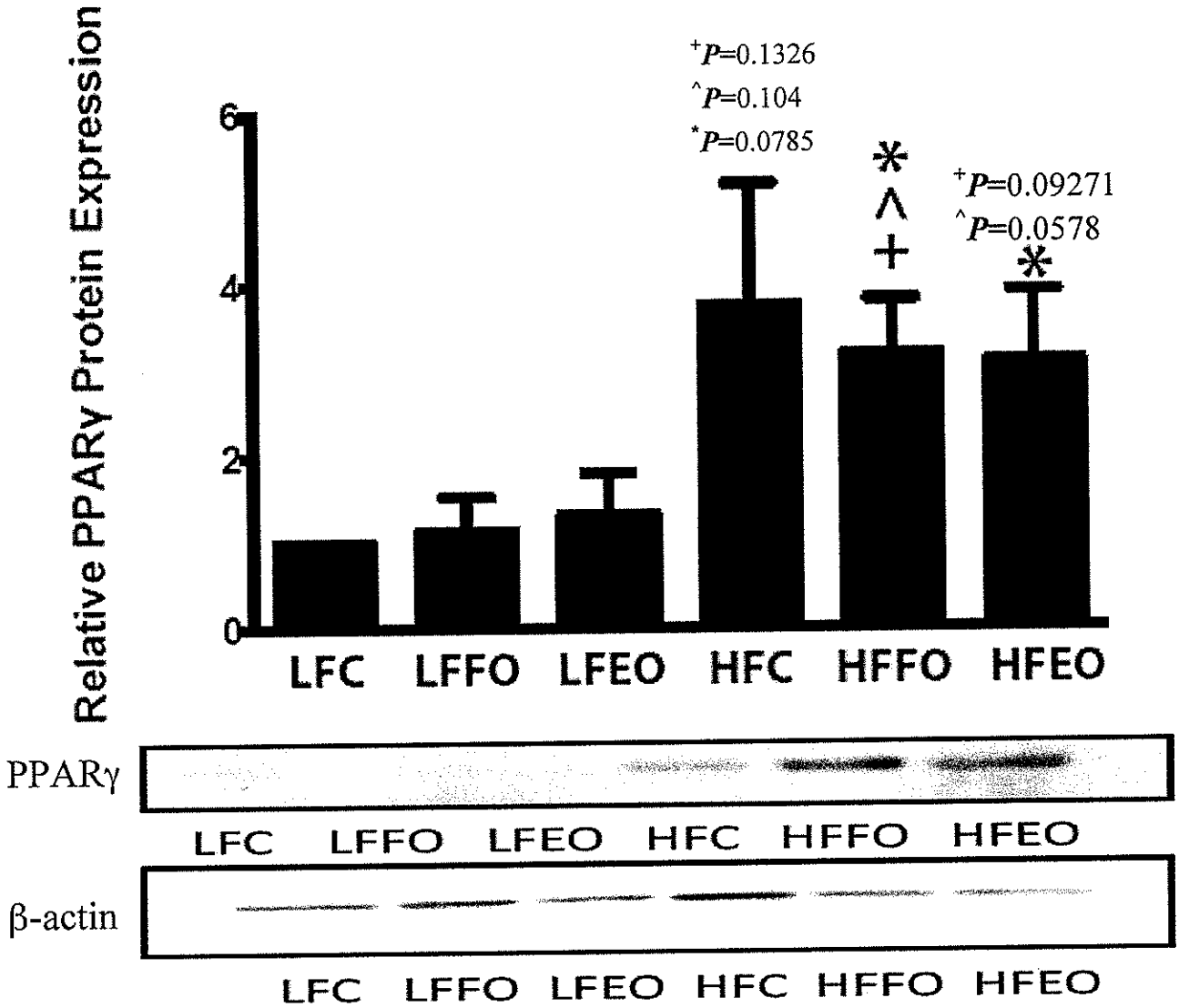

Figure 10: High fat feeding increases PPARγ expression and BiP are elevated with high fat feeding, but, *Echium* oil may nullify high fat-induced BiP expression.
Western blotting analysis of PPARγ, after normalization with respective β-Actin levels. Values presented were relative to protein expression in LFC or HFC. Data from (A) to (F) signify means ± SEM. *$P<0.05$ to LFC, ^$P<0.05$ to LFFO, +$P<0.05$ to LFEO, #$P<0.05$ to HFC, and β$P<0.05$ to HFFO. Significance determined by individual T-tests comparing protein values to the LFC and HFC.

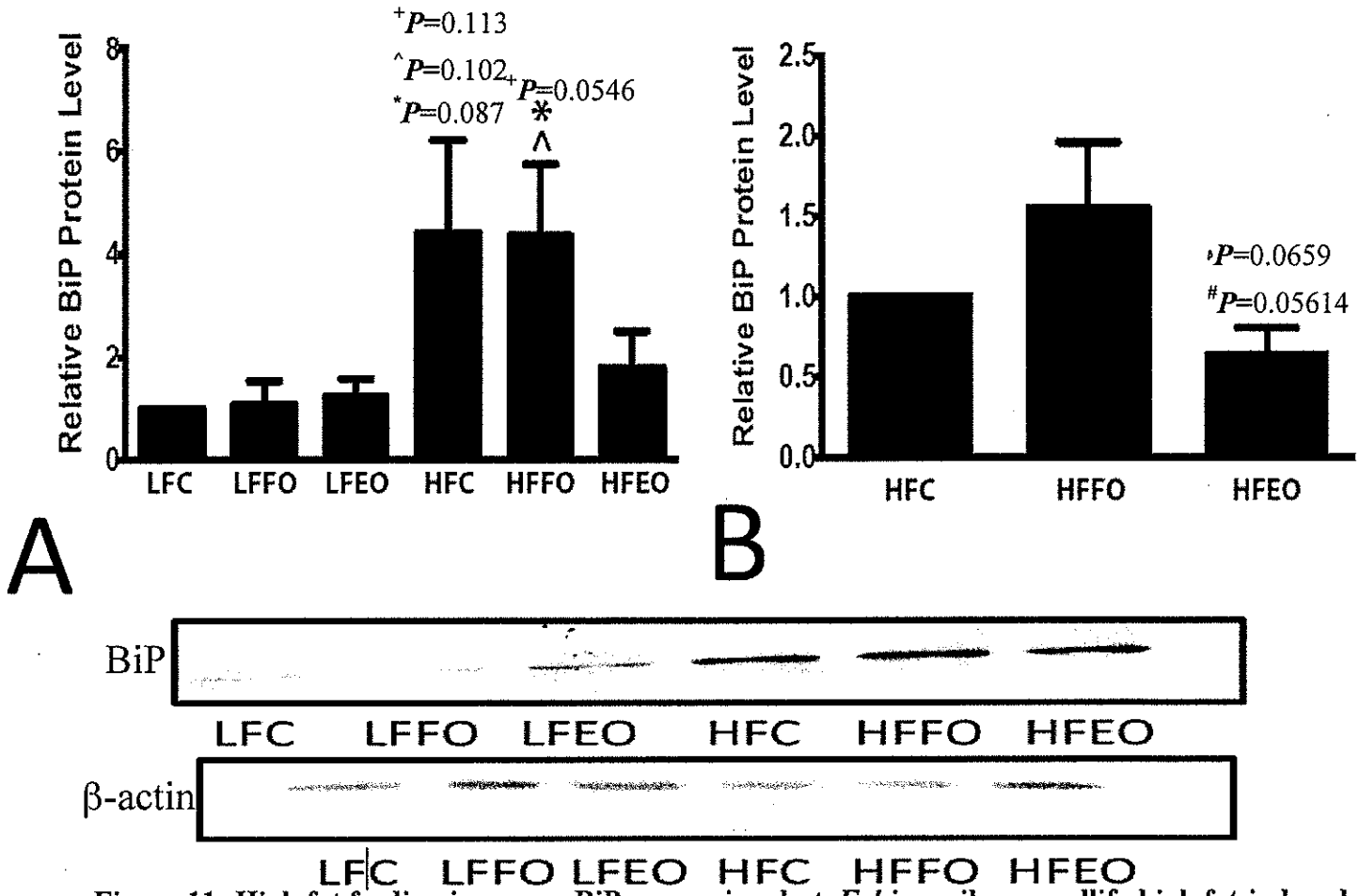

Figure 11: High fat feeding increases BiP expression, but, *Echium* oil may nullify high fat-induced BiP expression.

(**A** to **B**) Western blotting analyses of BiP expression after normalization with respective β-Actin levels. Values presented were relative to protein expression in LFC or HFC. Data from (A) to (F) signify means ± SEM. **P*<0.05 to LFC, ^*P*<0.05 to LFFO, +*P*<0.05 to LFEO, #*P*<0.05 to HFC, and β*P*<0.05 to HFFO. Significance determined by individual T-tests comparing protein values to the LFC and HFC.