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#### Impact of whole egg consumption on nutrient homeostasis in metabolic disease

by

#### **Cassondra Saande**

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

### DOCTOR OF PHILOSOPHY

Major: Nutritional Sciences

Program of Study Committee: Kevin Schalinske, Co-major Professor Matthew Rowling, Co-major Professor Lorraine Lanningham-Foster Rudy Valentine Manju Reddy Peter Clark

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2019

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## LIST OF ABBREVIATIONS

1,25D	1,25-dihydroxycholecalciferol
10% EGG	Diet containing 10% protein (w/w) from whole egg
2.5% EGG	Diet containing 2.5% protein (w/w) from whole egg
20% EGG	Diet containing 20% protein (w/w) from whole egg
25(OH)D	25-hydroxycholecalciferol
5-CH <sub>3</sub> -THF	5-methyltetrahydrofolate
5% EGG	Diet containing 5% protein (w/w) from whole egg
ADA	American Diabetes Association
AI	Adequate Intake
AS160	Akt substrate 160
BHMT	Betaine-homocysteine S-methyltransferase
С	Casein-based diet
C+Cho	Casein-based diet supplemented with choline
CAS	Casein-based diet
CAS+D	Casein-based diet supplemented with vitamin D <sub>3</sub>
CBS	Cystathionine $\beta$ -synthase
CVD	Cardiovascular disease
СҮР	Cytochrome p450
DBP	Vitamin D-binding protein
EDL	Extensor digitorum longus
EP	Egg protein-based diet

FGF-23 Fibroblast growth factor 23



FR	Folate-restricted
FR-C	Folate-restricted casein-based diet
FR-C+C	Folate-restricted casein-based diet supplemented with cholin
FR-EP	Folate-restricted egg protein-based diet
FR-WE	Folate-restricted whole egg-based diet
FS	Folate-sufficient
FS-C	Folate-sufficient casein-based diet
FS-C+C	Folate-sufficient casein-based diet supplemented with cholin
FS-EP	Folate-sufficient egg protein-based diet
FS-WE	Folate-sufficient whole egg-based diet
GLUT4	Glucose transporter type 4
HbA1C	Glycosylated hemoglobin
HDL	High-density lipoprotein
HOMA	Homeostatic model assessment of $\beta$ -cell function
HOMA	R Homeostatic model assessment of insulin resistance
IOM	Institutes of Medicine
IP	Intraperitoneal
IR β	Insulin receptor β
IRS-1	Insulin receptor substrate 1
ITT	Insulin tolerance test
LDL	Low-density lipoprotein
MTHFF	5,10-methylenetetrahydrofolate reductase
NHANI	S National Health and Nutrition Examination Survey



- PDCAAS Protein digestibility-corrected amino acid score
- PEMT Phosphatidylethanolamine N-methyltransferase
- PTH Parathyroid hormone
- RDA Recommended Dietary Allowance
- RXR Retinoid X receptor
- T2D Type 2 diabetes mellitus
- TMAO Trimethylamine N-oxide
- VDR Vitamin D receptor
- VDRE Vitamin D response elements
- WE Whole egg-based diet
- ZDF Zucker diabetic fatty



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#### ABSTRACT

Metabolic diseases, such as obesity and type 2 diabetes (T2D), are characterized by aberrant nutrient metabolism, such as disrupted metabolism of vitamin D and methyl groups. Whole eggs are a source of several nutrients, including vitamin D, B vitamins and choline, which may assist in the maintenance of micronutrient balance. Additionally, the high-quality protein content of whole eggs may contribute to satiety and body weight management. However, the relation between egg consumption and measures such as insulin sensitivity, glycemic control and cardiovascular risk factors in individuals with obesity, T2D and other metabolic abnormalities remains inconsistent. The objectives of the studies described in this dissertation were to evaluate the impact of whole egg consumption on 1) vitamin D homeostasis and body weight gain in T2D rats, 2) metabolic biomarkers of insulin resistance in T2D rats and 3) homocysteine metabolism in a rat model of hyperhomocysteinemia.

The first study described in this dissertation compared dietary whole egg to supplemental cholecalciferol with respect to vitamin D balance, weight gain, and body composition in T2D rats. Male Zucker diabetic fatty (ZDF) rats (n = 24) and their lean controls (n = 24) were randomly assigned to one of 3 dietary treatment groups: a casein-based diet (CAS), a dried whole egg-based diet (WE), or a casein-based diet containing supplemental cholecalciferol (CAS+D) at the same level of cholecalciferol provided by the dried whole egg-based diet (37.6 µg/kg diet). All diets provided protein at 20% (w/w) and were matched for lipid quantity to account for the additional lipid contributed by the whole egg. Rats were fed their respective diets for 8 weeks. Weight gain and percent body fat were reduced by approximately 20% and 11%, respectively, in ZDF rats fed WE compared



to ZDF rats fed CAS or CAS+D. ZDF rats fed CAS had 21% lower serum 25hydroxyvitamin D [25(OH)D] concentrations than lean rats fed CAS. In ZDF rats, WE consumption increased serum 25(OH)D concentrations 130% compared to CAS, whereas consumption of CAS+D increased serum 25(OH)D concentrations by 35% compared to CAS. Our data suggest that dietary consumption of whole egg is more effective than supplemental cholecalciferol in maintaining vitamin D status in T2D rats. Furthermore, whole egg consumption reduced weight gain and body fat percentage in obese T2D rats, without an effect on body weight parameters in the lean phenotype. These data may support new dietary recommendations targeting body weight management and prevention of vitamin D insufficiency in T2D.

The objective of the second study was to perform a follow-up, dose-response study to determine the minimal amount of dietary whole egg effective at maintaining vitamin D balance and attenuating the obese phenotype in T2D rats. A secondary objective of this study was to determine the effect of varying concentrations of whole egg on serum trimethylamine N-oxide (TMAO), a candidate cardiovascular disease risk factor. Male ZDF rats (*n*= 40) and their lean controls (*n*=40) were randomly assigned to a diet containing 20% casein (CAS), 20% egg protein (20% EGG), 10% egg protein (10% EGG), 5% egg protein (5% EGG) or 2.5% egg protein (2.5% EGG) for 8 weeks. All diets contained 20% total protein (w/w). The 20% EGG diet maintained vitamin D balance in ZDF rats, whereas the 10, 5 and 2.5% EGG diets did not prevent vitamin D insufficiency. Body weight gain was reduced by 29% and 31% in ZDF rats consuming 20% EGG and 10% EGG diets, and by 16% and 12% in ZDF rats consuming 5% EGG and 2.5% EGG diets compared to the CAS diet. All EGG diets increased serum TMAO, regardless of genotype, although a



greater increase was observed in the ZDF genotype. These data demonstrate that whole egg consumption attenuates weight gain in a dose-dependent manner in T2D rats. Additionally, egg consumption increases circulating TMAO concentrations in both lean and T2D rats, with a heightened TMAO response in T2D rats.

In the third study described in this dissertation, the effect of dietary whole egg on metabolic biomarkers of insulin resistance was evaluated in T2D rats. Male ZDF rats (n=12) and their lean controls (n=12) were randomly assigned to a casein- or whole egg-based diet. At week 5 of dietary treatment, mean blood glucose over the course of an insulin tolerance test was 32% higher in ZDF rats fed the whole egg-based diet compared to ZDF rats fed the casein-based diet. After 7 weeks of dietary treatment, whole egg consumption increased fasting blood glucose by 35% in ZDF rats. Furthermore, insulin-stimulated phosphorylation of key proteins in the insulin signaling pathway did not differ in skeletal muscle of ZDF rats fed casein- and whole egg-based diets. In lean rats, no differences were observed in insulin tolerance or skeletal muscle insulin signaling, regardless of experimental dietary treatment. These data suggest that whole body insulin sensitivity may be impaired by whole egg consumption in T2D rats, although no changes were observed in skeletal muscle insulin signaling that could explain this finding.

The objective of the final study presented in this dissertation was to determine the effect of whole eggs and egg components (i.e. egg protein and choline) with respect to homocysteine balance in a folate-restricted rat model characterized by moderate hyperhomocysteinemia. Furthermore, this study sought to determine the differential effects of whole eggs, egg protein or supplemental choline on the hepatic expression and activity of betaine-homocysteine *S*-methyltransferase (BHMT) and cystathionine  $\beta$ -synthase



(CBS), key enzymes in homocysteine metabolism. Male Sprague Dawley rats (N=48) were randomly assigned to a casein-based diet (n=12), a casein-based diet supplemented with choline (1.3%, w/w; n=12), an egg protein-based diet (n=12), or a whole egg-based diet (n=12). At week 2, half of the rats in each of the 4 dietary groups were provided a folaterestricted (FR; 0 g folic acid/kg) diet and half continued on the folate-sufficient (FS; 0.2 g folic acid/kg) diet for an additional 6 weeks. All diets contained 20% (w/w) total protein. Folate-restricted casein-fed rats exhibited a 53% increase in circulating homocysteine concentrations compared to FS rats fed a casein-based diet. In contrast, consumption of egg protein prevented hyperhomocysteinemia in FR rats compared to FR rats fed the casein-based diet. Hepatic BHMT activity was increased by 45% and 40%, respectively, by the egg protein-based and whole egg-based diets compared to the casein-based diets. These data demonstrate that dietary intervention with egg protein prevented elevated circulating homocysteine concentrations in a rat model of hyperhomocysteinemia, due in part to upregulation of hepatic BHMT. These data may support the inclusion of egg protein for dietary recommendations targeting hyperhomocysteinemia prevention.



#### **CHAPTER 1. GENERAL INTRODUCTION**

#### Introduction

The increasing prevalence of metabolic diseases, such as obesity, type 2 diabetes (T2D) and cardiovascular disease, is a critical public health issue. In the United States, approximately two thirds of adults are overweight or obese (1), one in ten have diabetes (2), and cardiovascular disease remains the leading cause of death (3). An individual's dietary patterns directly contribute to these disorders and can strongly influence health outcomes. Furthermore, it is well-documented that these disease states can alter nutrient metabolism. Thus, understanding the interactions between dietary components and health outcomes is important in the prevention and treatment of metabolic disease.

Eggs have a high nutrient-to-energy density ratio, and are a valuable source of protein and essential nutrients, including vitamin D, choline and B vitamins (4). Additionally, the 2015 Dietary Guidelines for Americans include eggs as part of a healthy eating pattern, in combination with a variety of vegetables, fruits, whole grains and low-fat dairy (5). As an affordable, nutrient-rich source of high-quality protein, whole eggs could offer several benefits, such as weight management and the maintenance of micronutrient balance, during the progression of metabolic disease. However, there is still uncertainty surrounding the potential health effects of dietary whole eggs in individuals with metabolic disease (6–9); therefore, further evaluation is necessary to inform dietary recommendations.

The overall objectives of this research were to examine various metabolic responses to whole egg consumption in metabolic disease. The studies presented in this dissertation focus specifically on the impact of dietary whole eggs on micronutrient balance, body weight



management, and insulin resistance in rodent models of obesity, T2D and hyperhomocysteinemia, a metabolic disorder associated with cardiovascular disease.

#### **Dissertation Organization**

This dissertation consists of seven chapters with a general introduction, literature review, four manuscripts, and general conclusions. The first manuscript titled "Dietary whole egg consumption attenuates body weight gain and is more effective than supplemental cholecalciferol in maintaining vitamin D balance in type 2 diabetic rats" has been published in the Journal of Nutrition. This study compared whole egg consumption to supplemental cholecalciferol with respect to vitamin D homeostasis in a rat model of T2D. The work presented in the second manuscript was a follow-up, dose-response study investigating various concentrations of dietary whole egg with respect to vitamin D balance and weight gain in T2D rats and has been accepted for publication in the Journal of Nutrition for publication. The third manuscript titled "Whole egg consumption impairs insulin sensitivity in a rat model of obesity and type 2 diabetes" has been published in *Current Developments in Nutrition*. The aim of this study was to investigate the impact of whole egg consumption on insulin sensitivity and skeletal muscle insulin signaling using a rat model of T2D. The final manuscript evaluated the impact of dietary whole egg and egg components (i.e. egg protein and choline) with respect to homocysteine balance in a folate-restricted rat model of hyperhomocysteinemia. This work has been published in the Journal of Nutrition. All literature cited is based on the format of Journal of Nutrition and listed at the end of each chapter.



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#### **CHAPTER 2. LITERATURE REVIEW**

#### **Type 2 Diabetes Mellitus**

#### **Diagnostic Criteria**

An estimated 30 million adults are living with diabetes in the U.S. (1), with type 2 diabetes (T2D) accounting for 90-95% of all cases (2). T2D is a multifactorial disease characterized by a progressive loss of  $\beta$ -cell mass and/or function, in combination with insulin resistance, which manifests as hyperglycemia. Diagnostic criteria for T2D include fasting plasma glucose  $\geq$ 126 mg/dL, 2 hour plasma glucose  $\geq$ 200 mg/dL during a 75 g oral glucose tolerance test, glycosylated hemoglobin A1C (HbA1C)  $\geq$ 6.5%, or a random plasma glucose of  $\geq$ 200 mg/dL in a patients with classic symptoms of hyperglycemia (2). Diagnosis requires unequivocal hyperglycemia or at least two abnormal tests (2). It is also estimated that 84 million adults in the U.S. have prediabetes (1), which is classified by the presence of impaired fasting glucose (fasting plasma glucose 100-125 mg/dL), impaired glucose tolerance, or HbA1C of 5.7-6.4% (2). Individuals with prediabetes are at risk for the development of T2D and cardiovascular disease (CVD).

#### Pathophysiology

The development of T2D is triggered by a complex interplay between environmental factors and genetic predisposition. Key risk factors for T2D include the following: overweight and obesity, physical inactivity, family history of T2D, prior history of gestational diabetes, age 45 or older, and non-white race or ethnicity (3). Excess weight gain and obesity are among the most important predictors of T2D development, and their association with insulin resistance is likely a driving factor (4–6).



In a healthy individual in the postprandial state, glucose metabolism by the  $\beta$ -cell triggers insulin secretion. Glucose enters the  $\beta$ -cell via the glucose transporter 2 (GLUT 2) and then undergoes glycolysis, leading to an increase in ATP within the cell (7). The rise in intracellular ATP inhibits the ATP-sensitive K<sup>+</sup> channel, causing depolarization of the  $\beta$ -cell. This activates voltage-gated Ca<sup>2+</sup> channels, causing Ca<sup>2+</sup> influx and an increase in intracellular Ca<sup>2+</sup> (7). The elevation in intracellular Ca<sup>2+</sup> leads to the exocytosis of insulin contained within insulin secretory granules, releasing insulin into the bloodstream (7). Insulin binding to the insulin receptor at target tissues activates the receptor, which transduces its signal to downstream effectors through a cascade of phosphorylation events (7). In skeletal muscle and adipose tissue, insulin stimulates translocation of the glucose transporter 4 (GLUT 4) from the cytosol to the plasma membrane, thereby promoting glucose uptake and storage (7). In a state of insulin resistance and  $\beta$ -cell dysfunction these processes are impaired, resulting in an inability to maintain blood glucose control (8,9).

It is widely agreed that obesity is a major risk factor in the development of insulin resistance, and numerous studies suggest that chronic low-grade inflammation plays an important role in this process (10,11). Adipose tissue modulates metabolism by secreting several cytokines and hormones. The proper balance of these cytokines and hormones is altered in overweight and obese individuals, activating pro-inflammatory pathways that suppress insulin signaling and lead to dysfunction of the pancreatic islet  $\beta$ -cells (12). Increased concentrations of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1), and decreased concentrations of the anti-inflammatory factor adiponectin, are commonly observed in states of obesity and insulin resistance (11,13).



In insulin resistance individuals, a progressive decline in  $\beta$ -cell function characterizes the transition from normal to impaired glucose tolerance and then to overt T2D. In response to a decrease in insulin sensitivity, the pancreatic  $\beta$ -cells increase insulin release in attempt to maintain normal glucose tolerance (8,14). This adaptive response involves an increase in  $\beta$ cell mass and activity (15). However, as insulin resistance progresses, the  $\beta$ -cell is overstimulated, which results in declining  $\beta$ -cell mass and function, eventually leading to apoptosis (16,17). The decreased insulin release owing to  $\beta$ -cell dysfunction, combined with impaired insulin sensitivity reduced glucose uptake, results in persistent hyperglycemia and T2D (8).

#### **Prevention and Treatment via Lifestyle Modification**

The American Diabetes Association (ADA) recommends referral of prediabetic patients to an intensive lifestyle intervention with the objectives to achieve and maintain a 7-10% weight loss and to increase moderate intensity physical activity to a minimum of 150 minutes/week (18,19). Dietary patterns such as a Mediterranean eating plan or a low-fat, low-calorie eating plan should be encouraged to assist in weight loss and diabetes prevention (20–22). Although low-carbohydrate diets have shown beneficial short-term effects on glycemic control in some studies (23), further studies including long-term outcomes are needed to determine whether low-carbohydrate diets are beneficial for patients with prediabetes (24). Emphasis should be placed on increasing intake of fruits, vegetables, whole grains, legumes and nuts while minimizing intake of refined foods (25). As evidenced by observational studies, higher intake of particular dietary components, including nuts, berries, yogurt, coffee and tea,



is associated with reduced diabetes risk, whereas intake of red meats and sugar-sweetened beverages are associated with an increased risk (26–29).

It is well-established that the onset of T2D can be prevented or delayed through lifestyle modifications, such as dietary modification, physical activity and weight loss. Several studies have shown long-term diabetes risk reduction in patients previously involved in shorter-term lifestyle interventions. In the Finnish Diabetes Prevention Study, overweight, glucose intolerant individuals assigned to a lifestyle intervention group exhibited significantly greater weight reduction, as well as improvements in glycemia and lipemia, compared to the control group after 3 years of follow-up (30). After 7 years of total follow-up, diabetes risk was reduced by 43% in individuals in the lifestyle intervention group, which was associated with reduced weight loss and fat intake and increased physical activity and intake of dietary fiber (31). In the Da Qing Diabetes Study, a Chinese cohort of individuals with impaired glucose tolerance were assigned to either a control group or to diet only, exercise only, or a combination of diet and exercise intervention for a period of 6 years (32). During the 6-year active intervention period, diabetes incidence was 51% lower in the intervention groups compared to the control group, and 43% lower during a 20-year follow-up period. These differences in diabetes incidence occurred in the absence of significant body weight differences between control and intervention groups (33). Finally, in the U.S. Diabetes Prevention Program Outcomes Study, diabetes incidence was reduced by 58% after 2.8 years of intensive lifestyle intervention (34). Diabetes incidence was reduced by 34% and 27% in the lifestyle intervention group at 10 and 15 years of follow-up, respectively, indicating long-lasting preventative effects of lifestyle intervention (34,35).



In individuals with diagnosed T2D, weight loss and weight maintenance are important in diabetes management, as even modest weight loss has been shown to improve glycemic control (36,37). For example, in a multi-center, randomized, controlled trial in type 2 diabetic subjects, individuals assigned to intensive lifestyle intervention experienced an 8.6% weight loss after 1 year follow-up, compared to 0.7% in the group assigned to general diabetes education, as well as a significant reduction in HbA1C (38). After 4 years of follow-up, the intensive lifestyle intervention group was significantly more likely to experience complete or partial remission of T2D (39). For overweight or obese patients with T2D, it is recommended to achieve and maintain a weight loss of >5% through diet, physical activity and behavioral therapy (40). The ADA recommends adherence to healthful eating patterns and engagement in 150 minutes/week of moderate physical activity to achieve and maintain weight management goals, attain glycemic targets and prevent or delay complications in individuals with diagnosed T2D (41). Reducing overall carbohydrate intake using approaches such as minimizing added sugars and refined grains and emphasizing non-starchy vegetables, has demonstrated considerable evidence for meeting glycemic targets and is achievable with numerous eating patterns (19). A variety of eating patterns have shown success in diabetes management, including the Mediterranean diet, Dietary Approaches to Stop Hypertension, low-carbohydrate diets and plant-based diets (42–46). Overall, nutrient-density and portion control should be emphasized to improve glycemia and weight management.

#### Vitamin D

#### **Biosynthesis and Metabolism**

Vitamin D is a steroid hormone that can be produced endogenously upon sunlight exposure to the skin, or can be obtained through dietary sources or supplementation.



Endogenous production of vitamin D3 occurs when ultraviolet B photons penetrate the epidermis, resulting in photolysis of cutaneous 7-dehydrocholesterol to previtamin D3. Previtamin D3 then isomerizes to vitamin D3 (cholecalciferol), which is absorbed into the circulation (47–49). Vitamin D is obtained from the diet either in the form of vitamin D3, found in foods of animal origin, or vitamin D2 (ergocalciferol), found in plants and fungi (48). Regardless of whether it is obtained through sunlight exposure, dietary sources or supplementation, vitamin D is biologically inert and must undergo two hydroxylation reactions to be converted to the biologically active form.

Vitamin D metabolites are lipophilic and therefore require carrier proteins for transport in the circulation, primarily vitamin D-binding protein (DBP). Vitamin D3 absorbed into circulation is transported by DBP to the liver, where it undergoes hydroxylation to 25hydroxyvitamin D3 (25(OH)D) by 25-hydroxylase (Figure 2-1). There are several cytochrome p450 (CYP) enzymes capable of 25-hydroxylase activity, including CYP27R1 and CYP27A1 (50). Following hydroxylation in the liver, 25(OH)D is secreted back into the circulation where it is bound by DBP. DBP has a high affinity for vitamin D3 metabolites, and nearly all circulating 25(OH)D is found in a complex with DBP (51). The 25(OH)D-DBP complex is then delivered to the kidney by receptor-mediated endocytosis via the membrane-associated proteins megalin and cubilin in the renal proximal tubule and the intracellular adaptor protein disabled-2 (Dab2) (52). 25(OH)D internalized into the renal proximal tubule can undergo hydroxylation to 1,25-dihydroxyvitamin D3 (1,25D; calcitriol), the biologically active form of vitamin D, by 25-hydroxyvitamin D 1 $\alpha$ -hydroxlase (CYP27B1), or reabsorption into circulation (Figure 2-1) (48). The presence of 25-hydroxyvitamin D 1α-hydroxlase has also been reported in extrarenal tissues, such as the colon, pancreas, brain and placenta, although



1,25D is predominantly synthesized in the kidney (53). Lastly, vitamin D catabolism and inactivation is carried out by the enzyme 24-hydroxylase (CYP24A1) (Figure 2-1). Both 25(OH)D and 1,25D are targets for 24-hydroxylase, which is able to catalyze hydroxylation reactions on the side chain of both forms of the vitamin. The 24- and 23- hydroxylated products are then targeted for excretion (50).



Figure 2-1: Vitamin D Metabolism (50). CYP, cytochrome p450, FGF23, fibroblast growth factor 23, PTH, parathyroid hormone, VDR, vitamin D receptor,  $1\alpha$ ,25-(OH)2D3, 1,24-dihydroxyvitamin D3; 25-OH-D3, 25-hydroxyvitamin D3.

#### **Mechanism of Action**

Synthesis of the active hormone 1,25D is a highly regulated process, which is influenced by circulating concentrations of calcium and phosphorus, parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23) (48). The classical, hormonal actions of 1,25D are related to the maintenance of bone health via regulation of circulating calcium and phosphorus. To maintain plasma calcium within a normal range, 1,25D acts on the intestines, bones and kidneys to increase calcium absorption, mobilize calcium from bone and stimulate



renal reabsorption of calcium (54). CYP27B1, responsible for 1,25D synthesis, is subject to upregulation by PTH, low plasma calcium and low plasma phosphorus, ensuring calcium homeostasis (50). In contrast, FGF23, stimulated by elevated circulating phosphorus, and 1,25D inhibit CYP27B1 (48).

The genomic actions of 1,25D are mediated by the vitamin D receptor (VDR), a ligandactivated transcription factor localized in the nuclei of target cells (55). Binding of 1,25D to VDR triggers the formation of a heterodimer with the retinoid X receptor (RXR). The VDR-RXR heterodimer then recognizes and binds vitamin D response elements (VDREs) in the DNA sequence of genes regulated by vitamin D, thereby regulating gene transcription (55). Regulation of gene expression by 1,25D is well-established in the classical vitamin D target tissues, such as the intestines, kidney, bone and parathyroid. It also plays a role in a diverse range of biological responses, including the regulation of immune function, cell proliferation, differentiation and apoptosis (55,56).

#### Status

Circulating concentrations of 25(OH)D are considered the best indicator of vitamin D status, and represent the combined contributions of cutaneous vitamin D synthesis, dietary intake and supplementation (57). The defined cutoff values for vitamin D status, which include deficiency, insufficiency, sufficiency and toxicity, remain a subject of debate amongst the scientific community, and guidelines regarding vitamin D status differ between the Institutes of Medicine (IOM) and the Endocrine Society (Table 2-1) (58).

The 2011 IOM Committee concluded that a serum 25(OH)D level of > 20 ng/mL (> 50 nmol/L) is sufficient to provide maximal calcium absorption, minimize risk of rickets in children, and minimize risk of osteomalacia adults (57). In contrast, the Endocrine Society



concluded that a serum 25(OH)D concentration of at least 30 ng/mL (75 nmol/L) is optimal (59). It is important to note that guidelines set forth by the IOM rely on bone health as an indicator of vitamin D adequacy for the general population (57), whereas the objective of the Endocrine Society Clinical Practice Guideline was to provide recommendations to clinicians for the evaluation, prevention and treatment of vitamin D deficiency, with a focus on patients at risk for deficiency (59). Some argue that the IOM cutoff of 20 ng/mL may not be sufficient to provide the potential benefits of vitamin D (58). For example, the PTH response to varying levels of serum 25(OH)D may depend on both gender and age, with certain individuals requiring serum 25(OH)D > 30 ng/mL to minimize PTH concentrations (60). Additionally, studies have demonstrated reduced fracture risk with a mean serum 25(OH)D of 30 ng/mL, whereas risk reduction has not been shown with a serum 25(OH)D of 20 ng/mL (61,62). Lastly, suboptimal vitamin D status is associated with various chronic diseases, including obesity, type 2 diabetes and chronic kidney disease, and a higher dose of vitamin D may be required to provide potential nonskeletal benefits (63,64).

Vitamin D Status	Institutes of Medicine		Endocrine Society	
vitainin D Status –	ng/mL	nmol/L	ng/mL	nmol/L
Deficient	< 12	< 30	< 20	< 50
Insufficient	12 - 20	30 - 50	21 - 29	53 - 73
Sufficient	> 20	> 50	30 - 100	75
Toxic	> 50	> 125		

Table 2-1: Vitamin D status based on circulating concentrations of 25-hydroxycholecalciferol.

The RDA for vitamin D for adults is set at 600 IU (15  $\mu$ g) (57). It is recognized that vitamin D can be obtained through both sunlight exposure and dietary intake. However, there are numerous factors that impede cutaneous vitamin D synthesis, including latitude, skin



pigmentation, age, sunscreen and sun exposure behaviors, such as a person's attire, physical activity and working environment. As such, recommendations assume minimum sun exposure and are developed using the estimated dietary intakes of vitamin D to achieve serum 25(OH)D concentrations of 20 ng/mL, deemed sufficient for the maintenance of bone health (57).

Vitamin D insufficiency, following the IOM definition of 12 - 20 ng/mL (30 - 50 nmol/L) has been reported to affect an estimated 32% of the U.S. population (65). Furthermore, based on NHANES 2001-2006 data, an estimated 10% of the U.S. population presented with serum 25(OH)D < 30 nmol/L, putting them at risk for fractures and skeletal abnormalities such as rickets and osteomalacia (65). Individuals at particularly high risk for deficiency include those with increased skin pigmentation or insufficient sun exposure, pregnant and lactating women, and the elderly (66-68). The 2015 Dietary Guidelines for Americans identified vitamin D as a nutrient of public health concern, as vitamin D intake falls below the Estimated Average Requirement set forth by the IOM, and underconsumption is associated with adverse health outcomes (69). Indeed, 69% of vitamin D-containing supplement users and 95% of non-supplement users in the U.S. have usual vitamin D intakes that fall below the Estimated Average Requirement (70).

The Upper Limit for vitamin D intake is set at 4000 IU/d, a level at which sustained, long-term intake will not cause harm to the general population (57). However, evidence regarding the health effects of long-term, moderately high intakes of vitamin D is lacking, as most available evidence is based on exposures of less than 6 months. Although conditions of vitamin D toxicity are unlikely from a combination of routine sources, cases of vitamin D toxicity have been reported following prolonged high-dose supplementation. In a risk assessment based on human clinical trials, Hathcock et al. concluded that that an intake of



10,000 IU/d was not associated with adverse outcomes (71), while toxic side effects have been documented at doses at or above 50,000 IU/d for periods ranging from weeks to months (57). Vitamin D toxicity is characterized by hypercalcemia, owing to increased bone resorption (72). As circulating calcium increases, renal function decreases while CVD risk increases, making the condition life threatening if left untreated (57).

#### Vitamin D and Type 2 Diabetes

Accumulating evidence suggests that vitamin D insufficiency may play a role in T2D development, though the potential benefits of vitamin D supplementation in T2D prevention and treatment remains a subject of debate. Receptors for 1,25D have been found in the  $\beta$ -cell, liver, muscle and adipose tissue, and the enzymes necessary for vitamin D activation are also expressed in these tissues (73). Results from in vitro and in vivo studies have shown impaired glucose-stimulated insulin secretion under vitamin D-deficient conditions, and restored insulin secretion with vitamin D supplementation (74–77). Some of these studies also demonstrated impaired glucose tolerance with vitamin D deficiency (75).

Epidemiological studies show a relatively consistent association between vitamin D deficiency and the prevalence of T2D (78,79). However, it is important to note many confounding factors, such as physical activity, adiposity and dietary habits, that are linked to vitamin D status and could affect T2D risk. In the Nurses' Health Study, total vitamin D intake was not associated with T2D incidence after adjustment for potential confounding factors (80). However, T2D risk was 33% lower in women with the highest combined intake of calcium and vitamin D (>1200 mg/day and >800 IU/day, respectively) compared to women with the lowest combined intake of calcium and vitamin D (<600 mg/day and <400 IU/day,



respectively) (80).

Observational studies indicate an inverse association between vitamin D status, insulin sensitivity and  $\beta$ -cell function, though these associations are not always consistent (81–84). Furthermore, a number of studies report improvements in  $\beta$ -cell function, insulin sensitivity and glycemia following intervention with vitamin D supplementation (85-88). In a randomized, controlled trial in patients with T2D, individuals that received 4000 IU vitamin D3 daily for 2 months had a significant reduction in HbA1C compared to individuals that received a placebo, although no differences in HOMA-IR were observed (88). A placebocontrolled trial in adults at high risk for T2D found that supplementation with 2000 IU/day of vitamin D3 for 16 weeks resulted in improved  $\beta$ -cell function in the supplemental vitamin D3 group (87). In this study, the effects of vitamin D on insulin sensitivity were insignificant, indicating that vitamin D may predominantly effect the  $\beta$ -cell (87). Other studies in healthy adults or T2D patients also report no changes in insulin sensitivity following supplementation (89,90). In contrast, two clinical trials in centrally obese or insulin resistance subjects demonstrated improved insulin sensitivity following short-term vitamin D supplementation (85,86). A recent meta-analysis of randomized controlled trials of vitamin D supplementation in adults with T2D reported a modest reduction in HbA1C, but no differences in fasting blood glucose, following treatment with vitamin D (91). Similarly, in a meta-analysis performed by Wu et al., vitamin D supplementation reduced HbA1C but had no effect on fasting blood glucose (92). While research suggests that vitamin D may play a role in diabetes prevention, considerable homogeneity exists between studies, both regarding study design, participants and outcomes.



#### **Dietary Sources**

As described previously, vitamin D can be acquired through sunlight exposure or through the diet. Fatty fish, such as salmon and tuna, are among the best naturally occurring dietary sources of vitamin D, followed by liver and other organ meats, egg yolks and cheeses (93). Certain varieties of mushrooms also contain vitamin D in varying amounts (93). Foods of animal origin provide vitamin D in the form of vitamin D3 and its metabolite, 25(OH)D, whereas plants and fungi provide vitamin D2 (93). Notably, few other foods naturally contain vitamin D and foods naturally high in vitamin D are not frequently consumed. Therefore, in cases of insufficient cutaneous synthesis, the US population is largely dependent on food fortification and supplementation to maintain adequate vitamin D status (94). The primary vitamin D-fortified food source in the U.S. is milk. This fortification was initiated on a voluntary basis in the 1920's after vitamin D was identified as critical to the prevention of rickets (57). Other vitamin D fortified foods include ready-to-eat breakfast cereals, as well as certain brands of orange juice, yogurt and margarine (Table 2-2) (93).

Systematic reviews of randomized controlled trials have provided evidence that vitamin D fortification in the food supply increases serum 25(OH)D in both children and adults (95–97). Recent evidence from Finland demonstrated the effectiveness of food fortification policy as a strategy to prevent vitamin D deficiency in the general population (98). In this Finnish cohort, 91% of non-supplement users reached serum 25(OH)D concentrations >50 nmol/L through consumption of vitamin D-fortified milk products and fat spreads, compared to 44% pre-fortification (98). Similarly, in a randomized controlled trial, Madsen et al. reported that vitamin D fortification of milk and bread maintained wintertime vitamin D status >30 nmol/L and >50 nmol/L in 99% and 84% of individuals, respectively (99). In addition to food fortification, animal feeding practices can increase both the vitamin D3 and 25(OH)D content



of foods such as meat, poultry and eggs, and may provide an additional means to improve vitamin D status in the general population (100–103).

Food	Vitamin D content (IU)
Fortified dairy products	
Milk, 1 c	115
Yogurt (fortified), 1 c	115
Other fortified foods	
Orange juice, 1 c	100
Ready-to-eat cereals (Cheerios), 1 oz	38
Ready-to-eat cereals (Total), 1 oz	100
Eggs, 1 large	44
Seafood	
Salmon, sockeye, canned, 1 oz	238 - 243
Trout, rainbow, cooked, 1 oz	224
Swordfish, cooked, 1 z	204
Salmon, pink, canned, 1 oz	159 – 164
Salmon, sockeye, cooked, 1 oz	149
Sturgeon, cooked, 1 oz	146
Mackerel, cooked, 1 oz	131
Mackerel, canned, 1 oz	83
Tuna, light, canned in oil, 1 oz	78
Herring, cooked, 1 oz	62
Sardines, canned in oil, 1 oz	56
Tilapia, cooked, 1 oz	42
Tuna, white, canned in water, 1 oz	
Tuna, light, canned in water, 1 oz	13

Table 2-2: Food Sources of Vitamin D\*

\*Adapted from Scientific Report of the 2015 Dietary Guidelines Committee (70)

Currently, the United States Department of Agriculture Food Composition Database does not take the 25(OH)D content of animal-based foods into account. Given the frequent consumption of animal-based food products in the US, this may lead to an underestimation of the population's vitamin D intake (104). Accurate assessment of the population's current vitamin D intake is critical prior to the development of food fortification strategies to prevent



deficiency. Evidence suggests that intake of 25(OH)D is ~5 times more potent in raising serum 25(OH)D compared to an equivalent amount of vitamin D3 (105). However, estimates of the potency factor vary from ~1.4 to 10, and there is not yet a consensus on the potency factor that should be used (106–109). Although the results of current food fortification programs provide compelling evidence for the use of vitamin D as a potential fortificant, a better understanding of the vitamin D content of foods, as well as simulations regarding the impact of vitamin D fortification, are required before dietary intervention.

#### Hyperhomocysteinemia

#### **Homocysteine Metabolism**

Homocysteine is a sulfur-containing amino acid derived from the metabolism of the essential amino acid methionine. Methionine is activated by methionine adenosyltransferase (MAT) to form *S*-adenosylmethionine (SAM), the universal methyl donor required for almost all transmethylation reactions. All SAM-dependent transmethylation reactions generate *S*-adenosylhomocysteine (SAH), which is then hydrolyzed by SAH hydrolase (SAHH) to form adenosine and homocysteine (110) (Figure 2-2). Homocysteine produced by SAM-dependent transmethylation must be remethylated back to methionine or irreversibly catabolized to cysteine by transsulfuration. Homocysteine remethylation occurs via both folate-dependent and independent mechanisms (Figure 2-2).





Figure 2-2: Folate, methyl group and homocysteine metabolism. BHMT, betaine homocysteine S-methyltransferase; CBS, cystathionine  $\beta$ -synthase; DMG, dimethylglycine; MAT, methionine adenosyltransferase; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; SAH. S-adenosylhomocysteine; SAHH. Sadenosylhomocysteine hydrolase; SAM, S-adenosylmethionine; THF, tetrahydrofolate; 5-CH<sub>3</sub>-THF, 5-methyltetrahydrofolate, X, methyl group acceptor.

The folate-dependent remethylation pathway utilizes the donation of a methyl group by 5-methyltetrahydrofolate (5-CH3-THF) via the vitamin B12-dependent enzyme methionine synthase (MS). Folate-independent remethylation is catalyzed by betaine-homocysteine Smethyltransferase (BHMT), a reaction that utilizes betaine, derived from the oxidation of choline, as a methyl donor. Alternatively, the irreversible catabolism of homocysteine is initiated by vitamin B6-dependent cystathionine  $\beta$ -synthase (CBS) to form cystathionine, which is further metabolized to cysteine (110) (Figure 2-2).



#### Pathogenesis

Hyperhomocysteinemia is a condition characterized by abnormally high concentrations of circulating homocysteine. Normal serum/plasma homocysteine concentrations range from 5-15 µmol/L, whereas concentrations of 16-100 µmol/L and >100 µmol/L represent moderate and severe hyperhomocysteinemia, respectively (111). Several factors have been associated with the development of hyperhomocysteinemia, such as genetics, chronic disease, male gender, advancing age and nutritional deficiencies (112–114). Moderate elevations in circulating homocysteine occur in 5-7% of the general population and are typically attributed to environmental and genetic factors, whereas cases of severe hyperhomocysteinemia are rare and can be attributed to major genetic mutations in key enzymes implicated in homocysteine metabolism (115). Hyperhomocysteinemia is recognized as an independent risk factor for cardiovascular disease; thus, maintenance of homocysteine balance carries important public health implications.

Several polymorphisms in genes encoding enzymes responsible for homocysteine metabolism have been described, including mutations in the 5,10-methylenetetrahydrofolate reductase (MTHFR) and CBS genes. MTHFR catalyzes the conversion of 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate (5-CH3-THF), which serves as a methyl donor in the folate-dependent remethylation of homocysteine to methionine. Normal MTHFR activity is critical in maintaining sufficient 5-CH3-THF concentrations to prevent the accumulation of homocysteine. The MTHFR gene has at least two functional polymorphisms, the most common of which encodes a cytosine to thymine substitution at nucleotide 677 (C677T) (116). The MTHFR C677T polymorphism results in reduced enzyme activity concurrent with moderately elevated circulating homocysteine concentrations (117). CBS


deficiency is an autosomal recessive disorder that impairs homocysteine disposal via the transsulfuration pathway. Mutations in the CBS gene cause homocystinuria, a condition characterized by high concentrations of homocysteine in the urine (118). In the absence of CBS, homocysteine accumulates in tissues and is exported to the blood, resulting in severe hyperhomocysteinemia. Circulating homocysteine concentrations have been reported to increase 10-20-fold in untreated patients (119). Clinical features of CBS-deficient patients include mental retardation, ectopic lenses and a high incidence of vascular pathology, where about 50% of untreated patients experience a thrombotic event by age 30 (120,121). The estimated frequency of CBS deficiency is 1:100,000 in western countries (122).

Maintenance of homocysteine balance is dependent on the vitamins folate, vitamin B6 and vitamin B12, which function as cofactors and substrates in homocysteine metabolism, as well as the essential nutrient choline. Several studies report an inverse association between vitamin B6, folate and vitamin B12 status and circulating concentrations of homocysteine (123–126). Vitamin B6 is required for the activity of CBS, the enzyme responsible for the irreversible catabolism of homocysteine. Vitamin B6 is widespread in the food supply, making deficiency uncommon. However, the prevalence of vitamin B6 deficiency is increased in certain groups, such as alcoholics or individuals with liver disease (127). In the folatedependent homocysteine remethylation reaction catalyzed by methionine synthase, 5-CH3-THF functions as a methyl donor and vitamin B12 serves as a key cofactor required for methionine synthase activity. A deficiency in either of these vitamins impairs homocysteine remethylation via methionine synthase. It is estimated that the prevalence of hyperhomocysteinemia (defined as > 13  $\mu$ mol/L) was reduced by ~50% following the 1996 Food and Drug Administration mandate requiring folic acid fortification of enriched grain



products in the United States (128). As such, current cases of hyperhomocysteinemia due to nutritional deficiency are likely related to a deficiency in vitamin B12. A vitamin B12 deficiency impacts homocysteine metabolism directly by impairing methionine synthase activity, which, in turn, traps methyl groups in the form of 5-CH3-THF (129). Vitamin B12 is found exclusively in foods of animal origin, placing vegans at risk for deficiency (130). In a study evaluating B vitamin status and circulating homocysteine, hyperhomocysteinemia was observed in 66% of individuals adhering to a vegan diet (131). Additionally, vitamin B12 absorption requires the release of protein-bound vitamin B12 by hydrochloric acid and gastric protease, followed by the binding of free vitamin B12 to intrinsic factor, which is released from the parietal cells of the stomach. Thus, patients with conditions such as pancreatic exocrine insufficiency and atrophic gastritis, a condition affecting 10-30% of older adults, are at risk for hyperhomocysteinemia subsequent to vitamin B12 malabsorption (132). It is estimated that over 12% of individuals over the age of 60 are vitamin B12 deficient, which may explain the increase in circulating homocysteine concentrations associated with aging (126,133). Lastly, plasma homocysteine is inversely associated with dietary intakes of choline and betaine, which function as a methyl donors in the folate-independent remethylation of homocysteine via BHMT (134–136).

In addition to genetic and nutritional etiologies of hyperhomocysteinemia, elevated circulating homocysteine is also associated with male gender and chronic disease. Hyperhomocysteinemia is strongly associated with end-stage renal disease, significantly increasing the risk of vascular comorbidities in affected patients (137,138). The exact mechanism underlying this association is not completely understood; however, it has been hypothesized that increased concentrations of protein-bound homocysteine may impair the



glomerular filtration rate, decreasing renal clearance of homocysteine (139). Other disease states associated with high circulating concentrations of homocysteine include hypothyroidism, estrogen deficiency, and the use of several medications, such as phenytoin, sulfzalazine and methotrexate. These medications may impair normal homocysteine metabolism by depleting folate stores or by impairing the synthesis of vitamin cofactors involved in homocysteine remethylation and transsulfuration (140). Lastly, a number of studies report gender effects on total homocysteine, with men exhibiting significantly higher circulating homocysteine concentrations compared to women (141–143).

## Association with Cardiovascular Disease

The relationship between hyperhomocysteinemia and cardiovascular risk was first described in patients with homocystinuria due to a hereditary deficiency in CBS. The high incidence of vascular pathology in homocystinuric children, combined with data from animal studies, led McCully and Wilson to hypothesize that elevated circulating homocysteine concentrations may cause arteriosclerosis (144). Since then, numerous reports have established increased risk of several cardiovascular pathologies, including coronary artery disease, myocardial infarction and stroke, in patients suffering from both moderate and severe forms of hyperhomocysteinemia. Findings from an early meta-analysis indicated that 10% of the populations' risk of coronary artery disease was attributable to circulating homocysteine (145). A more recent meta-analysis found a 32% increase in ischemic heart disease risk and a 59% increased risk of stroke associated with each 5 µmol/L elevation in circulating homocysteine (146). In a statistical analysis of the Multi-Ethnic Study of Atherosclerosis and National Health and Nutrition Examination Survey III datasets, hyperhomocysteinemia (> 15 µmol/L) was



significantly associated with cardiovascular events and mortality (147). In a prospective cohort of middle-aged women, elevated homocysteine was an independent risk factor for myocardial infarction incidence and mortality (148). Although the mechanisms underlying the relationship between hyperhomocysteinemia and cardiovascular disease have not been fully elucidated, factors that likely play a role include oxidative stress, dysregulated methylation, and posttranslational modification by homocysteinylation (149). Homocysteine oxidation results in the production of superoxide and hydroxyl radicals, reactive oxygen species known to induce endothelial cell injury (150,151). Altered homocysteine and methyl group metabolism affects the transmethylation capacity of the cell and there is evidence that hyperhomocysteinemia results in DNA and protein hypomethylation, thereby regulating gene expression and modifying protein function (152-156). Lastly, homocysteine thiolactone, a thioester metabolite of homocysteine, has been shown to target and covalently bind numerous circulating proteins, modifying their structure and/or function (157,158). The aforementioned mechanisms may result in endothelial dysfunction, increased collagen synthesis leading to decreased elasticity of the arterial wall, and smooth muscle cell proliferation, factors implicated in the pathogenesis of cardiovascular disease (149,159).

### Treatment

The demonstration that hyperhomocysteinemia is an independent risk factor for cardiovascular disease has generated interest in homocysteine-lowering therapies as a means to reduce cardiovascular disease-associated morbidity and mortality. Treatment strategies for patients with hereditary homocystinuria due to CBS deficiency are as follows: 1) increase residual CBS activity with pharmacological doses of vitamin B6, 2) restrict dietary methionine to decrease load on the transsulfuration pathway, 3) provide oral betaine to increase



homocysteine remethylation to methionine (160). CBS deficient patients can be classified as either responsive or non-responsive to treatment with high-dose vitamin B6 (120). In vitamin B6-unresponsive patients, CBS deficiency is managed by methionine restriction and betaine supplementation (160). For individuals with moderate hyperhomocysteinemia, a reduction in circulating homocysteine can be accomplished by increased intake of the B vitamins folate, vitamin B6 and vitamin B12, either by increasing dietary intake, supplementation, or both. The current Recommended Dietary Allowance (RDA) for these vitamins is 400 µg of dietary folate equivalents, 1.3 mg of vitamin B6 and 2.4 µg of vitamin B12 for non-pregnant, non-lactating adults. The RDA for vitamin B6 increases to 1.5 mg and 1.7 mg, respectively, for females and males over 51 years of age (161). Sources of these vitamins include fortified grains and cereals, dark leafy green vegetables, and legumes as a source of folate; fortified breakfast cereals, fish, beef, poultry and starchy vegetables as a source of vitamin B6; and fish, meat, poultry, eggs, milk, and fortified breakfast cereals as a source of vitamin B12 (161).

Several epidemiological studies have shown associations between dietary intake patterns and circulating homocysteine (123,126,162,163), and dietary interventions have been successful at reducing plasma homocysteine (164–168). In the Framingham Heart Study, intake of multivitamin supplements, breakfast cereals and leafy green vegetables was associated with reduced concentrations of circulating homocysteine (162). In a randomized, controlled trial of subjects with plasma homocysteine concentrations  $\geq 9 \ \mu mol/L$ , folic acid supplementation, consumption of folic acid-fortified breakfast cereals, and increased consumption of folate-rich foods decreased plasma homocysteine by 21, 24 and 9%, respectively (168). Decreases in plasma homocysteine have also been reported in dietary interventions aimed at increasing fruit and vegetable intake (164–166). Vitamin therapy with



supplemental folic acid, vitamin B6 and vitamin B12 has been highly successful at lowering circulating homocysteine. Numerous clinical trials have shown homocysteine-lowering benefits of B vitamin therapy, with reported reductions up to 39% (169–177).

Results from the Homocysteine Studies Collaboration meta-analysis predicted an 11% and 19% reduction in risk of ischemic heart disease and stroke, respectively, per 3 µmol/L reduction in homocysteine (178). In patients with severe hyperhomocysteinemia due to CBS deficiency, treatment with various combinations of vitamin B6, folic acid, vitamin B12, dietary methionine restriction and betaine reduced homocysteine concentrations from severe to moderately elevated, and markedly reduced vascular events (179). However, B vitamin therapy has not substantially improved cardiovascular outcomes in patients with moderate hyperhomocysteinemia, despite successful reductions in circulating homocysteine. For example, in the Vitamin Intervention for Stroke Prevention (VISP) Trial, moderate reductions in total homocysteine following vitamin therapy with folic acid, vitamin B6 and vitamin B12 had no beneficial effect on vascular outcomes (180). Likewise, homocysteine lowering with supplemental folic acid, vitamin B6 and vitamin B12 had no impact on the risk of cardiovascular events in vascular disease patients enrolled in the Heart Outcomes Prevention Evaluation 2 (HOPE-2) Trial (170). Similar conclusions were reached by other large, randomized clinical trials [NORVIT (173), SEARCH (174), WENBIT (175), WAFACS (176), HOST (177)]. In contrast, a meta-analysis investigating the effect of B vitamin therapy on cerebrovascular risk reported reduced risk of overall stroke following homocysteine-lowering with B vitamin supplementation (125). A recent Cochrane review found that homocysteinelowering interventions (vitamins B6, B9 or B12 given alone or in combination) did not reduce



risk of myocardial infarction; however, homocysteine-lowering interventions were associated with a small but statistically significant reduction in stroke (181).

There are several possible explanations for observed cardiovascular outcomes in clinical trials of homocysteine-lowering therapies. Many of the clinical trials recruited patients with existing cardiovascular disease as a form of secondary prevention; therefore, conclusions cannot be made regarding the efficacy of homocysteine-lowering therapy as a form of primary prevention. Additionally, cardiovascular disease comprises numerous conditions affecting the heart and blood vessels and, as such, response to treatment may differ depending on the clinical endpoint. Moreover, as posited by Bostom et. al., the presence of folic acid fortification may reduce the ability to detect differences between placebo and treatment groups (172). Factors such as medication use, duration of homocysteine-lowering therapy, and duration of followup may also play a role. Indeed, additional analyses that focused solely on stroke outcomes from the HOPE-2 trial demonstrated a reduced risk of overall stroke in individuals receiving combined folic acid, vitamin B6 and vitamin B12 therapy (171). Subgroup analyses indicated a larger risk reduction in patients with baseline homocysteine concentrations in the highest quartile (> 13.8 µmol/L) and in patients residing in countries without folic acid fortification (171). In a meta-analysis by Wang et. al., primary prevention with folic acid supplementation significantly reduced risk of stroke (182)

### **Dietary Whole Eggs**

# **Nutritional Value of Eggs**

It is widely recognized that eggs are a source of high quality protein and contain a high nutrient-to-energy density ratio. One large egg contains just 70 kcal, while providing nutrients



such as choline, riboflavin, folate, vitamin B12, vitamin A and vitamin D (Table 2-3) (183). Eggs are also a highly bioavailable source of the carotenoids lutein and zeaxanthin, antioxidants that may play a role in disease prevention (184). Additionally, eggs are readily available, affordable, easy to prepare, and thus play an important role in maintaining nutritional adequacy in groups such as the elderly, low-income families, and individuals restricting caloric intake. Song and Kerver reported that eggs contributed 20-30% of the RDA for vitamins A, E and B12 among egg consumers in the National Health and Nutritional Examination Survey III, whereas non-consumers were less likely to meet the RDA for these vitamins (185). Based on the National Health and Nutrition Examination Survey (NHANES) 2001-2008 24 h diet recall data, approximately 20% of the population report consuming eggs on a given day (186). Several studies estimate daily egg consumption of ~0.5 servings/d in the U.S. population (187–189).

Eggs are one of the best sources of dietary choline, providing 117 mg per large egg (Table 2-3) (190). Choline is an essential nutrient required for neurotransmitter synthesis, cell signaling, lipid transport and methyl group metabolism. Low choline status results in an inability to remethylate homocysteine via the folate-independent pathway catalyzed by BHMT and is associated with elevations in circulating homocysteine. Thus, maintaining adequate choline stores is important in preventing hyperhomocysteinemia and the associated cardiovascular risk. Choline can be obtained via dietary intake or de novo synthesis, although de novo synthesis alone is not sufficient to meet human needs (191). Endogenous biosynthesis of choline is catalyzed by phosphatidylethanolamine N-methyltransferase (PEMT), resulting in the methylation of phosphatidylethanolamine to form phosphatidylcholine. Genetic polymorphisms, such as polymorphisms in the PEMT gene, affect demands for dietary choline,



resulting in significant variation in the dietary choline requirement (192). The recommended adequate intake (AI) for choline for adults is 425 mg/d for women, 450 mg/d for pregnant women, 550 mg/d for lactating women and 550 mg/d for men (191). Several studies demonstrate that the majority of the population is not meeting the recommended AI for choline (135,193,194), although others report sufficient intakes (195). Of note, the AI set for choline does not take into account genetic polymorphisms that increase dietary methyl group requirements, and it is estimated that up to 50% of the population may require choline intakes above the AI (196).

Eggs are also a source of vitamin D, providing both vitamin D3 and 25(OH)D, as described previously (Table 2-3). Both vitamin D3 and 25(OH)D can be efficiently transferred from the hen's feed to the egg yolk in a dose-dependent manner, making eggs a good candidate for biofortification (103). Indeed, studies have demonstrated that supplementation of hen feed with varying amounts of vitamin D3 and 25D results in a marked increase in the vitamin D3 and 25(OH)D content of eggs (103). Additionally, eggs naturally contain a higher amount of 25(OH)D content of eggs (103). Additionally, eggs naturally contain a higher amount of 25(OH)D than several other animal-based foods, and 25(OH)D is known to raise serum 25(OH)D more readily than vitamin D3 (104,197). One whole, large egg (50 g) contains approximately 1.25  $\mu$ g (50 IU) vitamin D3 and 0.325  $\mu$ g (13 IU) 25(OH)D (104). Adjusting for 25(OH)D potency using a factor of 5, the whole eggs provide 1.625  $\mu$ g (65 IU) of 25(OH)D. Therefore, when taking into account 25(OH)D potency, consumption of one large egg meets 19% of the RDA for vitamin D (57,104).



Nutrient	1 large egg (50.0 g)	Vitamin and Mineral % RDA <sup>b</sup>
Energy (kcal)	72	—
Protein (g)	6.25	—
Total lipid (g)	4.74	—
Fatty acids, total saturated (g)	1.556	—
Fatty acids, total monounsaturated (g)	1.821	
Fatty acids, total polyunsaturated (g)	0.952	
Cholesterol (mg)	185	
Calcium (mg)	28	3
Iron (mg)	0.88	men 11
		women 5
Magnesium (mg)	6	2
Phosphorus (mg)	98	17
Zinc (mg)	0.65	7
Riboflavin	0.194	16
Vitamin B-6 (mg)	0.072	6
Folate, DFE <sup>b</sup> (µg)	18	5
Vitamin B-12 (µg)	0.35	15
Vitamin A, RAE <sup>b</sup> (µg)	80	10
Vitamin D (IU)	41	7
Choline (mg)	117	23

Table 2-3: Nutritional Composition of Whole Eggs<sup>a</sup>

<sup>a</sup> Adapted from the United States Department of Agriculture National Nutrient Database (198).

<sup>b</sup>DFE, dietary folate equivalent, RAE, retinol activity equivalent, RDA, recommended dietary allowance.

# Egg Consumption and Health

Satiety

It is well established that dietary protein is associated with increased measures of satiety, and increased intake of satiating foods may provide a means to limit energy intake and promote weight loss. One large egg contains 6.28 g of protein, meeting 12% of the daily value



based on a 2000 kcal diet (198). The protein digestibility-corrected amino acid score (PDCAAS) for eggs is 118, higher than that of many protein sources, including beef and soy with PDCAAS scores of 92 and 91, respectively (199). Numerous studies have demonstrated a satiating effect of dietary whole eggs or egg protein, in both rats and humans (200–203). Using a 3-way crossover design, Fallaize et al. demonstrated that consumption of an egg breakfast resulted in greater satiety in healthy men, as assessed by visual analog scales, compared to a cereal or croissant breakfast (204). Furthermore, the egg breakfast resulted in lower energy intake at lunch and dinner compared to the cereal and croissant breakfasts (204). In overweight and obese subjects fed a breakfast consisting of eggs or an isocaloric bagel breakfast, the egg breakfast resulted in higher satiety and reduced energy intake at lunch and in the next 36 hours (202). Additionally, Ratliff et al. reported that consumption of an egg breakfast resulted in a suppressed ghrelin response in healthy male and female subjects compared to an isocaloric bagel breakfast (203). Of note, Nielsen et al. demonstrated that measures of satiety and ab libitum energy intake did not differ between subjects fed macronutrient-balanced, isocaloric meals based in vegetable protein (fava beans and split peas), meat (veal and pork) or eggs, indicating that different protein sources have comparable effects on appetite (205).

A limited number of studies have investigated the impact of habitual egg intake on body weight regulation. In an 8 week dietary intervention study, overweight and obese participants were assigned to an egg or bagel breakfast, with or without concomitant energy restriction (206). Consumption of the egg breakfast resulted in a greater reduction in BMI, waist circumference and body fat and a greater weight loss compared to the bagel breakfast in the groups assigned to energy-restricted diets, whereas no differences in body weight



parameters were observed between egg and bagel breakfasts in the non-energy-restricted groups (206). In obese Chinese adolescents assigned to an egg or steamed bread breakfast for 3 months, consumption of an egg breakfast resulted in significant weight loss (207). In contrast, Pearce et al. and Fuller et al. did not observe differences in weight loss following 12 weeks and 21 weeks of energy restriction, respectively, between type 2 diabetic subjects fed high-egg or low-egg diets (208,209). Likewise, Rueda and Kholsa found no differences in body weight regulation in university students assigned to a breakfast with or without eggs over a 14 week semester (210). It appears that the short-term satiating effect of eggs does not always translate into long-term weight loss, although research on this topic is limited and additional studies are needed.

# Cardiovascular Health

The hypothesis that dietary cholesterol may contribute to cardiovascular disease risk was first proposed in the Framingham Heart Study, which provided evidence for a relationship between serum cholesterol and coronary heart disease (211,212). On average, daily cholesterol intake ranges from 200-350 mg/d in the U.S., depending on factors such as gender and age (213). Egg consumption contributes significantly to dietary cholesterol intake, with one large egg providing approximately 200 mg cholesterol (214,215). In 1968, the American Heart Association published recommendations to limit cholesterol consumption to <300 mg/d and to limit egg consumption to no more than 3 eggs/wk, based on the theory that reducing dietary cholesterol would result in reduced circulating cholesterol and a subsequent reduction in cardiovascular disease risk (216). However, scientific evidence for the recommended cholesterol restriction from studies using animal models often required pharmacological doses to elicit hypercholesterolemia, and epidemiological studies failed to account for confounding



factors, such as saturated fat intake (217). A number of epidemiological studies report that dietary cholesterol is no longer associated with coronary heart disease when dietary fiber and saturated fat are included in the analysis (218–221). For example, in the Seven Countries Study, the correlation between cholesterol intake and coronary heart disease mortality became insignificant after adjusting for saturated fat (221,222). Furthermore, research demonstrating that human subjects could compensate for an increase in cholesterol intake by decreasing cholesterol absorption and/or endogenous cholesterol synthesis and by increasing bile acid excretion cast doubt on the recommendation to limit egg consumption (223-225). In the following years, numerous studies investigated the effect of dietary cholesterol on plasma cholesterol concentrations and cardiovascular risk. Today, an extensive body of research indicates that dietary cholesterol does not significantly contribute to cardiovascular disease risk. In light of these findings, the Dietary Guidelines for Americans did not include a recommendation to limit dietary cholesterol consumption in the 2015 edition (69). However, recent findings using pooled data from 6 prospective cohort studies in the U.S., with a mean follow-up of 17.5 years, have challenged this recommendation (226). The study found that, for each additional 300 mg of cholesterol or half egg consumed, there was an increased risk of incident cardiovascular disease and all-cause mortality (226).

Meta-analyses have demonstrated that increased cholesterol intake is associated with marginal increases in plasma cholesterol (227). Howell et al. reported a change of 2.2 mg/dL in plasma cholesterol per 100 mg/d change in dietary cholesterol (228). However, the increase in plasma total cholesterol is concomitant with increases in both low-density lipoprotein (LDL) cholesterol and high-density lipoprotein (HDL) cholesterol. As a result, the LDL:HDL ratio, a major determinant of cardiovascular disease risk, remains unchanged or only minimally altered



(227). It is important to note that there is considerable heterogeneity in the response to dietary cholesterol in humans (223). Circulating cholesterol concentrations are influenced by cholesterol absorption, endogenous cholesterol synthesis, and rates of bile acid synthesis and excretion (229). In a study quantifying cholesterol absorption in men and women using duel stable isotopic tracers, cholesterol absorption ranged from 29-80%, with a mean absorption of 56% (230). The majority of human subjects compensate for increased cholesterol intake by effective feedback control mechanisms. These individuals are classified as "hypo-responders" to dietary cholesterol, as increased intake does not significantly increase circulating cholesterol concentrations (227). In contrast, individuals who experience significant changes in plasma cholesterol in response to cholesterol intake are classified as "hyper-responders" (227). McNamara et al. examined individual responses to low (~250 mg/d) and high (~800 mg/d) cholesterol diets and found a 26% reduction in endogenous cholesterol synthesis as well as reduced cholesterol absorption in subjects whose plasma cholesterol remained unchanged in response to high dietary cholesterol intake (223). Likewise, Bruno et al. reported that an intake of 3 eggs/d for a period of 4 wk downregulated cholesterol synthesis while maintaining the LDL:HDL ratio (231). In pre-menopausal women, additional dietary cholesterol provided by eggs resulted in no changes in LDL cholesterol or HDL cholesterol in hypo-responders, whereas both LDL and HDL cholesterol were increased in hyper-responders (232). Importantly, the LDL: HDL ratio remained unchanged in both groups (232). Other studies have also reported no change or minimal changes to the LDL:HDL ratio following a period of egg consumption (233,234). The finding that dietary cholesterol has little impact on the LDL:HDL ratio may help explain why cholesterol consumption has little impact on cardiovascular disease risk.



Studies regarding the relation between egg consumption and cardiovascular disease have focused largely on the cholesterol content of eggs; however, eggs have recently received attention for their phosphatidylcholine and choline content as well. It has been suggested that dietary choline may potentially elicit negative health effects through production of trimethylamine-N oxide (TMAO), a metabolite that has emerged as a potential predictor of cardiovascular risk (235). TMAO can be found in the diet in the preformed state, or it can be produced by the liver following the metabolism of its precursors, choline and carnitine, by the intestinal microbiota (235). Findings by Tang et al. and Miller et al. demonstrate that consumption of 2 or more eggs increases plasma concentrations of TMAO (236,237). In contrast, DiMarco et al. reported no changes in plasma TMAO following intake of 2-3 eggs/d for 4 wk (238). Circulating concentrations of TMAO were shown to predict cardiovascular disease risk in cardiac patients (236) and were predictive of myocardial infarction and stroke (236). Similarly, serum TMAO concentrations were associated with the number of infarcted coronary arteries in patients undergoing cardiovascular surgery (239). It remains to be determined whether TMAO plays a causative role in cardiovascular disease progression, or is simply a marker of cardiovascular pathology. It is important to note that fish is a food naturally high in TMAO, and fish consumption is associated with reduced cardiovascular risk (240). Furthermore, dietary choline intake is associated with several health benefits, and foods containing TMAO precursors provide many important nutrients (54). Thus, further studies are warranted before any conclusions regarding diet, TMAO and cardiovascular risk can be made.

To date, the majority of scientific evidence documents no effect of egg consumption on cardiovascular disease risk. Hu et al. reported no difference in the relative risk of coronary heart disease in participants consuming less than 1 egg/wk compared to participants consuming



 $\geq$  1 egg/d (241). In a prospective study of healthy, middle-aged men, no associations between egg consumption and incidence of cardiovascular disease, stroke, or myocardial infarction were identified (242). Likewise, consumption of up to 1 egg/d was not associated with cardiovascular mortality in a Mediterranean population (243). In a large Chinese cohort, moderate egg consumption was associated with reduced cardiovascular risk (244). A metaanalysis conducted by Alexander et al. found that daily egg intake is not associated with coronary heart disease risk, and may be associated with a reduction in stroke risk (245). Similarly, consumption of 1 egg/d did not increase risk of cardiovascular disease in the Guangzhou Biobank Cohort Study, and was associated with a reduced risk of stroke (246). Lastly, a dose response meta-analysis of prospective cohort studies did not find any link between daily egg consumption and risk of coronary heart disease or stroke (247). However, as mentioned previously, a recent analysis of pooled data from 6 prospective U.S. cohorts reported a significant, dose-dependent association between egg consumption and cardiovascular disease risk (226). Thus, the associations between consumption and heart health remain controversial and likely depend on an individual's lifestyle and genetics, as well as the presence of cardiovascular risk factors.

### *Type 2 Diabetes*

The relation between egg consumption and health outcomes in type 2 diabetics and individuals at risk for T2D is unclear and requires further assessment. Although some studies suggest that egg consumption may increase T2D risk, others find no association or a beneficial impact on T2D risk factors (248–252). There does appear to be an association between egg consumption and cardiovascular comorbidity in the diabetic population (245,247,253),



although this association has not been observed in all studies (254,255). In a meta-analysis of prospective cohort studies, diabetics consuming >1 egg/d had a hazard ratio of 1.69 for overall cardiovascular disease risk compared to diabetics consuming  $\leq 1 \text{ egg/wk}$  (253). However, Tran et al. caution against drawing broad conclusions regarding the role of egg consumption in cardiovascular disease risk among diabetics, stating that shared risk factors between cardiovascular disease and type 2 diabetes make it extremely difficult to interpret results from epidemiological studies examining this complex relationship (255). Notably, a number of studies have demonstrated that dietary cholesterol is poorly absorbed in obese and insulin resistant individuals (256,257), suggesting that any potential relationship between egg intake and cardiovascular disease risk among diabetics may not be related to the cholesterol content of eggs. Furthermore, numerous prospective studies examining egg consumption in insulin resistant and type 2 diabetic individuals report favorable cardiometabolic outcomes, or no adverse effects (208,209,258–261). For example, Pearce et al. reported reduced total cholesterol, non-HDL cholesterol and triglycerides in type 2 diabetic and glucose intolerant individuals consuming an energy-restricted, high-protein diet containing 2 eggs/d (208). Likewise, Blesso et al. found that consumption of 3 eggs/d for 12 wk improved lipoprotein profiles in individuals with metabolic syndrome (258). Of note, many of the studies reporting favorable metabolic outcomes combined egg consumption with carbohydrate or energy restriction.

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# CHAPTER 3. DIETARY WHOLE EGG CONSUMPTION ATTENUATES BODY WEIGHT GAIN AND IS MORE EFFECTIVE THAN SUPPLMENTAL CHOLECALCIFEROL IN MAINTAINING VITAMIN D BALANCE IN TYPE 2 DIABETIC RATS

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whole egg attenuates body weight gain and is more effective than supplemental cholecalciferol in maintaining vitamin D balance in type 2 diabetes. *J Nutr* 2017, 147: 9,

1715-21, reprinted by permission of Oxford University Press.

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# Abstract

**Background:** Type 2 diabetes (T2D) is characterized by vitamin D insufficiency owing to excessive urinary loss of 25-hydroxycholecalciferol (25(OH)D). We previously reported that a diet containing dried whole egg, a rich source of vitamin D, was effective at maintaining circulating 25(OH)D concentrations in T2D rats. Furthermore, whole egg consumption reduced body weight gain in T2D rats.

**Objective:** This study was conducted to compare whole egg consumption to supplemental cholecalciferol with respect to vitamin D balance, weight gain, and body composition in T2D rats.

**Methods:** Male Zucker diabetic fatty (ZDF) rats (n= 24) and their lean controls (n=24) were obtained at 5 wk of age and randomly assigned to 3 treatment groups: a casein-based diet (CAS), a dried whole egg-based diet (WE), or a casein-based diet containing supplemental cholecalciferol (CAS+D) at the same level of cholecalciferol provided by the dried whole egg-based diet (37.6  $\mu$ g/kg diet). Rats were fed their respective diets for 8 wk. Weight gain and



food intake were measured daily, circulating 25(OH)D concentrations were measured by ELISA, and body composition was analyzed by dual X-ray absorptiometry.

**Results:** Weight gain and percent body fat were reduced by approximately 20% and 11%, respectively, in ZDF rats fed WE compared to ZDF rats fed CAS or CAS+D. ZDF rats fed CAS had 21% lower serum 25(OH)D concentrations than lean rats fed CAS. In ZDF rats, WE consumption increased serum 25(OH)D concentrations 130% compared to CAS, whereas consumption of CAS+D increased serum 25(OH)D concentrations 35% compared to CAS.

**Conclusion:** Our data suggest that dietary consumption of whole egg is more effective than supplemental cholecalciferol in maintaining circulating 25(OH)D concentrations in T2D rats. Moreover, whole egg consumption attenuated weight gain and reduced percent body fat in ZDF rats. These data may support new dietary recommendations targeting prevention of vitamin D insufficiency in T2D.

## Introduction

Although vitamin D insufficiency is common globally, it is highly predominate in type 2 diabetes (T2D), affecting 70-90% of the T2D population (1-3). Vitamin D insufficiency is defined as circulating 25-hydroxycholecalciferol (25(OH)D) concentrations between 30-50 nmol/L (12-20 ng/mL), whereas deficiency is defined as serum 25(OH)D concentrations below 30 nmol/L (12 ng/mL). Evidence from prospective studies suggests a correlation between inadequate vitamin D concentrations and T2D (4-6). Specifically, vitamin D deficiency may be a factor in the development of insulin resistance as well as the pathogenesis of T2D by affecting either insulin sensitivity,  $\beta$ -cell function or both (7-9); however, other studies have found little or no association between T2D and these measures (10, 11). Furthermore, we have



urinary excretion of circulating 25(OH)D and vitamin D binding protein (DBP), thereby exacerbating vitamin D deficiency (12-14). Therefore, there is a critical need to identify dietary intervention strategies to prevent and/or treat vitamin D deficiency in the diabetic population.

Dietary vitamin D exists in two forms, ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3). The most abundant dietary form is cholecalciferol, which is metabolized in the liver to 25(OH)D by humans and animals; therefore, animal-based foods are a source of 25(OH)D as well as cholecalciferol. The precursor form of active vitamin D is 25(OH)D, which, in the circulation, reflects an individual's vitamin D status. Vitamin D recommendations for diabetics are inconsistent. Randomized clinical trials focusing on T2D outcomes vary with respect to vitamin D dose and regimen, ranging from 1000-6000 IU/ d to 20,000-40,000 IU /wk with study durations lasting from several months to years. Results from these studies differ with respect to improvements in fasting blood glucose, glycated hemoglobin (HbA1c), and insulin sensitivity (15-19). While the current RDA for vitamin D in adults is 600 IU/d, supplementation guidelines remain an intense topic of debate. Although an intake of 600 IU/d is sufficient to support musculoskeletal health, more studies are needed to clearly assess the impact of supplementary cholecalciferol on chronic diseases (20). As reviewed by Mathieu (21), growing evidence supports the adoption of the international guidelines on supplementation of cholecalciferol at 500-1000 IU/d to prevent vitamin D deficiency and reduce the risk of T2D onset.

Treatment of T2D is primarily focused on lifestyle modifications, including improvements in diet and physical activity, to promote weight loss and improve blood glucose control. We have previously shown that dietary resistant starch was an effective dietary strategy for maintaining vitamin D balance by protecting renal health, thereby preventing



urinary excretion of 25(OH)D and DBP. In contrast, the present study utilized dried whole eggs to focus on increasing dietary consumption of vitamin D as a means to improve vitamin D status. Whole eggs are an excellent source of vitamin D, in the form of both 25(OH)D and cholecalciferol, which is found exclusively in the yolk (22). Promoting egg consumption has been a controversial diet recommendation for individuals with T2D because of the rich cholesterol content of eggs. Because diabetics are at an increased risk for cardiovascular disease (CVD), they have been encouraged to limit the number of eggs they consume. To date, there are a number of studies that contradict the relation between egg consumption and chronic disease (23-27). More importantly, recent revisions to the Dietary Guidelines for Americans no longer include recommendations to limit intake of dietary cholesterol, a decision based on the growing body of research showing that dietary cholesterol intake has little effect on serum cholesterol concentrations and subsequent health risks (28). Furthermore, numerous human studies report that egg consumption is associated with increased satiety, which leads to reduced overall caloric intake (29-33). Some human studies also report that egg consumption promotes weight loss; however, the literature regarding the effect of egg consumption on body weight management remains inconsistent (34, 35). Nevertheless, a growing body of research demonstrates several benefits of whole egg consumption, such as the high nutrient content and satiating effect of whole eggs; thus, dietary whole egg consumption may be beneficial in the diabetic population (36-38).

We previously reported that a dried whole egg-containing diet is a highly effective strategy to maintain circulating 25(OH)D concentrations in T2D rats (39). Additionally, whole egg consumption reduced weight gain in diabetic rats. Thus, the primary objectives of this follow-up study were 1) to compare the vitamin D provided by whole eggs to a diet



supplemented with cholecalciferol in maintaining serum vitamin D balance and 2) to further investigate the effect of whole egg consumption on weight gain and body composition in T2D rats.

#### **Materials and Methods**

Animals and Diets. All animal studies were approved by the Institutional Animal Care and Use Committee at Iowa State University and were performed according to the Iowa State University Laboratory Animal Resources Guidelines. Male Zucker diabetic fatty (ZDF; fa/fa) rats (n= 24) and lean (fa/+) control rats (n= 24) were purchased at 5 wk of age (Charles River Laboratories). Rats were housed individually in plastic cages in a temperature-controlled room with a 12-h light-dark cycle. All diets were formulated and pelleted by Research Diets, Inc. Dried whole egg was purchased from Rose Acre Farms and sent to Research Diets, Inc. for diet formulation. All rats were acclimated to a semi-purified diet (AIN-93G) for 1 wk. Rats were randomly assigned to 1 of 3 experimental diets (Table 3-1): a casein-based diet (CAS), a dried whole egg-based diet (WE), or a casein-based diet containing supplemental cholecalciferol (CAS+D) provided at the same level of cholecalciferol supplied by the WE diet  $(37.6 \ \mu g/kg \ diet)$ . Vitamin mix in all diets provided 25  $\mu g$  vitamin D/kg diet. The whole egg diet contained an additional 12.6 µg cholecalciferol/kg diet, thus, the WE diet provided a total of 37.6 µg cholecalciferol/kg diet. This level was matched in the CAS+D diet with the addition of 12.6 µg cholecalciferol. All diets provided protein at 20% (w/w) and were matched for lipid content (18.3%) via the addition of corn oil to the CAS and CAS+D diets, accounting for the additional lipid provided by the dried whole egg. Rats were given ad libitum access to food and water for 8 wk. Food intake was recorded daily for each rat beginning at wk 3 of the study. Pelleted diets were weighed and distributed daily. Consumption was defined as the difference



in pellet weight within a 24 h period. Prior to sacrifice, rats were placed in metabolic cages for 12 h, during which urine was collected and then stored at -80° C for subsequent analysis. Rats were anesthetized via a single intraperitoneal injection of ketamine:xylaxine (90:10 mg/kg body weight). Whole blood was collected via cardiac puncture and blood glucose was measured using a glucometer (Bayer Healthcare). Body fat, lean body mass, bone mineral density and bone mineral content were measured post-necropsy using dual energy x-ray absorptiometry (DEXA).

*Biochemical Analysis.* Analysis of serum and urinary creatinine was measured using commercially available colorimetric kits (Cayman Chemical). Urinary total protein concentrations were measured using a bicinchoninic acid assay (Thermo Scientific Pierce), serum concentrations of 25(OH)D were analyzed using a commercial enzyme immunoassay kit (Immunodiagnostic Systems), and urinary concentrations of 25(OH)D and DBP were also analyzed using a commercial enzyme immunoassay kit (Immunodiagnostic Systems), and urinary concentrations of 25(OH)D and DBP were also analyzed using a commercial enzyme immunoassay kit (Immunodiagnostic Systems and Life Diagnostics, respectively) as previously described (12, 13, 40). Authenticity of all kits for use on rodent biological samples has been verified by the manufacturer.

*Statistical Analysis.* All data were analyzed using SigmaPlot 9.0 (Systat Software Inc.). Mean values were evaluated for statistically significant differences (P < 0.05) using a two-way ANOVA (genotype x diet) followed by the Fisher's Least Significant Difference (LSD) post hoc test for multiple comparisons. Nonparametric analysis was utilized when normality failed or variances were unequal using a Kruskal-Wallis one-way ANOVA on Ranks.



#### Results

Whole egg consumption reduced total body weight and cumulative weight gain despite increased food intake in ZDF rats. Lean and ZDF rats initially gained the same amount of weight across all dietary groups within a given genotype. As expected, ZDF rats fed the CAS and CAS+D diets gained more weight throughout the study compared to all lean control rats (Figure 3-1A). However, ZDF rats fed WE exhibited a plateau in cumulative weight gain beginning on d 10 and an approximate 20% reduction in weight gain compared to ZDF rats fed CAS and CAS+D after 8 wk of dietary treatment. Furthermore, cumulative weight gain in ZDF rats fed WE was statistically equivalent to all lean control rats beginning on d 22, and for the remainder of the study. Although ZDF rats fed WE gained less weight than ZDF rats fed CAS and CAS+D, total food intake in ZDF rats fed WE was approximately 7% higher compared to ZDF rats fed CAS and CAS+D (Table 3-2). Beginning at wk 5, ZDF rats fed WE had higher weekly food intake per 100 g body weight compared to ZDF rats fed CAS and CAS+D, whereas weekly food intake (g/100 g body weight) did not differ in lean rats regardless of diet (Figure 3-1B). Moreover, cumulative weight gain and total food intake did not differ between the lean control rats fed either CAS, CAS+D or WE. There were no differences in the food efficiency ratio within the ZDF genotype. In contrast, the food efficiency ratio [(weight gain, g/food intake, g) x 100].) was 12% lower in lean rats fed WE compared to lean rats fed CAS (Table 3-2).

# ZDF rats fed WE exhibited a lower body fat percentage than ZDF rats fed CAS and CAS+D.

Percent body fat and percent lean body mass are presented in Figure 3-2. Percent body fat did not differ between dietary groups within lean control rats. In contrast, WE consumption in ZDF rats reduced body fat percentage by 8 and 13%, respectively, compared to ZDF rats fed CAS



and CAS+D. Lean body mass was increased by 11% in ZDF rats fed WE compared to ZDF rats fed CAS+D, whereas lean body mass did not differ, regardless of diet, within the lean rats. Bone mineral density did not differ across all dietary groups and genotypes (Table 3-2). Bone mineral content, expressed as a percentage of body weight, did not differ between dietary groups within the lean control rats. Because bone mineral content was corrected for body weight, bone mineral content was 7 and 9% higher, respectively, in WE-fed ZDF rats than in ZDF rats consuming CAS or CAS+D.

*The WE diet elevated circulating 25(OH)D concentrations to a greater extent than the CAS+D diet.* Serum 25(OH)D concentrations of all treatment groups are shown in Figure 3-3. As expected, ZDF rats fed CAS had lower (21%) serum 25(OH)D concentrations than their lean counterparts fed CAS. The WE diet increased 25(OH)D concentrations by 130% compared to ZDF rats fed CAS, whereas CAS+D increased circulating 25(OH)D by only 35% compared to ZDF rats fed CAS. Likewise, serum 25(OH)D concentrations of lean rats fed CAS+D and the WE diet were increased by 19% and 113%, respectively, compared to lean rats fed CAS. When compared to CAS+D, WE increased serum 25(OH)D concentrations by 80% and 70% in lean and ZDF rats, respectively.

*Serum and urinary biochemical measurements.* The presence of hyperglycemia in ZDF rats confirmed the diabetic state; however, blood glucose did not differ between dietary groups within the lean or ZDF genotype. In lean rats fed WE, serum insulin was lower than all other dietary groups. The homeostatic model assessment of insulin resistance (HOMA-IR) was decreased by 80 and 71%, respectively, in lean rats fed WE compared to lean rats fed CAS and



CAS+D. HOMA-IR values did not differ between dietary groups within the ZDF genotype. Urinary output, urinary 25(OH)D, urinary DBP and serum creatinine were increased in ZDF rats compared to lean rats. Urinary creatinine excretion was reduced in ZDF rats fed CAS and CAS+D by approximately 67% and 79%, respectively, compared to all lean rats. In contrast, urinary creatinine excretion did not differ in ZDF rats fed the WE diet compared to all lean rats. Urinary total protein excretion did not differ between lean and ZDF rats. WE consumption was without effect on urinary measures within the lean or ZDF genotype. Likewise, there were no differences in serum creatinine within lean or ZDF rats (Table 3-3).

#### Discussion

We have previously shown that a dried whole egg-based diet is a highly effective strategy for maintaining serum 25(OH)D concentrations in rats with T2D (39). The present study demonstrates that vitamin D derived from whole egg may be more effective than an equivalent amount of supplemental cholecalciferol added to a casein-based diet at maintaining serum 25(OH)D concentrations. Serum 25(OH)D concentrations were markedly higher in both lean and ZDF rats fed WE compared to rats fed CAS+D. Consumption of the WE diet in ZDF rats resulted in elevated serum 25(OH)D despite urinary losses due to the presence of diabetic nephropathy. All ZDF rats exhibited excessive urinary excretion of 25(OH)D regardless of dietary group, which suggests that the increase in serum 25(OH)D in ZDF rats fed the WE diet was due to a mechanism other than attenuated urinary losses. The difference in serum 25(OH)D concentrations between the WE and CAS+D diets may be due to the potency of 25(OH)D contained within whole eggs. In support of this theory, Cashman et al. carried out a human study comparing orally supplemented 25(OH)D to cholecalciferol and found that oral supplementation with 25(OH)D raised serum 25(OH)D concentrations five times more than an



oral cholecalciferol supplement per microgram consumed (41). As reviewed by Ovesen et al., a number of studies have reported 25(OH)D to be more potent than the equivalent amount of cholecalciferol in raising serum concentrations of 25(OH)D, however, the exact potency factor remains undetermined (42).

Nutritionally, eggs boast a number of benefits; they are rich in high quality protein, contributing to satiety; contain a high nutrient-to-energy density ratio, and are inexpensive and easy to prepare (24, 43). Furthermore, egg consumption has been shown to increase circulating HDL-cholesterol concentrations, which is associated with lower CVD risk (44-46). Despite these advantages, there remains a negative perception toward egg consumption for individuals with diabetes. Previous studies have suggested that high egg consumption may be associated with higher CVD outcomes in people with T2D, a population already at risk for CVD (47-49); however, more recent studies contradict this finding. A randomized control trial found that consuming 2 eggs per d for 3 mo did not negatively affect the lipid profile of diabetics (23). A similar study reported that egg consumption, in combination with healthy dietary changes, improved glucose homeostasis, as well as lipid profiles in a diabetic population (45). Furthermore, the 2015 Dietary Guidelines for Americans no longer include recommendations to limit intake of dietary cholesterol as a direct result of the decades of research demonstrating little effect of dietary cholesterol on serum cholesterol concentrations and subsequent health risks (50). Taken together, egg consumption, as a source of vitamin D, represents a reliable dietary intervention strategy for maintaining serum 25(OH)D concentrations in diabetics without posing additional heart health risks.

We previously reported that whole egg consumption attenuated weight gain in ZDF rats fed a dried whole egg-based diet compared to ZDF rats fed a casein-based diet (39). In the



present study, ZDF rats fed WE exhibited a marked reduction in cumulative body weight gain compared to ZDF rats fed CAS and CAS+D. Furthermore, cumulative body weight gain in ZDF rats fed WE was the same as all lean control rats; thus, the reduction in cumulative body weight gain by WE is genotype specific, only occurring in the obese, diabetic state. The observed decrease in body weight in ZDF rats fed WE was, in part, due to a decrease in body fat percentage compared to ZDF rats fed CAS and CAS+D. In our recent unpublished observations using a diet-induced model of obesity in Sprague Dawley rats, cumulative weight gain was decreased by 23% in diet-induced obese rats fed a dried whole egg-based diet compared to diet-induced obese rats fed a casein-based diet. Moreover, diet-induced obese rats fed the dried whole egg-based diet gained the same amount of weight as control rats fed caseinand dried whole egg-based diets. These findings support the concept that whole egg consumption reduces weight gain in an obese state in both genetic and diet-induced models, whereas whole egg consumption is without effect on body weight in a lean phenotype. Previous studies have attributed differences in body weight following a whole egg-based diet to increased satiety, while others have found no difference in food intake (29-33). However, in the present study, we report an increase in food intake in ZDF rats fed WE, suggesting that the reduction in body weight in the ZDF genotype is likely the result of a mechanism other than satiety. Furthermore, we found no difference in the food efficiency ratio in ZDF rats within any of the dietary groups. Others have suggested dietary fat as a potential mechanism and there is evidence to support that dietary fat composition may influence final body weight or weight gain in an obese state, depending on the ratio of unsaturated to saturated fatty acids (51, 52). Other potential mechanisms include changes in thermogenesis or energy expenditure and alterations in the gut microbiome. Several rodent and human studies have found an association



between obesity and modifications to the intestinal microbiota; thus, it is possible that a component of the WE diet interacts with the intestinal microbiota in an obese state only (53, 54). Further studies are needed to elucidate the mechanism by which whole egg consumption attenuates weight gain in both the genotype- and diet-induced obese phenotype.

In conclusion, the present study demonstrates that dietary consumption of whole egg may be more effective than supplemental cholecalciferol in maintaining normal circulating 25(OH)D concentrations in T2D. Furthermore, whole egg consumption results in reduced body weight gain in obese, type 2 diabetic rats. Future dose response studies are required to identify the minimal amount of dietary whole egg required to maintain vitamin D homeostasis and attenuate body weight gain in obesity and T2D. Our findings support the concept that inclusion of whole eggs in the diet is an important recommendation for maintenance of vitamin D balance in T2D.

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C.J.S and S.K.J. performed all aspects of animal maintenance, administration of experimental diets, laboratory experiments and drafted the original version of this manuscript. K.E.H. and C.H.R assisted in animal maintenance and laboratory procedures. K.L.S. and M.J.R. assisted with the study design and edits to the manuscript. All authors read and approved the final version of this manuscript.

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# **Tables and Figures**



Figure 3-1: Cumulative body weight gain (A) and food intake (B) in lean control and Zucker diabetic fatty (ZDF) rats fed a casein-based (CAS), whole egg-based (WE), or casein-based diet including supplemental cholecalciferol (CAS+D) for 8 wk. Data are mean values  $\pm$  SEMs; n = 8. Values without a common letter differ (P < 0.05). (B) For clarity, P values reported are for wk 8. G; genotype, D; diet, G x D; genotype x diet (interaction).





Figure 3-2: Percent body fat and lean body mass of lean control and Zucker diabetic fatty (ZDF) rats following 8 wk dietary treatment with a casein-based (CAS), whole egg-based (WE), or casein-based diet including supplemental cholecalciferol (CAS+D). Data are means  $\pm$  SEMs; n = 8. Bars without a common letter differ (P < 0.05). Capital letters indicate differences in lean body mass and lower case letters indicate differences in percent body fat. An asterisk denotes a difference in lean body mass between ZDF rats fed WE and ZDF rats fed CAS+D when analyzed by a one-way ANOVA within the ZDF genotype. G; genotype, D; diet, G x D; genotype x diet (interaction).





Figure 3-3: Circulating 25-hydroxycholecalciferol (25(OH)D) concentrations of lean control and Zucker diabetic fatty (ZDF) rats following 8 wk dietary treatment with a casein-based (CAS), whole egg-based (WE), or casein-based diet including supplemental cholecalciferol (CAS+D). Data are means  $\pm$  SEMs; n = 8. Bars without a common letter differ (P < 0.05). G; genotype, D; diet, G x D; genotype x diet (interaction).



Ingredient	CAS	CAS+D	WE
		g/kg	
Casein (vitamin-free)	200	200	0
Dried standard whole egg, Type 350 <sup>2,3,4</sup>	0	0	435
Cornstarch	417	417	365
Glucose monohydrate	150	150	150
Mineral Mix (AIN 93)	35	35	35
Vitamin Mix (AIN 93)	10	10	10
Cholecalciferol, 100,000 IU/g	0	0.00504	0
Biotin, 1%	0	0	0.4
Corn oil	183	183	0
Choline bitartrate	2	2	2
L-Methionine	3	3	3

Table 3-1 Composition of the casein-based diet (CAS), casein-based diet including supplemental cholecalciferol (CAS+D) and whole egg-based diet (WE) fed to lean control and Zucker diabetic fatty rats for 8 wk<sup>1</sup>.

<sup>1</sup>All diets were formulated by and purchased from Research Diets Inc.

<sup>2</sup> Whole egg was purchased from Rose Acre Farms and sent to Research Diets Inc. for diet formulation.

<sup>3</sup> Total protein and lipid content provided by 435 g of whole egg were 46% (200 g) and 42% (183 g), respectively.

<sup>4</sup> Total cholecalciferol provided by the casein-based diet, casein-based diet including supplemental cholecalciferol and whole egg-based diet were 25, 37.6 and 37.6  $\mu$ g/kg diet, respectively.



Table 3-2 Final body weight, total food intake, bone mineral density, bone mineral content, and food efficiency ratio of lean control and Zucker diabetic fatty rats (ZDF) fed a casein-based diet (CAS), a casein-based diet including supplemental cholecalciferol (CAS+D) and a whole egg-based diet (WE) for 8 wk<sup>1</sup>.

	Lean		ZDF			Р			
	CAS	CAS+D	WE	CAS	CAS+D	WE	Genoty pe	Diet	Genotype x Diet
Final Body Weight (g)	351 ± 5°	338 ± 11°	$322 \pm 19^{\circ}$	$425 \pm 5^{a}$	$419 \pm 5^{a}$	$383 \pm 5^{\mathrm{b}}$	< 0.001	0.003	0.62
Total Food Intake (g)	$419 \pm 6^{\circ}$	$430 \pm 13^{\circ}$	$400 \pm 9^{\circ}$	$748 \pm 14^{\mathrm{b}}$	751 ± 21 <sup>b</sup>	$803 \pm 12^{a}$	< 0.001	0.419	0.006
Bone Mineral Density (g/cm <sup>2</sup> )	$0.20 \pm 0.003$	0.19 ± 0.004	$0.19\pm0.005$	$0.19 \pm 0.003$	0.18 ± 0.003	0.19 <u>+</u> 0.007	0.188	0.057	0.188
Bone Mineral Content (% BWT)	$2.59\pm0.03^{\rm a}$	$2.65 \pm 0.03^{a}$	$2.68 \pm 0.03^{a}$	$2.15 \pm 0.05^{\circ}$	2.11± 0.03°	$2.29 \pm 0.02^{b}$	< 0.001	0.002	0.089
Food Efficiency Ratio	$25 \pm 0.5^{a}$	$24 \pm 0.9^{a}$	$22 \pm 0.7^{\text{b}}$	$10 \pm 0.4^{\circ}$	$11 \pm 0.9^{\circ}$	9 ± 0.6°	0.037	< 0.001	0.715

<sup>1</sup> Data are means  $\pm$  SEMs; n=8. Mean values within a row without a common letter are statistically significant (P < 0.05).

	Lean			ZDF			Р		
	CAS	CAS+D	WE	CAS	CAS+D	WE	Genotype	Diet	Genotype x Diet
Urinary Output (mL)	8.7 ± 3.6 <sup>b</sup>	$6.8 \pm 1.5^{b}$	$3.1 \pm 1.0^{b}$	$14.6 \pm 1.5^{a}$	$12.9 \pm 1.8^{a}$	19.9 <u>+</u> 4.0 <sup>a</sup>	< 0.001	0.71	0.043
Urinary Total Protein (mg/ 12 h)	$29 \pm 3$	48 ± 13	53 ± 5	38 ± 4	$32 \pm 6$	37 ± 7	0.189	0.29	0.12
Urinary Creatinine (mg/12 h)	$3.3 \pm 0.3^{a}$	$3.8 \pm 0.5^{a}$	$2.8 \pm 0.5^{a}$	$1.1\pm0.2^{\rm b}$	$0.7 \pm 0.1^{b}$	1.6 <u>+</u> 0.4 <sup>ab</sup>	< 0.001	0.991	0.029
Serum Creatinine (mg/dL)	$2 \pm 0.3^{b}$	$2 \pm 0.3^{b}$	$2 \pm 0.2^{b}$	$17 \pm 4^{a}$	$21 \pm 7^{a}$	$21 \pm 6^{a}$	< 0.001	0.968	0.929
Urinary 25(OH)D (pmol/mg creatinine)	66 ± 15.9 <sup>b</sup>	64 ± 21.1 <sup>b</sup>	$46 \pm 7.6^{b}$	$1610 \pm 691^{a}$	$1700 \pm 654^{a}$	1990 <u>+</u> 1070 <sup>a</sup>	< 0.001	0.95	0.938
Urinary DBP (µg/12 h)	$0.910 \pm 0.19^{\text{b}}$	$0.983 \pm 0.28^{b}$	1.01 ±0.17 <sup>b</sup>	$993 \pm 213^{a}$	$749 \pm 253^{a}$	1270 <u>+</u> 443 <sup>a</sup>	< 0.001	0.523	0.523
Blood glucose (mg/dL)	$256 \pm 21^{b}$	291 ± 17 <sup>b</sup>	$284 \pm 24^{\mathrm{b}}$	$688 \pm 41^{a}$	$560 \pm 67^{a}$	$693 \pm 43^{a}$	< 0.001	0.261	0.093
Serum Insulin (ng/mL)	$3.8 \pm 0.7^{a}$	$2.3 \pm 0.2^{a}$	$0.8 \pm 0.4^{\mathrm{b}}$	$2.9 \pm 0.4^{a}$	$3.7 \pm 0.4^{a}$	$2.5 \pm 0.7^{a}$	0.082	0.004	0.018
HOMA-IR (%)	$54 \pm 9^{ab}$	$38 \pm 4^{\text{b}}$	11 ± 13°	$113 \pm 21^{a}$	$117 \pm 21^{a}$	99 <u>+</u> 29ª	< 0.001	0.161	0.647

Table 3-3 Biochemical measurements of lean control and Zucker diabetic fatty (ZDF) rats fed a casein-based diet (CAS), a casein-based diet including supplemental cholecalciferol (CAS+D) and a whole egg-based diet (WE) for 8 wk<sup>1</sup>.

<sup>1</sup> Data are means  $\pm$  SEMs; n = 8. Mean values within a row without a common letter are statistically significant (P < 0.05).

# CHAPTER 4. DIETARY WHOLE EGG REDUCES BODY WEIGHT GAIN IN A DOSE-DEPENDENT MANNER IN ZUCKER DIABETIC FATTY RATS

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A manuscript accepted for publication in the *Journal of Nutrition*. Cassondra J. Saande, Joseph L. Webb, Paige E. Curry, Matthew J. Rowling and Kevin L. Schalinske

#### Abstract

**Background:** We previously reported that a whole egg-based diet attenuated weight gain in type 2 diabetic (T2D) rats and more effectively maintained vitamin D status than an equivalent amount of supplemental cholecalciferol.

**Objective:** The objective of this study was to determine the lowest dose of whole egg effective at maintaining vitamin D homeostasis and attenuating the obese phenotype in T2D rats.

**Methods:** Zucker diabetic fatty (ZDF) rats (n= 40; 6 wk of age; prediabetes) and their lean controls (n=40; 6 wk of age) were randomly assigned to a diet containing 20% casein (CAS), or 20, 10, 5 or 2.5% protein from whole egg (20% EGG, 10% EGG, 5% EGG, 2.5% EGG, respectively). All diets contained 20% total protein (w/w). All rats received their respective diets for a period of 8 wk, a stage of growth and development which translates to adolescence in humans, reaching 14 wk of age, a point at which ZDF rats exhibit overt T2D. Weight gain was measured 5 d/wk and circulating 25-hydroxycholecalciferol [25(OH)D] was measured by enzyme-linked immunoassay. Mean values were compared by a 2-way analysis of variance.

**Results:** The 20% EGG diet maintained serum 25(OH)D at 30 nmol/L in ZDF rats, whereas the 10, 5 and 2.5% EGG diets did not prevent insufficiency, resulting in serum 25(OH)D concentrations of 24 nmol/L in ZDF rats. Body weight gain was reduced by 29% (P < 0.001)



and 31% (P < 0.001) in ZDF rats consuming 20 and 10% EGG diets, and by 16% (P = 0.004) and 12% (P = 0.030) in ZDF rats consuming 5 and 2.5% EGG diets compared to CAS.

**Conclusion:** Whole egg-based diets exerted a dose-dependent response with respect to attenuating weight gain. These data may support dietary recommendations aimed at body weight management in individuals predisposed to obesity and T2D.

#### Introduction

Over 30 million people in the U.S. suffer from diabetes, with type 2 diabetes (T2D) accounting for approximately 90-95% of all reported cases (1). Overweight, obesity and excessive weight gain substantially increase diabetes risk, and body weight management is an effective strategy for T2D risk reduction (2–5). Dietary recommendations for type 2 diabetes are focused primarily on body weight management, attaining glycemic targets, and preventing or delaying the onset of T2D complications, including diabetic nephropathy (6–8). Vitamin D insufficiency and deficiency, defined by the Institutes of Medicine as circulating 25-hydroxycholecalciferol [25(OH)D] concentrations of 30-50 nmol/L and <30 nmol/L (9), respectively, are highly prevalent in T2D (10,11). Furthermore, low circulating vitamin D concentrations are exacerbated by the presence of diabetic nephropathy, owing to excessive urinary excretion of 25(OH)D (12).

Numerous studies report an association between suboptimal vitamin D status and T2D prevalence and incidence, as well as decreased insulin sensitivity and impaired  $\beta$ -cell function (13–17). Because cutaneous vitamin D synthesis is often limited, the population is largely dependent on food fortification and dietary intake of vitamin D-containing foods (18). Eggs are one of few naturally-occurring dietary sources of vitamin D and make an important contribution to vitamin D intake in the U.S. population due to their frequent consumption (19).



Vitamin D from eggs is provided in the forms of cholecalciferol (vitamin D3) as well as 25(OH)D, the circulating form of vitamin D that is representative of an individual's status (20). We previously reported that a whole egg-containing diet was more effective than a diet containing the equivalent amount of supplemental cholecalciferol at maintaining vitamin D status in T2D rats (21). The efficacy of the whole egg-based diet in improving vitamin D status may be due to the 25(OH)D content of whole egg, as several studies have reported that 25(OH)D is more potent in raising serum 25(OH)D concentrations compared to an equivalent amount of cholecalciferol (22).

In our previous studies, we also observed reduced body weight gain in obese rats with T2D consuming a whole egg-based diet (21,23). A limited number of studies have found significant weight loss following a period of daily egg intake (1-2 eggs/d) in human subjects (24–26), suggesting an impact of egg consumption on energy intake and/or body weight regulation. There is strong, consistent evidence that T2D onset can be prevented or delayed through lifestyle modifications resulting in weight loss, including dietary modification (8,27–29). Furthermore, recommendations for diabetes management in individuals with existing T2D support lifestyle intervention to achieve weight loss and weight maintenance (30,31).

The goal of this follow-up dose-response study was to determine the minimal amount of dried whole egg effective at maintaining vitamin D status and attenuating the development of the obese phenotype in T2D rats, as our previous studies demonstrated that dietary whole egg diet was successful at both reducing weight gain and maintaining vitamin D homeostasis. Although whole egg consumption offers several benefits for individuals with T2D, including a high nutrient-to-energy density and high-quality protein, the impact of egg consumption on cardiovascular disease risk remains controversial for this population (32–35). It has been



suggested that the phosphatidylcholine and choline content of eggs may contribute to cardiovascular disease susceptibility, due to the production of trimethylamine N-oxide (TMAO) following the intake of choline-containing foods (36,37), as several studies have reported an association between increased circulating TMAO and cardiovascular disease risk (37–41). Thus, a secondary objective of this study was to characterize the effect of varying concentrations of dietary whole egg on circulating TMAO in lean and T2D rats.

#### **Materials and Methods**

Animals and Diets. All animal studies were approved by the Institutional Animal Care and Use Committee at Iowa State University and were performed according to the Iowa State University Laboratory Animal Resources Guidelines. Male Zucker diabetic fatty (ZDF; fa/fa) rats (n=40) and lean (fa/+) control rats (n=40) were purchased at 5 wk of age (Charles River Laboratories). Rats were housed individually in plastic cages in a temperature-controlled room with a 12-h light-dark cycle. All diets were formulated by Research Diets, Inc. (New Brunswick, NJ). Dried whole egg was purchased from Rose Acre Farms and sent to Research Diets, Inc. for diet formulation. All rats were acclimated to a modified semi-purified diet (AIN-93G) for 1 wk. At 6 wk of age, an age at which ZDF rats exhibit prediabetes (42), rats were randomly assigned to 1 of 5 experimental diets (Table 4-1). All diets provided protein at 20% (w/w) from either casein, whole egg, or a combination of casein and whole egg. The 5 experimental diets were as follows: a diet containing 20% (w/w) casein (CAS), a diet containing 20% (w/w) protein from whole egg (20% EGG), a diet containing 10% casein and 10% protein from whole egg (10% EGG), a diet containing 15% casein and 5% protein from whole egg (5% EGG), or a diet containing 17.5% casein and 2.5% protein from whole egg (2.5% EGG). All diets were matched for total lipid quantity (16.6%) via the addition of corn



oil to all of the diets that included casein as a protein source. Rats were given *ad libitum* access to food and water for 8 wk, such that the dietary treatment period began when ZDF rats were in a prediabetic state and continued until 14 wk of age, an age at which ZDF rats exhibit overt T2D, diabetic nephropathy and urinary loss of 25(OH)D (42-44). Body weights and food intake were recorded 5 d/wk. Prior to sacrifice, rats were placed in metabolic cages for 12 h, during which urine was collected and then stored at -80° C for subsequent analysis. Rats were anesthetized via a single intraperitoneal injection of ketamine:xylaxine (90:10 mg/kg body weight). Whole blood was collected via cardiac puncture, centrifuged in separation tubes, and the resultant serum was stored at -80°C. Euthanasia was achieved by exsanguination. Body fat, lean body mass, bone mineral density, and bone mineral content were measured post-necropsy by dual energy x-ray absorptiometry (DXA; Delphi W Hologic, Inc). DXA measurements were standardized by placing animals in a prone position; each animal's nose, front left paw, and back right paw were positioned in the same region marked on the scanning platform with tape. All animals were scanned following a 7 minute whole-body DXA scan by a certified DXA operator.

*Biochemical Analysis*. Serum glucose was measured using a colorimetric kit (Wako Diagnostics; cat. no. 439-90901). Urinary total protein concentrations were determined using a bicinchoninic acid assay (Thermo Scientific Pierce; cat. no. 23225). Urinary creatinine was measured using a commercially available colorimetric kit (Cayman Chemical; cat. no. 500701). Serum and urinary concentrations of 25(OH)D were analyzed using a commercial enzyme immunoassay kit (Immunodiagnostic Systems; cat. no. AC-57SF1). All analyses were performed according to the manufacturer's instructions and all samples were assayed in



duplicate. For all biochemical analysis, absorbance was measured using the Synergy H1 Hybrid Reader (BioTek) and analyzed using the Gen5<sup>™</sup> Microplate Reader Software (BioTek).

Choline Metabolites Panel. LC-MS/MS was used to measure free choline, betaine, and dimethylglycine, as previously described (30), with modifications to include measurements of methionine and TMAO. Briefly, 100mL of 0.1% formic acid in acetonitrile and 5mL of internal standard mix was added to 50mL of plasma. Internal standard mix contained choline D13 (CDN Isotopes), betaine D3 (CDN Isotopes), dimethylglycine D3 (CDN Isotopes), methionine D3 (Santa Cruz Biotechnology), and trimethylamine N-oxide (TMAO) D9 (Cambridge Isotopes). After vortexing and centrifugation, 5mL of clear supernatant was injected on a Syncronis Silica column (150 x 2.1mm, 5mm) with matching guard column (ThermoFisher Scientific). Metabolites were separated under isocratic conditions using 19% of 15mM ammonium formate with 0.1% formic acid, and 81% acetonitrile with a flow rate of 0.5mL/min. Calibration curves were generated by serial dilutions of unlabeled metabolites in water with addition the 5mL of internal standard mix. Positive ion SRM transitions were m/z76 - 58 and m/z 85 - 66 for TMAO and TMAO D9; m/z 104-60 and m/z 117-69 for choline and choline D13; m/z 104-58 and m/z 107-61 for DMG and DMG D3; m/z 118-58 and m/z121-61 for betaine and betaine D3; and m/z 150-133 and m/z 153-135 for methionine and methionine D3. Assay imprecision was less than 5% for each metabolite based on in-house human plasma controls.



*Statistical Analysis.* All data were analyzed using SPSS Statistics Software Version 23 (IBM). Mean values were evaluated for statistically significant differences (P < 0.05) using a two-way ANOVA (genotype x diet) followed by the Fisher's Least Significant Difference (LSD) post hoc test for multiple comparisons. An analysis of simple effects was performed when the interaction term was statistically significant. When normality failed or variances were unequal a nonparametric analysis was performed using the Kruskal-Wallis 1-factor ANOVA on ranks.

#### Results

*Consumption of the EGG diets resulted in a dose-dependent decrease in cumulative weight gain in ZDF rats.* There was a significant interaction between diet and genotype on cumulative weight gain; therefore, an analysis of simple effects was performed. There was a simple effect of diet on cumulative weight gain within the ZDF genotype (P < 0.001), but not the lean genotype (P = 0.337) (Figure 4-1A). As expected, weight gain was higher in ZDF rats fed CAS compared to all lean rats throughout the study. On d 9, weight gain on d 9 was approximately 14% lower in all ZDF rats fed EGG diets compared to ZDF rats fed CAS. From d 23 onwards, ZDF rats fed 20% EGG and 10% EGG diets. By the end of the study, consumption of the 20% EGG and 10% EGG diets. By the end of the study, consumption of the 20% EGG and 10% EGG diets decreased cumulative weight gain by 29% and 31%, respectively, in ZDF rats fed CAS. Additionally, cumulative body weight gain was 16% and 12% lower, respectively, in ZDF rats fed the 5% EGG and 2.5% EGG diets compared to ZDF rats fed CAS.

Likewise, there was a significant interaction between diet and genotype on final body weight, followed by a simple effect of diet within the ZDF genotype (P < 0.001), but not the



lean genotype (P = 0.472) (Table 4-2). Consumption of the 20% EGG and 10% EGG diets decreased final body weight by approximately 11% in ZDF rats compared to ZDF rats fed the CAS, 5% EGG and 2.5% EGG diets. Final body weight did not differ between lean rats, regardless of dietary treatment group (Table 4-2).

*Rats fed EGG diets exhibited higher food intake than rats fed the CAS diet.* There was a significant effect of both diet and genotype on food intake (Figure 4-1B). There was a main effect of diet on food intake, which was higher in all rats fed any of the EGG diets compared to the CAS diet. There is variation in day-to-day food intake; however, when averaged over the week, food intake per 100 g body weight in wk 1-4 was lower in ZDF rats fed CAS compared to ZDF rats fed the 10, 5 and 2.5% EGG diets, but did not differ from ZDF rats fed the 20% EGG diet. In wk 5, average food intake did not differ in ZDF rats fed CAS compared to ZDF rats fed any of the EGG diets. In weeks 6-8, average food intake was lower in ZDF rats fed CAS compared to ZDF rats fed any of the EGG diets. Food intake during the final wk of the study was approximately 36% higher with consumption of the 20%, 10% 5% and 2.5% EGG diets in ZDF rats compared to ZDF rats fed the ZDF rats fed the CAS diet (Figure 4-1).

*Body Composition*. Body composition parameters are presented in Table 4-2. Significant main effects of both diet and genotype were observed on the percentage of body fat and lean body mass. Percent body fat was increased by 194% in ZDF rats compared to lean rats, whereas the percentage of lean body mass was 41% lower in ZDF rats compared to lean rats. No differences in percent body fat or lean body mass were observed within the lean or ZDF genotype across the different diets. Likewise, significant main effects of both diet and genotype were observed



on grams of body fat, whereas only a main effect of genotype was observed on grams of lean mass. Grams of body fat were 229% higher and grams of lean mass were 35% lower in ZDF rats compared to lean rats. There was a significant effect of genotype on bone mineral density, which was 5% lower in ZDF rats compared to lean rats. No differences in bone mineral density were observed within a given genotype across the different diets. In contrast, there was a significant effect of diet on bone mineral content. In ZDF rats, bone mineral content was decreased by approximately 9% in ZDF rats fed 20% EGG and 10% EGG compared to ZDF rats fed CAS, 5% EGG and 2.5% EGG diets. No differences in bone mineral content were observed within the lean genotype.

*Circulating 25(OH)D concentrations*. There were significant main effects of both diet and genotype on serum concentrations of 25(OH)D (Figure 4-2). As expected, circulating 25(OH)D concentrations were 31% lower in ZDF rats fed CAS compared to lean rats fed CAS. When compared with CAS, consumption of the 20% EGG diet increased serum 25(OH)D by 35 and 40% in lean and ZDF rats, respectively. ZDF rats fed the 10% EGG, 5% EGG and 2.5% EGG diets exhibited serum 25(OH)D concentrations that did not differ from lean CAS-fed rats. However, serum 25(OH)D concentrations in ZDF rats fed 10% EGG, 5% EGG and 2.5% EGG also did not statistically differ from serum 25(OH)D in ZDF rats fed CAS. For lean rats, consumption of the 10% EGG, 5% EGG and 2.5% EGG and 2.5% EGG diets resulted in serum 25(OH)D concentrations that did not differ from lean CAS. For lean rats, consumption of the 10% EGG, 5% EGG and 2.5% EGG diets resulted in serum 25(OH)D concentrations that did not differ from lean CAS. For lean rats, consumption of the 10% EGG, 5% EGG and 2.5% EGG diets resulted in serum 25(OH)D concentrations that did not differ from lean CAS. For lean rats, consumption of the 10% EGG, 5% EGG and 2.5% EGG diets resulted in serum 25(OH)D concentrations that did not differ from lean CAS or lean 20% EGG groups.

*Serum and urinary biochemical measurements*. As expected, serum glucose concentrations were approximately 145% higher in ZDF rats compared to lean rats (Table 4-3). Serum glucose



did not differ between any of the dietary treatment groups within the lean or ZDF genotype. The presence of polyuria, proteinuria, and reduced creatinine clearance in ZDF rats confirmed a state of diabetic nephropathy. Urinary output and urinary total protein were increased by 536% and 296%, respectively, in ZDF rats compared to lean rats (Table 4-3). Urinary creatinine clearance was decreased by 20% in ZDF rats compared to creatinine clearance in the lean genotype (Table 4-3). Urine output, urinary total protein, and urinary creatinine concentrations did not differ between any of the dietary treatment groups within a given genotype (Table 4-3). Lastly, urinary excretion of 25(OH)D was increased by 800% in ZDF rats compared to lean rats, confirming the loss of vitamin D in the urine of ZDF rats. No differences in urinary 25(OH)D were observed between dietary treatment groups within the lean or ZDF genotype (Table 4-3).

*Choline metabolites panel.* Results of the choline metabolites panel are presented in Table 4-4. There was a significant interaction between diet and genotype on serum concentrations of TMAO. The simple effect of diet on serum TMAO was significant in both the lean (P = 0.032) and ZDF (P < 0.001) genotype. The simple effect of genotype on serum TMAO was significant within the 20% EGG (P = 0.002), 10% EGG (P < 0.001) and 5% EGG (P = 0.018) and 2.5% EGG (P = 0.013) diets, but not the CAS diet (P = 0.886). For ZDF rats, consumption of the 20% EGG, 10% EGG, 5% EGG and 2.5% EGG diets resulted in serum TMAO in ZDF rats fed CAS. In lean rats, consumption of the 20% EGG and 10% EGG diets increased serum TMAO by 225 and 100%, respectively, compared to serum TMAO in lean rats fed CAS. Serum


TMAO did not differ between ZDF rats fed CAS and lean rats fed the CAS, 5% EGG and 2.5% EGG diets.

There was also a significant interaction between diet and genotype on serum concentrations of methionine. The simple effect of diet on serum methionine was significant in both the lean (P < 0.001) and ZDF (P = 0.015) genotype and the simple effect of genotype on serum methionine was significant within all 5 dietary treatment groups. Circulating concentrations of methionine were 34% lower in ZDF rats compared to lean rats. Furthermore, there was an inverse relationship between serum methionine and the percentage of whole egg in the diet. There were significant main effects of both diet and genotype on serum concentrations of betaine. Circulating betaine concentrations were decreased by 21% in the ZDF genotype and were higher in the 20% EGG (P < 0.001), 10% EGG (P < 0.001) and 5% EGG (P < 0.001) diets compared to CAS. Lastly, there was a main effect of genotype on both serum choline and serum dimethylglycine. Circulating concentrations of choline were 20% higher in ZDF rats compared to lean rats, whereas circulating concentrations of dimethylglycine were 20% lower in ZDF rats.

#### Discussion

We previously reported attenuated body weight gain in T2D rats consuming a whole egg-based diet, concomitant with an increase in food intake (21). In the current study, we show a dose-dependent reduction in weight gain in ZDF rats fed 20% EGG, 10% EGG, 5% EGG and 2.5% EGG diets, which translates to a human intake of approximately 14, 7, 3.5 and 1.75 eggs/d, respectively, when calculated based on a 2,000 kcal/d diet. Notably, all EGG diets resulted in reduced weight gain in ZDF rats compared to the CAS diet. In contrast to our findings, Pearce et al. reported no differences in weight loss following 12 wk consumption of



energy restricted diets with or without 2 eggs/d in human subjects with T2D (46). Similarly, final body weights did not differ in T2D subjects after 3 mo consumption of energy-restricted high egg ( $\geq 12$  eggs/wk) or low egg ( $\leq 2$  eggs/wk) diets (47). However, in a randomized, controlled, crossover trial of adults with type 2 diabetes, consumption of 2 egg/d for 12 wk significantly reduced body mass index, waist circumference and percent body fat from baseline, compared to exclusion of dietary eggs (26). There are notable differences between our animal studies and these human studies, such as age at the onset of dietary intervention and the stage of diabetes development. In the current study, growing rats were fed their respective diets from 6 wk of age (prediabetes) until 14 wk of age, a point where obesity and T2D is clearly indicated in the ZDF rat (43,48), whereas adult human subjects presented with obesity and T2D prior to dietary intervention with whole egg. Thus, there is a significant difference between a prevention study design in an animal model as compared to a treatment study design in human studies. Our findings suggest that egg consumption may reduce weight gain during the development of obesity and T2D. Notably, daily consumption of a hard-boiled egg breakfast for 3 mo resulted in significant weight loss in obese Chinese adolescents compared to a breakfast consisting of steamed bread (25), which indicates that egg consumption can positively impact body weight management in humans during a period of growth and development.

Despite a reduction in weight gain, we did not observe reduced food intake in ZDF rats fed all four EGG diets compared to ZDF rats fed CAS, which is in agreement with our previous results (21). This observation, together with leptin receptor deficiency in the ZDF rat, suggests that the differences in weight gain in ZDF rats fed the EGG diets were not related to satiety. However, the exact mechanism underlying the observed reductions in weight gain remains to



be determined. Whole eggs are a complex food matrix containing many components that could potentially contribute to the observed reduction in weight gain, including lipids, amino acids/peptides, vitamins, miRNAs, and bioactive compounds. Potential mechanisms responsible for the differential response to weight gain in lean and ZDF rats following whole egg consumption include alterations in energy expenditure, the composition of the gut microbiome, gene expression and nutrient absorption. The lipid composition of the EGG diets differed from that of the CAS diet, contributing phospholipid species and a distinct fatty acid profile, and it has been reported that the dietary lipid composition can alter rates of lipid oxidation, fat deposition and weight gain (49–51). Loh et al. found that the amount and type of dietary fat influenced weight gain, energy expenditure and carcass lipid content in obese Zucker rats, but had a negligible effect on lean control rats (49). It has also been shown that alterations in dietary fat and protein correspond to changes in the gut microbiome (52,53). Furthermore, several studies have reported differences in gut microbiota signatures between lean and obese patients (54) and dietary factors and the gut microbiota may interact to modulate energy metabolism and obesity (55). Therefore, an interaction between egg consumption and the gut microbiota may impact weight gain in ZDF rats. Additionally, eggs contain miRNAs, which can influence gene expression and impact human health. Baier et al. reported that egg miRNAs are bioavailable and modulate mRNA translation in peripheral blood mononuclear cells in healthy adults following consumption of one hard-boiled egg (56). Lastly, various peptides with  $\alpha$ -glucosidase inhibitory activity have been isolated from egg white (57,58). These compounds inhibit the hydrolysis of dietary carbohydrates, thereby preventing their absorption, and could contribute to a reduction in body weight.



In our previous studies, we reported that a diet containing 20% protein (w/w) from whole egg markedly improved vitamin D status in ZDF rats (21,23), and may be more effective than an equivalent dose of supplemental cholecalciferol for maintaining serum 25(OH)D concentrations (21). In agreement with our previous findings, serum 25(OH)D concentrations were significantly higher in both lean and ZDF rats consuming the 20% EGG diet compared to their CAS-fed counterparts. However, in both lean and ZDF rats, consumption of the 10% EGG, 5% EGG and 2.5% EGG diets did not increase circulating concentrations of 25(OH)D compared to lean and ZDF rats fed CAS. As we've reported previously, there were no differences in urinary excretion of 25(OH)D across dietary treatment groups within a given genotype, indicating that the differences in serum 25(OH)D are not related to differences in urinary vitamin D losses (21). While egg consumption makes up a large component of the population's dietary vitamin D intake, habitual dietary intakes of vitamin D fall below the estimated average requirement for the majority of Americans (19,59,60), as well as internationally (61,62). Taken together, these findings suggest that increasing the intake of non-fortified, naturally occurring food sources of vitamin D, such as eggs, may not be a useful singular strategy for improving vitamin D status in individuals with T2D. The inclusion of eggs in a well-balanced diet that contains other sources of vitamin D remains a useful strategy to maintain vitamin D homeostasis. Moreover, eggs are a good candidate for vitamin D fortification (63) and several studies suggest that food fortification represents an effective method to meet the vitamin D needs of the population (64–68). Numerous studies have demonstrated that the cholecalciferol and 25(OH)D content of eggs can be substantially increased by vitamin D supplementation of the hens' feed (63,69–71), providing scope to meet dietary recommendations (63). Moreover, Hayes et al. (72) found that consumption of 7



vitamin D-enriched eggs per wk was protective against declining serum 25(OH)D concentrations in the winter. To date, there are no studies investigating whether vitamin D-enriched eggs effectively maintain vitamin D status in rats or humans with T2D.

The phosphatidylcholine and choline content of eggs has emerged as a potential link between egg consumption and cardiovascular disease (CVD) risk, due to the hepatic production of trimethylamine N-oxide (TMAO) following their metabolism by the intestinal microbiota to trimethylamine (37,41). Numerous studies have identified an association between circulating TMAO concentrations, CVD risk and major adverse cardiovascular events (73,74). Furthermore, high plasma choline and betaine, precursors of TMAO, are associated with risk of major adverse cardiovascular events (75). Elevated TMAO has been reported in rodent models of diabetes and human subjects (76–78) and T2D appears to strengthen the relation between TMAO and adverse cardiovascular events (41,79). In the present study, higher levels of egg consumption resulted in higher TMAO production in both lean and ZDF rats. Additionally, we observed a heightened TMAO response to whole egg consumption in ZDF rats compared to lean rats. Whether TMAO is a biomarker of cardiovascular pathology or plays a mediating role in CVD progression remains to be determined. Importantly, several studies also report protective effects of circulating TMAO (80). The potential relation between choline, TMAO and CVD risk in individuals with diabetes requires careful assessment in future research, particularly as choline is an essential nutrient with important roles in human health (81).

There are several limitations to this study. The ZDF rat is an extreme model of obesity and T2D, which develops due to a genetic mutation of the leptin receptor in the brain, resulting in hyperphagia. Leptin receptor deficiency is rare in humans (82); thus, the etiology of obesity



and T2D differs between the rat model used in this study and the human condition. Secondly, although body fat and lean body mass was measured by DXA, identifying the distribution of body fat would aid in the interpretation of these results. In addition, female rats were not included in this study and the observed results may differ in females. Future studies will include measurement of fat pad weights to determine whether differences in body fat distribution exist between dietary treatment groups and will focus on both male and female rats to determine whether sex-specific differences exist. Finally, identifying a mechanism underlying the reduction in body weight gain in ZDF rats fed a whole egg-based diet was out of the scope of this study; however, future studies will be directed at elucidating potential mechanisms.

These data demonstrate that the consumption of diets containing 20, 10, 5 and 2.5% protein from whole egg reduce weight gain in growing, obese rats with T2D in a dosedependent fashion. These findings have significant translational implications for humans, as intake of whole eggs in doses as low as 2.5% dietary protein from whole eggs, which translates to less than 2 eggs/d, may help attenuate weight gain in individuals predisposed to obesity and type 2 diabetes. However, long-term studies investigating egg consumption as a strategy to prevent excessive weight gain in a human population are warranted. Additionally, this study demonstrated that a diet containing 20% protein from whole egg effectively maintained vitamin D status in T2D rats, whereas diets containing 10, 5 and 2.5% egg protein did not prevent vitamin D insufficiency. Lastly, we observed an increase in serum TMAO following consumption of whole egg-based diets, with an augmented response in the ZDF genotype. Future research regarding the relation between egg consumption, TMAO, and CVD risk in individuals with T2D is needed.



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#### **Tables and Figures**



Figure 4-1: Cumulative body weight gain (A) and food intake (B) in lean and ZDF rats fed CAS, 20% EGG, 10% EGG, 5% EGG and 2.5% EGG diets for 8 wk. Mean values at day 58 (A) or day 53 (B) without a common letter differ (P < 0.05). Body weights and food intake were averaged over each wk (B). Abbreviations are: CAS, casein-based diet; 20% EGG, diet containing 20% (w/w) protein from whole egg; 10% EGG, diet containing 10% casein and 10% protein from whole egg; 5% EGG, diet containing 15% casein and 5% protein from whole egg; 2.5% EGG, diet containing 17.5% casein and 2.5% protein from whole egg (2.5% EGG); and ZDF, Zucker diabetic fatty.



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Figure 4-2: Circulating 25(OH)D concentrations in lean and ZDF rats after 8 wk dietary treatment with CAS, 20% EGG, 10% EGG, 5% EGG and 2.5% EGG. Data are mean values  $\pm$  SEMs; n = 8. Bars without a common letter differ (P < 0.05). CAS, casein-based diet, 20% EGG, diet containing 20% (w/w) protein from whole egg; 10% EGG, diet containing 10% casein and 10% protein from whole egg; 5% EGG, diet containing 15% casein and 5% protein from whole egg; 2.5% EGG, diet containing 17.5% casein and 2.5% protein from whole egg (2.5% EGG); ZDF, Zucker diabetic fatty, 25(OH)D, 25-hydroxycholecalciferol.



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Ingredient (g/kg)	CAS	20% EGG	10% EGG	5% EGG	2.5% EGG
Casein (vitamin-free)	200	0	100	150	175
Dried standard whole egg, Type 350 <sup>2,3,4</sup>	0	405	203	102	51
Cornstarch	434	395	414	424	429
Glucose monohydrate	150	150	150	150	150
Mineral Mix (AIN 93)	35	0	0	0	0
Mineral Mix (S10022C) <sup>4</sup>	0	3.5	3.5	3.5	3.5
Vitamin Mix (AIN 93)	10	10	10	10	10
Biotin, 1% (w/v)	0	0.4	0.4	0.4	0.4
Corn oil	166	0	83	124	145
Choline bitartrate	2	2	2	2	2
L-Methionine	3	3	3	3	3

Table 4-1: Composition of the CAS, 20% EGG, 10% EGG, 5% EGG and 2.5% EGG diets fed to lean control and Zucker diabetic fatty rats for 8 wk<sup>1</sup>.

<sup>1</sup>All ingredients were purchased from Envigo with exception of dried whole egg (Rose Acre Farms), as well as L-methionine and choline bitartrate (Sigma Aldrich). All diets were formulated by and purchased from Research Diets Inc. Abbreviations are: CAS, casein-based diet; 20% EGG, diet containing 20% (w/w) protein from whole egg; 10% EGG, diet containing 10% casein and 10% protein from whole egg; 5% EGG, diet containing 15% casein and 5% protein from whole egg; 2.5% EGG, diet containing 17.5% casein and 2.5% protein from whole egg (2.5% EGG).

<sup>2</sup> Whole egg was purchased from Rose Acre Farms and sent to Research Diets Inc. for diet formulation.

<sup>3</sup> Total protein and lipid content provided by 405 g of whole egg were 49% (200 g) and 41% (166 g), respectively.

<sup>4</sup> Custom mineral mix formulated to match all diets for mineral content.



Table 4-2: Percent body fat, percent lean mass, bone mineral density and bone mineral content of lean control and Zucker diabetic fatty rats (ZDF) fed CAS, 20% EGG, 10% EGG, 5% EGG and 2.5% EGG for 8 wk<sup>1</sup>.

			Lean					ZDF				Р	
	CAS	20% EGG	10% EGG	5% EGG	2.5% EGG	CAS	20% EGG	10% EGG	5% EGG	2.5% EGG	Genotype	Diet	Genotype x Diet
Final Body Weight (g)	$330 \pm 8.4^{\rm bc}$	315 ± 7.6°	$336 \pm 12^{bc}$	331 <u>+</u> 8.4 <sup>bc</sup>	$322 \pm 8.2^{bc}$	395 <u>+</u> 10 <sup>a</sup>	345 ± 6.2 <sup>b</sup>	332 ± 7.3 <sup>bc</sup>	373 <u>+</u> 8.3ª	$380 \pm 7.6^{a}$	< 0.001	0.002	0.001
Body Fat (g)	$59 \pm 6^{b}$	42 ±3 <sup>b</sup>	$54 \pm 5^{b}$	$50\pm5^{\mathrm{b}}$	$54 \pm 4^{b}$	$\frac{196 \pm 8^{a}}{8^{a}}$	$151 \pm 11^{a}$	$155 \pm 8^{a}$	$168 \pm 7^{a}$	$183 \pm 8^{a}$	< 0.001	0.001	0.059
Body Fat (%)	17.8 ± 1.7 <sup>b</sup>	$13.2 \pm 0.7^{b}$	$15.8 \pm 1.0^{b}$	15.1 ± 1.5 <sup>b</sup>	$16.8 \pm 1.2^{b}$	49.9 ± 1.7 <sup>a</sup>	$43.5 \pm 3.0^{a}$	$46.5 \pm 1.8^{a}$	$45.0 \pm 1.2^{a}$	$48.2 \pm 1.7^{a}$	< 0.001	0.013	0.966
Lean Mass (g)	$260 \pm 9^{b}$	$266 \pm 8^{b}$	$267 \pm 9^{b}$	$272 \pm 5^{\mathrm{b}}$	$256 \pm 8^{\mathrm{b}}$	$173 \pm 10^{a}$	$174 \pm 8^{a}$	$162 \pm 7^{a}$	$182 \pm 3^{a}$	$175 \pm 4^{a}$	< 0.001	0.422	0.581
Lean Mass (%)	$78.8 \pm 1.1^{a}$	$84.3 \pm 1.0^{a}$	$79.5 \pm 1.0^{a}$	$82.3 \pm 1.2^{a}$	$79.3 \pm 0.9^{a}$	43.8 ± 1.7 <sup>b</sup>	$50.5 \pm 2.6^{\text{b}}$	48.7 <u>+</u> 1.7 <sup>b</sup>	$49.0 \pm 1.1^{b}$	$46.2 \pm 1.4^{b}$	< 0.001	0.001	0.687
Bone Mineral Density (g/cm <sup>2</sup> )	$0.1737 \pm 0.003^{ab}$	0.1686 ± 0.003 <sup>abc</sup>	0.1736 <u>+</u> 0.003 <sup>ab</sup>	0.1758 <u>+</u> 0.003 <sup>a</sup>	$0.1691 \pm 0.002^{abc}$	0.1641 <u>+</u> 0.004 <sup>cd</sup>	$0.1636 \pm 0.002^{cd}$	$0.1608 \pm 0.003^{d}$	0.1665 ± 0.002 <sup>bcd</sup>	$0.1628 \pm 0.003^{cd}$	< 0.001	0.292	0.646
Bone Mineral Content (g)	$10.7 \pm 0.34^{a}$	$10.3 \pm 0.35^{ab}$	$10.7 \pm 0.34^{a}$	$\begin{array}{c} 10.8 \pm \\ 0.26^a \end{array}$	$10.3 \pm 0.30^{ab}$	$10.8 \pm 0.21^{\circ}$	9.9 ± 0.15 <sup>b</sup>	9.8 ± 0.21 <sup>b</sup>	$11 \pm 0.23^{a}$	$10.7 \pm 0.19^{a}$	0.845	0.012	0.110

<sup>1</sup>Values are means  $\pm$  SEMs; n=8. Mean values within a row without a common letter are statistically significant (P < 0.05). CAS, caseinbased diet, 20% EGG, diet containing 20% (w/w) protein from whole egg; 10% EGG, diet containing 10% casein and 10% protein from whole egg; 5% EGG, diet containing 15% casein and 5% protein from whole egg; 2.5% EGG, diet containing 17.5% casein and 2.5% protein from whole egg (2.5% EGG).

	Lean							ZDF	Р				
	CAS	20% EGG	10% EGG	5% EGG	2.5% EGG	CAS	20% EGG	10% EGG	5% EGG	2.5% EGG	Genotype	Diet	Genotype x Diet
Serum Glucose (mg/dL)	209 <u>+</u> 19 <sup>b</sup>	$205 \pm 15^{\mathrm{b}}$	247 ± 21 <sup>b</sup>	244 ± 23 <sup>b</sup>	$228 \pm 18^{\mathrm{b}}$	$572 \pm 61^a$	$501 \pm 39^{a}$	$576 \pm 51^{a}$	611 <u>+</u> 61 <sup>a</sup>	$518 \pm 44^{a}$	< 0.001	0.348	0.797
Urinary Output (mL)	$2.9 \pm 1^{b}$	$2.0 \pm 0.3^{\text{b}}$	$2.5\pm0.4^{\mathrm{b}}$	$2.8 \pm 0.4^{b}$	$4.0 \pm 2^{b}$	$13 \pm 2^{a}$	$17 \pm 1^{a}$	$17 \pm 2^{a}$	$21 \pm 3^{a}$	$21 \pm 2^{a}$	< 0.001	0.057	0.182
Urinary Total Protein (mg/12 hr)	35.6 ± 2.5 <sup>b</sup>	52.1 ± 5.2 <sup>b</sup>	$54.2 \pm 2.6^{b}$	47.9 ± 3.0 <sup>b</sup>	$40.9 \pm 4.6^{b}$	139 <u>+</u> 28 <sup>a</sup>	$189 \pm 15^{a}$	$174 \pm 19^{a}$	$215 \pm 33^{a}$	$194 \pm 23^{a}$	< 0.001	0.145	0.388
Urinary Creatinine (mg/12 hr)	$2.8 \pm 0.4^{ab}$	$2.7 \pm 0.3^{ab}$	$3.2 \pm 0.2^{a}$	$3.1 \pm 0.2^{a}$	$3.0 \pm 0.6^{a}$	$1.9 \pm 0.3^{b}$	$2.3 \pm 0.3^{ab}$	$2.5 \pm 0.4^{ab}$	$2.6 \pm 0.5^{ab}$	$2.8 \pm 0.3^{ab}$	0.030	0.460	0.940
Urinary 25(OH)D (pmol/mg creatinine)	19.3 ± 3.2 <sup>b</sup>	$17.8 \pm 1.3^{b}$	$17.0 \pm 1.0^{b}$	$20.0 \pm 1.8^{b}$	35.2 ± 11 <sup>b</sup>	169 ± 33ª	$249 \pm 69^{a}$	$144 \pm 14^{a}$	$\frac{245 \pm}{37^a}$	$204 \pm 33^{a}$	< 0.001	0.353	0.399

Table 4-3: Biochemical measurements of lean control and Zucker diabetic fatty (ZDF) rats fed CAS, 20% EGG, 10% EGG, 5% EGG and 2.5% EGG for 8 wk<sup>1</sup>.

<sup>1</sup> Values are means  $\pm$  SEMs; n = 8. Mean values within a row without a common letter are statistically significant (P < 0.05). CAS, casein-based diet, 20% EGG, diet containing 20% (w/w) protein from whole egg; 10% EGG, diet containing 10% casein and 10% protein from whole egg; 5% EGG, diet containing 15% casein and 5% protein from whole egg; 2.5% EGG, diet containing 17.5% casein and 2.5% protein from whole egg (2.5% EGG).



Table 4-4: Circulating concentrations of methionine, choline, betaine, dimethylglycine and TMAO of lean control and Zucker diabetic fatty (ZDF) rats fed CAS, 20% EGG, 10% EGG, 5% EGG and 2.5% EGG for 8 wk<sup>1</sup>.

	Lean						ZDF					Р		
	CAS	20% EGG	10% EGG	5% EGG	2.5% EGG	CAS	20% EGG	10% EGG	5% EGG	2.5% EGG	Genotype	Diet	Genotype x Diet	
Serum Methionine (nmol/mL)	93.6 ±2.1ª	$66.9 \pm 2.2^{\circ}$	$71.6 \pm 2.7^{\circ}$	84.8 ± 2.1 <sup>b</sup>	$82.0 \pm 1.4^{b}$	$56.2 \pm 2.0^{d}$	$47.5 \pm 1.2^{e}$	51.7 ± 2.3 <sup>de</sup>	$54.4 \pm 1.2^{d}$	$56.7 \pm 2.8^{d}$	< 0.001	< 0.001	< 0.001	
Serum Choline (nmol/mL)	29.9 ± 2.2 <sup>bc</sup>	25.3 ± 1.4°	29.1 ± 2.6 <sup>bc</sup>	38.9 <u>+</u> 5.3 <sup>ab</sup>	$26.0 \pm 1.5^{\circ}$	$38.0 \pm 4.6^{ab}$	27.7 ± 1.5 <sup>bc</sup>	41.1 ± 7.9 <sup>ab</sup>	$34.4 \pm 5.6^{bc}$	$39.1 \pm 2.8^{a}$	0.020	0.133	0.190	
Serum Betaine (nmol/mL)	89.9 <u>+</u> 3.2 <sup>cd</sup>	122 <u>+</u> 7.1 <sup>ab</sup>	117 <u>+</u> 4.9 <sup>ab</sup>	137 <u>+</u> 6.6 <sup>a</sup>	102 <u>+</u> 5.9 <sup>bc</sup>	$60.4 \pm 10^{d}$	$103 \pm 5.8^{\rm bc}$	$104 \pm 13^{bc}$	$\frac{111 \pm}{20^{bc}}$	72.3 ± 4.3 <sup>d</sup>	< 0.001	< 0.001	0.892	
Serum Dimethylglycine (nmol/mL)	$6.5 \pm 0.2^{ab}$	$6.9 \pm 0.2^{a}$	$5.9 \pm 0.4^{abc}$	$6.9 \pm 0.5^{ab}$	$5.9 \pm 0.2^{\rm abc}$	$4.5 \pm 0.5^{d}$	$4.8\pm0.2^{\rm cd}$	$5.8 \pm \\ 0.7^{bcd}$	5.3 ± 0.7 <sup>cd</sup>	5.1 ± 0.4 <sup>cd</sup>	< 0.001	0.585	0.124	
Serum TMAO (nmol/mL)	1.2 ± 0.1 <sup>e</sup>	$3.9 \pm 0.4^{abc}$	$2.4 \pm 0.1^{bcd}$	$1.8 \pm 0.1^{cde}$	$1.8 \pm 0.2^{de}$	1.1 ± 0.1°	$6.7 \pm 1.3^{a}$	$6.8 \pm 0.8^{\mathrm{a}}$	$3.9 \pm 0.8^{abc}$	$\begin{array}{c} 4.0 \pm \\ 0.8^{ab} \end{array}$	< 0.001	< 0.001	0.010	

<sup>1</sup> Values are means  $\pm$  SEMs; n = 8. Mean values within a row without a common letter are statistically significant (P < 0.05). CAS, casein-based diet, 20% EGG, diet containing 20% (w/w) protein from whole egg; 10% EGG, diet containing 10% casein and 10% protein from whole egg; 5% EGG, diet containing 15% casein and 5% protein from whole egg; 2.5% EGG, diet containing 17.5% casein and 2.5% protein from whole egg (2.5% EGG); TMAO, trimethylamine N-oxide.



# CHAPTER 5. WHOLE EGG CONSUMPTION IMPAIRS INSULIN SENSITIVITY IN A RAT MODEL OF OBESITY AND TYPE 2 DIABETES

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Whole egg consumption impairs insulin sensitivity in a rat model of obesity and type 2 diabetes.

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### Abstract

**Background:** The literature regarding the relation between egg consumption and type 2 diabetes (T2D) is inconsistent and there is limited evidence pertaining to the impact of egg consumption on measures of insulin sensitivity.

**Objective:** The objective of this study was to investigate the effect of dietary whole egg on metabolic biomarkers of insulin resistance in T2D rats.

**Methods:** Male Zucker diabetic fatty rats (*n*=12; 6 wk of age) and their lean controls (*n*=12; 6 wk of age) were randomly assigned to a casein- or whole egg-based diet. At wk 5 of dietary treatment, an insulin tolerance test (ITT) was performed on all rats and blood glucose was measured by glucometer. After 7 wk of dietary treatment, rats were anesthetized and whole blood was collected via a tail vein bleed. Following sedation, the extensor digitorum longus muscle was removed before and after an intraperitoneal insulin injection and insulin signaling in skeletal muscle was analyzed by western blot. Serum glucose and insulin were analyzed by ELISA for calculation of the homeostatic model assessment of insulin resistance (HOMA-IR).

**Results:** Mean ITT blood glucose over the course of 60 min was 32% higher in ZDF rats fed the whole egg-based diet compared to ZDF rats fed the casein-based diet. Furthermore, whole egg consumption increased fasting blood glucose by 35% in ZDF rats. Insulin-stimulated phosphorylation of key proteins in the insulin signaling pathway did not differ in skeletal muscle



of ZDF rats fed casein- and whole egg-based diets. In lean rats, no differences were observed in insulin tolerance, HOMA-IR and skeletal muscle insulin signaling, regardless of experimental dietary treatment.

**Conclusions:** These data suggest that whole body insulin sensitivity may be impaired by whole egg consumption in T2D rats, although no changes were observed in skeletal muscle insulin signaling that could explain this finding.

#### Introduction

The increasing prevalence of type 2 diabetes (T2D) is a critical public health issue and insulin resistance is a key contributor to T2D development (1,2). Insulin resistance is a condition characterized by hyperinsulinemia; hyperglycemia; and impaired glucose and insulin tolerance (3). Diet is an important modifiable risk factor for insulin resistance and the progression of T2D. Therefore, understanding the relation between dietary components, such as whole egg, and insulin resistance is essential for developing future dietary recommendations for the millions of individuals with existing T2D, as well as those that are at high risk for developing T2D.

Insulin mediates its metabolic effects by binding to the insulin receptor, thereby modifying the activity and/or intracellular location of proteins involved in the insulin signaling pathway. Insulin binding to the insulin receptor triggers autophosphorylation of the insulin receptor  $\beta$  (IR  $\beta$ ) subunit, which activates the receptor and initiates a cascade of phosphorylation events (4). Key events in the insulin signaling cascade include the activation of the insulin receptor substrate 1 (IRS-1) via tyrosine phosphorylation;

serine/threonine phosphorylation of Akt and its subsequent activation; phosphorylation of Akt substrate 160 (AS160) at serine/threonine residues and translocation of the glucose transporter type 4 (GLUT4) from intracellular vesicles to the plasma membrane, resulting in increased glucose



uptake in skeletal muscle and adipose tissue (5–7). Defects in insulin function through the sequential action of the insulin receptor, IRS-1, Akt, AS160 and GLUT4 have been reported in metabolic disorders associated with insulin resistance, such as obesity and T2D (8,9). Impaired insulin signaling at any of these key steps reduces the ability of insulin to promote glucose uptake and utilization.

Limited and inconsistent findings have been reported on the relation between egg consumption and T2D. Whereas some studies suggest that egg consumption increases the risk of T2D (10–12), others report a null association or a beneficial impact on T2D risk and outcomes (13–18). A meta-analysis found no association between egg consumption and T2D risk in countries outside of the U.S., but found a modest increase in T2D risk that was restricted to U.S. studies, suggesting that these results may be confounded by factors such as dietary behaviors of the U.S. population (19). Results from a recent human study suggest that the apparent association between egg consumption and T2D risk in the U.S. population and T2D risk in the U.S. population may be due to an interaction between meat and egg intake, and not egg intake alone (20).

It is widely recognized that obesity is a major risk factor for insulin resistance, which precedes the onset of overt diabetes (1–3). We previously reported that a whole egg-based diet attenuates cumulative body weight gain in the Zucker diabetic fatty (ZDF) rat, a well-characterized genetic model of obesity and T2D (21,22). The observed attenuation in body weight gain was attributed, in part, to an 8% reduction in body fat in ZDF rats consuming a whole egg-based diet (21). Furthermore, we extended this research to a diet-induced model of obesity and demonstrated that whole egg consumption in diet-induced obese rats markedly reduces weight gain compared to diet-induced obese rats fed a casein-based diet (unpublished observations; CJ Saande, SK Jones, KE Hahn, CH Reed, MJ Rowling, KL Schalinske, 2017). There is very limited evidence regarding



the association between egg consumption and measures of insulin sensitivity (14,23,24) and, to our knowledge, the impact of whole egg consumption on insulin signaling has not been examined. Thus, the objective of this study was to investigate whether the previously observed reductions in adiposity in ZDF rats fed a whole egg-based diet are related to improved insulin sensitivity and enhanced insulin signaling.

#### **Materials and Methods**

*Rats and Diets.* All animal studies were approved by the Institutional Animal Care and Use Committee at Iowa State University (IACUC # 1-18-8674-R; approval date 01/12/18) and were performed according to the Iowa State University Laboratory Animal Resources Guidelines. Male Zucker diabetic fatty (ZDF; fa/fa) rats (n= 12) and lean (fa/+) control rats (n=12) were purchased at 5 wk of age (Charles River Laboratories). Rats were housed two per cage with a 12-h light-dark cycle in a temperature controlled room. All rats were acclimated to a semipurified diet (AIN-93G) for one wk. Following acclimation, rats were randomly assigned to 1 of 2 experimental diets (Table 5-1): a case in-based diet (n=12) or a whole egg-based diet (n=12). Both diets provided protein at 20% (w/w) and were matched for lipid content (17.7% total lipid) via the addition of corn oil to the casein-based diet to account for the additional lipid contribution of the whole egg. Diets were prepared weekly and rats were given ad libitum access to food and water for a period of 7 wk. Body weight and food intake were recorded 5 days/wk. Prior to sacrifice, food was withheld for 4 h and rats were anesthetized via a single intraperitoneal (IP) injection of ketamine:xylazine (90:10 mg/kg body weight). Following sedation, whole blood was collected via a tail vein bleed and blood samples were stored on ice until centrifugation. The extensor digitorum longus (EDL) muscle was removed from one leg prior to an insulin injection to account for basal differences in insulin signaling. All rats were then given an IP insulin injection (Sigma; 10 U/kg body weight) and the



EDL muscle was removed from the other leg 10 min post-insulin injection to allow sufficient time for insulin signaling to occur (25–28). Immediately following tissue removal, muscle samples were snap-frozen in liquid nitrogen and stored at -80°C for subsequent analysis. The epididymal fat pad was removed and weighed. A total of 24 rats were euthanized; euthanasia was achieved by exsanguination. Whole blood was centrifuged in separation tubes and the resultant serum was stored at -80°C.

*Insulin tolerance tests.* Insulin tolerance tests (ITT) were performed at wk 5 of experimental dietary treatment. Rats were fasted for a period of 4 h prior to insulin tolerance testing and given an IP insulin injection (0.5 U/kg body weight). Blood samples were collected from the tail vein immediately prior to the insulin challenge, as well as 15, 30, 45 and 60 min thereafter. Blood sampling was performed by making a nick with a sterilized razor blade toward the end of the tail and blood glucose was measured with the use of a glucometer (Bayer Healthcare). When blood glucose was above the detection limit (600 mg/dL), the maximum value of 600 mg/dL was used.

*Serum glucose and serum insulin.* Serum collected on the final day of the study was used for analysis of fasting glucose, fasting insulin and calculation of the homeostatic model assessment of insulin resistance (HOMA-IR). Serum glucose was measured using a commercially available colorimetric kit (Wako Diagnostics). Analysis of serum insulin was measured by a commercially available immunoassay kit for the detection of insulin in rat sera (EMD Millipore).

*Western blot analysis.* Extensor digitorum longus muscles were homogenized in 800 µL of lysis buffer [Tris-hydrochloric acid (pH 7.8, 50 mM), Ethylenediaminetetraacetic acid (EDTA; 1 mM)



Ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA; 1 mM), Glycerol (10%, w/v), Triton-X 100 (1%, w/v), Dithiothreitol (DTT; 1 mM)] containing phosphatase (Sigma) and protease (Thermo Scientific) inhibitors. Samples were then centrifuged at 4000 x g for 15 min at 4°C and the supernatant was collected. Protein concentrations were determined using a bicinchoninic acid assay (Pierce) according to the manufacturer's instructions. A total of 20 µg protein was loaded and run on a 4-15% gradient sodium dodecyl sulfate polyacrylamide gel (Bio-Rad). Following separation, proteins were transferred onto a polyvinylidene difluoride membrane (EMD Millipore) and blocked at room temperature for 1 h in Tris-buffered saline with 0.05% tween (TBST) and 5% non-fat dry milk. Membranes were incubated in p-IGFI Receptor B<sup>Tyr1135/1136</sup>/Insulin Receptor  $\beta^{\text{Tyr1150/1151}}$ , p-Akt<sup>Ser473</sup>, Akt and p-AS160<sup>Thr642</sup> antibodies (Cell Signaling) at 1:1000 overnight at 4 °C. Following incubation with primary antibody, membranes were washed and incubated with an anti-rabbit secondary antibody (Cell Signaling) at 1:5000 for one hour at room temperature. Membranes were incubated in enhanced chemiluminescent substrate (SuperSignal West Pico PLUS Sensitivity Substrate or SuperSignal West Femto Maximum Sensitivity Substrate; Thermo Scientific) for 5 min prior to imaging with the ChemiDoc XRS detection imaging system (Bio-Rad). Densitometry was determined using Image Lab software (BioRad) and raw data was normalized to total protein.

*Statistical analysis.* All data were evaluated for statistically significant differences (P < 0.05) with the use of SPSS Statistics Software Version 23 (IBM). Body and epididymal fat pad weights, food intake and serum parameters were analyzed with the use of a 2-factor ANOVA (diet x genotype). An analysis of main effects was performed when the interaction between diet and genotype was not statistically significant. Insulin tolerance test data was analyzed by a 3-factor, repeated



measures ANOVA (time x diet x genotype) and statistically significant two-way interactions were followed by an analysis of simple main effects. Western blot data was analyzed with the use of a 3-factor mixed ANOVA to determine the effects of insulin, diet and genotype on insulin signaling. All pairwise comparisons were performed using the Fishers least significant difference post hoc test.

#### Results

*Body and relative adipose tissue weights*. As expected, there was a significant main effect of genotype on initial and final body weight. ZDF rats had a higher mean initial body weight compared to their lean counterparts and body weight was 13% higher in ZDF rats compared to lean rats on the final day of the study. Diet was without effect on final body weight in both lean and ZDF rats (Table 5-2). Likewise, there was a significant main effect of genotype on relative adipose tissue weight [epididymal fat pad weight (g/ 100 g body weight)]. The ZDF genotype was associated with a 74% higher mean relative adipose tissue weight than the lean genotype. No significant differences in relative adipose tissue weight were observed across diets within lean or ZDF rats (Table 5-2).

*Food intake*. Main effects analysis indicated a significant effect of genotype on food intake and total energy intake. ZDF rats exhibited an 86% higher mean total food intake compared to lean rats (Table 5-2). Likewise, total energy intake was 86% higher in ZDF rats compared to lean rats (Table 5-2). There was no effect of diet on total food intake or total energy intake.

*Insulin tolerance test*. Analysis of ITT blood glucose concentrations revealed a significant effect of time on circulating glucose concentrations, demonstrating that insulin effectively lowered blood



glucose. There was also a significant effect of genotype and diet, as well as significant diet\*genotype and time\*genotype two-way interactions. As expected, there was a simple main effect of genotype (P < 0.001) on blood glucose, indicating markedly higher blood glucose in ZDF rats compared to lean rats at each time point (Figure 5-1). A simple main effect of time was also observed in the ZDF genotype (P = 0.001), but not the lean genotype (P = 0.836). Lastly, a simple main effect of diet was observed in the ZDF genotype (P < 0.001), but not the lean genotype (P = 0.836). Lastly, a simple main effect of diet was observed in the ZDF genotype (P < 0.001), but not in the lean genotype (P = 0.987). With the exception of baseline blood glucose, ZDF rats fed the whole egg-based diet exhibited approximately 38% higher blood glucose concentrations from the 15-60 min time points compared to ZDF rats fed the casein-based diet. In contrast, blood glucose did not differ between dietary treatment groups in lean rats at any of the time points (Figure 5-1).

Serum glucose, serum insulin, HOMA-IR and HOMA- $\beta$ . There was a significant main effect of genotype on serum glucose, serum insulin and the HOMA-IR. As expected, mean serum glucose, serum insulin and HOMA-IR values were 244, 629 and 234% higher, respectively, in the ZDF genotype compared to the lean genotype (Table 5-3). Diet was without effect on serum glucose concentrations within the lean genotype; however, serum glucose concentrations were increased by 35% in ZDF rats fed the whole egg-based diet compared to ZDF rats fed the casein-based diet (Table 5-3). No differences in serum insulin concentrations were observed across dietary groups within the lean genotype, whereas serum insulin was 68% higher in ZDF rats fed the casein-based diet compared to ZDF rats fed the whole egg-based diet. There was no effect of diet on the HOMA-IR within the lean or ZDF genotype (Table 5-3). Lastly, there was a significant main effect of diet on the homeostatic model assessment of  $\beta$ -cell function (HOMA- $\beta$ ). The whole egg-based diet



was associated with a mean decrease of 44% in HOMA- $\beta$  compared to the casein-based diet (Table 5-3).

*Insulin signaling pathway*. Insulin increased phosphorylation of the IR  $\beta^{Tyr1150/1151}$  by 291% in lean rats fed the whole egg-based diet compared to IR  $\beta^{Tyr1150/1151}$  phosphorylation prior to insulin (Figure 5-2); however, post-insulin IR  $\beta^{Tyr1150/1151}$  phosphorylation did not reach statistical significance (P = 0.215) in lean casein-fed rats compared to pre-insulin p- IR  $\beta^{Tyr1150/1151}$ . No differences in p-IR  $\beta^{Tyr1150/1151}$  were observed pre- or post-insulin in ZDF rats, regardless of dietary treatment (Figure 5-2). In lean rats fed the casein- and whole egg-based diets, the post-insulin ratio of p-Akt<sup>Ser473</sup>: total Akt was increased 17-fold and 18-fold, respectively, compared to the pre-insulin ratio (Figure 5-3). Pre- and post-insulin p-Akt<sup>Ser473</sup>: total Akt did not differ in ZDF rats, regardless of dietary treatment. However, in ZDF rats fed the whole egg-based diet, the post-insulin p-Akt<sup>Ser473</sup>: total Akt ratio did not statistically differ from the lean genotype (Figure 5-3). No differences in post-insulin p-AS160<sup>Thr642</sup> were observed, regardless of diet or genotype (data not shown).

#### Discussion

The relation between egg consumption and T2D remains contradictory and evidence is limited regarding potential mechanisms that may explain the reported associations between dietary egg intake, glycemic control and incident diabetes. The present study aimed to examine the effects of egg consumption on insulin tolerance and insulin signaling in vivo using a rat model of obesity and T2D. While egg consumption impaired glycemic control in ZDF rats during an insulin tolerance test, no differences were observed in skeletal muscle insulin signaling between ZDF rats



fed casein- and whole egg-based diets. Although skeletal muscle is the primary site of insulinstimulated glucose disposal, glucose metabolism by the liver and adipose tissue also contributes to whole body glucose homeostasis (29–31). The relative contribution of these tissues to systemic glucose metabolism, as well as differences in timing between insulin tolerance testing and skeletal muscle collection for insulin signaling analysis, may explain the differential results observed between whole body insulin tolerance and skeletal muscle insulin signaling. Very few studies have investigated the effect of egg consumption on direct measures of insulin sensitivity (23). In the present study, we report higher blood glucose during an insulin tolerance test in ZDF rats consuming a whole egg-based diet compared to ZDF rats fed a casein-based diet. In support of this finding, egg consumption was inversely associated with insulin sensitivity and the metabolic clearance rate of insulin in a cross-sectional analysis of a non-diabetic population, though these associations became insignificant after adjustment for body mass index and dietary cholesterol (23). Likewise, Djousse et al. reported an increase in fasting blood glucose and insulin resistance, as measured by HOMA-IR, across varying amounts of egg consumption in a prospective cohort of older adults (14). However, the authors noted that the magnitude of difference, although statistically significant, was not likely to be of clinical significance (14). Here, we report higher fasting blood glucose in ZDF rats after 7 wk of dietary treatment with the whole egg-based diet, but no differences in HOMA-IR, a model used to quantify insulin resistance, between ZDF rats fed casein- and whole egg-based diets.

In the early stages of insulin resistance, enhanced pancreatic insulin secretion attempts to compensate for reduced responsiveness to insulin in peripheral tissues as a means to maintain normal glucose tolerance. A physiologic approach to accomplish this goal is by enhanced  $\beta$ -cell mass and activity (32,33). As insulin resistance progresses, compensatory hyperinsulinemia is



unable to maintain normal blood glucose concentrations. Insulin secretion is continuously stimulated by hyperglycemia, and  $\beta$ -cell structure and function becomes compromised, ultimately leading to apoptosis (33). In ZDF rats,  $\beta$ -cell mass decreases between ages 6-12 wk of age, and is significantly reduced at 12 wk (34–36). The observed loss of  $\beta$ -cell mass has been attributed an increase in cell death (34,35).  $\beta$ -cell dysfunction in ZDF rats is accompanied by a progressive decline in circulating insulin concentrations, beginning at 7 wk of age (34,36). We report significantly lower serum insulin, concomitant with higher serum glucose, in ZDF rats fed the whole egg-based diet compared to ZDF rats fed the casein-based diet after 7 wk of dietary treatment (13 wk of age). Additionally, consumption of a whole egg-based diet was associated with decreased HOMA- $\beta$ , an index of  $\beta$ -cell function, suggesting impaired insulin production and secretion in rats fed the whole egg-based diet. It is possible that ZDF rats fed the whole egg-based diet exhibit a higher rate of decline in  $\beta$ -cell function, potentially explaining these differences. In cultured  $\beta$ -cells, cholesterol accumulation results in apoptosis and impaired glucose-stimulated insulin secretion (37–40). The cholesterol content of whole egg may play a role in the observed reduction in serum insulin; however, whether whole egg consumption impacts β-cell function in ZDF rats remains to be determined.

Aberrant insulin signaling in skeletal muscle and adipose tissue impairs insulin-mediated translocation of GLUT4 and subsequent glucose uptake. To our knowledge, there are no previous studies examining the effect of egg consumption on insulin signaling. In the present study, phosphorylation of IR  $\beta^{Tyr1150/1151}$  was not significantly increased in ZDF rats following an insulin injection, regardless of experimental dietary treatment. This result is consistent with findings from numerous human studies, which show reduced tyrosine phosphorylation of the insulin receptor and its subsequent kinase activity in states of insulin resistance (41–46). The serine/threonine



kinase Akt is activated by insulin-stimulated phosphorylation at both Thr308 and Ser473 and plays a key role in the regulation of glucose uptake into insulin responsive tissues (47). As expected, we report a marked increase in the ratio of p-Akt<sup>Ser473</sup>: total Akt in lean rats in response to insulin. Conversely, the p-Akt<sup>Ser473</sup>: total Akt ratio was not significantly increased by insulin in ZDF rats fed both casein- and whole egg-based diets. In agreement with this finding, several studies report defective Akt phosphorylation and kinase activity in insulin resistant subjects compared to lean controls (48–52). Phosphorylation of AS160, a downstream substrate of Akt, links insulin signaling to GLUT4 translocation and impaired insulin-stimulated AS160 phosphorylation has been reported in skeletal muscle of diabetic human subjects (52,53). In contrast to these findings, we did not observe differences in post-insulin p-AS160<sup>Thr642</sup> between lean and ZDF rats, regardless of dietary treatment group.

Eggs are a source of high-quality protein, and several human studies report an association between egg consumption, increased satiety and reduced caloric intake (54–57). Egg consumption has also been shown to promote weight loss in a limited number of human studies (58,59). In contrast to our previous findings (21,22), we did not observe a reduction in body weight gain in ZDF rats fed a whole egg-based diet. Moreover, relative adipose tissue weight not differ between ZDF rats, regardless of dietary treatment. It is well-documented that weight loss is a highly effective strategy to improve insulin sensitivity and glycemia, both in the prevention and treatment of T2D (60,61). Furthermore, numerous human studies report improved glycemic control in type 2 diabetics following adherence to low-carbohydrate, low-glycemic index and high-protein diets (62,63). Indeed, beneficial impacts off egg consumption on blood glucose control have been shown in human subjects when combined with energy or carbohydrate restriction (13,24,64,65). For example, Pearce et al. reported improvements in glycemic and lipid profiles in type 2 diabetics



following consumption of a hypoenergetic, high-protein diet containing 2 eggs/d (13). In individuals with metabolic syndrome, Blesso et al. found a reduction in HOMA-IR following consumption of a carbohydrate-restricted diet including 3 eggs/d (24). In the current study, rodent diets were matched for macronutrient content and there were no differences in final body weight between ZDF rats fed casein-based and whole egg-based diets. Taken together, these findings suggest that reported improvements in glycemic control associated with egg consumption may be related to changes in dietary macronutrient content and/or improved body weight management, and not a direct effect of egg consumption on skeletal muscle insulin signaling.

A limitation of this study is the quantity of dried whole egg used in the whole egg-based diet, which exceeds the amount of whole egg that would typically be consumed in a human diet. The quantity of dried whole egg was determined such that the whole egg- and casein-based diets were matched for protein content. Additionally, analysis of  $\beta$ -cell mass and glucose-stimulated insulin secretion would provide insight into whether  $\beta$ -cell function declines more rapidly in ZDF rats fed the whole egg-based diet. Lastly, insulin signaling was only analyzed in the EDL muscle. The EDL is frequently used in analysis of skeletal muscle insulin signaling (7,66–69). However, it is possible that sensitivity for phosphoregulation by insulin may differ in other muscle groups. Future studies will include analysis of skeletal muscle groups composed of different fiber types, as well as additional tissues, to provide a more comprehensive examination of insulin signaling.

In summary, these data suggest that whole egg consumption may impair insulin sensitivity in T2D rats. Although consumption of a whole egg-based diet negatively impacted whole body insulin sensitivity in ZDF rats, we were unable to identify changes in skeletal muscle insulin signaling that could explain this finding. Future studies investigating the impact of whole egg consumption on  $\beta$ -cell function may offer a potential explanation for the reduction in fasting serum



insulin in ZDF rats fed a whole egg-based diet. Furthermore, dose-response studies are warranted to determine whether the observed impairment in insulin sensitivity is maintained at a lower dose of whole egg.

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C.J.S. designed the study and performed all aspects of animal maintenance, preparation of experimental diets, insulin tolerance testing and laboratory experiments, as well as drafted the original version of this manuscript. M.A.S. assisted in animal maintenance and preparation of experimental diets. J.L.W. assisted with insulin tolerance testing. R.J.V., M.J.R. and K.L.S. assisted with the study design. All authors read and approved the final manuscript.

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# **Tables and Figures**

Figure 5-1: Insulin tolerance test blood glucose in lean and Zucker diabetic fatty rats fed a caseinbased or whole egg-based diet for 5 wk. Data are means  $\pm$  SEMs; *n*=3-6. Data within the same time point without a common letter differ (*P* < 0.05). Three-factor repeated measures ANOVA: Time, *P* < 0.001; Diet, *P* = 0.027; Genotype, *P* < 0.001; Time\*Diet, *P* = 0.662; Time\*Genotype *P* = 0.031; Diet\*Genotype *P* = 0.025; Time\*Diet\*Genotype, *P* = 0.572.





Figure 5-2: Skeletal muscle p-IR  $\beta^{\text{Tyr1150/1151}}$  (A) and representative western blot images of skeletal muscle p-IR  $\beta^{\text{Tyr1150/1151}}$  and total protein (B) pre- and post-insulin injection in lean and Zucker diabetic fatty rats fed a casein-based or whole egg-based diet for 7 wk. Data are expressed relative to pre-insulin p-IR  $\beta^{\text{Tyr1150/1151}}$  in lean rats fed the casein-based diet. Data are means  $\pm$  SEMs; *n*=5-6. Bars without a common letter differ (*P* < 0.05). Three-factor mixed ANOVA: Insulin, *P* = 0.029; Diet, *P* = 0.492; Genotype, *P* = 0.874; Insulin\*Diet, *P* = 0.297; Insulin\*Genotype *P* = 0.169; Diet\*Genotype *P* = 0.723; Insulin\*Diet\*Genotype, *P* = 837. LC-Pre, Lean Casein Pre-insulin; LC-Post, Lean Casein Post-insulin; ZC-Pre, ZDF Casein Pre-insulin; ZC-Post, ZDF Casein Post-insulin; LWE-Pre, Lean Whole Egg Pre-insulin; LWE-Post, Lean Whole Egg Pre-insulin; ZWE-Post, ZDF Whole Egg Post-insulin.





Figure 5-3: The ratio of skeletal muscle p-Akt<sup>Ser473</sup>: total Akt (A) and representative western blot images of skeletal muscle p-Akt<sup>Ser473</sup>, total Akt and total protein (B) pre- and post-insulin injection in lean and Zucker diabetic fatty (ZDF) rats fed a casein-based or whole egg-based diet for 7 wk. Data are expressed relative to the pre-insulin p-Akt<sup>Ser473</sup>:total Akt ratio in lean rats fed the caseinbased diet. Data are means  $\pm$  SEMs; *n*=5-6. Bars without a common letter differ (*P* < 0.05). Threefactor mixed ANOVA: Insulin, *P* < 0.001; Diet, *P* = 0.53; Genotype, *P* = 0.157; Insulin\*Diet, *P* = 0.571; Insulin\*Genotype *P* = 0.11; Diet\*Genotype *P* = 0.535; Insulin\*Diet\*Genotype, *P* = 0.609. LC-Pre, Lean Casein Pre-insulin; LC-Post, Lean Casein Post-insulin; ZC-Pre, ZDF Casein Preinsulin; ZC-Post, ZDF Casein Post-insulin; LWE-Pre, Lean Whole Egg Pre-insulin; LWE-Post, Lean Whole Egg Post-insulin; ZWE-Pre, ZDF Whole Egg Pre-insulin; ZWE-Post, ZDF Whole Egg Post-insulin.



Ingredient (g/kg) <sup>1</sup>	Casein	Whole Egg
Casein (vitamin free)	200	0
Dried whole egg	0	413
Cornstarch	423	387
Glucose monohydrate	150	150
Mineral Mix (AIN 93)	35	35
Vitamin Mix (AIN 93)	10	10
Biotin 1%	0	0.4
Corn oil	177	0
Choline bitartrate	2	2
L-Methionine	3	3
Macronutrients (kcal/kg)		
Protein	800	800
Lipid	1593	1593
Carbohydrate	2292	2148
Total Energy	4685	4541

Table 5-1: Composition of the casein-based diet and whole egg-based diet fed to lean control and Zucker diabetic fatty rats for 7 wk.

<sup>1</sup>All ingredients were purchased from Envigo with the exception of dried whole egg (Rose Acre Farms) as well as L-methionine and choline bitartrate (Sigma-Aldrich).

 $^2$  Total protein and lipid content provided by 413 g of dried whole egg was 48.4 (200g) and 42.9% (177g), respectively.



Table 5-2: Body and adipose tissue weights and total food intake of lean and Zucker diabetic fatty (ZDF) rats fed a casein-based or whole egg-based diet for 7 wk.

	Le	an	ZI	DF	Р			
	Casein	Casein Whole Egg		Casein Whole Egg		Diet	Genotype x Diet	
Initial Body Weight <sup>1</sup> (g)	$157 \pm 5^{a}$	$155 \pm 6^{a}$	$191 \pm 6^{b}$	$191 \pm 4^{b}$	< 0.001	0.877	0.824	
Final Body Weight <sup>1</sup> (g)	$329 \pm 7^{a}$	$334 \pm 5^{a}$	$378 \pm 4^{\text{b}}$	$371 \pm 8^{b}$	< 0.001	0.897	0.368	
Epididymal Fat Pad Weight <sup>1</sup> (g/100 g body weight)	$0.47 \pm 0.04^{a}$	$0.50 \pm 0.08^{a}$	$0.86 \pm 0.08^{b}$	$0.83 \pm 0.03^{b}$	<0.001	0.992	0.642	
Total Food Intake <sup>2</sup> (g)	$990 \pm 28^{a}$	$930 \pm 29^{a}$	1843 ± 135 <sup>b</sup>	$1732 \pm 163^{b}$	< 0.001	0.449	0.818	
Total Energy Intake <sup>2</sup> (kcal)	$4639 \pm 130^{a}$	$4224 \pm 132^{a}$	$8636 \pm 632^{b}$	7865 ± 740 <sup>b</sup>	<0.001	0.265	0.729	

<sup>1</sup> Data are means  $\pm$  SEMs; *n*=6. Data within the same row without a common letter differ (*P* < 0.05).

<sup>2</sup> Data are means  $\pm$  SEMs; *n*=3. Total food intake per cage (2 rats per cage). Data within the same row without a common letter differ (*P* < 0.05).



Table 5-3: Fasting serum glucose, fasting serum insulin, HOMA-IR and HOMA- $\beta$  of lean and Zucker diabetic fatty (ZDF) rats fed a casein-based or whole egg-based diet for 7 wk<sup>1</sup>.

	Lear	n	Z	ZDF	P		
	Casein	Whole Egg	Casein	Whole Egg	Genotype	Diet	Genotype x Diet
Serum glucose (mg/dL)	$124 \pm 13^{\circ}$	189 <u>+</u> 19 <sup>c</sup>	$457 \pm 31^{b}$	$618 \pm 86^{a}$	< 0.001	0.026	0.317
Serum Insulin (ng/mL)	$0.3 \pm 0.1^{\circ}$	$0.4 \pm 0.1^{\circ}$	$3.2 \pm 0.4^{a}$	$1.9 \pm 0.6^{b}$	< 0.001	0.116	0.078
HOMA-IR	$2.1 \pm 0.46^{b}$	$4.0 \pm 1.2^{b}$	$82 \pm 9.3^{a}$	$59 \pm 20^{a}$	< 0.001	0.344	0.267
HOMA-β (%)	$51 \pm 13^{ab}$	$32 \pm 13^{\text{b}}$	$72 \pm 13^{a}$	$37 \pm 12^{ab}$	0.331	0.046	0.554

<sup>1</sup> Data are means  $\pm$  SEMs; *n*=6. Data within the same row without a common letter differ (*P* < 0.05).



# CHAPTER 6. DIETARY EGG PROTEIN PREVENTS HYPERHOMOCYSTEINEMIA VIA UPREGULATION OF HEPATIC BETAINE HOMOCYSTEINE S-METHYLTRANSFERASE ACTIVITY IN FOLATE-RESTRICTED RATS

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### Abstract

**Background:** Hyperhomocysteinemia is associated with increased cardiovascular disease risk. Whole eggs contain several nutrients known to impact homocysteine regulation, including sulfur amino acids, choline, and B vitamins.

**Objective:** The objective of this study was to determine the effect of whole eggs and egg components (i.e. egg protein and choline) with respect to 1) homocysteine balance and 2) the hepatic expression and activity of betaine-homocysteine *S*-methyltransferase (BHMT) and cystathionine  $\beta$ -synthase (CBS) in a folate-restricted rat model of hyperhomocysteinemia.

**Methods:** Male Sprague Dawley rats (N=48; 6 wk of age) were randomly assigned to a caseinbased diet (C; n=12), a casein-based diet supplemented with choline (C+Cho; 1.3%, w/w; n=12), an egg protein-based diet (EP; n=12), or a whole egg-based diet (WE; n=12). At wk 2, half of the rats in each of the 4 dietary groups were provided a folate-restricted (FR; 0 g folic acid/kg) diet and half continued on the folate-sufficient (FS; 0.2 g folic acid/kg) diet for an additional 6 wk. All diets contained 20% (w/w) total protein. Serum homocysteine was measured by HPLC and BHMT



and CBS expression and activity were evaluated using RT-qPCR, western blot and enzyme activity. A 2-factor ANOVA was used for statistical comparisons.

**Results:** Rats fed FR-C exhibited a 53% increase in circulating homocysteine concentrations compared to rats fed FS-C (P < 0.001). In contrast, serum homocysteine did not differ between rats fed FS-C and FR-EP (P = 0.078). Hepatic BHMT activity was increased by 45% and 40%, respectively, by the EP (P < 0.001) and WE (P = 0.002) diets compared to the C diets.

**Conclusions:** Dietary intervention with egg protein prevented elevated circulating homocysteine concentrations in a rat model of hyperhomocysteinemia, due in part to upregulation of hepatic BHMT. These data may support the inclusion of egg protein for dietary recommendations targeting hyperhomocysteinemia prevention.

#### Introduction

The metabolism of folate, methyl groups, and homocysteine are interrelated processes that play a critical role in maintaining optimal health. Perturbation of these pathways can result in hyperhomocysteinemia, which has been implicated as a risk factor for cardiovascular disease (CVD) (1–4). Elevated circulating homocysteine concentrations are associated with increased risk of several cardiovascular pathologies, including myocardial infarction, stroke, and atherosclerosis (1,5). Therefore, maintenance of homocysteine balance may play a role in the prevention of cardiovascular disease-associated morbidity and mortality.

Homocysteine is a sulfur-containing amino acid derived from the metabolism of the indispensable amino acid methionine. Homocysteine metabolism occurs via three pathways: 1) folate-dependent remethylation to methionine, a reaction catalyzed by the vitamin B-12-dependent enzyme methionine synthase; 2) folate-independent remethylation catalyzed by betaine-homocysteine S-methyltransferase (BHMT); and 3) irreversible catabolism by the vitamin B-6-



dependent enzyme cystathionine  $\beta$ -synthase (CBS) (6) (Figure 6-1). The etiology of hyperhomocysteinemia has been attributed to several factors, including genetics, chronic disease, sex, age, and nutritional deficiencies. Genetically inherited mutations in genes encoding the enzymes CBS and 5,10-methylenetetrahydrofolate reductase (MTHFR) result in an inability of the cell to adequately metabolize homocysteine (Figure 6-1) (7–9). Hyperhomocysteinemia is also associated with chronic diseases, such as renal disease, diabetes, hypertension, and hypothyroidism, as well as male sex and older age (10–12). Additionally, nutritional deficiencies in folate, vitamin B-12, and vitamin B-6 impair homocysteine regulation (13–15). Several studies have reported an inverse correlation between B vitamin intake and CVD risk (14,16–22).

Homocysteine metabolism relies on several nutrient substrates and cofactors; therefore, dietary composition plays an important role in maintaining homocysteine balance. Whole eggs are a significant source of folate, vitamin B-12 and vitamin B-6 (23). Furthermore, egg yolk is a rich source of choline, utilized as a methyl donor in the folate-independent remethylation of homocysteine (6,23). Lastly, egg protein provides a balanced content of the sulfur amino acids methionine and cysteine (23), and variations in dietary sulfur amino acid content have been reported to regulate homocysteine metabolism (24,25). In our preliminary studies, we have consistently found that hyperhomocysteinemia was prevented in folate-restricted rats fed an egg protein-based diet, concomitant with an increase in hepatic BHMT activity. Thus, the aim of this study was to expand upon our preliminary findings by investigating the impact of dietary whole eggs and egg components (i.e. choline and egg protein) on 1) circulating homocysteine concentrations and 2) hepatic expression and activity of BHMT and CBS in a folate-restricted rat model of moderate hyperhomocysteinemia.



### **Materials and Methods**

**Rats and Diets.** All animal studies were approved by the Institutional Animal Care and Use Committee at Iowa State University and were performed according to the Iowa State University Laboratory Animal Resources Guidelines. Male Sprague Dawley rats (N=48) were purchased at 5 wk of age (Envigo). Rats were housed 2 per cage in ventilated cages (Innovive) with a 12-h lightdark cycle in a temperature-controlled room. All rats were acclimated to a semi-purified diet (AIN-93G) for 1 wk. Following acclimation, rats were randomly assigned to 1 of 4 experimental diets (n= 12 rats per group): a casein-based diet (C); a casein-based diet supplemented with choline (1.3%, w/w, C+Cho); an egg protein-based diet (EP); or a whole egg-based diet (WE). At wk 2, half of the rats in each dietary group were provided the same treatment diet, but with the omission of folate (folate-restricted, FR; 0 g folic acid/kg) from the vitamin mix, and half of the rats continued on the original folate-sufficient (FS; 0.2 g folic acid/kg) diet for the remainder of the 8 wk study period. This resulted in a total of 8 experimental dietary groups: a folate-sufficient caseinbased diet (FS-C); a folate-restricted casein-based diet (FR-C); a folate-sufficient casein-based diet supplemented with choline (FS-C+Cho); a folate-restricted casein-based diet supplemented with choline (FR-C+Cho); a folate-sufficient egg protein-based diet (FS-EP); a folate-restricted egg protein-based diet (FR-EP); a folate-sufficient whole egg-based diet (FS-WE); and a folaterestricted whole egg-based diet (FR-WE) (Table 6-1). Supplemental choline in the casein-based diet was matched to the choline content provided by the whole egg-based diet (1.3%, w/w). FR was achieved with a custom-formulated vitamin mix (Envigo) devoid of folate. FR rats were provided diets and drinking water without added antibiotics to elicit a moderate degree of hyperhomocysteinemia, as we have previously reported (26). All diets provided protein at 20% (w/w) and were matched for lipid quantity (17.7% total lipid) via the addition of corn oil to the C, C+Cho and EP diets to account for the additional lipid contribution of the whole egg. It is important



to note that all diets were matched for total lipid quantity, but differed in lipid composition. Dried whole egg also contributed phospholipid species to the WE diets, primarily phosphatidylcholine and phosphatidylethanolamine, as well as cholesterol a fatty acid profile that differs from that of corn oil (23,27,28). All diets were prepared weekly and rats were given ad libitum access to food and water for the duration of the study. Body weights were recorded 5 d/wk. Prior to sacrifice, food was withheld for 12 h to ensure that all rats were in a fasted state. Rats were anesthetized via a single intraperitoneal injection of ketamine:xylazine (90:10 mg/kg body weight). Whole blood was collected via cardiac puncture, centrifuged at 4,000 x g for 15 min, and the resulting serum fraction was stored at -80°C. The liver was removed, snap-frozen in liquid nitrogen, and stored at -80°C for subsequent analysis.

*Enzyme-linked immunoassays.* Liver samples were homogenized in 5 vol of phosphate buffered saline [PBS; NaCl (138 mM), KCl (2.7 mM), Na2KPO4 (10 mM), KH2PO4 (1.8 mM)], centrifuged at 4,000 x g for 15 min at 4°C, and the resulting supernatant was stored at -80°C for subsequent analysis. Hepatic concentrations of SAM and SAH were measured using competitive enzyme immunoassay kits appropriate for the detection and quantification of SAM and SAH in tissue lysates (Cell BioLabs; catalog no. STA-671-C).

*Serum homocysteine and cysteine.* Serum total homocysteine and cysteine were measured by high-pressure liquid chromatography with post-column fluorescence detection, as previously described (29).



RNA Isolation and Quantitative Real-Time PCR. Total liver RNA was extracted using the Quickgene 810 Nucleic Acid Isolation System with a Quickgene RNA Tissue Kit (Autogen). Total RNA yield and purity was measured by spectrophotometry (Nanodrop 2000; Thermo Fisher Scientific). Single-stranded cDNA (1.8  $\mu$ g/20  $\mu$ L reaction) was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR reactions were performed in quadruplicate using Forget-Me-Not<sup>™</sup> EvaGreen<sup>®</sup> detection reagents (Biotium) for the detection of Bhmt and Cbs with the LightCycler 96 Real-Time PCR System (Roche). The primer sets specific for Bhmt (forward primer: TCACTGCAGCGAAGAGAAAG, reverse primer: CGGTCCCTGAATGGTTCTATG) and Cbs (forward primer: CTTAGCAGTTCCTCCTCACATC, reverse primer: AGGTAGACATGACCACAGGTA) were normalized to Gapdh (forward primer: AGGTCGGTGTGAACGGATTTG, reverse primer: GGGGTCGTTGATGGCAACA). Amplification efficiencies of target and reference gene assays were verified using a dilution series of sample cDNA and efficiency was calculated from the slope of the regression line. Data were analyzed using the  $\Delta\Delta CT$  method (CTsample – CTGapdh =  $\Delta CT$ ;  $\Delta CT sample - \Delta CT control = \Delta \Delta CT$  (30).

*Western blot analysis.* Liver samples (50 mg) were homogenized in 1 mL of radioimmunoprecipitation assay (RIPA) buffer [Tris-HCl (50mM, pH 7.4), NaCl (150 mM), sodium deoxycholate (0.5, w/v) NP-40 (1%, w/v), SDS (0.1%, w/v)] with protease inhibitors (Thermo Fisher Scientific). Samples were then centrifuged at 4,000 x g for 15 min at 4°C and the supernatant was collected. Protein concentrations were determined using a bicinchoninic acid assay (Pierce) according to the manufacturer's instructions. Liver lysates were diluted to 3  $\mu$ g/ $\mu$ L in loading buffer and a total of 45  $\mu$ g protein was loaded onto a 4-20% SDS polyacrylamide gel



(Bio-Rad) for separation of proteins by electrophoresis. Following separation, proteins were transferred (100 min; 400 mA) to a nitrocellulose membrane. All membranes were stained with Ponceau S to verify equal loading and transfer. Membranes were washed with PBS and incubated with primary antibodies (BHMT, cat. no sc-69708; CBS, cat. no sc-133154;  $\alpha$  Tubulin, cat. no sc-5286; Santa Cruz Biotechnology) overnight at 4°C. Following 3 washes with PBS, membranes were incubated with secondary antibody (IRDye 800CW Goat anti-Mouse, cat. no 925-32210) for 1 h at room temperature. Membranes were washed 4 times in PBS prior to imaging via digital fluorescence detection using an Odyssey CLx imaging system (Li-Cor). Net intensity of each band was determined using Image Studio<sup>TM</sup> 2.0 software and normalized to  $\alpha$  Tubulin.

*Enzyme Activity Assays.* BHMT and CBS activities were measured by high-pressure liquid chromatography, as previously described (31).

*Statistical analysis.* All data were analyzed with the use of SPSS Statistics Software Version 23 (IBM). The assumption of homogeneity of variances was assessed by Levene's test for equality of variances, normality was assessed by Shapiro-Wilk's test and the data was checked for outliers using Grubb's test. Mean values were evaluated for statistically significant differences (P < 0.05) with the use of a 2-factor ANOVA (experimental diet x folate status). An analysis of main effects was performed when the interaction effect of experimental diet and folate status was not statistically significant, followed by the Fisher's Least Significant Difference post hoc test for pairwise comparisons. In cases of unequal variance where there was normality and equal sample size, a two-way ANOVA was run on the original data. In cases of unequal variance where



normality was violated, data was log transformed and test comparisons were run to verify that the original data and the transformed data yielded the same result.

#### Results

*Cumulative body weight gain.* Cumulative body weight gain and final body weight did not differ in rats fed the C, C+Cho or WE diets, regardless of folate status. Rats fed the FS-EP diet gained 15% less weight than those fed the FR-EP diet (Table 6-2).

*Hepatic SAM and SAH.* There was no effect of experimental diet or folate status on hepatic concentrations of SAM or SAH (Table 6-2). Likewise, no differences were observed in the ratio of hepatic SAM:SAH across any of the experimental dietary treatment groups (Table 6-2).

### Egg protein consumption prevented hyperhomocysteinemia in rats fed a folate-restricted diet.

There was no statistically significant interaction between diet and folate status on serum homocysteine concentrations (P = 0.352). An analysis of main effects indicated a significant impact of folate status on circulating homocysteine concentrations. Serum homocysteine concentrations did not differ between any of the FS dietary treatment groups. As expected, circulating homocysteine concentrations were increased by 53% in rats fed the FR-C diet compared to rats fed the FS-C diet (Figure 6-2A). Likewise, rats fed the FR-C+Cho and the FR-WE diets exhibited 42% and 38% higher serum homocysteine concentrations, respectively, compared to rats fed the FS-C diet. In contrast, circulating homocysteine concentrations did not differ between rats fed FR-EP and all FS dietary treatment groups (Figure 6-2A).



*Circulating cysteine concentrations.* There were no differences in circulating cysteine concentrations across any of the dietary treatment groups, regardless of folate status (Figure 6-2B).

*Hepatic CBS transcript abundance, protein abundance and enzyme activity.* No differences were observed in hepatic Cbs mRNA, CBS abundance, or enzyme activity, regardless of experimental diet or folate status (Figure 6-3).

*Hepatic BHMT transcript and protein abundance.* Quantitative real-time PCR analysis revealed no differences in hepatic Bhmt mRNA, regardless of experimental diet or folate status (Figure 6-4A). Likewise, there was no effect of experimental diet or folate status on BHMT abundance (Figure 6-4B).

*Hepatic BHMT activity was upregulated by EP and WE diets.* Main effects analysis indicated a significant effect of experimental diet on BHMT activity. The EP and WE diets resulted in a mean increase of 45% (P < 0.001) and 40% (P = 0.002), respectively, in BHMT activity compared to either C diet, regardless of folate status (Figure 6-4D). Pairwise comparisons showed a 36% and 32% increase, respectively, in BHMT activity in rats fed FS-EP and FR-WE diets compared to rats fed FS-C. No differences were observed in BHMT activity between rats fed FS-EP, FR-EP, FS-WE and FR-WE diets (Figure 6-4D).

#### Discussion

CVD is responsible for approximately 1 in 3 deaths in the United States and hyperhomocysteinemia is associated with increased CVD risk (32); thus, dietary strategies to maintain homocysteine balance may have implications for CVD prevention. The present study



demonstrates that dietary egg protein prevents elevated concentrations of circulating homocysteine in a folate-restricted rat model of hyperhomocysteinemia. The observed maintenance of homocysteine homeostasis with dietary egg protein may related to a compensatory increase in the hepatic activity of BHMT, as hepatic BHMT activity was higher in rats fed EP diets. This would suggest an increase in folate-independent remethylation of homocysteine to methionine. In vitro studies have demonstrated that BHMT and MS contribute equally to homocysteine remethylation in the liver (33). In these studies, MS impairment in vitro led to increased BHMT activity (33). In contrast to these findings, we did not observe altered BHMT abundance or activity in response to folate restriction. Rodent studies have shown that inhibition of BHMT activity results in hyperhomocysteinemia, demonstrating that BHMT is an important factor in maintaining normal circulating homocysteine concentrations (34,35). Therefore, it is not surprising that upregulation of BHMT activity by the EP diets would have a positive impact on circulating concentrations of homocysteine.

BHMT is regulated by both dietary and hormonal factors. Numerous studies have reported that glucocorticoids and insulin play important roles in the regulation of BHMT expression and activity (36–39). There is also considerable evidence that BHMT regulation is altered by dietary factors, such as intake of sulfur amino acids, choline and betaine (31,40–44). In the present study, hepatic BHMT activity was upregulated by both EP and WE diets, whereas the C+Cho diet was without effect on BHMT activity. Several studies report elevated hepatic BHMT gene expression and activity in states of methionine deficiency and methionine excess (40,42,44,45). Excess dietary cysteine and cystine have also been shown to upregulate hepatic BHMT (40,46). Furthermore, variations in methionine and cysteine intake have been reported to regulate flux through homocysteine remethylation and transsfulfuration pathways in human subjects (24,25). It is



possible that the sulfur amino acid content of egg protein and whole egg may be responsible for the observed changes in hepatic BHMT, but this remains to be determined. Regardless, upregulation of BHMT activity by the WE diets did not result in improved homocysteine balance. The differential response in serum homocysteine concentrations between FR-EP and FR-WE may be due to other constituents of the egg yolk.

Regulation of CBS includes transcriptional regulation by factors such as hormones and growth factors, redox regulation, and allosteric activation by SAM (47,48). The lack of effect on hepatic transcript level, protein abundance and activity of CBS across any of the experimental dietary treatment groups indicated that the prevention of hyperhomocysteinemia in rats fed the FR-EP diet was not attributable to increased catabolism of homocysteine via the transsulfuration pathway. This finding is in agreement with the observation that circulating concentrations of cysteine, the initial product of the transsulfuration reaction, did not differ across any of the dietary treatment groups.

The present study suggests that consumption of egg protein protects against increases in serum homocysteine in a folate-restricted model of hyperhomocysteinemia, owing in part to upregulated hepatic BHMT activity. Taken together, these data may support the inclusion of egg protein in dietary recommendations for maintenance of homocysteine balance in groups at-risk for elevated circulating homocysteine, such as lacto-ovo vegetarians, the elderly, and individuals with polymorphisms in the CBS and MTHFR enzymes. However, the relationship between dietary egg protein and homocysteine balance remains to be determined in a human population. Future dose-response studies are needed to determine the minimal amount of egg protein required to maintain homocysteine balance.



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Figure 6-1: Folate, methyl group and homocysteine metabolism. BHMT, betaine homocysteine *S*-methyltransferase; CBS, cystathionine  $\beta$ -synthase; DMG, dimethylglycine; MAT, methionine adenosyltransferase; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; SAH, *S*-adenosylhomocysteine; SAHH, *S*-adenosylhomocysteine hydrolase; SAM, *S*-adenosylmethionine; THF, tetrahydrofolate; 5-CH<sub>3</sub>-THF, 5-methyltetrahydrofolate, X, methyl group acceptor.



**Tables and Figures** 

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Figure 6-2: Circulating homocysteine (A) and cysteine (B) concentrations of FS and FR rats fed a C, C+Cho, EP or WE diet for 8 wk. Data are means  $\pm$  SEMs; *n*=6. Bars without a common letter differ (*P* < 0.05). FS, folate sufficient; FR, folate restricted, C, casein-based diet; C+Cho, casein-based diet supplemented with choline; EP, egg protein-based diet; WE, whole egg-based diet.





Figure 6-3: Hepatic *Cbs* mRNA (A), hepatic CBS protein abundance (B), representative western blots of CBS and  $\alpha$  Tubulin (C), and enzyme activity (D) of FS and FR rats fed a C, C+Cho, EP or WE diet for 8 wk. *Cbs* mRNA and CBS abundance are expressed relative to rats fed the FS-C diet. Data are means  $\pm$  SEMs; *n*=6. Bars without a common letter differ (*P* < 0.05). FS, folate sufficient; FR, folate restricted, C, casein-based diet; C+Cho, casein-based diet supplemented with choline; EP, egg protein-based diet; WE, whole egg-based diet.





Figure 6-4: Hepatic *Bhmt* mRNA (A), hepatic BHMT protein abundance (B), representative western blots of BHMT and  $\alpha$  Tubulin (C), and enzyme activity (D) of FS and FR rats fed a C, C+Cho, EP or WE diet for 8 wk. *Bhmt* mRNA and BHMT abundance are expressed relative to rats fed the FS-C diet. Data are means  $\pm$  SEMs; *n*=6. Bars without a common letter differ (*P* < 0.05). FS, folate sufficient; FR, folate restricted, C, casein-based diet; C+Cho, casein-based diet supplemented with choline; EP, egg protein-based diet; WE, whole egg-based diet.



Ingredient <sup>1</sup>	FS-C	FS-C+Cho	FS-EP	FS-WE	FR-C	FR-C+Cho	FR-EP	FR-WE
				g/kg				
Casein	200	200	0	0	200	200	0	0
Dried whole egg <sup>2</sup>	0	0	0	413	0	0	0	413
Egg white solids	0	0	200	0	0	0	200	0
Cornstarch	423	412	423	387	423	412	423	387
Glucose monohydrate	150	150	150	150	150	150	150	150
Corn oil	177	177	177	0	177	177	177	0
Mineral mix (AIN93)	35	35	35	35	35	35	35	35
Vitamin mix (AIN93)	10	10	10	10	0	0	0	0
FR Vitamin mix	0	0	0	0	10	10	10	10
Biotin	0	0	0.4	0.4	0	0	0.4	0.4
Choline bitartrate	2	13	2	2	2	13	2	2
L-Methionine	3	3	3	3	3	3	3	3

Table 6-1: Composition of FS and FR C, C+Cho, EP or WE diets fed to Sprague Dawley rats for 8 wk.<sup>3</sup>

<sup>1</sup>All ingredients were purchased from Envigo with the exception of dried whole egg (Rose Acre Farms), as well as L-methionine and  $\frac{1}{5}$ choline bitartrate (Sigma-Aldrich).

<sup>2</sup>Total protein and lipid content provided by 413 g of dried whole egg were 48.4 (200 g) and 42.9% (177 g), respectively.

<sup>3</sup> FS, folate sufficient; FR, folate restricted, C, casein-based diet; C+Cho, casein-based diet supplemented with choline; EP, egg proteinbased diet; WE, whole egg-based diet.

Parameter	FS-C	FS-C+Cho	FS-EP	FS-WE	FR-C	FR-C+Cho	FR-EP	FR-WE	<i>P</i> -value Diet	<i>P</i> -value Folate Status	<i>P</i> -value Diet x Folate Status
Initial Body Weight (g)	166 <u>+</u> 10	167 <u>+</u> 8	169 <u>+</u> 8	171 <u>+</u> 4	176 <u>+</u> 4	174 <u>+</u> 3	161 <u>+</u> 9	174 <u>+</u> 2	0.676	0.489	0.560
Final Body Weight (g)	379 <u>+</u> 9ª	385±13ª	339±11 <sup>b</sup>	373±9ª	383±17ª	379±5ª	$361\pm8^{ab}$	$376\pm7^{a}$	0.013	0.456	0.582
Cumulative Weight Gain (g)	213±4 <sup>a</sup>	218±13ª	170±8 <sup>b</sup>	$202\pm7^{a}$	$207 \pm 14^{a}$	205±4ª	200±10 <sup>a</sup>	$202\pm6^{a}$	0.022	0.724	0.095
Hepatic SAM (nmol/g)	43.5±7.0	50.0±10.5	38.3±14.6	41.1±9.3	54.7±9.2	39.0±11.8	35.3 <u>+</u> 8.0	59.6±12.8	0.58	0.614	0.573
Hepatic SAH (nmol/g)	70.5 <u>+</u> 4.7	62.3±9.9	69.0 <u>+</u> 9.1	65.8±12.6	59.7 <u>+</u> 7.8	41.7±5.7	75.7 <u>+</u> 9.1	63.8 <u>+</u> 9.2	0.155	0.290	0.458
SAM:SAH Ratio	0.6±0.1	1.0±0.3	0.8±0.5	0.9 <u>±</u> 0.3	1.0 <u>±</u> 0.2	1.1 <u>±</u> 0.4	$0.5 \pm 0.1$	1.0±0.2	0.584	0.706	0.688

Table 6-2: Body weight parameters and hepatic concentrations of *S*-adenosylmethionine (SAM) and *S*-adenosylhomocysteine (SAH) in Sprague Dawley rats fed a C, C+Cho, EP or WE diet and their respective FR diets for 8 wk.<sup>2</sup>

<sup>1</sup>Values are means  $\pm$  SEMs; *n*=6. Mean values within a row without a common letter are statistically significant (*P* < 0.05). <sup>2</sup> FS, folate sufficient; FR, folate restricted, C, casein-based diet; C+Cho, casein-based diet supplemented with choline; EP, egg protein-based diet; WE, whole egg-based diet.



## **CHAPTER 7. GENERAL CONCLUSIONS**

### **Overall Summary and Conclusions**

Metabolic diseases such as obesity, type 2 diabetes (T2D) and cardiovascular disease are characterized by alterations in nutrient metabolism. It is recognized that an individuals' dietary patterns can assist in preventing or delaying the onset of metabolic disease or, in contrast, can directly contribute to disease development and progression. Whole eggs are a nutrient-dense food, providing high-quality protein and an array of vitamins and minerals, and can play both nutritional and functional roles in the diet (1,2). Potential benefits of egg consumption during the progression of metabolic disease include body weight management and the maintenance of micronutrient balance, including vitamin D and methyl groups (2–4). However, there remains controversy surrounding the potential adverse effects of egg consumption, such as impaired blood glucose regulation and cardiovascular disease risk, in individuals with metabolic disease (5–9). The studies presented in this dissertation, utilizing rat models of metabolic disease, demonstrate that egg consumption may contribute to the maintenance of body weight and micronutrient balance, but may also impair insulin sensitivity.

The first two studies described in this dissertation examined whole egg as a naturally occurring dietary source of the micronutrient vitamin D. Suboptimal vitamin D status is highly prevalent in individuals with T2D (10–12), and is exacerbated by the presence of diabetic nephropathy owing to urinary loss of vitamin D (13,14). Results from the studies described in this dissertation demonstrate that the consumption of whole eggs was effective at maintaining vitamin D status in T2D rats. Notably, a diet containing 20% (w/w) protein from whole egg maintained vitamin D balance more effectively than a diet containing an equivalent amount of supplemental cholecalciferol. However, consumption of lower doses of whole egg, at 10, 5 and



2.5% protein from whole egg, translating to approximately 7, 3.5 and 1.75 eggs/day in humans, did not prevent vitamin D insufficiency in T2D rats. These data suggest that consumption of 1-2 eggs/day, without additional measures to improve vitamin D status, may not be an effective means to prevent insufficiency in individuals with T2D. However, accumulating evidence suggests that vitamin D-fortified eggs may be protective against declining concentrations of circulating 25(OH)D (15–17). Thus, studies evaluating the effect of fortified egg consumption on vitamin D homeostasis in T2D are warranted.

An interesting outcome of the vitamin D studies was a significant reduction in weight gain in obese, T2D rats fed a diet containing 20% protein from whole egg. This attenuation of weight gain was, in part, due to a reduction in body fat percentage. Furthermore, a dose-dependent attenuation of weight gain was observed in T2D rats fed diets containing 20, 10, 5 and 2.5% protein from whole egg. Despite reduced weight gain in T2D rats fed varying concentrations of dietary whole egg, we did not observe a corresponding reduction in food intake, indicating that the observed reduction in weight gain was not related to measures of satiety.

Obesity and excessive weight gain are major risk factors in the development of insulin resistance and T2D (18–21); thus, we investigated whether the attenuation of weight gain in T2D rats consuming a diet containing 20% protein from whole egg was related to measures of insulin sensitivity and skeletal muscle insulin signaling. Insulin sensitivity was impaired in T2D rats fed a whole egg-based diet; however, no differences in skeletal muscle insulin signaling were observed between T2D rats fed casein- and whole egg-based diets. In addition, fasting serum glucose was increased, whereas fasting serum insulin and the homeostatic model assessment of  $\beta$ -cell function were decreased in T2D rats fed a whole egg-based diet.



The final study described in this dissertation focused on other components of whole egg that may modulate the metabolic disorder hyperhomocysteinemia, which is associated with cardiovascular disease risk. Eggs contain choline, B vitamins and sulfur amino acids, nutrients which play important roles in the regulation of methyl group metabolism. Our results indicate that dietary egg protein may help prevent elevations in circulating homocysteine in a rodent model of hyperhomocysteinemia, due to upregulation of hepatic betaine-homocysteine *S*-methyltransferase activity, an enzyme important in the maintenance of homocysteine balance.

The findings from these studies suggest that inclusion of whole eggs in the diet may assist in body weight maintenance in individuals at risk for the development of obesity and T2D. Future studies will aim to elucidate the mechanism underlying the observed reduction in weight gain. Additionally, whole eggs are an important source of nutrients, such as B vitamins, choline and vitamin D, and, in combination with a healthy eating pattern, may contribute to vitamin D and homocysteine balance in individuals with metabolic disease. However, egg consumption may impair insulin sensitivity and blood glucose regulation in individuals with pre-existing T2D, though future studies are warranted to determine the impact of lower concentrations of dietary whole egg on metabolic biomarkers of insulin sensitivity.

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