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The Regulation of Fuel Metabolism and Substrate Availability During the Prolonged Fast
of the Northern Elephant Seal

A dissertation submitted in partial satisfaction of the requirements
for the degree

Doctor of Philosophy

in

Quantitative and Systems Biology

by

Jose Abraham Viscarra

Committee in charge:

Professor Rudy M. Ortiz, Chair
Professor Shannon Atkinson
Professor Daniel Crocker
Professor David Ojcius
Professor Nestor Oviedo

2013

The Dissertation of Jose Abraham Viscarra is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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Rudy Ortiz, Chair

University of California, Merced

2013

To my family and friends
for their love and support

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List of Original Papers

The following original papers were submitted during the completion of this study and were used as the basis for this dissertation:

1) **Viscarra JA**, JP Vazquez-Medina, D E Crocker, R M Ortiz
Glut4 is Upregulated Despite Decreased Insulin Signaling during Prolonged Fasting in Northern Elephant Seal Pups
American Journal of Physiology – Regulatory, Integrative and Comparative Physiology. 300: R150–R154, 2011. PMCID: PMC3023278

2) **Viscarra JA**, CD Champagne, D E Crocker, R M Ortiz
5'AMP-activated Protein Kinase Activity is Increased in the Northern Elephant Seal During Prolonged Fasting-Induced Insulin Resistance
Journal of Endocrinology. 209: 317-325, 2011. PMCID: PMC3250370

3) **Viscarra JA**, JP Vazquez-Medina, R Rodriguez, CD Champagne, SH Adams, DE Crocker, RM Ortiz
Decreased expression of adipose CD36 and FATP1 contributes to increased plasma free fatty acids during prolonged fasting in northern elephant seal pups.
Journal of Experimental Biology. 215: 2455-64, 2012.

4) **Viscarra JA** and RM Ortiz
Cellular mechanisms regulating fuel metabolism in mammals: role of adipose tissue and lipids during prolonged food deprivation.
Metabolism: Clinical and Experimental. In Press, 2013.

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List of Abbreviations

AMPk, AMP kinase
ATGL, Adipose Triglyceride Lipase
BCAA, Branched chain amino acid
CNS, Central nervous system
CD36, Fatty acid Translocase
DAG, Diacylglycerol
DHA, Dihydroxyacetone
DGAT, Diglyceride acyltransferase
EGP, Endogenous glucose production
FA; Fatty acid
FAS, Fatty acid Synthase
FATP, Fatty acid Transport Protein
FFA; Free fatty acidG3P, Glycerol-3-phosphate
GK, Glycerol Kinase
GLUT4, Glucose transporter type 4
HSL, Hormone-sensitive Lipase
LCFA, Long chain fatty acid;
LPL, Lipoprotein Lipase
MAG, Monoacylglycerol
MGAT, Monoglyceride acyltransferase
MGL, Monoglyceride Lipase
PEPCK-c, Phosphoenolpyruvate Carboxykinase-cytosolic
PPAR, Peroxisome Proliferator –activated Receptor
RBC, Red blood cells
RQ, Respiratory Quotient
SNS, Sympathetic nervous system
TAG, Triacylglycerol

Acknowledgements

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Publications

Viscarra JA and RM Ortiz
Cellular mechanisms regulating fuel metabolism in mammals: role of adipose tissue and lipids during prolonged food deprivation.
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Field of Study

Major Field: Quantitative and Systems Biology (Emphasis in Physiology)

Studies in Physiology
Professor Rudy M. Ortiz

Abstract

The Regulation of Fuel Metabolism and Substrate Availability During the Prolonged Fast of the Northern Elephant Seal

Jose Abraham Viscarra
Doctor of Philosophy
University of California, Merced
2013
Advisor: Rudy Ortiz, PhD

Food deprivation in mammals results in profound changes in fuel metabolism and substrate regulation. Among these changes are decreased reliance on the counter-regulatory dynamics by insulin-glucagon due to reduced glucose utilization, and increased concentrations of lipid substrates in plasma to meet the energetic demands of peripheral tissues. Prolonged food deprivation also increases lipid oxidation and utilization, which may contribute to the onset of the insulin resistance associated with fasting. Because insulin resistance promotes the preservation of glucose and oxidation of fat, it has been suggested to be an adaptive response to food deprivation. As the primary storage site of lipid substrates, adipose serves as a primary contributor to the regulation of metabolism in food deprived states. Through its regulation of lipolysis, adipose influences the availability of carbohydrate, lipid, and protein, and so, may potentially be a key regulator of fasting metabolism.

The northern elephant seal pup (*Mirounga angustirostris)* naturally undergoes a 2-3 month post-weaning fast during which it depends primarily on the oxidation of fatty acids to meet its energetic demands. The concentration of non-esterified fatty acids (NEFA) increases and is associated with the development of insulin resistance-like symptoms in late-fasted pups. Additionally, plasma NEFA concentrations respond differentially to an intravenous glucose tolerance test (ivGTT) depending on fasting duration suggesting that impaired insulin action may play a key role in the regulation of metabolic substrates in prolong-fasted animals. However, because fasting mammals also exhibit hypoinsulinemia, the insulin resistance-like conditions they experience may actually result from reduced pancreatic sensitivity/capacity, necessitating further assessment to better understand these dynamic responses.

We have previously reported that adipose Glut4 expression and AMP kinase (AMPK) activity increase and plasma glucose decreases in fasting seals suggesting that AMPK activity contributes to the regulation of metabolism via Glut4 during insulin resistance-like conditions in late fasted pups. Therefore the goals of this study were: (1) to assess the impact of fasting on the regulation of metabolic substrates and thereby identify the mechanisms that allow fasting-adapted mammals to tolerate prolonged food deprivation, and (2) to assess the insulin sensitivity status of fasting elephant seals pups to determine whether fasting results in insulin resistance or pancreatic dysfunction.

To accomplish this we infused early- and late-fasted seals with either glucose (0.05 g/kg) or insulin (0.065 U/kg), and a separate group of late-fasted seals with low (10 pM/kg) or high (100 pM/kg) dosages of glucagon-like peptide-1 (GLP-1) immediately

following a glucose bolus (0.5g/kg), and measured the systemic and cellular responses. Because GLP-1 facilitates the glucose-stimulated insulin secretion, these infusions provide a method to assess pancreatic capacity and responsiveness. Adipose tissue and plasma samples collected prior to the infusions were used to determine the effects of fasting duration. Samples collected during the infusions were used to assess insulin sensitivity and pancreatic function.

Fasting was associated with an increased NEFA:glycerol ratio in plasma and an increased DAG:TAG ratio in adipose. Furthermore, fasting decreased the expressions of fatty acid transporters and hormone sensitive lipase, and increased the expression of adipose triglyceride lipase. This suggests that increased plasma NEFA results from: (1) decreased tissue NEFA uptake, and (2) the transition to partial hydrolysis. Insulin infusions increased the phosphorylation of insulin receptor and Akt in adipose and muscle; however the timing of the signaling response was blunted in adipose. Despite the dose-dependent increases in insulin and increased glucose clearance (high dose), both GLP-1 dosages produced increases in plasma cortisol and glucagon, and may have contributed to the glucogenic role of GLP-1.

Results suggest that long-term fasting induces shifts in the regulation of lipolysis and lipid metabolism that contribute to the onset of insulin resistance in adipose, all-the-while maintaining insulin sensitivity in muscle. Furthermore, fasting does not impair pancreatic capacity, but does lead to decreased glucose tolerance. Impaired glucose tolerance may facilitate the onset of a whole-body insulin resistance-like condition despite the maintenance of skeletal muscle insulin sensitivity that serves to promote the preservation of metabolic substrates, especially glucose, while allowing for the continued development of fasting elephant seal pups.

Introduction

Mammalian response to food deprivation

The typical mammalian response to food deprivation involves significant changes in the catabolism of metabolic substrates. These changes have been designated as the three phases of starvation, each phase characterized by the predominant utilization of a single class of substrate: 1) carbohydrate, 2) lipid, and 3) protein (1-3) (Fig. 1). Carbohydrate stores are limited to the glycogen in liver and skeletal muscle and so are depleted quickly, allowing phase one to only last a matter of hours. Because lipid stores tend to be significantly larger than carbohydrate stores, the majority of the time that a mammal can endure food deprivation will be spent in phase two (1-3). Protein is stored in the form of lean tissue, so a transition to phase 3, resulting from the improper regulation of substrates or simply inadequate lipid stores, leads to tissue degradation (cachexia) for energy, followed shortly by organ failure and death (1, 4, 5).

Starving vs. Fasting

Mammals enduring food deprivation will quickly transition to reliance on lipid oxidation to meet energetic demands (1-3). With this transition comes the need to alter the mechanisms regulating metabolism in order to help preserve metabolic substrates, while maintaining their availability in circulation. The ideal metabolic shift involves decreased reliance on glucose by peripheral tissues, so as to maintain glucose availability for tissues that do not readily metabolize lipids (e.g., CNS, RBC). This reduction in glucose utilization would then lead to decreased reliance on branched chain amino acids (BCAA) for gluconeogenesis and thus decreased protein catabolism (4). Mammals adapted to prolonged food deprivation, like seals or bears, appear to exhibit this ideal situation as they rely almost exclusively on lipid oxidation and decrease protein utilization during fasting (6, 7). However, mammals not adapted to fasting lifestyles, like humans, continue to utilize protein and glucose (i.e., maintain RQ over 0.8 (8)) even when in phase two. Because humans exhibit similar changes to their endocrine response as fasting-adapted mammals, changes in the cellular machinery within peripheral tissues during food deprivation may be responsible for the ability of adapted mammals to properly regulate the availability and utilization of metabolic substrates.

The Northern Elephant Seal

The northern elephant seal (*Mirounga angustirostris*) has been the subject of extensive study for many years due to its adaptive nature to conditions that would be considered pathophysiological in other mammals, especially humans. Chronic hyperglycemia, obesity, intermittent but prolonged food deprivation, and extended bouts of hypoxia are all potentially detrimental, but are *natural* components of the elephant seal's life history (9-15). A vast majority of the previous work done with elephant seals

has focused on measuring plasma concentrations of numerous hormones and biochemical parameters, with virtually no work on cellular mechanisms. Though we now know a significant amount about the *normal* fasting biochemical/hormonal profiles of these animals, little is known about how these hormones interact with specific tissues or what contributions cellular mechanisms make to the regulation of metabolism during the fast.

Biochemistry of the fast.
After birth, seal pups nurse for approximately 28 days during which their body fat composition increases to about 50% due to the high fat content of their mothers' milk (9). They are abruptly weaned after those 28 days, and left to begin their 2-3 month-long fast. Like the adults, they do not eat or drink anything during this time and rely on the oxidation of fatty acids for approximately 90% of their energy (9). For this reason, the concentration of free fatty acids (FFA), as well as other lipid substrates, increases substantially in plasma during the fast (9, 16-18). Though carbohydrate only contributes to approximately 5% of their metabolic requirements, fasting northern elephant seals maintain a state of relative hyperglycemia (13), which decreases about 20% with fasting (10, 12, 15). Because there is no exogenous glucose intake during this time, this state of relative hyperglycemia is maintained exclusively through endogenous glucose production (EGP) (10). Interestingly, the rate of EGP maintained by the seal is much greater than that of other mammals of similar mass, under fed or fasting conditions (10). Despite their excessive fat mass and chronically elevated lipid and glucose levels, northern elephant seals do not experience any detrimental consequences to their health, and oddly appear to thrive under these conditions, suggesting that they have evolved robust physiological and cellular mechanisms that have allowed them to adapt to such extreme circumstances.

Endocrine response to fasting
Regardless of the fact that elephant seal pups have to complete their development while fasting, they exhibit an endocrine response to food deprivation similar to that observed in the adult elephant seals, as well as other mammals (2, 12, 16, 19). Because they do not depend on glucose metabolism to a great extent, counter-regulatory dynamics by hormones like insulin, which promotes glucose uptake, and glucagon, which promotes glucose production, are decreased (10, 12, 19). Though insulin is low immediately after weaning, it decreases further with the fast, while slight increases in glucagon are observed (10, 19). Additionally, glucagon-like peptide 1 (GLP-1), a gut hormone which facilitates insulin secretion in response to feeding, also decreases with fasting. Cortisol concentration in plasma increases substantially with fasting (10, 17, 19), and because it is known to exhibit great influence over substrate availability and utilization (20-23), is thought to play a prominent role in the regulation of metabolism during the pups' fast. Additionally, because cortisol is also known to impede insulin signaling (20), it has been suggested that it may contribute to the development of insulin resistance-like conditions observed in fasting seal pups.

Insulin Resistance

Insulin resistance results when the cellular response to insulin at target tissues is reduced. There are many ways by which insulin resistance can manifest but the most common is that induced by hypercaloric diets, high in fat, that lead to the ectopic accumulation of lipid in peripheral tissues. Because it is commonly associated with obesity and type 2 diabetes, insulin resistance is often regarded as a negative condition. However, insulin resistance has also been proposed to be a mammalian adaptation to prolonged food deprivation (18, 24, 25). Insulin resistance is a common consequence of fasting (26) and, though the exact mechanisms by which it manifests are still unclear, it is thought that its primary cause is similar to that of diet-induced insulin resistance (e.g. increased availability/utilization of lipids) (18, 27, 28). However, unlike the complications that accompany diet-induced insulin resistance, the decreased glucose uptake and utilization resulting from impaired insulin signaling would be beneficial to fasting mammals in that it would preserve the limited carbohydrate substrates for tissues that do not readily oxidize lipids. Thus insulin resistance would promote the ideal situation discussed above by favoring lipid utilization and reducing the catabolism of lean tissue (29).

Insulin resistance in fasting elephant seals

Due to the multitude of similarities between the endocrine/hormonal profiles of elephant seals pups and that of insulin resistant mammals, we and others have suggested that northern elephant seals develop fasting-induced insulin resistance (10, 16, 18). Furthermore, because of the benefits of insulin resistance described, we have also suggested that it may be part of the adaptive cellular mechanisms employed by fasting seals to regulate metabolic substrates. Our preliminary analyses of these cellular mechanisms demonstrated reductions in adipose insulin signaling activity along with reductions in the expression of peroxisome proliferator-activated receptor gamma (PPARγ; transcriptions factor whose expression correlates positively with insulin sensitivity). We also observed increased activation of AMP kinase (AMPk; protein kinase that serves as a cellular energy monitor) and increased expression of glucose transporter 4 (GLUT4; prototypical insulin-responsive glucose transporter) suggesting that insulin-independent mechanisms may regulate substrate availability in fasting elephant seal pups (18, 19, 30).

At the systemic level, our preliminary analyses also demonstrated that late fasted seal pups exhibit reduced capacity to clear a glucose bolus, and display noticeable increases in plasma FFA instead of the expected insulin-induced decrease (19). Reduced insulin signaling and glucose clearance, along with the inability to inhibit lipolysis, highly suggest reductions in insulin action. However, because late-fasted seals also exhibit a blunted pancreatic response (i.e. reduced insulin secretion) as a result of the glucose bolus, the changes observed may instead result from impaired glucose tolerance or pancreatic beta cell damage. In either case, the increased availability/utilization of lipids may play a direct role as ectopic lipid accumulation is known to impair insulin signaling in peripheral tissues, while increased lipid oxidation is known to impair insulin production and secretion from pancreatic beta cells.

Purpose of Study

  The purpose of this study was to identify the adaptive cellular mechanisms employed by northern elephant seal pups to regulate the availability and utilization of metabolic substrates during their prolonged fast. Because fasting elephant seals pups exhibit the typical insulin resistant phenotype, we suspect that they develop fasting-induced insulin resistance as part of their adaptive mechanisms to promote the oxidation of lipid and preservation of lean tissue. However, as this insulin resistant phenotype may also be due to pancreatic dysfunction, we must determine their insulin sensitivity status and whether or not they experience any impairment to insulin secretion as a result of fasting. Furthermore, because the onset of insulin resistance and pancreatic dysfunction may be closely tied to changes in lipid handling, we must examine the contribution of adipose tissue to the development of insulin-resistance-like conditions. Therefore, the following specific aims will address the hypothesis that northern elephant seal pups develop fasting-induced insulin resistance, and utilize insulin-independent mechanisms to regulate the availability of metabolic substrates during their prolonged fast.

Specific Aim 1. To determine the effect of fasting on lipid handling mechanisms within adipose tissue and determine their contribution to the onset of insulin resistance-like conditions in elephant seal pups.

Specific Aim 2. To determine whether decreased insulin signaling in late fasted seals is a result of impaired pancreatic function or loss of insulin sensitivity.

Literature Review

In the past, research concerning metabolic regulation during food deprivation focused on hormonal control at the systemic level, allowing for the characterization of the typical endocrine response to fasting (1, 2, 5). However, because most endocrine factors that regulate metabolism postprandially have reduced roles in food-deprived mammals (31), considerable investigation has focused on the contributions of intracellular mechanisms of substrate regulation to metabolism (29, 32-34). Though the majority of this work has been done in humans and rodents during feeding or short term fasting, data from mammals that endure prolonged bouts of food deprivation, like seals, suggests that lipid substrates may have the same regulatory effects (18, 35, 36). As the principal storage site of lipids, the preferred fuel during fasting, adipose must contribute to food-deprived conditions, and thus, understanding its contributions, from the systemic to the molecular level, is important to assess metabolic regulation during fasting in mammals.

Mechanisms Regulating Substrate Availability

Most mammals suppress sympathetic nervous system activity (SNS) (37, 38), various endocrine factors that regulate postprandial metabolism (31), and the activity of adipokines (39), in order to reduce energy expenditure under food deprived states. Food deprivation also increases the concentrations of slow-acting hormones like cortisol or biomolecules like retinoic acid that control the expression of genes within the liver, adipose, and other peripheral tissues to generate metabolism-regulating proteins like lipases or fatty acid transporters (40, 41). These proteins are responsible for shifting metabolism and maintaining the availability and utilization of substrates within a tolerable range. Therefore, the regulation of their activity and expression is of critical importance to the survival of the organism.

Lipolysis

Intracellular lipases are responsible for breaking down the stored TAG molecules to release three FA and glycerol. Adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoglyceride lipase (MGL) are the principal lipases. Each removes a single FA in a stepwise fashion, converting the TAG to diacylglycerol (DAG), then monoacylglycerol (MAG), and finally free glycerol. This ensures a steady supply of substrates for ATP generation, but also allows for the utilization of glycerol in either hepatic lipogenesis or gluconeogenesis (42). Because hepatic synthesis of FFA is reduced during food deprived conditions (43), essentially all of the FFA in circulation result from adipose tissue lipolysis.

ATGL is the rate-limiting enzyme in TAG hydrolysis (44). ATGL expression increases during food deprivation in humans and rats (34, 45) and so may be responsible for the increased circulating FFA concentration during fasting. AMP-activated protein kinase (AMPk) activity has also been reported to increase with fasting in rats and elephant seals (19, 30, 32), and because its upregulation increases ATGL

expression *in vitro* (46, 47), it is likely responsible for the increased ATGL expression *in vivo* during fasting. Though its affinity for TAG molecules is much less than that of ATGL, HSL is capable of hydrolyzing TAG, DAG, and MAG molecules (48). As its name implies, HSL is responsive to various hormones, such as catecholamines and insulin and so can also increase or decrease lipolysis depending on the needs of the organism. Insulin decreases substantially during fasting in mammals (31, 49) while the concentration of catecholamines (e.g. epinephrine, norepinephrine) increases (50), thus promoting increased lipolysis through HSL. However, HSL lipolytic activity decreases when AMPk is chronically activated (51), likely to limit the pro-lipolytic effects of catecholamines.

The chronic activation of AMPk during fasting could therefore be a major part of the adaptive mechanisms used by mammals, as it could inhibit HSL and increase expression of ATGL, and thereby promote partial hydrolysis of stored TAG. Increased ATGL activity would maintain rates of lipolysis, while decreased HSL activity would reduce DAG hydrolysis, preventing premature depletion of lipid stores (Fig. 2). This would increase the FFA:glycerol turnover ratio, potentially reducing the amount of free glycerol available in plasma for subsequent conversion to carbohydrate via gluconeogenesis. Interestingly, decreased HSL and increased ATGL activities are also associated with the dysregulation of fat metabolism seen in high fat diet-induced obesity in mice (51). The only thing separating the two conditions would seem to be the impairment of AMPk activity due to obesity.

The effects of fasting on MGL have not been thoroughly investigated so these data are scarce. However, in the postabsorptive state, MGL is responsible for catalyzing the final step in the separation of glycerol and fatty acids by hydrolyzing MAG (52). Several MAG species have been shown to promote lipid storage and reduce energy expenditure by binding to and activating the cannabinoid receptors in rats and humans (53). Decreased MGL activity and maintained endocannabinoid signaling may be beneficial to hibernators, since they decrease their metabolic rate and do not maintain normothermic rates of energy expenditure (53). However, mammals maintaining their body temperature and metabolic rate during food deprivation may increase MGL activity, since increased lipid storage would not benefit their survival under these circumstances (29). Alternatively, if increased AMPk activity results in the accumulation of DAG molecules, then MGL activity would not be as crucial because a limited amount of MAG would be produced and available for further metabolism.

Fatty Acid Uptake

Fatty acid transporters are the primary mediators of long chain fatty acid (LCFA) uptake into cells and therefore have substantial control over the availability of FA in circulation. Fatty acid translocase (CD36), fatty acid binding protein (FABP), and fatty acid transport protein 1 (FATP1) are the three principal transporters regulating FA uptake by cells (54). Retinoic acid has been reported to regulate the expression of CD36 and FATP1 through the retinoic acid receptor (RAR) and peroxisome proliferator-activated receptor gamma (PPARγ) in cells and diabetic rats (55, 56). Regulation of the transporters appears to be tissue specific as fasting increases their expression in muscle (57), but decreases their expression in the liver and adipose tissue (58, 59). This

differential expression suggests that regulation of the transporters during fasting involves the activation of different subtypes of RAR and PPAR within the different tissues. Additionally, the differential expression of fatty acid transporters is likely associated with the availability of energy stores within the tissues, as muscle stores are limited compared to liver and adipose tissue. This may also result from the need to maintain elevated concentrations of FFA in circulation during fasting to support a lipid-based metabolism. The liver and adipose tissue actively participate in the futile cycling of FA (re-esterification of FFA into TAG) even when attempting to maintain elevated plasma FFA (60). Because both tissues account for approximately 70% of fatty acid uptake in the postabsorptive state (61), decreased expression content may be the principal mechanism contributing to a decrease in FA uptake under prolonged food deprived conditions.

Hepatic Re-esterification

While most tissues will oxidize FA to generate ATP, a very small fraction of the plasma FFA are re-esterified into TAG by the liver, packaged into very low density lipoproteins (VLDL), and returned into circulation (62). In the postabsorptive state, this process could also entail the *de novo* synthesis of FA by fatty acid synthase (FAS) (43). However, FAS expression and activity decrease with fasting as a result of decreased insulin (63), so FFA released by adipose tissue should account for the majority of the FA re-esterified into TAG by the liver. Synthesis of glycerol-3-phosphate (G3P) is also necessary for hepatic re-esterification because G3P serves as the backbone of the TAG molecule (64). This involves the phosphorylation of free glycerol by glycerol kinase (GK), or *de novo* synthesis of G3P by phosphoenolpyruvate carboxykinase-cytosolic (PEPCK-C)(65). The fasting induced increase in plasma cortisol promotes hepatic PEPCK-C expression (41), which may predominantly contribute to G3P synthesis via glyceroneogenesis. However, because PEPCK-C is also involved in gluconeogenesis (66), its increased expression may facilitate both processes. Though endogenous glucose production (EGP) has been reported to decrease with fasting duration in seals, rats, and dogs (4, 67, 68), a basal level must be maintained to ensure that glucose is available for tissues that do not rely on lipid oxidation (e.g., CNS & RBC). Therefore, because TAG synthesis must be balanced against gluconeogenesis, the liver contributes to a limited amount of FFA re-esterification.

Adipose Tissue Re-esterification

Fatty acid re-esterification is predominantly mediated by adipose tissue, which internalizes FFA as well as the FA released from VLDL-TAG by lipoprotein lipase (LPL) (60). However, like the FA transporters, LPL decreases as a result of fasting (69), likely in an attempt to maintain plasma FFA concentration elevated. Similar to re-esterification in the liver, a pool of fatty acid acceptors is necessary to synthesize the TAG molecule (64). Because GK activity in adipose tissue of humans and rodents is relatively very low, even in the fed state (70, 71), G3P must be derived from either conversion of glucose to dihydroxyacetone (DHA) and then G3P (72), or by glyceroneogenesis using branched chain amino acids (BCAA) or TCA cycle intermediates (73). In rats fasted for 48 hours, adipose tissue glucose uptake decreases by 68% while PEPCK-C activity increases by 400% (74), therefore suggesting that

glyceroneogenesis may be the preferred method of G3P synthesis during short term fasting.

Contrary to what is seen in the liver, cortisol decreases the expression of PEPCK-C in adipose tissue (73, 75). This differential regulation of PEPCK-C expression may be indicative of the decreased need for TAG storage in adipocytes and increased need for FA mobilization during prolonged food deprivation. Alternatively, because up to 40% of the FFA released by lipolysis are re-esterified by adipocytes (76), a shift to partial hydrolysis during fasting could allow for the accumulation of DAG and MAG within adipose tissue that could serve as fatty acid acceptors (Fig. 2). This would allow for reduced reliance on glyceroneogenesis because remodeling of MAG and DAG by monoglyceride acyltransferase (MGAT) and diglyceride acyltransferase (DGAT) (77) could achieve TAG synthesis. Both MGAT and DGAT activities increase in adipose tissue of marmots prior to hibernation (78) demonstrating that these enzymes are involved in the preservation of lipid substrates in food-deprived mammals. Additionally, short-term fasting in rodents increases DGAT expression in adipose tissue due to relatively low levels of carbohydrate (79) suggesting that the same increase may be seen in adipose tissue of prolong-fasting mammals. This process could potentially reduce the impact of futile cycling on energy stores by: **1)** reducing the amount of FFA released through lipolysis, **2)** keeping a pool of acylglycerols in adipocytes to serve as FA acceptors, and **3)** reducing the need for glyceroneogenesis.

Significance and Scope

Mammals adapted to fasting lifestyles can depend primarily on lipid metabolism and still maintain tight control of both substrate availability and utilization. Because they do so despite experiencing decreased nervous system activity as well as decreased endocrine regulation, they offer the unique opportunity to investigate the cellular contributions to systemic metabolic regulation. As stated earlier, fasting seals experience cellular and biochemical changes similar to that seen in obese humans, and appear to develop fasting-induced insulin resistance. Unlike humans, the seal maintains control of its metabolism, and appears to benefit from the reduced insulin action. Therefore, delineating the mechanisms that allow seals, and other fasting-adapted mammals, to maintain control of metabolism has the potential to improve our understanding of the cellular perturbations that lead to metabolic derangement in humans.

Methodology

Experimental Design

Because our preliminary studies demonstrated significant changes in cellular insulin signaling, as well as in AMPk activation, at 7 weeks postweaning, our analysis of the fasting induced changes will consist of comparisons between early (2 weeks postweaning) and late (7 weeks postweaning) fasted pups. We are limited in the types of tissue that we can collect due to the protected status of these animals. However, we were able to assess their insulin sensitivity status by infusing early and late fasted seals with insulin and comparing rates of glucose clearance as well as insulin signaling activity in peripheral tissues (adipose, tissue). Furthermore, we were able to assess pancreatic function in late fasted seals by performing GLP-1-GTTs and comparing changes to that of GTT alone. Because GLP-1 facilitates pancreatic insulin secretion in response to elevated glucose in circulation, it allowed us to determine whether the reduction in plasma insulin in late fasted seals resulted from pancreatic dysfunction or just reduced glucose tolerance.

Overall Strategy

Specific Aim 1. To determine the effect of fasting on lipid handling mechanisms within adipose tissue and determine their contribution to the onset of insulin resistance-like conditions in elephant seal pups, we examined:
1) Expression of lipid handling proteins in adipose tissue
2) Concentrations of TAG and DAG in adipose tissue
3) Concentrations of lipid-regulating hormones and lipid substrates in plasma
4) Activity of lipases in adipose tissue and plasma
5) Acylcarnitine profiles in plasma

Specific Aim 2. To determine whether decreased insulin signaling in late fasted seals is a result of impaired pancreatic function or loss of insulin sensitivity, we examined the following parameters in response to a either an insulin infusion or a GLP-1-GTT:
1) Expression/phosphorylation of insulin signaling proteins in adipose tissue and muscle
2) Concentrations of metabolism-regulating hormones in plasma
3) Concentrations of metabolic substrates in plasma
4) Rates of plasma glucose clearance

Sample Collection

All procedures were reviewed and approved by the Institutional Animal Care and Use Committees of both the University of California Merced and Sonoma State University. All work was realized under the National Marine Fisheries Service marine mammal permit #87-1743.

Northern elephant seal (*Mirounga angustirostris*) pups were studied at Año Nuevo State Reserve (30 km north of Santa Cruz, CA) during their natural postweaning fast while they are still on land. Elephant seal pups were isolated during the procedures to avoid interruption by the much larger adults. Body mass was measured using a hanging-load cell suspended from a tripod. After weighing, pups were initially sedated with 1 mg/kg Telazol (tiletamine/zolazepam HCl, Fort Dodge Labs, Ft Dodge, IA) administered intramuscularly. Once immobilized, an 18 gauge, 3.5 inch spinal needle was inserted into the extradural spinal vein. Blood glucose was measured using a commercially available blood glucose monitor (OneTouch Ultra2; LifeScan, Inc., Milpitas, CA). Pre-infusion blood samples were collected in chilled, EDTA-treated vacutainer sample tubes containing a protease inhibitor cocktail (PIC; Sigma-Aldrich, St. Louis, MO), and kept on ice until they could be centrifuged. A pre-infusion adipose tissue biopsy was collected by first cleaning a small region in the flank of the animal near the hind flipper, with alternating wipes of isopropyl alcohol and betadine, followed by a subcutaneous injection of 2-3 mL of lidocaine (Henry Schein, Melville, NY). A small (<1.5 cm) incision was made using a sterile scalpel, and a blubber biopsy (ca. 100-200 mg) was collected with a sterile biopsy punch needle (Henry Schein). Biopsies were rinsed with cold, sterile saline, placed in cryogenic vials, immediately frozen by immersion in liquid nitrogen, and stored at -80°C until later analyses.

Manipulations

Glucose tolerance tests: Pups were sampled at two time periods: early (2-3 wk postweaning; n=5) and late (6-8 weeks postweaning; n=8). Following initial sample collection, animals were infused with a mass-specific dose of glucose (0.5 g/kg) administered through the spinal needle over a 2 minute period (12, 14). Continued immobilization was maintained with ~100 mg bolus intravenous injections of ketamine, as needed. Subsequent blood samples were collected at 5, 10, 15, 20, 30, 45, 60, 90, 120, and 150 minutes post-infusion, measured for glucose, and placed on ice until they could be processed. Subsequent adipose biopsies were collected at 60 and 150 minutes post-infusion and stored as described above.

Insulin infusions: To determine the effects of prolonged fasting on peripheral insulin signaling activity and function, ten fasting seal pups (early n=5 (2 weeks), late n=5(7weeks)) were infused with a mass-specific dose of insulin (0.065 U/kg) (Humulin; Eli Lilly, Indianapolis, IN). Following the infusion, blood samples were collected at 5, 10, 20, 30, 60, 90, and 120 minutes. Subsequent adipose and opportunistic muscle biopsies were collected at 60 and 120 minutes. Procedures were terminated at 120 mins (instead of 150 mins like in the GTT or GLP-GTT) due to concerns over the safety of the animals. Immediately following the collection of the 120 min samples, glucose was infused slowly to assist in the restoration of preinfusion levels.

Glucagon-like Peptide-1 + GTT: To determine whether fasting resulted in reduced insulin production (pancreatic capacity) or just reduced insulin secretion (pancreatic glucose intolerance) we infused GLP-1 during a glucose tolerance test (GTT) in late fasted seals. Elephant seal pups were administered either a low (LDG; 10 pmol/kg; n=3) or high (HDG; 100 pmol/kg; n=4) dose of GLP-1 (Sigma, St Louis, MO) immediately following a glucose bolus (0.5 g/kg) (i.v.) infused within 2 mins. GLP1+GTT manipulations were performed only on late-fasted animals, because we and others have demonstrated that the insulin resistance-like conditions develop with fasting duration (12, 16, 18, 19, 30). Following the infusions, blood samples were collected at 10, 20, 30, 60, 90, 120, and 150 minutes. Subsequent adipose biopsies were collected at 60 and 150 minutes.

Analyses

Sample Preparation.
Blood samples were centrifuged for 15 min at 3000 x g at 4°C, and the plasma was transferred to cryo-vials, frozen by immersion in liquid nitrogen, and immediately stored at -80°C. Frozen tissue samples were homogenized in 250 μL of hypotonic buffer containing a protease and phosphatase inhibitor cocktail (PIC; Sigma). The homogenate was then centrifuged at 16,100 x g for 15 min and the aqueous layer was aliquoted into a separate tube. 150 μL of TBS containing 1% v/v Triton X-100, 1% w/v SDS and 1% v/v PIC was added to the pellet and sonicated, the resulting suspension was then centrifuged at 16,100 x g for 15 min and the aqueous layer was again transferred to a separate tube. Total protein content in both cytosolic and membrane bound fractions was measured by Bradford assay (Bio-Rad Laboratories, Hercules, CA).

Western Blot.
Thirty μg of total protein was resolved in 4-15% Tris-HCl SDS gradient gels. Proteins less than 100 kDa were electroblotted using the Bio-Rad Trans Blot SD semi-dry cell onto 0.45 μm nitrocellulose membranes. Proteins larger than 100 kDa were electroblotted using the Bio-Rad Mini Protean Transfer apparatus onto 0.45 μm nitrocellulose membrane. Membranes were blocked with 3% bovine serum albumin (BSA) in PBS containing 0.05% v/v of Tween 20 (PBS-T), and incubated overnight with primary antibodies (diluted 1:500 to 1:5000) against Actin, Akt2, Adipose triglyceride lipase (ATGL), carnitine palmitoyl transferase 1 (CPT1), carnitine palmitoyl transferase 2 (CPT2), fatty acid translocase (CD36), fatty acid transport protein (ACSVL5), fatty acid synthase (FAS), glucose transporter 4 (GLUT4), hormone sensitivity lipase (HSL), lipoprotein lipase (LPL), peroxisome proliferator-activated receptor gamma (PPARγ), phosphoenol pyruvate carboxykinase-cytosolic (PEPCK-c), phospho-AMP kinase (p-AMPk) (Santa Cruz Biotechnology, Santa Cruz, CA), p-IR-β/IR-β, p-IRS-1/IRS-1, phosphatidylinositol-3 kinase (PI3k), and β-actin (Assay designs, Ann Arbor, MI). Primary antibodies were validated for use in elephant seal tissues by preadsorption with a blocking peptide and by using cell lysates recommended by the manufacturers as positive controls. Because skeletal muscle was collected opportunistically and in small amounts, we prioritized our measurements to Akt/phospho-Akt and IR-β/phospho-IR-B, which allowed us to assess activation of insulin receptor signaling. Membranes were washed,

incubated with HRP-conjugated secondary antibodies (Pierce, Rockford, IL), re-washed, and developed using the Pierce ECL Western Blotting Substrate (Thermo Scientific). Blots were visualized using a Kodak Image Station 440CF (Kodak Digital Sciences) and quantified by using CareStream Molecular Imaging software. In addition to consistently loading the same amount of total protein per well (25 μg), densitometry values were further normalized by correcting for the densitometry values of Actin or β-Actin (19).

Plasma Analyses
The plasma concentrations of triglycerides (TG; Cayman Chemical, Ann Arbor, MI), glycerol (Cayman), glucose (Cayman), and non-esterified fatty acids (NEFA; Wako Chemicals; Richmond, VA) were measured with colorimetric kits. Plasma IGF-1 (DSL, Inc., Webster, TX), leptin (guinea pig; Millipore), insulin (porcine insulin, Millipore, Billerica, MA), glucagon (Millipore), and cortisol (Siemens, USA) were measured using radioimmunoassay kits. Plasma adiponectin (canine adiponectin, Millipore) was measured using an enzyme immunoassay kit. Glucagon-like peptide 1 (Millipore) was measured using a fluorometric assay kit. All kits (with the exception of GLP-1) have been previously validated for use with elephant seal plasma (17-19, 30, 80). The GLP-1 assay was validated for use with elephant seal plasma by performing linearity of dilution as well as spike and recovery assessments. All samples were analyzed in duplicate and run in a single assay with intra-assay percent coefficient of variability of < 10% for all assays.

Acylcarnitine Profiling
Plasma samples were packaged in dry ice and sent to Case Western Reserve University for comprehensive analyses of acylcarnitines, free carnitine, and total acylcarnitine. Carnitine concentrations were determined from 100 µL of plasma using HPLC-MS methods as previously described (81-83). The limit of detection (LOD) for individual acylcarnitines using this method is 20 nmol/L with the exception of acetylcarnitine, propionylcarnitine, and valproylcarnitine at 100 nmol/L. Acylcarnitine species with values below the LOD were not included in further analyses.

Lipase Activity
The activity of lipases in plasma and adipose was determined by performing a standard enzyme activity assay we developed for this specific purpose. For adipose, 200 mg of tissue were homogenized in 300 uL of lysis buffer (50mM Tris, 10% glycerol, 1% Triton X-100, 1% protease (Sigma) and phosphatase (Thermo) inhibitor cocktail), spun and the supernatant collected. Lipase activity in plasma was measured directly without any treatment of sample. First, 50 uL aliquots of plasma and adipose homogenates were added in duplicate to two sets of polystyrene tubes that were pre-chilled in an ice bath. Then, 25 uL of 12 mM triglycerides (Novartis, San Carlos, CA) were added to all tubes, keeping both sets of tubes in an ice bath (4°C) during the addition of triglycerides. One set of tubes was then incubated in a 37°C water bath for one hour to generate NEFAs, after which it was returned to the ice bath. The concentration of NEFA was determined in both sets of tubes (37°C and 4°C) as described above. The values obtained for the 4°C samples were subtracted from those incubated at 37°C to correct for any NEFA present

before the addition of triglycerides. The sensitivity of the assay was determined by the least detectable value following serial dilution of a plasma pool and calculated to be 0.003 mmol NEFA/ml/min.

Calculations
To assess the contributions of adipose and plasma lipase activity to the plasma pool of NEFA, a series of calculations were conducted using the following parameters. Plasma volume (PV) and adipose stores were estimated from age and body mass, respectively: PV (L) = 8.32-0.03*age (days) using approximate ages of the fasting pups (84). Total adipose tissue mass was calculated as 45% of the total mass (85). The baseline plasma NEFA pool was calculated by multiplying the time 0 concentration by the PV. The plasma NEFA pool following glucose administration was calculated by multiplying the area under the curve (AUC) of NEFA concentration following glucose administration by PV and dividing by 150 min (to account for the duration of the post-administration sampling period). Baseline contribution of plasma and adipose lipase activities to the plasma NEFA pool were determined by multiplying the time 0 values of lipase activities by PV or total adipose mass, respectively, and multiplying the resulting value by 150 min (to allow for comparison to the glucose-administration contributions). Contribution of plasma lipase activity to the change in plasma NEFA pool following glucose administration was calculated by multiplying PV by the AUC of plasma lipase activity from 0 to 150 mins. The contribution of adipose lipase activity to the change in plasma NEFA pool following glucose administration was calculated by multiplying the total adipose mass by the AUC of adipose lipase activity from 0 to 150. Percent uptake of NEFA was calculated by dividing the estimated tissue uptake (= lipase contribution – ΔNEFA pool) by the lipase contribution and multiplying by 100%. For baseline % uptake, it was assumed that ΔNEFA pool was at equilibrium (i.e. tissue uptake (efflux) was equal to lipase-mediated input (influx)).

Adipose Lipid Analyses
TAG and DAG were measured in adipose tissue using commercially available kits (TG, Cayman Chemicals; mouse DAG, CUSABIO, China). Tissue was processed as recommended by the manufacturer in the kit inserts. The DAG kit was validated by performing linearity of dilution as well as spike and recovery assessments. All samples were analyzed in duplicate and run in a single assay with intra-assay, percent coefficients of variability of < 10% for all assays.

Glucose Clearance Calculations
For the insulin infusions, the rate of glucose disappearance (K) was calculated using the linear regression as the negative slope of glucose concentrations from 0 min to 60 min post infusion. For the GLP-1 infusions, K was calculated assuming that equilibration of the injected glucose with the total body pool was achieved by 20 min (10, 12). Thus, K was calculated using the linear regression as the negative slope of glucose concentrations from 20 min to 150 min post infusion (12, 19). This method was also used to estimate K from previously reported mean values (86-89) and compared to those calculated here in response to the infusions.

Statistics

The baseline (or T0) measurements (plasma or tissue protein content) of the early and late groups were used to assess changes in variables as a function of fasting duration. Means (±SE) were compared by ANOVA using a Fisher's PLSD post-hoc test. Repeated Measures ANOVA was used to determine changes in parameters following the various infusions. Mean (± SE) area-under-the-curve (AUC) was calculated for each variable measured during the GTTs and used to determine integrated changes between early and late fasting groups. Adipose and skeletal muscle target protein content was normalized by expressing as percent change vs. the early fast mean value, and compared by ANOVA to determine changes in response to the separate infusions. Changes were considered significantly different at $p < 0.05$. Statistical analyses were performed with StatView® software (SAS Institute Inc., Cary, NC).

Results

Analysis of lipid handling mechanisms
Effects of Fasting on Body Mass and Lipid Metabolites
Mean body mass of pups sampled late in the fast was 25% lower than that of pups
sampled early in the fast (122 ± 5 vs. 92 ± 6 kg). The concentration of plasma TGs
increased by approximately 39% (Table 1) with fasting. The concentration of glycerol did
not change as a result of fasting. Fasting was associated with an 82% (Table 1) increase
in mean plasma NEFA concentration. Fasting increased the ratio of NEFA to glycerol
44% (Table 1).

Fasting alters adipose lipid mobilizing proteins
Mean adipose ATGL expression was 46% higher in late- vs. early-fasted animals (Fig. 3).
Conversely, adipose expressions of the fatty acid transporters, CD36 and FATP1, HSL,
and PEPCK-c were decreased 53%, 32%, 25%, and 28%, respectively, with fasting (Fig.
3). Neither the insulin nor the GLP-1 infusions resulted in significant time-effect changes
in protein expression of lipid mobilizing proteins.

Effects of Glucose Administration on Lipid Metabolites
In early-fasted pups, mean plasma TG concentrations decreased 27% with glucose
administration before returning to pre-GTT levels at 150 min, while concentrations
increased 22% in late-fasted pups and remained elevated throughout the measurement
period (Fig. 4A). In early-fasted pups, mean plasma glycerol concentrations decreased
38% as a result of the glucose administration before returning to basal levels at 150 min
(Fig. 4B). Plasma glycerol concentration did not change as a result of the GTT late in the
fast (Fig. 4B). Glucose administration decreased mean plasma NEFA by 55% in early-
fasted pups, and increased it by 27% in late fasted pups (Fig. 4C). In both periods, mean
plasma NEFAs returned to baseline at 150 min (Fig 4C). The ratio of NEFA to glycerol
did not change as a result of the glucose administration during either sampling period
(Fig. 4D).

Lipase Activities
Fasting increased mean plasma lipase activity 2-fold (0.8 ± 0.2 vs. 2.0 ± 0.2 nmol NEFA
produced/mL of plasma/min) (Fig. 5A). Mean plasma lipase activity increased 50% as a
result of the glucose administration in late-fasted animals and returned to basal levels by
150 min; however, the change in lipase activity following the GTT was not significant in
early fasted animals (Fig. 5A). Mean adipose lipase activity decreased 50% with fasting
(3.4 ± 0.5 vs. 1.4 ± 0.2 nmol NEFAs produced/g of adipose tissue/min) (Fig. 5B). Mean
adipose lipase activity levels were suppressed by 99% as a result of the GTT in early-
fasted seals and remained 60% lower than baseline at 150 min (Fig. 5B). Mean adipose
lipase activity increased nearly 2-fold as a result of the GTT in late-fasted seals, and
returned to baseline at 150 min (Fig. 5B).

Fasting increased the estimated total plasma NEFA pool at baseline and following glucose administration (Table 2). Fasting increased the baseline contribution of plasma lipases to the content of NEFA in circulation 2-fold, while decreasing that of adipose tissue by 66% (Table 2). Fasting also increased the estimated ivGTT-induced contribution of plasma lipases nearly 4-fold but not that of adipose lipases (Table 2). The contribution of adipose lipases to the circulating NEFA pool was higher than that of plasma lipases at baseline and following glucose administration early and late in the fast (Table 2). Fasting decreased the estimated % NEFA tissue uptake following ivGTT by 26%. Because only a single time point was available to calculate the baseline uptake, it was assumed that baseline lipase contributions were equal to tissue uptake and so the % contribution was 100% during both periods.

Plasma Acylcarnitines
To better understand how long-term fasting and insulin resistance impacts the metabolic fates of NEFA in seals, acylcarnitine metabolite profiling was conducted since changes in these molecules typically track alterations in tissue acyl-CoA status (90). The concentration of total carnitine and free carnitine did not change significantly with fasting, however the concentration of the calculated total acylcarnitines (Fig. 6) as well as the Acyl:Free carnitine ratio (0.51±0.03 vs. 1.25±0.18) increased in late fasted seals. Acetylcarnitine concentration increased significantly with fasting, and indices of incomplete NEFA β-oxidation (C6-C14-carnitines) were also increased significantly in late-fasted seals.   Long-chain acylcarnitine concentrations were higher in late-fasted animals (Table 3).

Plasma Apelin
The concentration of baseline plasma apelin decreased 22% with fasting (0.68 ± 0.02 vs. 0.53 ± 0.04 ng/mL) (Fig. 7). In response to glucose administration, mean plasma apelin increased 25% at 30 min in the early-fasted pups and 19% at 60 min in the late-fasted pups before returning to baseline levels at 120 min in both periods (Fig. 7). Plasma apelin AUC did not change as a result of fasting (118 ± 13 vs. 97 ± 11 ng/ml * min).

Effects of Glucose Administration on Adipose Protein Contents
While the adipose content of PEPCK-C did not change with glucose administration in early-fasted pups, PEPCK-C increased 18% at 60 min and returned to pre-GTT levels by 150 min in late-fasted pups (Fig. 8). In contrast, CPT I and II did not change with the late GTT, but both decreased 27% and 36%, respectively, at 150 min with the early GTT (Fig. 9A & B).

Analysis of insulin sensitivity and pancreatic function
Effect of fasting on body mass, plasma metabolites & adipose DAG:TAG
Mean body mass of late-fasted pups was 27% lower than early-fasted pups (127 ± 1 vs 93 ± 4 kg) (Table 5). Mean plasma glucose decreased 21% with fasting (9.6 ± 0.4 vs 7.5 ± 0.4 mM) (Table 5). Mean plasma TG (0.88 ± 0.09 vs 1.07 ± 0.14 mM) and NEFA (1.1 ± 0.1 vs 2.1 ± 0.3 mM) increased 22% and 90%, respectively, with fasting (Table 5). Mean

plasma glycerol did not change significantly with fasting (0.26 ± 0.06 vs 0.33 ± 0.10 mM); however, the NEFA to glycerol ratio increased 50% (4.2 ± 0.3 vs 6.4 ± 0.4) with fasting (Table 5). Fasting did not significantly change the absolute content of adipose DAG or TAG; however, adipose DAG:TAG ratio increased 55% with fasting (Table 5).

Fasting is associated with an insulin resistance-like endocrine profile
Mean plasma adiponectin (80.8 ± 3.7 vs 65.1 ± 3.6 ng/mL), GLP-1 (4.9 ± 1.3 vs 1.4 ± 0.9 pM), and insulin (3.2 ± 0.5 vs 2.0 ± 0.6 µU/mL) decreased 23%, 71% and 37%, respectively, with fasting (Table 5). Mean plasma cortisol nearly doubled with fasting (201 ± 13 vs 396 ± 21 nM) (Table 5). Mean plasma glucagon did not change significantly with fasting (11.6 ± 1.6 vs 12.9 ± 0.9 pM) (Table 5).

Fasting does not alter insulin-stimulated glucose clearance (K)
Mean plasma glucose was reduced by 30 mins and remained decreased throughout the sampling period during the insulin infusions in both the early- and late-fasted seals (Fig. 10A). A fasting effect on insulin-mediated glucose clearance was not detected (1.04 ± 0.18 vs 1.02 ± 0.16 mg/dL/min) suggesting that peripheral tissue insulin sensitivity is not compromised with fasting duration.

GLP-1-mediated increase in glucose clearance (K) is dose dependent
Peak plasma glucose was not significantly different between LDG and HDG infusions; however, HDG was associated with a 45% increase in mean plasma glucose compared to late fasting GTT (19) (161 ± 12 vs 109 ± 8 % from baseline) (Fig 10B). In both LDG and HDG, plasma glucose remained elevated for the duration of sampling period (Fig. 10B). Glucose clearance was increased 24% with HDG compared to LDG (0.86 ± 0.06 vs 0.69 ± 0.01 mg/dL/min) and 45% compared to late-fasting GTT (19) (0.59 ± 0.02) (Fig. 10C).

Insulin increases plasma cortisol & glucagon
Figure 11A confirms that the insulin infusions elevated circulating insulin levels for 60 mins at both infusion periods (Fig. 11A). A fasting effect on insulin AUC was not detected suggesting that the metabolism of insulin is not altered with fasting (Fig. 11A). Early insulin infusion elevated mean plasma cortisol at 30 min and at 60 min in the late infusion, with levels remaining elevated for the remainder of the study, reaching a peak at approximately a 200% increase at 90 min (Fig 11B) suggesting that the adrenal responsiveness to insulin is not impaired with fasting duration. Early insulin infusion elevated mean plasma glucagon at 60 min, while the late infusion elevated glucagon at 10 min, with mean circulating levels remaining elevated for the duration of the sampling period (Fig 11C). Neither the early nor the late insulin infusions induced time-effect changes in plasma GLP-1 concentration, so only the fasting associated changes are presented (Table 5).

GLP-1 promotes a glucogenic endocrine response, but increases plasma insulin
Figure 11D confirms that GLP-1 infusions maintained elevated circulating GLP-1 levels throughout the sampling period and that a dose-dependent response was achieved. Both LDG and HDG induced a dose-dependent increase in mean plasma cortisol by 10 min

with levels remaining elevated throughout the sampling period (Fig. 11E) despite our previous data demonstrating that GTT alone suppressed plasma cortisol within this time point suggesting that adrenal responsiveness is highly sensitive to GLP-1. HDG increased the peak plasma cortisol concentration by 430% compared to LDG (296 ± 65 vs 56 ± 21 % from baseline) (Fig. 11E). HDG and LDG increased mean plasma glucagon within 10 min with peak levels increased 51% and 34%, respectively, and remaining elevated throughout the sampling period (Fig. 11F). While a dose-dependent effect of GLP-1 on plasma glucagon was not detected, the trend is suggestive of an effect, which would imply that the pancreas is sensitive to GLP-1 levels. This is corroborated by the dose-dependent response of plasma insulin to GLP-1 (Fig. 11G). While all treatments (including late fasting GTT) stimulated insulin secretion, the LDG infusion did not further increase the GSIS beyond the late fasting GTT (Fig. 11G). However, HDG nearly doubled the increase in mean plasma insulin compared to LDG and late fasting GTT (19), with this increase persisting throughout the sampling period (Fig. 11G).

Insulin and GLP-1 infusions decrease plasma lipids
Insulin. The early infusion decreased mean plasma TAG 30% at 60 mins and returned to baseline levels at 120 mins; however, the late infusion did not significantly change plasma TAG (Fig. 12A). Early and late infusions increased mean plasma glycerol 38% and 23%, respectively, at 120 mins (Fig. 12B). The early infusion decreased mean plasma NEFA 65% at 30 mins and 75% at 60 mins before returning to baseline levels at 120 mins, while the late infusion decreased levels 39% at 30 mins, 44% at 60 mins, and 42% at 120 mins (Fig. 12C).

GLP-1 + GTT. Neither LDG nor HDG significantly changed mean plasma TAG (Fig. 12A). LDG decreased mean plasma glycerol 42% at 60 mins and 33% at 150 mins, and HDG decreased levels 28% at 60 mins and 29% at 150 mins (Fig. 12B). LDG increased mean plasma NEFA 17% at 30 mins with levels returning to baseline by 60 mins, while HDG decreased levels 26% at 30 mins and 30% at 150 mins (Fig. 12C).

Insulin infusion stimulates insulin signaling in adipose & muscle independent of fasting duration
Fasting was associated with a 40% and 28% decrease in insulin receptor phosphorylation in adipose and muscle, respectively (Fig. 13A & B). The early insulin infusion increased mean adipose insulin receptor phosphorylation 70% at 60 mins and was doubled at 120 mins (Fig. 13A). The late insulin infusion increased adipose insulin receptor phosphorylation 134% at 60 mins and 192% at 120 mins (Fig. 13A). The early insulin infusion increased mean muscle insulin receptor phosphorylation 35% at 60 mins and 72% at 120 mins (Fig. 13B). The late insulin infusion increased muscle receptor phosphorylation 45% at 60 mins and 113% at 120 mins (Fig. 13B). Despite the fasting-associated difference in receptor phosphorylation (at T0), the magnitude of phosphorylation at the post-infusion periods between early and late was not different suggesting that fasting duration did not compromise insulin-mediated receptor activation.

Fasting was not associated with significant changes in either adipose or muscle Akt phosphorylation. The early insulin infusion increased mean adipose Akt phosphorylation 33% at 60 mins and 21% at 120 mins (Fig. 13C). The late insulin infusion increased adipose Akt phosphorylation 19% at 120 mins (Fig. 13C). The early insulin infusion increased mean muscle Akt phosphorylation 51% at 60 mins and 47% at 120 mins, while the late infusion increased muscle Akt phosphorylation 26% at 60 mins and 48% at 120 mins (Fig. 13D).

Fasting was associated with a near doubling of baseline adipose AMPk phosphorylation (Fig. 13E). The early insulin infusion increased mean adipose AMPk phosphorylation 30% at 120 mins, while the late infusion increased AMPk phosphorylation 88% at 60 mins and 70% at 120 mins (Fig. 13E).

High dose GLP-1 stimulates adipose insulin signaling
LDG did not significantly change the phosphorylation of the insulin receptor or Akt (Fig. 13G & H). HDG increased the phosphorylation of adipose insulin receptor over 2-fold at 60 min and remained equally elevated at 120 min, while the phosphorylation of adipose Akt increased 24% at 60 mins (Fig. 13G & H).

Analysis of lipid handling mechanisms

For mammals that undergo prolonged periods of absolute food deprivation, like the elephant seal, the onset of insulin resistance may be an adaptive biological response to fasting. Reduced peripheral glucose uptake and increased concentration of NEFA in circulation, typically associated with insulin resistance (91-95), allow greater availability of glucose and ketone bodies to specific tissues that display limited metabolism of lipids (e.g. CNS, RBC, etc.), decreasing the need for gluconeogenesis from amino acids. Interestingly, elephant seals maintain rates of glucose production and use more glucose than expected for an animal enduring such a prolonged fast . These results suggest that the northern elephant seal pup possesses robust mechanisms to maintain elevated plasma NEFAs which may not be shared by other seals since fasting duration decreases NEFA concentration in grey and hooded seal pups (96, 97). These mechanisms include decreasing the expression of CD36 and FATP1 and increasing the expression of ATGL in adipose. Collectively, these mechanisms may reduce adipose NEFA uptake while promoting regulated TG hydrolysis. This shift in NEFA regulation may explain how the elephant seal pup is able to maintain its lipid stores for such a protracted period in spite of the increasing energetic demands associated with development during their post-weaning fast.

Regulation of fatty acid uptake

We have previously shown that, based on plasma glucose dynamics, elephant seal pups are sensitive to insulin at the beginning of their fast and develop glucose intolerance after several weeks of fasting (19). The lipid profiles exhibited by early-fasted seals in response to an ivGTT support this observation as they appear to be those of a *healthy* insulin-sensitive mammal (98, 99): glucose administration increases plasma insulin (19) and decreases circulating lipids by decreasing adipose lipase activity (98). In addition to insulin, a glucose-induced increase in plasma apelin likely contributes to the inhibition of adipose lipase activity (100). A major finding of the current study was that in contrast to early-fasted pups, ivGTT failed to reduce circulating TG and NEFA in glucose-intolerant late-fasted pups, and in fact modestly, but significantly, increased concentrations of the latter. The basis for this response remains to be fully elucidated, but could involve a lower ivGTT-induced inhibition of lipolysis and/or differences in tissue NEFA uptake.

The observed decline in adipose expression of the fatty acid transporters, CD36 and FATP1, in late-fasted pups could have contributed to reduced uptake of NEFA into adipocytes (101). The calculated tissue NEFA uptake during the glucose challenge agrees with this finding, as it amounted to only 74% of its value during the early fasting period. This suggests a reduction in NEFA uptake by all tissues in late-fasted seals, as the reduction in uptake would be negligible if only adipose tissue was affected (102). The total baseline contribution of lipase activity to plasma NEFA content decreased in late-

fasted elephant seal pups. This suggests a decrease in lipolysis like that observed in grey seal and hooded seal pups after a few days of fasting. However, the decrease in lipolysis in grey and hooded seal pups was accompanied by an expected decrease in plasma NEFA (96, 97), not an increase as seen in elephant seal pups. This suggests that other seals may not experience similar reductions in NEFA uptake with fasting duration as that seen in elephant seal pups. This also hints at differential regulation of plasma NEFA between different seal species which may involve changes in protein expression.

Acylcarnitine profiles

The increase in total acylcarnitine concentration in plasma as well as that of the acyl:free carnitine ratio demonstrate increased accumulation of fatty acylcarnitines in plasma of late fasted seals. The current detailed acylcarnitine profiling concurs with a prior report in fasting elephant seal pups that described only free and total acylated carnitine in which it was reported that the plasma acyl:free carnitine ratio rose toward the end of the fasting period, suggestive of a temporal change in the dynamics of mitochondrial β-oxidation during fasting in this species (103). The concentrations of acylcarnitines in plasma have been shown to parallel the accumulation of acyl-CoA at the tissue level (81, 90) and so the changes and patterns observed in acylcarnitines are representative of the changes in acyl-CoA pools.

Acetylcarnitine, which nearly doubled in late fasted seals, made up the majority of the acylcarnitines in plasma during both the early and late fasting periods and, because its representative of intracellular acetyl-CoA, suggests that fasting pups experience incomplete β-oxidation (81).   The rate of endogenous glucose production has been shown to remain elevated in late fasted pups (10) and so incomplete β-oxidation likely results from reduced tricarboxylic acid (TCA) cycle activity as oxaloacetate is preferentially used for gluconeogenesis. Decreased tiglylcarnitine and 3-hydroxy-isovalerylcarnitine suggest a decrease in the catabolism of branched chain amino acids (BCAA) isoleucine and leucine, respectively, and go along with previous findings demonstrating a reduction in protein catabolism in late fasted seals (6).

Medium-chained fatty acylcarnitines are usually byproducts of long- and very long-chain fatty acid oxidation (81) and so their increase in late fasted seals further demonstrates incomplete β-oxidation and reduced TCA cycle activity. The majority of the long chain fatty acylcarnitines increase, and nearly double when compared as a group, in late fasted elephant seal pups. Long chain fatty acids have been reported to inhibit adipose lipolysis (104) so this may explain the observed decrease in lipase activity in adipose of late fasted pups. Two specific long chain polyunsaturated fatty acids (PUFA), linoleic and linolenic, are known to decrease metabolic rate in hibernators (105). However linoleoyl, alpha- linolenoyl, and gamma-linolenoylcarnitine combined only make up approximately 6% of the total long chain acyl carnitines and less than 1% of the total carnitine species. Therefore the low concentrations of these two PUFA may be involved in the lack of suppression of the elephant seals metabolic rate during its prolonged fast.

Regulation of lipolysis

Similar to what is seen in grey seal, hooded seal, and northern fur seal pups, plasma lipase activity increases with fasting in northern elephant seal pups (96, 97, 106). This phenomenon may be exclusive to the fast of seal pups since lipase expression decreases with age in skeletal muscle of weddell seals (107) and LPL activity decreases in blubber and mammary tissue of lactating female northern elephant seals (108). Despite the increased activity of plasma lipases (e.g. LPL, hepatic lipase, pancreatic lipase) and reduced activity of adipose lipases, our calculations show that the majority of lipolysis still occurs in adipose. There is a lack of a change in plasma glycerol and 2-fold increase in the NEFA:glycerol ratio in late-fasted seals during the glucose challenge that seems to disagree with the maintenance of adipose lipolysis (109-112). However, chronic AMPK activation in adipose (19) has the potential to inhibit HSL activity while promoting ATGL activity and thereby prevents the complete hydrolysis of triglycerides leading to a disconnect in the relationship between plasma NEFA and glycerol.

The increased expression of ATGL and decreased expression of HSL observed in late fasted seals supports this shift in the regulation of lipolysis. Since ATGL only partially hydrolyzes TGs to generate one NEFA, increasing its activity should result in the accumulation of intracellular DAG (51, 113). DAG accumulation has been reported to increase inflammation and impair insulin signaling (114, 115). Therefore, the increase in the DAG:TAG ratio in late-fasted pups suggests that the DAG accumulation may be the principal factor contributing to the blunted adipose insulin signaling observed late fasted pups (19, 30). Furthermore, inhibition of the complete hydrolysis of TAG could also explain the lack of a significant contribution of glycerol to gluconeogenesis during the fast in adult, lactating elephant seals (16). Preventing the shuttling of glycerol into the gluconeogenic pathway would allow the re-esterification of NEFAs and thus maintenance of both adipose and plasma lipid substrates (e.g. TG and NEFA) required to support the energetic demands of fasting, developing pups.

Regulation of fatty acid reesterification

A survey of metabolically-important enzymes further highlights the concept that in long-term fasted seal pups, adipose acts as a critical NEFA supplier while down-regulating its capacity for NEFA storage. For instance, decreased expression of FAS in adipose of late fasted seals suggests that *de novo* fatty acid synthesis is minimized in adipose tissue, though the rise in malonylcarnitine and 3-hydroxy-butyrylcarnitines along with increased long-chain fatty acylcarnitines suggest an increase in hepatic fatty acid synthesis in late fasted seals (116-118). Because PEPCK-C expression is associated with glyceroneogenesis (119), the decreased expression of adipose PEPCK-C with fasting suggests that NEFA re-esterification is decreased, which may further contribute to increased plasma NEFA concentrations. However, increased PEPCK-C at 60 min during the late glucose challenge, which could have resulted from the suppression of plasma cortisol (19), suggests that NEFA re-esterification is increased in adipose in response to the glucose administration (119). Increased PEPCK-C in response to the late-fasting GTT may restore adipose TG content following the glucose-induced increase in adipose lipase activity since PEPCK-C expression returns to pre-GTT levels by 150 min. Thus, the mechanisms contributing to the increase in plasma NEFAs in late-fasted seals involve

well-regulated series of events that includes: 1) increased plasma lipase activity, 2) increased expression of adipose ATGL, 3) decreased expression of adipose CD36 and FATP1 resulting in reduced NEFA uptake, 4) increased secretion of adipose LPL, and 5) decreased PEPCK-C protein content (Table 4).

Analysis of insulin sensitivity and pancreatic function

This study investigated the insulin sensitivity status of fasting in a large mammal naturally adapted to prolonged bouts of absolute food and water deprivation, the northern elephant seal. Seal pups were infused with insulin and GLP-1 (GSIS facilitating hormone) at different stages of fasting to properly assess peripheral insulin signaling and pancreatic responsiveness to help elucidate the mechanisms that contribute to fasting-induced insulin resistance. Results demonstrate that the timing of insulin signaling is blunted with fasting in adipose, but not compromised in muscle suggesting that fasting-induced insulin resistance is tissue-specific. Furthermore, the intracellular accumulation of DAG is likely a contributing factor to the impairment of adipose insulin signaling. Despite the reduced levels of basal insulin receptor and Akt phosphorylation (indicative of down-regulation of insulin signaling) in late-fasted animals, insulin and GLP-1 infusions clearly demonstrated that skeletal muscle remained insulin sensitive with fasting duration. Another unique finding is the responsiveness of the adrenal gland to GLP-1 to overcome the glucose-mediated suppression of glucocorticoids and the ability of GLP-1 to increase secretion of cortisol and glucagon. Collectively, these results help elucidate the mechanisms employed by prolong-fasted seal pups to maintain relative hyperglycemia and muscle insulin signaling in the presence of elevated circulating NEFA. This is important because these results emphasize the differential regulation of the metabolic control of glucose during fasting as adipose exhibits an insulin resistant-like state while insulin sensitivity is maintained in skeletal muscle.

Maintenance of whole-body insulin sensitivity

Whole-body insulin resistance usually results from decreased insulin sensitivity in skeletal muscle because muscle is the main tissue responsible for insulin-stimulated glucose uptake (120, 121). Therefore, maintenance of insulin sensitivity in muscle and not in adipose with fasting duration is surprising. As mentioned previously, the fasting-associated down-regulation in basal insulin signaling in muscle is likely due to the hypoinsulinemia observed in late fasted animals. This reduction in insulin secretion likely facilitates the adaptation to an insulin resistance-like condition in late-fasted seals. Nonetheless, skeletal muscle remains sensitive to insulin as the phosphorylation of its receptor and Akt increase with sampling time and is not different between early- and late-fasted animals suggesting that fasting does not compromise insulin signaling in muscle. Furthermore, each infusion (LDG, HDG, ITT) resulted in incremental increases in plasma insulin and a corresponding increase in the rate of glucose clearance suggesting that the increase in insulin signaling was functional. In addition to allowing adipose to regulate the availability of metabolic substrates in circulation, decreased insulin secretion likely serves to decrease basal glucose uptake by skeletal muscle, thereby preserving glucose for glucose-dependent tissues and maintaining the anabolic properties of insulin to facilitate the continued development of the pups during their postweaning fast.

Comparing the values for glucose clearance (*K*) as a function of insulin area under the curve (AUC_{insulin}) calculated from the different infusions performed here with those for other mammals (mice, rats, humans) (Fig. 14A) illustrates that seal pups do not experience the typical whole-body insulin resistance commonly associated with fasting (121). The relationships demonstrate that the curve for seals is shifted to the left, which suggests that a given *K* is accomplished with a smaller secretory burst of insulin (represented by AUC_{insulin}) when compared to other mammals. Adult female elephant seals show a similar response to a glucose bolus (12), suggesting that this phenotype is not exclusive to the developing pups. This would then imply that, despite an insulin resistant-like phenotype during fasting, these animals retain peripheral insulin sensitivity to a greater extent than terrestrial mammals. This comparison further suggests that both early and late insulin infusions were able to maximally induce the activation of the insulin receptor, since the rate of glucose clearance appears to reach a plateau.

Tissue-specific insulin resistance

Although adipose does not contribute to glucose uptake to nearly the same extent as skeletal muscle (122), its functions as an endocrine organ (39) and its ability to regulate lipid availability (24) make it a potent regulator of insulin sensitivity. Examination of adipose insulin signaling in fasting animals demonstrated a blunted insulin signaling response to insulin infusion, consistent with the onset of insulin resistance (123) in the late-fasted seals. Similarly, the phosphorylation of insulin receptor did not result in the phosphorylation of Akt in adipose in response to the GLP-1 infusion in late-fasted animals further suggesting that prolonged food deprivation is associated with blunted insulin signaling in adipose. Furthermore, the early insulin infusion decreased plasma NEFA by more than 70%; however, the late insulin and HDG infusions only reduced their concentrations 40% and 20%, respectively, suggesting that fasting is associated with impaired insulin-mediated inhibition of lipolysis. Adipose insulin resistance is usually due to inflammation associated with obesity and the accumulation of excess lipids (124-126), but is typically not detected until it causes the loss of skeletal muscle insulin sensitivity (121). Therefore, the presence of adipose insulin resistance and a whole body insulin resistance-like phenotype, while muscle insulin sensitivity is maintained in late-fasted seals is unique among mammals. These evolved mechanisms likely allowed these animals to adapt to prolonged food deprivation.

Glucogenic effects of GLP-1

The LDG had little effect on plasma insulin, as plasma insulin levels were similar to the levels induced by GTT alone (19). However, the HDG nearly doubled the increase in plasma insulin secretion suggesting that the pancreas in these fasting-adapted mammals is sensitive to GLP-1 and the responsiveness is not impaired with fasting duration. This indicates that the fasting-induced insulin resistance and hypoinsulinemia in adapted mammals is the consequence of impaired peripheral insulin signaling and not a result of pancreatic dysfunction. More interestingly, while the HDG-mediated increase in plasma insulin was associated with an increase in the rate of glucose clearance, both the LDG and HDG increased plasma glucose compared to the GTT alone (124) suggesting that GLP-1induced glucogenic mechanisms were sufficiently greater than insulin-mediated

glucose clearance. Activation of adrenal GLP-1 receptor has been reported to increase glucocorticoid secretion (127, 128), resulting in a subsequent increase in endogenous glucose production (129). Therefore, the observed GLP-1 dose-dependent increase in plasma cortisol and the increase in plasma glucagon are likely responsible for the GLP-1-mediated glucogenic actions (Fig. 14B). Though it has been previously reported that cortisol has limited influence on glucose production in fasting elephant seals (10, 16), the observation of GLP-1-induced increase in plasma glucose in the presence of elevated insulin suggests that cortisol and glucagon may work synergistically to regulate glucose production during fasting. This is further demonstrated by the response to the insulin infusions, as we observe a gradual increase in both plasma cortisol and glucagon, likely in response to the rapid decrease in plasma glucose.

Summary

Delineating adaptive mechanisms

The focus of this dissertation was to assess the mechanisms adapted by mammals to cope with prolonged food deprivation and to determine the contribution of adipose tissue and insulin resistance to the regulation of said mechanisms. Because adipose tissue is known to exhibit prominent regulatory capabilities over metabolic substrates in circulation, and is the storage site for the primary metabolic substrate during fasting, we suspected that it could be a key regulator of fasting metabolism. Additionally, because insulin resistance is commonly associated with fasting and promotes the ideal fasting phenotype (i.e. increased plasma lipids, decreased glucose uptake) through its impairment of insulin signaling, we suspected that it could play an important role in the mechanisms used by fasting-adapted mammals to regulate metabolism. The northern elephant seal pup undergoes a prolonged postweaning fast during which it must maintain its metabolic rate to support the calorically expensive processes associated with development. For this reason, it makes an ideal model to assess the adaptive cellular mechanisms used by mammals to cope with food deprivation.

Changes in lipid handling

Results from the assessment of lipid handling mechanisms suggest that a metabolism that increasingly relies primarily on lipid oxidation contributes to the onset of fasting-induced insulin resistance (19). The increased plasma acetylcarnitine coupled to higher concentrations of medium-chain acylcarnitines in late-fasted seal pups suggests that a mismatch of NEFA and TCA cycle capacity elicits incomplete β-oxidation that accompanies worsening glucose tolerance and thus may facilitate the impairment of insulin activity. The gradual shift in the regulation of lipase activity during the course of the fast reduces the rate at which adipose TAG stores are depleted. Additionally, the decreased expression of CD36, FATP1, and PEPCK promote the transition to a state of decreased NEFA uptake and thus facilitate the maintenance of elevated concentrations of plasma NEFA. The chronic activation of AMPk (19, 30) along with the increased expression of adipose ATGL and inhibition of HSL in late fasted elephant seal pups promotes a transition to partial hydrolysis of stored triglycerides that could result in the accumulation of intracellular DAG. Additionally, the decreased expression of CD36, FATP, and PEPCK-c could potentially maintain elevated plasma NEFA while lipolysis actually decreased in late fasted seals. Though we put this forth as a mechanism by which fasting seals can reduce the futile cycling associated with TAG metabolism, DAG accumulation has been reported to increase inflammation and potentially impair insulin signaling (114, 115). Therefore, the 55% increase in the DAG to TAG ratio that we report suggests that the onset of adipose insulin resistance in fasting seal pups is due to DAG accumulation.

Insulin sensitivity status of fasting elephant seal pups

Results from the assessment of insulin sensitivity and pancreatic function demonstrated that prolonged food deprivation in the northern elephant seal pup is associated with tissue-specific reductions in insulin sensitivity in which muscle insulin signaling is maintained, but adipose signaling is blunted. This state of reduced insulin sensitivity in adipose appears to result from the chronic activation of AMPk resulting in the accumulation of DAG. While late fasting is characterized by an insulin resistant-like phenotype (i.e., elevated NEFA, relatively high fasting blood glucose etc.), pancreatic insulin secretion increases in response to GLP-1 stimulation, suggesting that the adaptation to prolonged food deprivation in these large mammals is achieved by maintaining the integrity of pancreatic function paired with a reduction in glucose tolerance. The insulin resistance-like state likely assists in the regulation of metabolic substrates (24) while permitting the continued development of postweaned pups. Despite the increase in plasma insulin and the associated increase in glucose clearance, the GLP-1-mediated increase in plasma cortisol and glucagon was sufficient to overcome these insulinogenic effects and increase plasma glucose levels. Thus, cortisol and glucagon maintain potent glucoregulatory capabilities during fasting. This glucogenic response to GLP-1 infusion suggests that GLP-1 may function as more than an insulin secretagogue and may actually be involved in regulating glucose production during fasting conditions via its effects on cortisol and glucagon.

Implications

The accumulation of DAG in adipose tissue of late fasted northern elephant seal pups results from changes to mechanisms regulating lipid metabolism that we had proposed to be adaptations to fasting. Therefore, this suggests that DAG accumulation could also be responsible for the onset of insulin resistance in other fasting mammals. Furthermore, because these mechanisms are inherently insulin-independent, it strengthens the suggestion that cellular mechanisms play a much more prominent role in the regulation of metabolic substrates during food deprivation.

Suggestions for future research

Analysis of these mechanisms in a different fasting mammal would confirm our suggestions that DAG accumulation and insulin resistance are components of mammalian adaptations to cope with prolonged food deprivation. Additionally, a more in depth analysis of GLP-1fucntion during fasting would allow for the determination of its role in the regulation of endogenous glucose production.

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Table 1. Fasting-associated changes of lipid metabolites in plasma and metabolite ratios of northern elephant seal pups

	Early	Late
Triglycerides (mM)	0.62 ± 0.02	0.87 ± 0.03 #
Glycerol (mM)	0.18 ± 0.01	0.24 ± 0.01
NEFA (mM)	0.85 ± 0.10	1.55 ± 0.15 #
NEFA: Glycerol	4.5 ± 0.3	6.5 ± 0.5 #

# denotes significant difference from early at $p < 0.05$

Table 2. Estimated contributions of lipases to NEFA pool and estimated % Uptake of NEFA in fasting northern elephant seal pups

		Adipose Contribution	Plasma Contribution	Total Contribution	Total NEFA Pool	Tissue Uptake	Estimated % Uptake
Baseline (mmol)	Early	27.1 ± 3.6	0.9 ± 0.3+	28.1 ± 3.7	6.0 ± 0.7	28.1 ± 3.7	100‡
	Late	9.3 ± 0.8#	1.8 ± 0.2#,+	11.2 ± 0.8	9.7 ± 0.9#	11.2 ± 0.8#	100‡
Glucose-Induced (mmol)	Early	10.6 ± 0.8	0.63 ± 0.10+	11.3 ± 0.9	3.8 ± 0.4	13.4 ± 1.2	119 ± 3
	Late	11.8 ± 1.6	2.32 ± 0.11 #,+	14.1 ± 1.5	11.1 ± 0.2#	12.7 ± 1.8	89 ± 7#

‡ Refer to methods
# denotes difference from Early at $p < 0.05$, + denotes difference from adipose at $p < 0.05$

Table 3. Mean concentrations (± SEM) of plasma fatty acylcarnitines of early (n=5) and late (n=8) fasted elephant seal pups grouped by associated metabolic pathways

Derived From	Chain length	Carnitine species	Early (nM)	Late (nM)
	Short	R-3-Hydroxy-Butyrylcarnitine	42 ± 2	126 ± 14 #
	Short	S-3-Hydroxy-Butyrylcarnitine	51 ± 1	101 ± 15 #
	Medium	Hexanoylcarnitine	33 ± 4	55 ± 7 #
	Medium	R-3-Hydroxy-Hexanoylcarnitine	22 ± 1	26 ± 1
	Medium	S-3-Hydroxy-Hexanoylcarnitine	3 ± 1	5 ± 1
	Medium	4-Methyl-Hexanoylcarnitine	3.4 ± 0.9	4.6 ± 0.3
	Medium	Octanoylcarnitine	21 ± 2	33 ± 4 #
	Medium	R-3-Hydroxy-Octanoylcarnitine	8 ± 1	8 ± 1
	Medium	S-3-Hydroxy-Octanoylcarnitine	5 ± 1	4 ± 1
	Medium	Decanoylcarnitine	16 ± 2	30 ± 4 #
	Medium	cis-4-Decenoylcarnitine	2.3 ± 0.4	3.2 ± 0.7
	Medium	R-3-Hydroxy-Decanoylcarnitine	3.9 ± 0.3	4 ± 0.2
	Medium	S-3-Hydroxy-Decanoylcarnitine	14 ± 1	17 ± 2
	Medium	Lauroylcarnitine	7 ± 2	12 ± 2
	Medium	S-3-Hydroxy-Lauroylcarnitine	2 ± 1	5 ± 2
Fatty Acid Metabolism	Medium	Myristoylcarnitine	53 ± 1	88 ± 3 #
	Medium	Myristoleoylcarnitine	12 ± 5	7 ± 2
	Medium	cis-5-Tetradecenoylcarnitine	64 ± 7	120 ± 6 #
	Medium	trans-2-Tetradecenoylcarnitine	41 ± 1	50 ± 1 #
	Medium	cis,cis-5,8-Tetradecadienoylcarnitine	15.2 ± 0.7	14.9 ± 0.4
	Long	Palmitoylcarnitine	130 ± 8	258 ± 8 #
	Long	Palmitoleoylcarnitine	41 ± 2	107 ± 8 #
	Long	trans-2-Hexadecenoylcarnitine	18 ± 1	24 ± 2 #
	Long	R-3-Hydroxy-Palmitoylcarnitine	39 ± 2	49 ± 2 #
	Long	S-3-Hydroxy-Palmitoylcarnitine	37 ± 1	45 ± 1 #
	Long	Stearoylcarnitine	84 ± 5	135 ± 13 #
	Long	Oleoylcarnitine	306 ± 18	620 ± 32 #
	Long	Linoleoylcarnitine	40 ± 1	46 ± 1 #
	Long	Alpha-Linolenoylcarnitine	16 ± 1	18 ± 1
	Long	Gamma-Linolenoylcarnitine	18.8 ± 0.4	18.4 ± 0.3
	Long	R-3-Hydroxy-Stearoylcarnitine	6 ± 1	10 ± 2
	Long	S-3-Hydroxy-Stearoylcarnitine	35 ± 1	36 ± 1
	Short	Isobutyrylcarnitine	410 ± 62	261 ± 32
	Short	Tiglylcarnitine	9 ± 1	6 ± 1 #
	Short	Valerylcarnitine	13 ± 2	14 ± 2
	Short	Isovalerylcarnitine	57 ± 18	48 ± 9
Amino Acid Metabolism	Short	3-Hydroxy-Isovalerylcarnitine	72 ± 8	34 ± 4 #
	Short	2-Methyl-Butyrylcarnitine	317 ± 60	491 ± 48
	Short	Glutarylcarnitine	21 ± 4	14 ± 3
	Short	3-Methyl-Glutarylcarnitine	5 ± 2	2 ± 2
	Medium	Phenylacetylcarnitine	113 ± 47	107 ± 30
	Medium	Phenylpropionylcarnitine	6 ± 0.3	5.5 ± 0.1
	Short	Propionylcarnitine	939 ± 307	1362 ± 784
	Short	Malonylcarnitine	55 ± 16	119 ± 9 #
	Short	Methyl-Malonylcarnitine	16 ± 1	14 ± 1
	Short	Succinylcarnitine	88 ± 11	77 ± 8
Other	Short	Butyrylcarnitine	96 ± 12	123 ± 10
	Short	Pivaloylcarnitine	14.7 ± 7	0.3 ± 0.1
	Medium	Adipoylcarnitine	14 ± 1	16 ± 2
	Medium	Benzoylcarnitine	18 ± 4	9 ± 1
	Medium	Suberoylcarnitine	25 ± 4	43 ± 7 #

# denotes difference from early at p<0.05.

Table 4. Summary of the effect of long-term fasting on measured parameters and potential effects on plasma NEFA

	Effect of Fasting	Effect on plasma FFA
Adipose Lipase Activity	↓	Prevents depletion of adipose TG stores, contributes to maintenance of plasma FFA
Plasma Lipase Activity	↑	Increases plasma FFA
Plasma Apelin	↓	Prevents inhibition of adipose lipolysis (indirectly increases plasma FFA)
ATGL	↑	Increases plasma FFA
HSL	↓	Likely inhibited by AMPK, contributes little to plasma FFA
LPL	↓	Decreases plasma FFA
CD36/FATP1	↓	Decreases FFA uptake (indirectly increases plasma FFA)
PEPCK-C	↓	Decreases FFA reesterification (indirectly increases plasma FFA)
FAS	↓	Decreases *de novo* lipogenesis (may decrease plasma FFA)
CPT I/CPT II	↔	Maintains capacity for oxidation (may decrease plasma FFA)

Table 5. Mean (±SE) body mass, plasma glucose and lipids, adipose diacylglyceride (DAG): triacylglyceride (TAG) ratio, and plasma hormones at early (2 weeks) and late (7 weeks) fasting periods from northern elephant seal pups

	Early	Late
Body mass (kg)	127 ± 1	$93 \pm 4^{\#}$
Glucose (mM)	9.6 ± 0.4	$7.5 \pm 0.4^{\#}$
Triglycerides (mM)	0.88 ± 0.09	$1.07 \pm 0.14^{\#}$
Glycerol (mM)	0.26 ± 0.06	0.33 ± 0.10
NEFA (mM)	1.1 ± 0.1	$2.1 \pm 0.3^{\#}$
NEFA : Glycerol	4.2 ± 0.3	$6.3 \pm 0.4^{\#}$
Intra-adipose DAG:TAG	0.45 ± 0.06	$0.70 \pm 0.04^{\#}$
Adiponectin (ng mL^{-1})	80.8 ± 3.7	$65.1 \pm 3.6^{\#}$
Cortisol (nM)	201 ± 13	$396 \pm 21^{\#}$
Glucagon (pM)	11.6 ± 1.6	12.9 ± 0.9
Glucagon-like Peptide-1 (pM)	4.9 ± 1.3	$1.4 \pm 0.9^{\#}$
Insulin (uU mL^{-1})	3.2 ± 0.5	$2.0 \pm 0.6^{\#}$

# denotes significant difference from early at $p < 0.05$

Figures

Postprandial	Postabsorptive (Phase 1)	Fasting (Phase 2)	Starvation (Phase 3)
Increased Glucose Increased Insulin Decreased Cortisol	Maintained Glucose Decreased Insulin Increased Cortisol	Decreased Glucose Decreased Insulin Increased Cortisol Increased ketoacids	Greatly Reduced Glucose Greatly Reduced Insulin Greatly Increased Cortisol Greatly Increased ketoacids
↓	↓	↓	↓
Metabolic substrates stored as glycogen, triglycerides and protein	Glycogen hydrolyzed to glucose, endogenous glucose production increased	Stored triacylglycerols hydrolyzed to fatty acids following depletion of hepatic glycogen	Depletion of stored lipid substrates
↓	↓	↓	↓
Mixed use of substrates for ATP generation	Reliance on glucose oxidation for ATP generation	Reliance on lipid oxidation for ATP generation	Reliance on lean tissue degradation and protein catabolism for conversion of amino acids to glucose and ATP generation

Figure 1

Figure 1. Comparison of the changes to key metabolic parameters in mammals under postprandial, postabsorptive, fasting, and starving conditions.
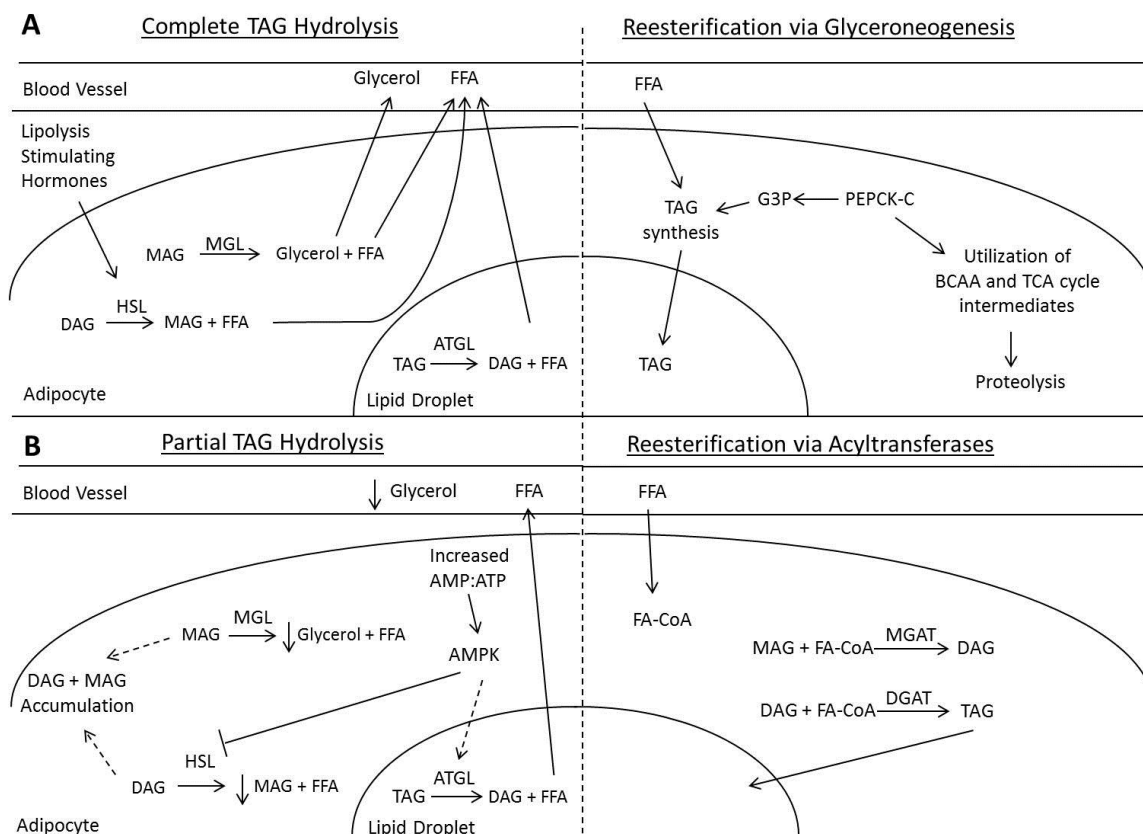
Figure 2. Schematic of the simplified **A)** complete hydrolysis of triacylglycerols and subsequent re-esterification, and **B)** the proposed partial hydrolysis of triglycerides and subsequent re-esterification of monoacylglycerols and diacylglycerols. Solid lines denote direct effects, dashed lines denote indirect effects. Short downward pointing arrows denote a decrease. Abbreviations: AMPK, AMP kinase; ATGL, adipose triglyceride lipase; BCAA, branched chain amino acids; DAG, diacylglycerol; DGAT, diglyceride acyltransferase; FA-CoA, fatty acyl-CoA; FFA, free fatty acid; G3P, glycerol-3-phosphate; HSL, hormone-sensitive lipase; MAG, monoacylglycerol; MGAT, monoglyceride acyltransferase; MGL, monoglyceride lipase; PEPCK-c, phosphoenolpyruvate carboxykinase cytosolic; TAG, triacylglycerol; TCA, tricarboxylic acid
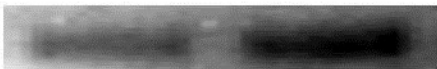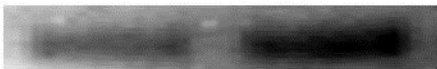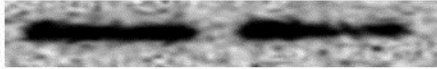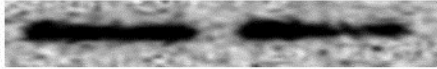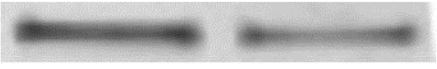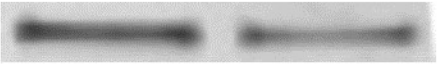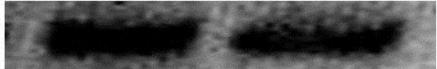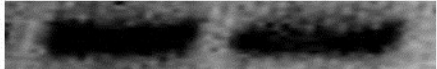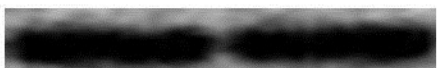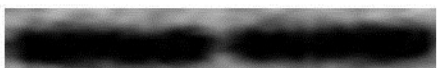
	Early	Late	% change
ATGL			$46 \pm 12^{\#}$
CD36			$-53 \pm 9^{\#}$
FAS			-15 ± 8
FATP1			$-32 \pm 4^{\#}$
HSL			$-25 \pm 6^{\#}$
LPL			-22 ± 12
PEPCK-c			$-28 \pm 7^{\#}$
Actin			

Figure 3

Figure 3. Mean (±SE) percent change of adipose lipid mobilizing proteins from early- (2 wks, n=5) and late-fasted (7wks postweaning, n=5) elephant seal pups. *Insert:* Representative western blots for each protein. # denotes significantly (p<0.05) different from early fasting. ATGL: adipose triglyceride lipase, CD36: fatty acid translocase, FAS: fatty acid synthase, FATP1: fatty acid transport protein 1, HSL: hormone-sensitive lipase, LPL: lipoprotein lipase, PEPCK-c: phosphoenol pyruvate carboxy kinase cytosolic.
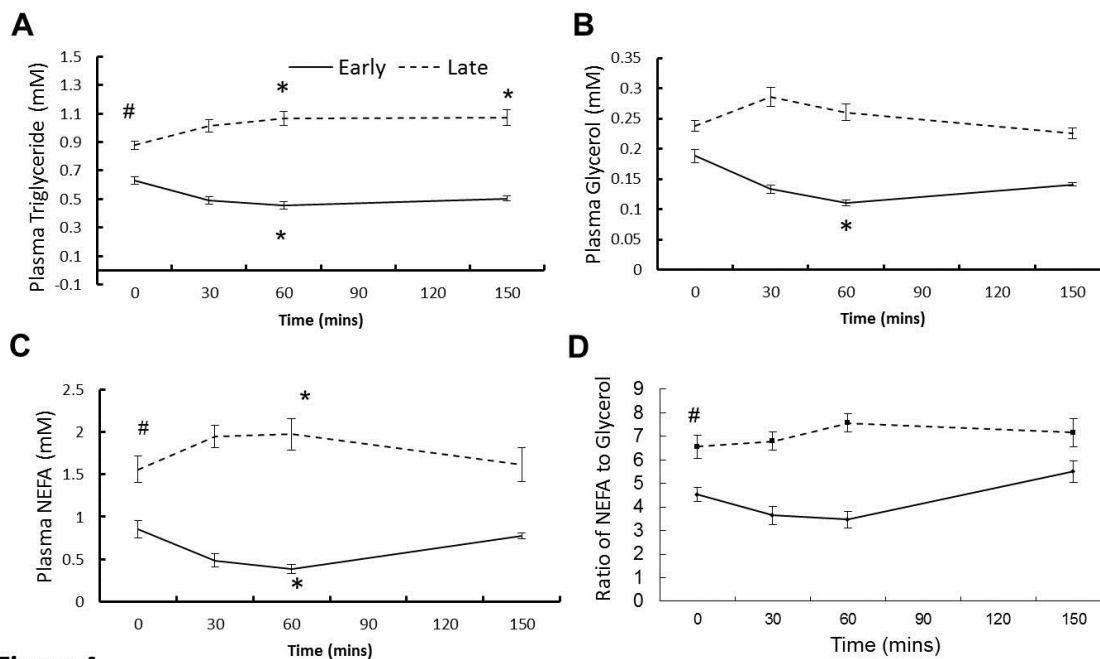
Figure 4. Mean (±SE) **A)** Plasma triglyceride, **B)** plasma glycerol, **C)** plasma NEFA, **D)** NEFA:Glycerol ratio in plasma of early (2 weeks; n=5) and late (6-8 weeks; n=8) fasted elephant seal pups during the course of the GTT. # denotes significant difference from early at $p<0.05$. * denotes significant difference from time 0 at $p<0.05$.
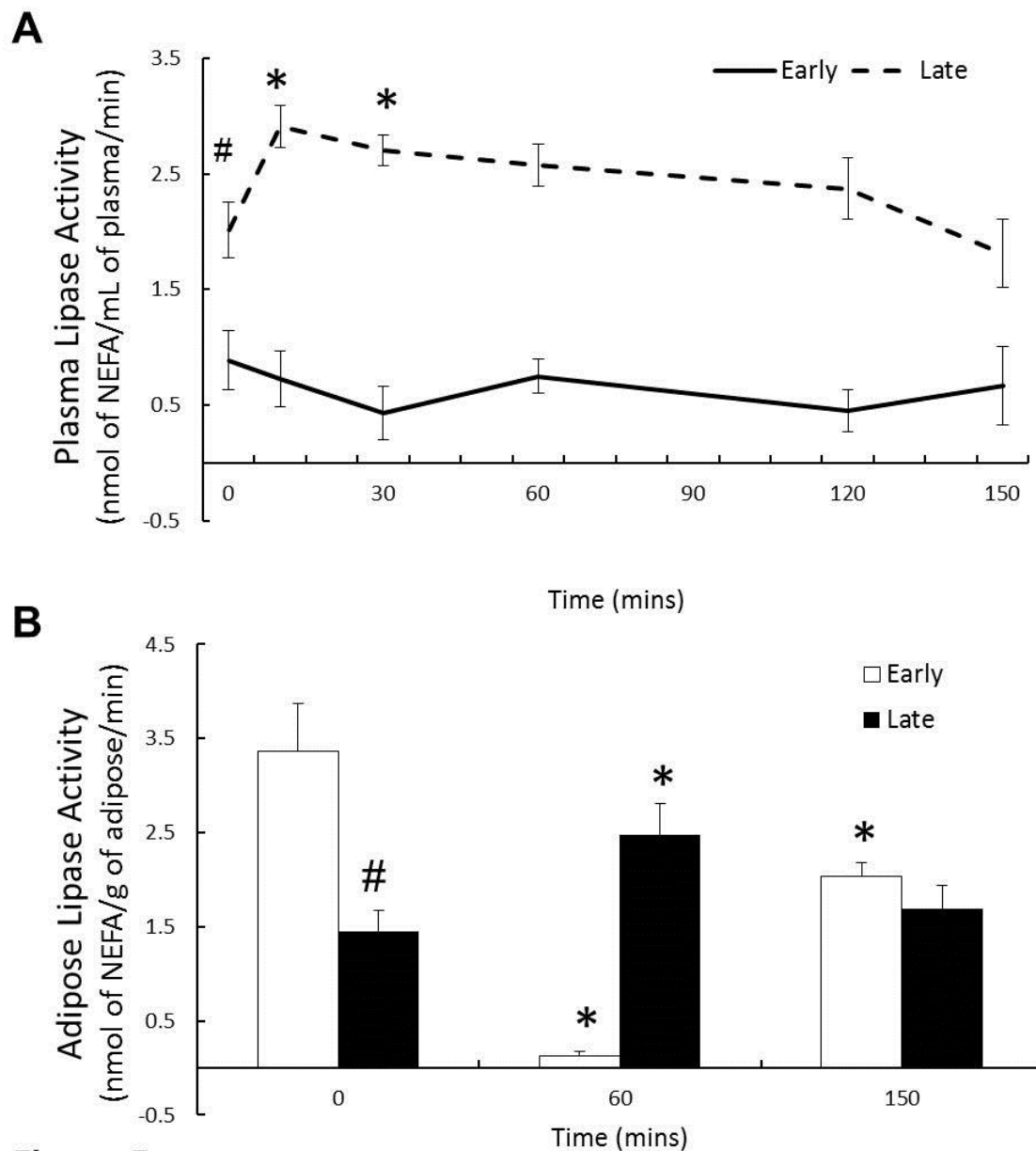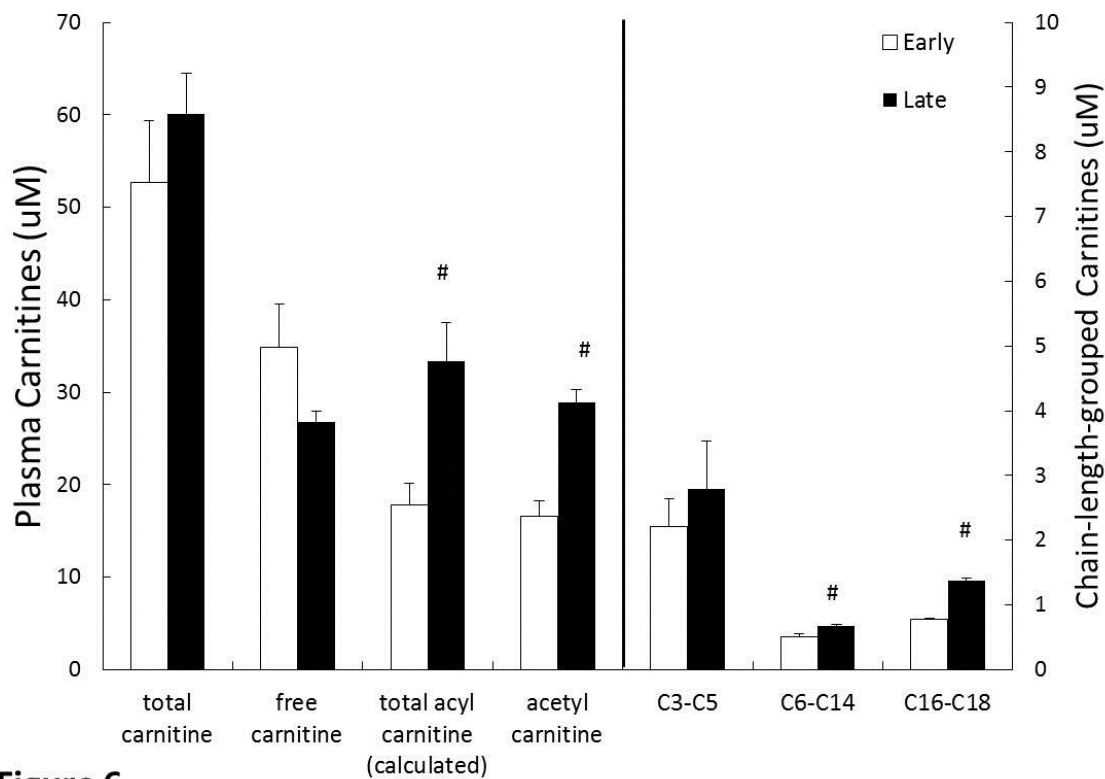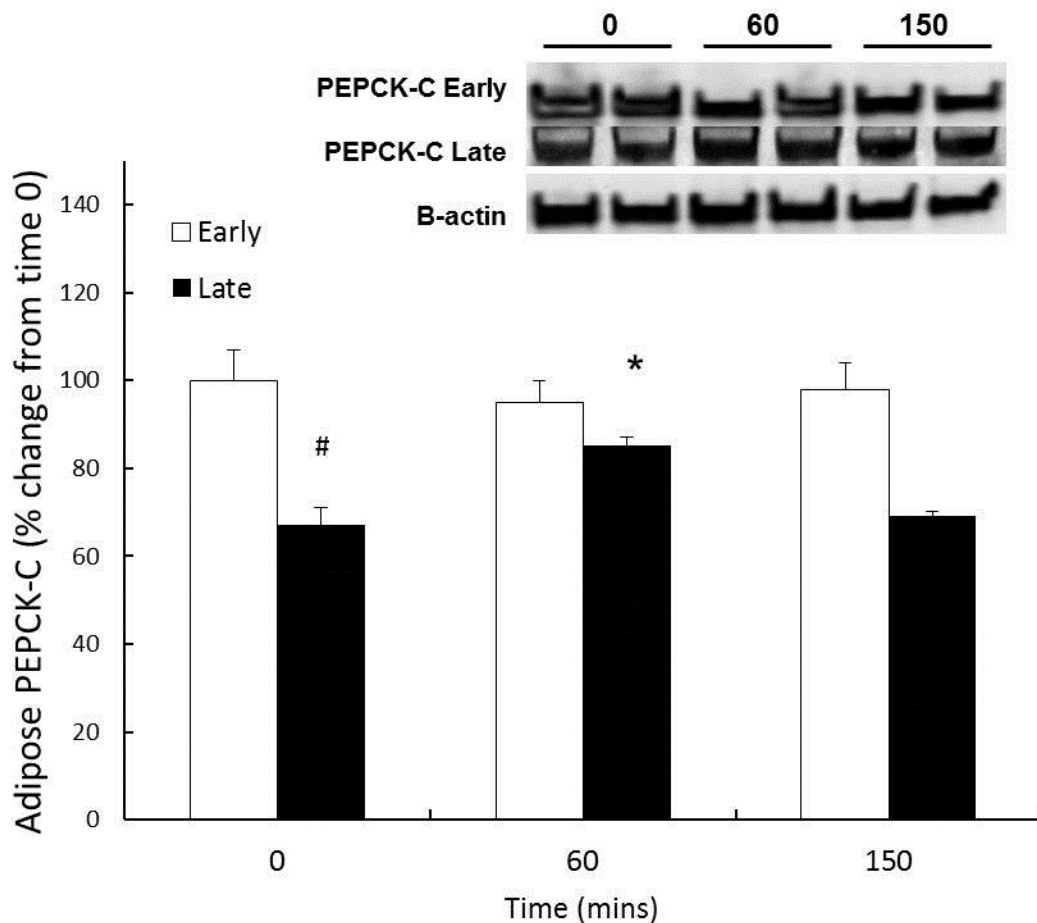
Figure 5. Mean (±SE) **A)** plasma lipase activity and **B)** adipose lipase activity in early (2 weeks; n=5) and late (6-8 weeks; n=8) fasted elephant seal pups during the course of the GTT. # denotes significant difference from early at p<0.05. * denotes significant difference from time 0 at p<0.05.

**Figure 6.** Mean (±SE) plasma carnitine concentrations of early (2 weeks; n=5) and late (6-8 weeks; n=8) fasted elephant seal pups. Chain length-grouped acylcarnitines use a secondary axis for clarity. # denotes significant change with fasting at p<0.05.

Figure 7

Time (mins)
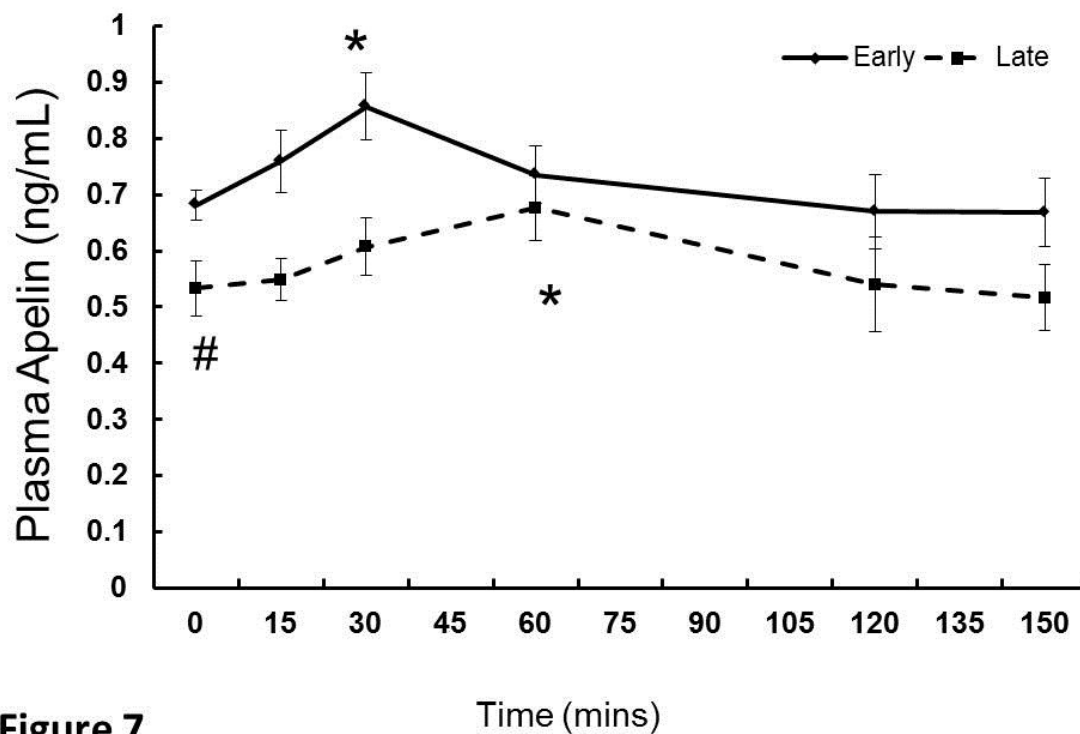
Figure 7. Mean (±SE) plasma apelin concentration of early (2 weeks; n=5) and late (6-8 weeks; n=8) fasted elephant seal pups during the course of the GTT. # denotes significant difference from early at $p<0.05$. * denotes significant difference from time 0 at $p<0.05$.

Figure 8

Figure 8. Mean percent change (±SE) of Phosphoenol pyruvate carboxy kinase in adipose of early (2 weeks; n=5) and late (6-8 weeks; n=8) fasted elephant seal pups during the course of the GTT and representative western blots. # denotes significant difference from early at p<0.05. * denotes significant difference from time 0 at p<0.05.
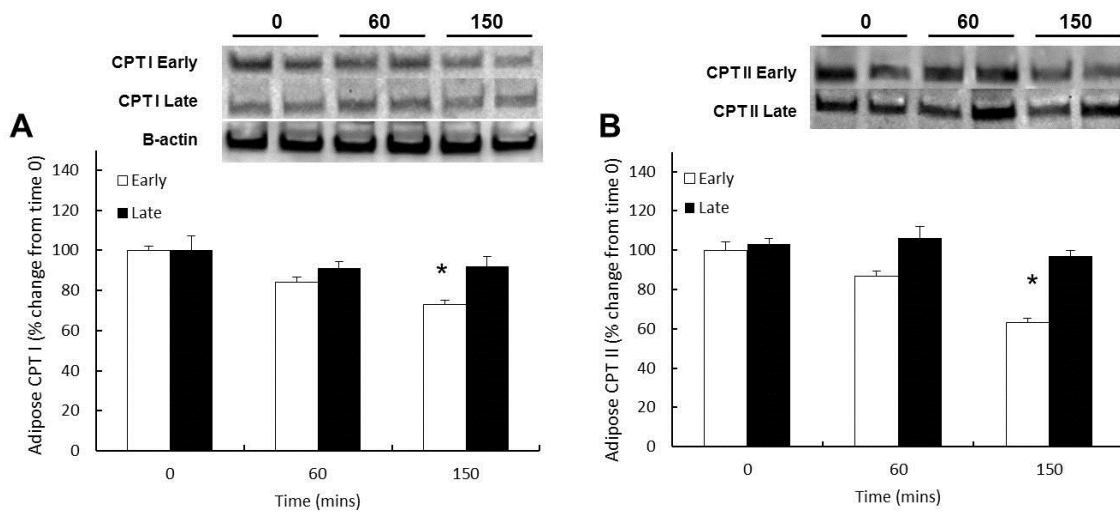
Figure 9

Figure 9. Mean percent change (±SE) of **A)** Carnitine Palmitoyl Transferase I and **B)** Carnitine Palmitoyl Transferase II in adipose of early (2 weeks; n=5) and late (6-8 weeks; n=8) fasted elephant seal pups during the course of the GTT and their representative western blots. * denotes significant difference from time 0 at $p<0.05$
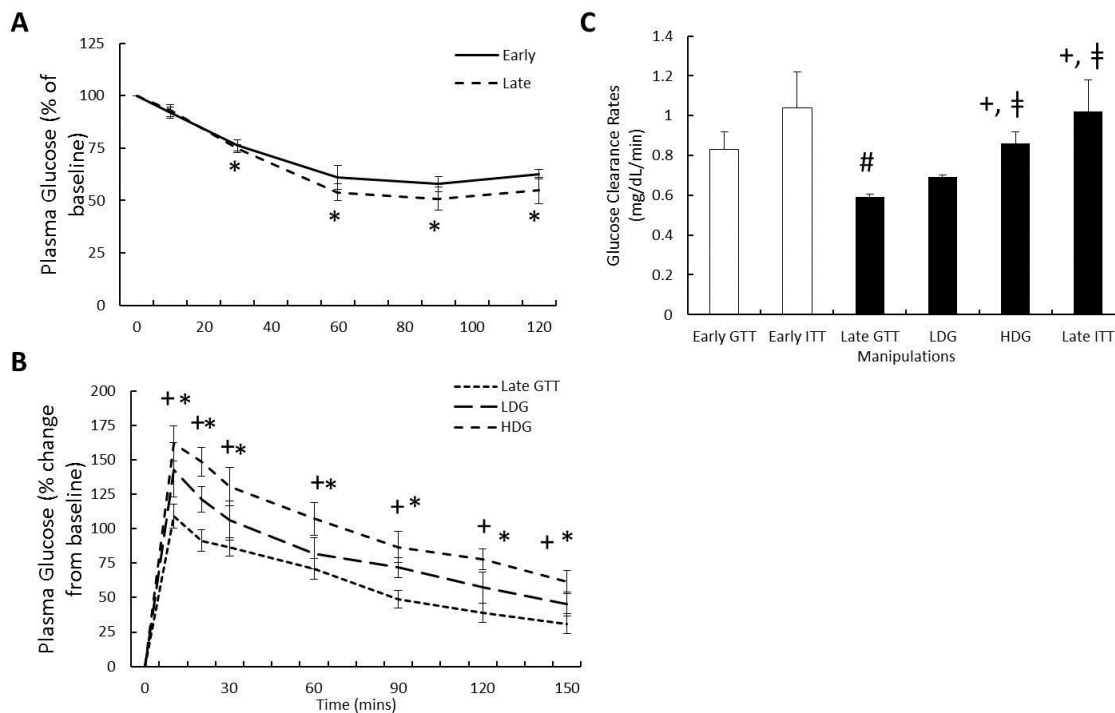
Figure 10

Figure 10. Mean (±SE) plasma glucose **A)** in response to early (n=5) and late insulin infusions (n=5), **B)** in response to low (LDG; n=3) and high dose (HDG; n=4) glucagon-like peptide-1 (GLP-1) infusions plasma (GLP-1), and **C)** the resulting glucose clearance rates (K) in response to the exogenous infusions. # denotes significantly ($p<0.05$) different from early fasting; * denotes significantly ($p<0.05$) different from baseline (T0); + denotes significantly ($p<0.05$) different from late fasting GTT; ‡ denotes significantly ($p<0.05$) different from LDG
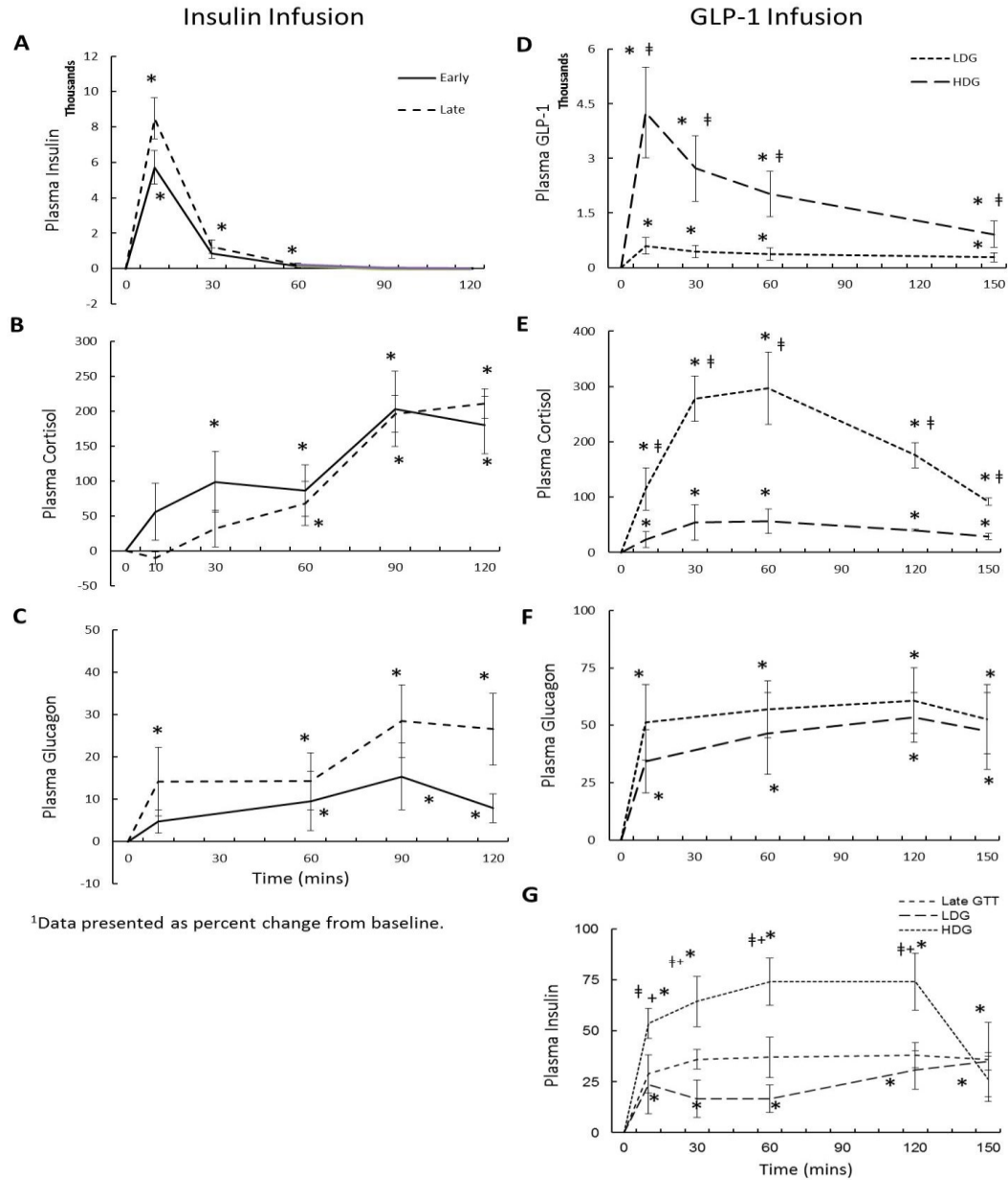
Figure 11

Figure 11. Mean (±SE) plasma **A)** insulin, **B)** cortisol, and **C)** glucagon in response to insulin infusions at early- (2 wks postweaning, n=5) and late, and **D)** glucagon-like peptide-1 (GLP-1), **E)** cortisol, **F)** glucagon, and **G)** insulin in response to low (LDG) and high dose (HDG) GLP-1 infusions in late-fasted (7 wks postweaning) elephant seal pups.- (7 wks postweaning, n=5) fasted elephant seal pups. * denotes significantly (p<0.05) different from baseline (T0); + denotes significantly (p<0.05) different from late fasting GTT; ‡ denotes significantly (p<0.05) different from LDG.
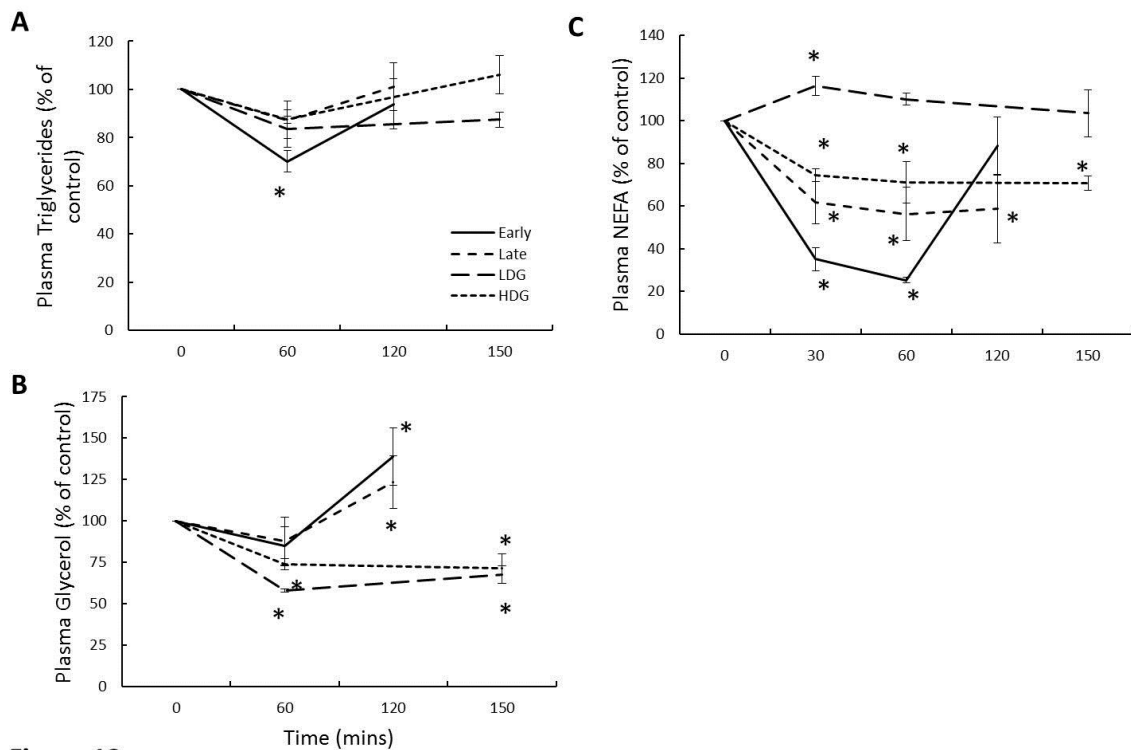
Figure 12. Mean (±SE) plasma **A)** triglycerides, **B)** glycerol, and **C)** NEFA in fasting elephant seal pups in response to early (n=5) and late (n=5) insulin infusions and low (LDG, n=3) and high dose (HDG, n=4)　GLP-1 infusions. # denotes significantly (p<0.05) different from early fasting; * denotes significantly (p<0.05) different from baseline (T0).
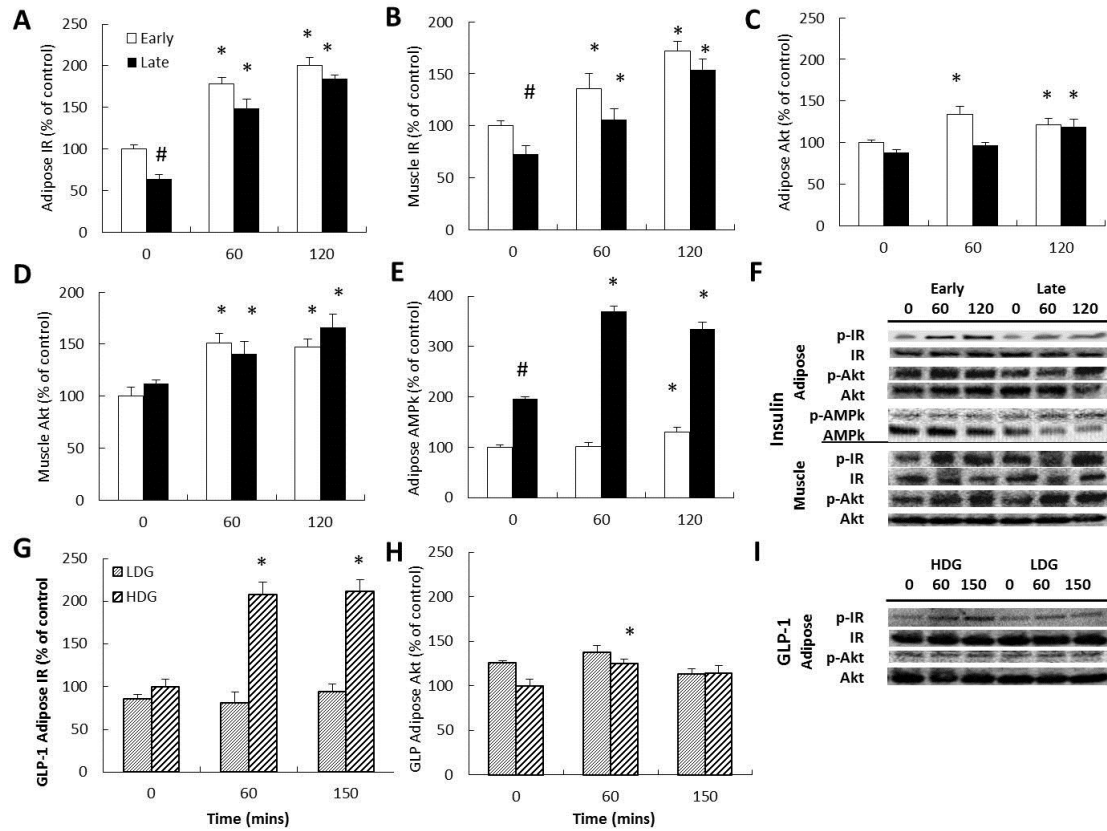
Figure 13

Figure 13. Mean (±SE) percent change of the phosphorylation of **A)** adipose insulin receptor, **B)** muscle insulin receptor, **C)** adipose Akt, **D)** muscle Akt, and **E)** adipose AMP kinase (AMPk) from early- (2 wks postweaning, n=5) and late-fasted (7 wks postweaning, n=5) elephant seal pups in response to insulin infusions. **F)** Representative Western blots for each protein from insulin infused animals. Mean (±SE) percent change of the phosphorylation of adipose **G)** insulin receptor and **H)** Akt in response to low (LDG; n=3) and high dose (HDG; n=4) GLP-1 infusions in late-fasted (7 wks postweaning) elephant seal pups. **I)** Representative Western blots for each protein from GLP-1 infused animals. # denotes significantly (p<0.05) different from early fasting; * denotes significantly (p<0.05) different from baseline (T0).
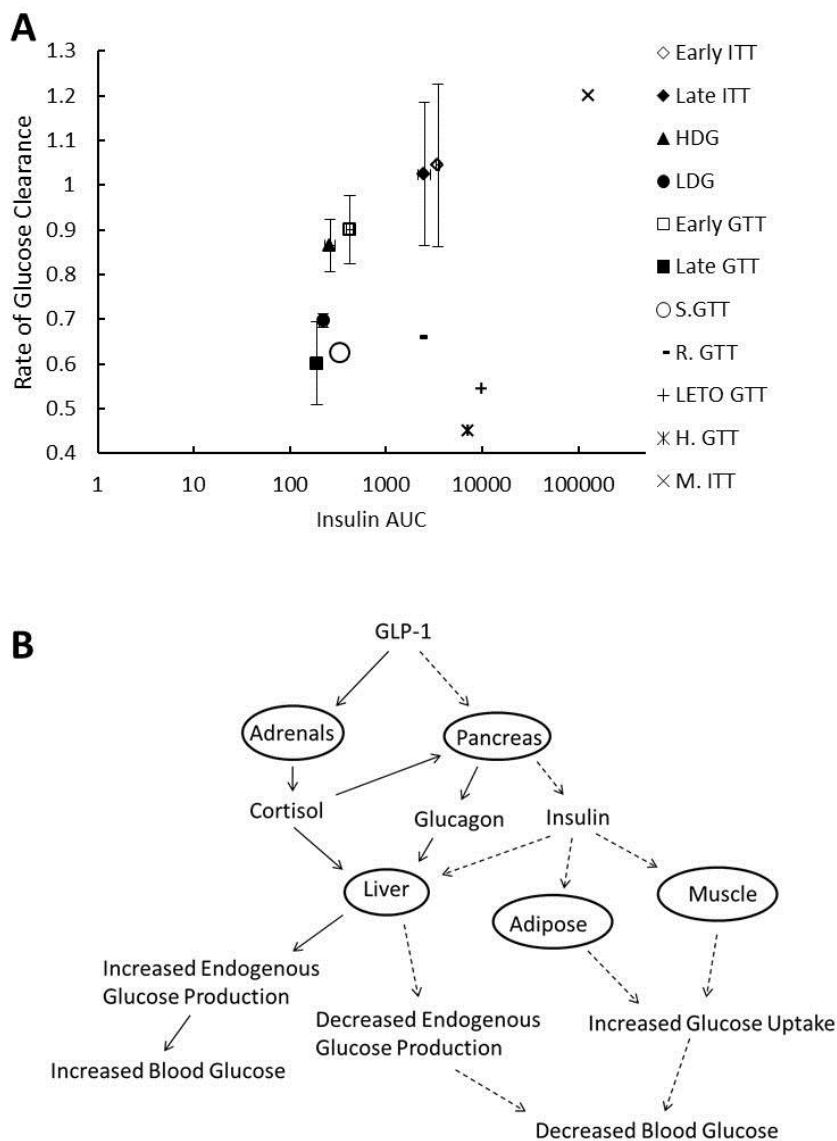
Figure 14

Figure 14. A) Relationship between mean insulin area under the curve ($AUC_{insulin}$) and mean glucose clearance (*K*) from the present study compared to values found in the literature for adult female elephant seals (S.GTT) (12), Sprague-Dawley rats (R.GTT) (86), Long Evans Tokushima Otsuka rats (LETO.GTT) (88), humans (H.GTT) (89), and mice (M.ITT) (87). **B)** Simplified schematic summarizing the effects of GLP-1 on the pancreas and adrenal glands with relation to their glucoregulatory capabilities. Solid lines are representative of steps leading to increased blood glucose, dashed lines are representative of steps leading to decreased blood glucose.