2011

Influencing Kidney Health and Function in Young Female Rats Through Dietary Alterations

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Influencing Kidney Health and Function in Young Female Rats Through Dietary Alterations

Joseph Christopher Gigliotti

**Dissertation submitted to the Davis College of Agriculture, Natural Resources and
Design in partial fulfillment of the requirements for a degree of**

**Doctor of Philosophy
in
Animal and Food Sciences**

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**Morgantown, West Virginia
2011**

Keywords: diet; omega-3 fatty acids; kidney; nephrocalcinosis

ABSTRACT

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Joseph C. Gigliotti

The occurrence of kidney failure in the US has drastically increased over the past decade. The decline in health associated with chronic kidney disease can be prevented if detected early in disease progression. However, substantial damage has often occurred before detection of renal disease is possible. Therefore, disease prevention (as in all diseases) is the optimal treatment.

In regards to the kidneys, dietary habits can have a profound influence on the function and overall kidney health. This is particularly evident in female rats due to their susceptibility to diet induced renal disease. In female rats, the initiation of chronic kidney disease typically involves nephrocalcinosis (NC). NC is characterized by increased renal calcium (Ca) content, and involves the pathological deposition of mineral deposits in renal tissue. Fluctuations in dietary Ca and phosphorus (P) content are the major consideration in managing NC. However, other dietary components can mediate the development and severity of this disease in rats.

Based on our study results, the source of dietary protein and lipid can influence the development of NC in female rats. Our initial study compared the nutritional quality of a novel protein source derived from krill to the milk protein, casein. Rats fed krill protein concentrate (KPC) had lower kidney weights ($P<0.001$), lower kidney Ca content ($P=0.002$), and lower incidence of renal damage than rats fed casein based diets. These changes occurred in the absence of fluctuating urinary mineral content, which is a major cause of NC. Further analysis showed that KPC contained residual krill oil, which is rich in omega-3 polyunsaturated fatty acids (ω -3 PUFAs). The next study involved isolation and characterization of krill oil. Krill oil is a unique lipid with high (>65%) polar lipid content including phospholipids, whereas other dietary sources of ω -3 PUFAs were found to be predominately triacylglyceride. In the final study, the characterized oils were added to casein-based diets and fed to female rats to determine if lipid content influences the development of NC. While rats fed KPC appeared to be protected from NC, rats fed the high fat diet as krill oil were the only rats who developed NC. The high inclusion of krill oil resulted in elevated dietary P content due to its high phospholipid content. Aside from NC, the renal fatty acid content was influenced by the diet and lead to an apparent decrease in urinary prostaglandin production in all rats consuming the long-chain ω -3 polyunsaturated fatty acids (ω -3 LCPUFAs) eicosapentaenoic (EPA) and docosahexaenoic acid (DHA). The consumption of the different ω -3 PUFA sources also resulted in reduced activity of the proinflammatory transcription factor NF κ B, and expression of the proinflammatory cyclooxygenase -2 (COX-II) and transforming growth factor-beta (TGF- β). Despite these observed results, no differences were noted in serum measures of kidney function. We concluded that changing the source of dietary protein and lipid can mediate renal health in female rats. It appears that ω -3 PUFAs do not influence the nephrocalcinogenic nature of casein. However, providing dietary ω -3 PUFAs resulted in decreased renal inflammatory activity as compared to rats fed corn oil. This

supports the benefit of ω -3 PUFA supplementation in reducing the onset of diseases caused by inflammatory processes, and suggests that different dietary sources of ω -PUFAs vary in regards to biological activity.

Acknowledgements

First, I would like to thank God for the many gifts he has presented me with throughout my life. In life there are things you can plan for and those you cannot, and I am eternally grateful for the way all things have turned out. I am grateful for meeting my beautiful wife Kristen, and having the opportunity to marry into a wonderful family. I am grateful to have learned from my parents; some through lectures and some through hardships. I have wonderful friends that make life and work very enjoyable, and for that I am also grateful.

I believe that the Davis College is a wonderful division of the college, with wonderful faculty and support staff. I have learned so much from various faculty members, information pertaining to both my future career and life in general. My supervisor Dr. Janet Tou has displayed both excellent professionalism and compassion throughout my work as both an undergraduate and graduate student. Through her guidance I feel that I have been able to flourish as a graduate student. Overall, I have obtained excellent training here at the Davis College; training that I will use for rest of my life. Those listed above are just a few of the many people whom have assisted me to what success I have and will accomplish. Thank you all.

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1.0. Literature Review

1.1 The Biochemistry of Omega-3 Polyunsaturated Fatty Acids (ω -3 PUFAs)

1.1.1 Essential Fatty Acids

Mammalian hepatocytes and adipocytes are able to synthesize a variety of fatty acids, which serve primarily as a metabolic energy source. However, α -linolenic acid (ALA; 18:3 ω -3) and linoleic acid (LA; 18:2 ω -6) are two PUFAs which cannot be synthesized *de novo*. Mammals do not have delta (Δ) 12 and Δ 15 desaturases, therefore double bonds cannot be placed beyond the Δ 9 carbon during fatty acid synthesis. The double bonds found in LA are at carbons Δ 9 and Δ 12, and carbons Δ 9, Δ 12, and Δ 15 in ALA. Therefore, ALA and LA are termed essential fatty acids (EFAs) and must be obtained from the diet.

Currently, it is recommended for women to consume ~1.1 and men ~1.6 g/d of ALA. LA requirements are ~ 12 g/d for women and ~17g/d for men [1]. Rodent EFA recommendations consist of 1.2g LA/kg diet and 2g ALA/kg [2]. However, these requirements were based on tissue ω -6 and ω -3 concentrations as opposed to tissue functionality. Therefore, the recommendations for EFAs may change once the relationship between tissue fatty acid content and tissue function is better understood.

1.1.2 Conversion of EFAs to long-chain polyunsaturated fatty acids (LCPUFAs)

Despite being termed essential, little biological activity has been directly attributed to ALA and LA. Instead, the essential fatty acids are elongated and desaturated to form their respective LCPUFAs. The formation of LCPUFAs from EFAs involves a series of steps, which occur primarily at the endoplasmic reticulum of hepatocytes (Figure 1).

The action of $\Delta 6$ desaturase is the committed step in EFA conversion to LCPUFAs [3]. Subsequent elongation and another desaturation by $\Delta 5$ desaturase results in the formation of arachadonic acid (AA; 20:4 ω -6) from LA, and EPA (20:5 ω -3) from ALA. DHA (22:6 ω -3) is formed from further metabolism of EPA, which includes: two elongation reactions, another desaturation by $\Delta 6$ desaturase, and relocation to the peroxisome for a single round of β -oxidation [4]. In mammalian tissues, AA predominates as the major ω -6 LCPUFA, while EPA and DHA are the primary ω -3 LCPUFAs [5].

Despite the biological importance attributed to these LCPUFAs, the conversion of the ω -3 EFA ALA into EPA and DHA appears to be limited. Factors limiting this conversion include: competition and substrate specificity for common elongation and desaturation enzymes [6], preference for ALA as a metabolic fuel [7], and LCPUFA induced suppression of liver desaturase enzyme expression [8]. The competition between LA and ALA is supported by a study in females subjects provided formulas consisting of constant LA (4% total energy) and variable ALA content (0-16% of total energy). The EPA content in

circulating lipid fractions increased from 0.2 to ~1.8% of total fatty acids with increasing ALA consumption. Despite the increasing presence of ALA, numerical increases in plasma AA were still observed [9]. Previous studies in men have shown that the oxidation of fatty acids is influenced by both the length of the ingested fatty acid and degree of saturation. By measuring the exhalation of $^{13}\text{CO}_2$ in men fed radiolabeled fatty acids, it was determined that the preference for fatty acid oxidation is $\text{C12:0} > \text{C18:3}(\omega\text{-3}) > 18:1t(\omega\text{-9}) > 18:1c(\omega\text{-9}) > 18:2(\omega\text{-6}) > 16:0 > 18:0$ [10]. Increased oxidation of ALA explains why only 15% of ALA is used to synthesize EPA and DHA in humans; with ALA derived DHA being undetectable in some studies [5, 11]. Similar results were found in rodent studies, suggesting that mammalian $\omega\text{-3}$ LCPUFA synthesis is limited and should be obtained in the diet [12].

1.1.3 Dietary sources of $\omega\text{-3}$ PUFAs

Research and health claims have promoted increased consumption of PUFAs. Plant oils are the richest source of the essential fatty acids LA and ALA, with flaxseed oil reported to be one of the richest sources of ALA [13]. However, the low mammalian *de novo* synthesis of the $\omega\text{-3}$ LCPUFAs EPA and DHA from ALA has led to the recommendation to increase dietary consumption of these LCPUFAs.

Recently, it has been suggested that healthy individuals should consume 400-500 mg $\omega\text{-3}$ LCPUFAs per day to decrease the risk for mortality associated with cardiovascular disease. This level of $\omega\text{-3}$ LCPUFA intake can be achieved in

the diet by following the American Heart Association's recommendation to consume two servings of fatty fish per week [14]. The recommended types of fish include tuna, salmon, and menhaden which are normally harvested from colder waters. Fatty fish are high in the ω -3 LCPUFAs EPA and DHA because their diets consist of various marine algae and planktonic crustaceans such as algae and krill. These planktonic organisms have a high capability for *de novo* ω -3 LCPUFAs synthesis, and depletion of fish stocks and concern of heavy metal contamination have led to an interest in the nutritional value of krill and algae [15, 16].

1.1.4 Cellular activities of LCPUFAs

Despite the host of benefits associated with ω -3 LCPUFA consumption, the cellular mechanisms behind their biological effects are still not fully understood. Originally, fatty acid trafficking was believed to be a relatively random and somewhat passive event. Information discovered during the past two decades suggests that fatty acid metabolism and cellular activity is tightly regulated [17], and that PUFAs are crucial components in cellular biology.

PUFAs and biological membranes

Fatty acids are typically cycled through several cellular compartments upon acquisition. The cellular fate of fatty acids depends on both the needs of the cell as well as the type of fatty acid present. As mentioned above, the oxidation of fatty acids depends on the length and desaturation of the fatty acid. However,

fatty acid oxidation typically accounts for less than 40% of detectable fatty acid utilization [10]. This suggests that storage as triglycerides and membrane incorporation are the major metabolic fates of dietary fatty acids.

A clear response to changes in dietary LCPUFA content is the resultant influence it has on cellular membrane fatty acid composition. While the saturated and monounsaturated fatty acid content of membranes remain constant (approximately 50 and 30%, respectively), the ω -6 and ω -3 PUFA content appears to be the most variable. This has important implications in cellular biology, since membrane fatty acid content influences membrane physiochemistry and subsequent cellular functions [18, 19].

The fatty acid content of the membrane can have profound influence on cellular activity and tissue function. A classic example for this interaction involves the activation of phospholipases. While several forms of phospholipases exist, all perform the similar action of cleaving membrane phospholipids upon stimulation by intracellular calcium. Phospholipase A₂ specifically targets the *sn*-2 position of membrane phospholipids, and results in the liberation of the AA or EPA that commonly resides there. Therefore, changing the membrane ω -6 and ω -3 PUFA content can influence which species of LCPUFA are released upon phospholipase activation. The activation of phospholipase A₂ during certain disease states suggests that liberated fatty acids play a key role in inflammatory processes [20].

The influence of PUFAs on NFκB activity

During the past 15 years, it has become evident that fatty acids influence gene expression through mediating the activity of various transcription factors [21]. Most studies addressing the influence of PUFAs on transcription factor activity is focused on metabolic processes. However, recent data suggests that PUFAs can also modulate the activity of transcription factors involved in inflammatory processes.

Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) is considered a key regulator of cell viability and inflammatory pathways. Activated NFκB upregulates the expression of various proinflammatory proteins in various cell types [22]. Therefore, NFκB is considered a master regulator of the inflammatory response and is a common target for steroidal anti-inflammatory drugs [24, 24].

Interestingly, fatty acids appear to play a role in NFκB activity. DHA supplementation, but not EPA, resulted in 60% decrease in NFκB activity, 50% reduction in COX-II expression [25], and greater than 25% reduction in vascular cellular adhesion molecule-1 (VCAM-1) expression in cultured endothelial cells [26]. In contrast, cultured immune cells and adipocytes display an increased NFκB activity when cultured with AA or palmitate [27, 28]. Therefore, the cellular and biological response depends on the species of fatty acid present and supports the recommendation to increase ω-3 LCPUFA consumption.

PUFAs and eicosanoid synthesis

As mentioned above, PUFAs influence the expression of COX-II through mediating NF κ B activation. The COX enzymes initiate the formation of prostaglandins, which are hormone like signaling compounds involved in virtually all aspects of physiology.

AA is the typical substrate for prostaglandin production. Activated phospholipase A₂ can result in liberated AA or EPA, which provides substrates for COX (which is located on the endoplasmic reticulum and nuclear envelope). COX converts AA to prostaglandin H₂ (PGH₂), which is an intermediate in the production of the 2-series prostaglandins (PGE₂, PGI₂, TXA₂, PGF_{2 α} etc.) by a respective synthase. Once produced, prostaglandins mediate various physiological responses by binding to select 7-transmembrane domain, G-protein coupled receptors. The physiological outcome is based upon the species of prostaglandin produced, as well as the nature of the prostaglandin receptor present on the target cells. The species of prostaglandin produced depends on the LCPUFA used as a substrate. The 2-series is derived from the ω -6 LCPUFA AA, while the 3-series of prostaglandins are derived from the ω -3 LCPUFA EPA. The 3-series of prostaglandins typically display different affinities for prostaglandin receptors as compared to the 2-series prostaglandins. Therefore, different cellular and physiological responses may occur to similar prostaglandins that differ in species. For example, PGE₃ differed from PGE₂ in inducing proliferation of cultured fibroblasts. PGE₂ resulted in fibroblast proliferation at 50ng/ml, while similar PGE₃ concentrations resulted in no difference in cell number. PGE₃ also

resulted in decreased expression of proinflammatory protein expression in both fibroblasts and macrophages as compared to cells exposed to PGE₂ [29].

Conclusion

LCPUFAs are important cellular compounds, as evident by the essential nature of their 18 carbon precursors. The reduction in inflammatory transcription factor activity and prostaglandin production supports the importance of ω -3 LCPUFAs in ameliorating inflammatory conditions. However, it is unclear if LCPUFAs influence non-pathological processes. Aside from mediating inflammatory processes, prostaglandins are involved in all aspects of physiology. For example, kidneys are heavily reliant on prostaglandin production while NF κ B mediates several forms of renal disease. Therefore, the dietary PUFA content may heavily influence renal physiology.

1.2 Renal Physiology

In general, the kidneys are viewed solely as excretory organs. While a major function of the kidneys is to filter wastes out of the circulation, the kidneys also play key roles in various physiological processes [30].

1.2.1 Renal Anatomy and Vasculature

The kidneys are bean shaped organs located between the 12th thoracic and the third lumbar vertebrae in humans. Each kidney (left and right) receives arterial blood from the descending aorta via the left or right renal artery, which enters the kidneys through the renal hilum (which is also the site of renal innervation). Inside the kidney the renal artery undergoes a series of divisions to form the segmental, interlobular, arcuate, and intralobular arteries as well as the afferent and efferent arterioles. The efferent arterioles further divide to form the peritubular capillaries and vasa recta, which adjoin to form venuoles and eventually merge to form the renal vein.

1.2.2 The nephron

The vasculature of the kidney provides necessary nutrients and gases to the surrounding tissue. In addition, the primary functions of the kidney are accomplished within specialized regions of the renal vasculature, and include regulation of blood volume, waste content, pH, electrolyte and mineral concentrations, and nutrient concentrations. By regulating these components of the blood, the kidneys are the primary organ responsible for maintaining a

constant composition of all extracellular fluids. In humans, it has been estimated that ~1700 liters of fluid flow through the kidney each day.

To accomplish this work load, each human kidney is composed of around 1 million nephrons (2 million in total). The nephron is composed of vascular and tubular portions which are involved in filtering the blood (Figure 2). Healthy individuals have around 2 million nephrons; yet, only ~10% of this capacity is needed to support life. Despite this large reserve capacity, millions of Americans suffer from inadequate renal function each year [31].

The structure and functions of the glomerulus

The primary structure of the nephron is the glomerulus. The glomerulus includes a number of cells / tissues that together perform the filtering responsibility of the kidney (as well as other processes). The glomerulus is composed of an afferent arteriole, Bowman's capsule, tubular capillaries (including the vasa recta), podocytes, and mesangium (Figure 3). Glomerular diseases (glomerulonephritis) are the primary cause of renal failure in the U.S., which suggests that this is a key structure in renal physiology [31].

The physiology of the glomerulus begins with the entry of blood into the glomerulus via the afferent arterioles. The afferent arteriole branches to form a "knot" of capillaries, which are completely encased by a structure called Bowman's capsule. These capillaries are extremely "leaky" due to the presence of fenestrations, which are porous structures in the walls of the capillaries (Figure 4). Fenestrations allow approximately 20% of the fluid portion of blood (plasma)

to be forced out of the capillaries into Bowman's capsule. This filtered fluid is termed filtrate since this is the portion of blood that was filtered. The remaining 80% of the blood not filtered leaves the capillaries via the efferent arteriole, and branches to form capillaries which surround the rest of the nephron [30].

The capillary structure inside Bowman's capsule appears as a "knot" due to the presence of podocytes and the mesangium (Figure 3). These components are considered pericapillary structures, since they are found amongst (mesangium) and/or physically attached to (podocytes) the capillaries. The podocytes "coat" the capillaries and assist with the filtering process, whereas the mesangium is a complex tissue with an undefined role in renal physiology. The mesangium is a matrix consisting of various collagens (and other structural components), the mesangial cells (which produce the matrix), and resident immune cells. Although the role of the mesangium in renal physiology is not completely understood, mesangial expansion is a common finding in glomerular diseases [32]. Mesangial expansion results in interference of normal glomerular processes, and is termed glomerular scarring (or glomerulosclerosis) upon histological evaluation.

Glomerular filtration occurs by only allowing a portion of the plasma to escape the capillaries and enter into Bowman's space (Figures 3 and 4). Normally the filtrate and blood have equal concentrations of small solutes such as organic nutrients and minerals, while the cell and protein content of the filtrate is negligible. This suggests that large plasma components such as cells and albumin are unable to escape the capillary lumen. Early research described that the glomerulus discriminates not only by size, but also by the charge of a molecule.

This filtering capacity appears to be largely mediated by the charge characteristics of the glomerular basement membrane. Loss of charge discrimination has been associated with renal disease in animal models, resulting in proteinuria and deposition of immune complexes within the glomerulus [33].

Reabsorption along the tubular portion of the nephron

Upon exiting the glomerular capillaries, the plasma filtrate is now subject to the biochemical processes of the tubular portion of the nephron. Small organic nutrients, such as glucose and amino acids, and minerals are as concentrated in the filtrate as found in the plasma. With ~1700 liters of blood flowing through the kidney daily, deficiencies of these solutes would quickly arise if they were not reabsorbed in the kidneys. Almost 100% of the sodium, glucose, amino acids, Ca, and water is reabsorbed from the filtrate by the kidneys. Aside from nutrient reabsorption, the kidneys play a key role in the maintenance of the pH and electrolyte content of the blood. Maintenance of these and other compounds can include reabsorptive and secretory processes, both of which are accomplished within the tubular portion of the nephron [30].

Upon leaving the glomerulus, the solute rich filtrate enters into the proximal convoluted tubule. The proximal convoluted tubule is where the majority of the glucose and amino acids are reabsorbed along with a majority of the sodium, chloride, and water. Reabsorption of glucose and amino acids is mediated by sodium-dependent cotransporters located on the apical membrane of the tubular epithelial cells. The organic nutrient transported from the tubular

lumen into the epithelial cell along with sodium. Since this process is continually occurring, the concentration of organic nutrients within the epithelial cell increases. High concentrations drive the diffusion of these nutrients out of the epithelial cell and into the peritubular capillaries, which allow for reentry into the general circulation [30].

Ca and P reabsorption

The reabsorption of Ca and P also occurs predominately in the proximal tubule. Tubular P is reabsorbed in a sodium dependent cotransport mechanism, while Ca reabsorption primarily occurs via paracellular pathway [48]. However, renal handling of Ca and P can also be influenced by hormonal signaling.

The major hormones involved in Ca and P metabolism are parathyroid hormone, vitamin D, and calcitonin [30]. Generally, parathyroid hormone and vitamin D are involved in increasing serum Ca levels by influencing release of Ca and P from bone, increasing intestinal absorption of Ca, and decreasing urinary excretion of Ca. Increased urinary reabsorption of Ca is mediated by parathyroid hormone induced increase in Ca channels in the distal nephron. Due to the toxic effects of elevated serum P, parathyroid hormone also stimulates the urinary excretion of P to counteract the increase in serum P induced by bone resorption. Calcitonin, on the other hand, is released from the thyroid gland in the presence of high serum Ca. The release of calcitonin triggers a general hypocalcemic response, which primarily involves decreased Ca release from bone.

After losing a large portion of the solutes (i.e. organic nutrients, Ca, P, etc.) in the proximal tubule, the remaining filtrate eventually descends from the cortex of the kidney (cortex includes the glomerulus and both the proximal and distal convoluted tubules) into the medullary tissue (medulla includes both the descending and ascending Loops of Henle and the collecting ducts). As the filtrate leaves the proximal tubule, it descends into the medulla via the descending limb of the Loop of Henle. The Loop of Henle has varying properties which contribute to the development and maintenance of the medullary characteristics. The descending limb is water permeable. Therefore, water diffuses out of the tubular lumen in response to the loss of solutes within the proximal tubule. The filtrate then continues through the ascending limb, where sodium-chloride is actively pumped out of the lumen and into the interstitial space and associated capillaries. The increased interstitial salinity is the driving force for water reabsorption from the kidney tubules. This provides an ability to regulate water excretion, which is a key function of the kidneys [30].

The Renin-Angiotensin-Aldosterone-System in Sodium and Water Reabsorption

Variable hydration states occur daily; yet extracellular volume remains relatively constant. This is accomplished by fluctuating the amount of water excreted or retained in urine. When there is an excess of water (overhydrated), then water reabsorption in the kidney is minimized and results in increased urinary volume (diuresis). The opposite occurs in states of dehydration, where the kidneys reabsorb water and urinary volume decreases (antidiuresis). This process

is initiated and carried out within the nephron and is referred to as the renin-angiotensin-aldosterone system (RAAS). RAAS's primary function is to maintain efficient blood flow through the kidney by modulating plasma hemodynamics [30].

The initiation of RAAS occurs with the juxtaglomerular apparatus, which is a structure composed of the afferent and efferent arterioles of the glomerulus and the ascending limb of Henle (Figure 5). Within the juxtaglomerular apparatus are a set of specialized cells which respond to changes in extracellular fluid components by releasing the protease renin. Angiotensinogen is continuously secreted by the liver and has little biological activity. The activation of angiotensinogen involves proteolytic cleavage by renin to produce angiotensin I. This proteolytic cleavage is the rate limiting step in the activation of the RAAS. Once angiotensin I is formed, it is rapidly converted to angiotensin II in pulmonary capillaries by angiotensin converting enzyme. The primary effects of angiotensin II include the secretion of aldosterone from the adrenal cortex, constriction of the efferent arterioles, and peripheral vasoconstriction. Taken together, the goal of the RAAS is to increase fluid flow through the kidney by increasing Na resorption, and redirecting blood flow through the glomerulus. The importance of the RAAS in whole body fluid content becomes evident when the mechanisms behind renin release are understood.

In the vasculature portion of juxtaglomerular apparatus, specialized granule cells act as baroreceptors which respond to changes in blood pressure. These cells produce and store renin, which is secreted in response to a drop in

blood flow. While renin is produced and secreted from the granule cells, other cells / tissues can also induce the secretion of renin. Within the ascending limb of Henle, which is in close proximity to the granule cells, are a group of cells known as the macula densa (Figure 5). While the granule cells were baroreceptors which were sensitive to changes in blood flow in the vasculature, the macula densa cells are sensitive to the NaCl content of the tubular fluid. These chemoreceptive cells induce a release of renin from the granule cells in response to a drop in NaCl.

The activation of the granule cells appears to be largely mediated by the production of prostaglandins by the stimulated macula densa. Rats fed a salt restricted diet have higher renal COX-II expression, which occurred specifically in the macula densa [34]. Furthermore, cultured mouse granule cells had increased renin mRNA and renin secretion when cultured with PGI₂ and PGE₂ [35]. Finally, neural stimulation can also induce the release of renin. The granule cells are innervated by the sympathetic nerves. The peripheral baroreceptors induce sympathetic nerve activity during hypotension, and results in an increase in renin excretion. The association of renal blood flow, tubular NaCl content, and systemic hypotension further supports the importance of the kidney in maintaining and regulating both renal and peripheral blood flow.

The distal portion of the nephron, starting with the ascending Loop of Henle, is water-impermeable. Therefore, the increased salinity outside of the tubule generates an osmotic gradient that can be utilized to reabsorb more water from with filtrate. In the presence of antidiuretic hormone (ADH), the distal portion becomes water permeable by the addition of aquaporins. This allows

water to leave the tubule due to the large interstitial sodium chloride concentration and reenter circulation via the peritubular capillaries / vasa recta. Therefore, this region of the nephron is where variable water excretion is controlled.

Tubular Secretion is Another Method to Remove Substances from the Blood

At this stage, the filtrate has lost much of its osmolarity due to solute reabsorption, (in the proximal tubule) and a portion of its water content (descending Loop of Henle). Another process begins in the distal convoluted tubule, and includes regulation of the unfiltered blood in the peritubular capillaries. By the process of tubular secretion, unwanted substances can still be removed despite not undergoing glomerular filtration. The plasma present in the peritubular capillaries are closely associated with the nephron tubules, as indicated in the process of reabsorption. In the distal tubule, plasma components such as potassium and protons can be pumped out of the capillaries and into the filtrate for excretion. This occurs due to the location of specific channels in the distal tubule epithelial cells. In the proximal tubule (where reabsorption occurs), the solutes were pumped into the cell and then diffused through channels located on the basolateral membrane facing the capillaries. These substances diffuse back into the circulation; however, the channels for potassium and protons in the distal tubule are located on the apical membrane. Therefore, potassium and protons secreted from the capillaries diffuse into the tubular lumen for excretion. The secretion of protons and the presence of bicarbonate transporters suggests that the kidneys are also involved in acid-base balance.

The fluid and solutes remaining in the filtrate at the end of the distal tubule are destined for excretion. The filtrate leaves the distal tubule and enters into a collecting duct. The collecting ducts route the filtrate from numerous nephrons to be collected in a calyx, which later empties into the ureter which leaves the kidney at the renal hilum. The ureter transports the filtrate to the bladder, where the urine is stored until excreted [30].

Typical measurements of renal function

Given the role of the kidney in maintaining blood composition, insufficient renal activity can influence multiple organ systems. Therefore, monitoring renal function is of great importance in both clinical and research settings.

Serum markers of renal function include creatinine, blood urea nitrogen (BUN), uric acid, total protein, albumin, Ca, and P. Serum creatinine levels can be combined with urinary excretion of creatinine to estimate glomerular filtration rate (GFR). Estimating GFR provides insight into the amount of fluid that is filtered by the kidney over a given time (ml/min). In humans, reduction in GFR allows for the severity of renal disease to be determined and is the standard method used to assess renal function. BUN is another common measurement, and also provides insight into the filtrating ability of the kidney. Urea is a waste product derived from the metabolism of amino acids, and is removed from the circulation through renal filtration. Therefore, increases in BUN signify renal deficiency. The remaining serum measures (total protein, albumin, Ca, and P) are

not specific to renal function. For example, alterations in serum protein and albumin could result from liver abnormalities and not glomerular damage resulting in increased excretion of albumin. Likewise, changes in serum Ca and P could result in pathologies associated with bone and or mineral metabolism (such as hyperparathyroidism).

Estimating GFR is by far the most common measure of renal function used. However, less than 10% of the nephrons present in healthy individuals are required to maintain blood composition [30]. This large renal capacity makes detection of early renal damage difficult using serum measurements. To address this issue, urinary composition can also be measured to determine renal health. The urinary markers are similar to those measured in serum and typically include albumin, pH, Ca, and P. Urinary albumin is a powerful measurement, because elevated urinary albumin (or total protein content) suggests damage has occurred to the filtrating apparatus of the kidney (i.e. glomerulus). As in the case of serum measures, urinary Ca, P, and pH could be the result of dysfunction in other organ systems. However, elevated urinary Ca (hypercalciuria), P (hyperphosphouria), and acidity are implicated in the etiology of certain renal diseases. Dietary components can influence the excretion of these compounds, and suggests that diet can have a profound influence on renal health.

1.3 The influence of dietary components on nephrocalcinosis in rats

1.3.1 Defining nephrocalcinosis

Nephrocalcinosis (NC) is a form of kidney disease that refers to the accumulation of Ca deposits in kidney tissue. NC has been documented in several animal species (including humans), however there may be differences in the etiology. Due to their sensitivity for NC, the female rat is a common animal model. NC can be induced in female rats with simple changes in dietary mineral, protein, and lipid content. NC has been shown to induce inflammatory processes that result in nephron damage and fibrosis, which are involved in the initiation of other forms of renal diseases. Therefore, NC is considered an initiating factor for the development of chronic renal disease and renal failure [36, 37].

NC typically develops within the tubular lumen, and appears to be dependent upon filtrate concentrations. It is believed that urinary compounds typically involved in NC (such as Ca, P, and oxalate) must become insoluble before deposition (or nucleation) can occur. *In vitro* studies have shown that Ca-based crystals adhere to anionic glycoproteins on the surface of tubular epithelial cells [38]. After a given exposure time, the epithelial cell engulfs the crystal as a clearance mechanism. However, free radical production and cytokine expression accompany this process, and leads to the formation of a site for nucleation [39, 40].

1.3.2 Dietary components and NC

The etiology of NC involves the precipitation of filtrate components followed by their attachment to the tubular epithelial cells. Therefore, factors influencing the solubility of urinary components (such as Ca, P, and oxalate) mediate the development of NC. These factors originate from the diet, and suggest that dietary alterations influence urinary composition and NC. This is expected since the main function of the kidney is to maintain blood composition through the production of urine.

Dietary mineral content

Variable consumption of Ca, P, and Mg can result in concomitant changes in their respective excretion. Surprisingly, increased consumption of dietary Ca is not directly associated with urinary Ca excretion and associated NC [41]. However, elevated dietary intake of P is tightly associated with both urinary P excretion [42] and severity of NC [43]. Several studies have also addressed the interaction of dietary Ca and P in NC, and reported that a dietary Ca:P ratio less than 1 is nephrocalcigenic [41]. However, it appears that the absolute amount of Ca and P is important and not just the ratio. Cockell et al., [44] found that the severity of NC increased with dietary Ca and P content despite a Ca:P ratio of 1.3.

Dietary protein content

Dietary protein has also been shown to influence urinary characteristics. In fact, Meyer et al., [45] concluded that changing the source of dietary protein

had a greater effect on NC than manipulations in dietary mineral content. This finding was later supported by Zhang and Beynen [46], who fed female rats diets varying in protein source (casein, soybean, or codmeal) and amount (18, 35, and 53%). They noted significant fluctuations in both urinary and renal content, with rats fed casein having the highest P excretion, most acidic urine, and highest kidney Ca content. Urinary acidification is a common finding in rats with high urinary P content [42, 47]. Urinary acidification could influence NC by decreasing proximal tubule P reabsorption and result in increased urinary P excretion [48, 49].

Dietary lipid content

Few publications exist studying the influence of lipids on diet induced NC. Schmiedl et al. [50] fed rats a hyperlipidemic-EFA deficient diet high in (1%) cholesterol and neutral fat (10%). As compared to rats fed a standard chow diet (5% lipid), rats fed the hyperlipidemic diet had more calcifications (mean of 2840 vs 392), and higher renal Ca and P content. Unlike urinary profiles mentioned above, the rats with NC did not excrete more P or an acidic urine. Instead, rats fed the hyperlipidemic diet had a more basic urine and higher excretion of oxalate leading to development of calcium oxalate (CaOx) deposition. CaOx is another Ca precipitate that can form in basic urine containing elevated oxalate concentrations [49]. CaOx is a common form of NC in humans and has potent proinflammatory signaling potential [39]. However, CaOx stone formation is typically associated with liver or GI dysfunction. The authors noted that the

hyperoxaluria reported was due to changes in liver oxalate synthesis, presumably due to the poor quality of the high fat diet.

Dietary PUFAs may also mediate NC through their proposed anti-inflammatory actions (Section 1.1.4). Furthermore, residual ω -3 PUFAs may explain why certain protein sources differ in their nephrocalcigenic ability [46]. ω -3 PUFA supplementation has shown beneficial effects in rodent models of hypercalcemic NC. Burgess et al., [51] and Schlemmer et al. [52] both reported decreased renal Ca content in Ca glubonate injected rats supplemented with EPA. However, Ca gluconate injections are hypercalcemic and induce NC by sustained hypercalciuria (a different mechanism than that normally occurring in rats). Additionally, no urinary values or serum values were given to determine if the observed NC was specific to the kidney. Therefore, it is unclear if PUFAs can mediate diet induced NC. Furthermore, different sources of ω -3 PUFAs exist and each may differ in ability to modulate the NC induced by feeding different dietary protein sources. Given their role in inflammatory processes and prostaglandin production, consuming diets differing in PUFA content may influence renal health and the development of NC.

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aortas and kidneys is reduced with n-3 fatty acid supplementation.

Prostaglandins Leukot Essent Fatty Acids, 59, 221-227.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

2.0 Study Objectives

Objective 1. To investigate the effect of consuming krill protein concentrate on renal and bone health.

Objective 2. To characterize krill oil that has been isolated using different processing methods.

Objective 3. To determine how different dietary sources of ω -3 PUFAs influence kidney health and function in female rats.

Gigliotti, J.C., Smith, A.L., Jaczynski, J., & Tou, J.C. (2011).

**Consumption of Krill Protein Concentrate Prevents Early Renal Injury and
Nephrocalcinosis in Female Sprague-Dawley Rats**

Journal of Urological Research, (2011), 39, 59-67

3.1 Background

We have previously shown that krill protein concentrate (KPC) has nutritional potential for human consumption [1]. However, these rats also had significantly lower kidney weights compared to rats fed casein, the standard protein source used in semipurified diets. Changes in kidney weight have been correlated with renal disease, and include potential alterations in mineral metabolism [2]. Therefore, the objective of this study was to address this change in body weight, and determine if changing the dietary protein source can influence measures of mineral metabolism and bone health.

3.2 Introduction

Diet-induced alterations in kidney health are common in female rats, with renal calcification (nephrocalcinosis) being a prime example [3, 4, 5]. The transition of nephrocalcinosis to kidney stones (nephrolithiasis) has been implicated in nephropathy and subsequent renal failure in rats [5]. Diet-induced alterations in the kidney function has been attributed to manipulating various components of the diet, including the dietary calcium:phosphorus ratio [6, 7, 8, 9, 10], and the dietary protein content [11, 12, 13]. Diets rich in phosphorus (P) or leading to increased P excretion have been reported as being an underlying cause of nephrocalcinosis [14]. However, Meyer et al. [11] reported that changing the source of dietary protein fed to female Wistar rats reduced calcium (Ca) deposition in the kidneys to a greater extent than manipulating dietary minerals. Together the study results suggest that the amount, as well as the source, of

dietary protein influences the development of nephrocalcinosis and nephrolithiasis [12, 13]. Additionally, different protein sources are associated with different fatty acids. The role of lipids in nephrocalcinosis and nephrolithiasis should be considered because fatty acids have been shown to differentially influence inflammatory pathways, and inflammation has also been implicated as an initiating factor in tissue calcification [15, 16, 17].

Nephrocalcinosis is commonly associated with increased urinary Ca excretion (hypercalcuria), and dietary proteins also influence the excretion of Ca. It has been proposed that higher consumption of dietary protein increases intestinal Ca absorption and this may increase urinary Ca excretion [18, 19]. The consumption of dietary protein has also been correlated with an increase in urinary acid excretion [20, 21, 22], which has been shown to reduce renal Ca reabsorption and possibly promote hypercalciuria [23]. These potential changes in kidney function may influence mineral balance and in turn, bone health. Calvo et al., [24] reported hypercalciuria in male Sprague-Dawley rats fed high protein diets; however, there was no effect on bone mineral content. Female rats display a higher sensitivity to alterations in renal handling of Ca [6] and therefore, in the present study female rats were used since they may provide a more sensitive model for mineral and subsequent bone loss.

In our study, female Sprague-Dawley rats fed protein derived from a pelagic source were compared to rats fed casein, a standard protein source used in commercial semi-purified rodent diets. Fish contain high-quality protein as well as other nutrients with potential health benefits. However, depletion of existing

fish stocks and environmental contaminants has raised public concern regarding fish consumption. A potential alternative is to promote consumption of under-utilized species such as krill. Based on body size, krill has the largest amount of protein (over 65% dry weight) among all organisms worldwide. Analysis of lipid content showed ~27% of total fatty acids in Antarctic krill (*Euphausia superba*) consists of the health benefiting omega-3 polyunsaturated fatty acids (ω -3 PUFAs) [1, 25]. Krill offers a promising high quality and sustainable source of protein for human consumption. Therefore, the objective of this study was to evaluate the effects of consuming KPC in regards to renal and bone health.

3.3 Materials and Methods

3.3.1 Diets and animal feeding study

All animal procedures were conducted in accordance with the guidelines set forth by the Institute of Laboratory Animal for the Care and Use of Laboratory Animals [26], and were approved by the Animal Care and Use Committee at West Virginia University.

Immature (age 28 d) female Sprague-Dawley rats (n=20) were purchased from Taconic Farms (Rockville, MD). Female rats were used due to their higher susceptibility to nephrocalcinosis compared to male rats [4]. Upon arrival, rats were individually housed in metabolic cages. All animals were maintained in a room at 21°C with a 12 h light/dark cycle. During a 14 d acclimation period, animals were given *ad libitum* access to deionized distilled water (ddH₂O) and AIN-93G diet (Harklan Teklad, IN). Following the 14 d acclimation period, rats

were randomly assigned (n=10 per group) to be fed *ad libitum* a low protein diet consisting of 10% crude protein supplied as casein or KPC for 4 weeks.

KPC was isolated from whole frozen Antarctic krill (*Euphausia superba*) purchased from Krill Canada (Langley, BC, Canada) using an isoelectric solubilization / precipitation method described previously in Gigliotti et al. [1].

The recovered KPC was 77.7% crude protein, 8.1% total lipid, and 4.4% total ash on a dry basis by proximate analysis. Freeze-dried KPC was stored at -20°C until mixed into the diet. Replacement of the protein as either KPC or casein at a level of 10% in AIN-93G diet was corrected for protein and lipids so that the diets were isocaloric and isonitrogenous. Ca and P contents of the diets were also matched to provide a 1.2 ratio (Table 1). Diets were kept stored at 5°C. Food consumption and ddH₂O intake were measured and replaced with fresh ddH₂O and assigned diet every 2 d.

3.3.2 Kidney mineral content

At the end of 4 weeks, rats were euthanized by CO₂ inhalation, and both kidneys excised. Kidneys were trimmed, decapsulated, and weighed separately. No statistical differences were found between the right and left kidney weights. Therefore, the left kidneys were assayed for mineral content and the right kidneys used for histological evaluation.

To determine kidney mineral concentrations, the left kidneys were dried overnight at 110°C in an oven and then ashed at 550°C in a muffle furnace (Lindberg 515A2, WI) for 24 h. To determine specific minerals, ashed samples

were dissolved in 70% nitric acid (2 mL) and neutralized in ddH₂O (5 mL). Samples were then filtered through Whatman no. 1 paper, diluted to volume (50 mL), and renal Ca and P content in the kidneys were measured using inductively coupled plasma spectrometry (ICP) (model P400, Perkin Elmer, Shelton, CN).

3.3.3 Kidney histology

The right kidneys (n=4) were dissected and immediately fixed in 10% formalin solution overnight and then transferred to 70% ethanol. Tissues were dehydrated through a series of increasing ethanol concentrations (70-100% in ddH₂O) to xylene, embedded in paraffin, and serially cut in 8µm thick sections using a microtome (American Optical Company, NY). Paraffin embedded sections were placed on moist glass slides and dried for mounting. Mounted sections were then deparaffinized in xylene, and rehydrated through a series of decreasing ethanol concentrations (100-70%) prior to staining.

Renal morphology and microtubular calcification was examined by standard hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining. PAS staining was performed following a commercially available protocol (Sigma-Aldrich, MO). Briefly, hydrated slides were incubated in Periodic Acid Solution for 5 minutes. After rinsing in ddH₂O, slides were immersed in Schiff's reagent for 15 minutes, washed in water, and then counterstained in Gill Hematoxylin solution for 90 seconds and cover slipped. All slides were analyzed under a Nikon TE 2000-S light microscope (Nikon Instruments, NY) at 4X magnification

by three trained individuals, and images captured using a PC interfaced with Q-Capture imaging software (Quantitative Imaging Corporation, BC, Canada).

3.3.4 Urinary assays of renal function and damage

Rats were individually housed in metabolic cages to collect urine for determination of renal function and damage. Urine samples were collected and centrifuged at 1,500 g for 10 min at 4°C to remove any debris. Urine samples were aliquoted into fresh tubes and stored at -80°C until assayed. Baseline urine consisted of pooled samples collected over 7 d of the first week of treatment. Final urine consisted of pooled samples collected over the final 7 d of the 4 week feeding study.

To determine renal function, urinary total protein and creatinine were determined using commercially available colorimetric assays (Cayman Chemical, MI). Serum creatinine concentrations were determined enzymatically using a commercially available Vet-16 rotor (Hemagen Diagnostics Inc., Columbia, MD). Glomerular filtration rate (GFR) was determined based on creatinine clearance (CrC) calculated as: $CrC = (UC/SC) \times V$, where UC is urinary creatinine concentration, V is the volume of measured urine excreted per minute, and SC is serum creatinine concentration. Renal damage was determined by measuring urinary n-acetyl glucosaminidase (NAG) activity, an early indicator of renal damage, using a commercially available colorimetric kit (Diazyme, CA). The optical densities of the urinary samples were determined at wavelength 505nm in a Beckman DU530 spectrophotometer (Beckman Coulter, CA). Urinary sodium

(Na) was determined by diluting the urine 1:40 (v/v) in ddH₂O and measuring the mineral concentration using the ICP. Urinary pH was determined using a standard pH meter (Beckman, CA).

3.3.5 Mineral balance

Mineral balance studies were performed by individually housing rats in metabolic cages to determine food intake and to collect feces and urine samples. Ca apparent absorption was calculated as $(\text{Ca intake} - \text{fecal Ca excretion}) / \text{Ca intake} \times 100$. Calcium retention was determined by calculating $\text{Ca intake} - (\text{fecal Ca excretion} + \text{urinary Ca excretion})$. P apparent absorption was calculated as $(\text{P intake} - \text{fecal P excretion}) / \text{P intake} \times 100$ and P retention was determined by calculating $\text{P intake} - (\text{fecal P excretion} + \text{urinary P excretion})$.

Ca and P intake was calculated by multiplying the amount of diet consumed and the dietary concentration of Ca (6 mg/g diet) or P (5 mg/g diet) as provided by diet manufacturer and confirmed by ICP. Urine and fecal samples consisted of pooled samples collected during the final 7 d of the 4 week feeding study. To determine urinary minerals, samples were diluted 1:40 in ddH₂O and Ca and P concentration measured by ICP. To determine fecal minerals, the fecal samples were freeze-dried for 48 h. Feces were ashed overnight in a muffle furnace (Lindberg 515A2, WI), dissolved in 70% nitric acid, diluted to 1:50 in ddH₂O and then Ca and P concentration determined by ICP.

3.3.6 Bone measurements

Both femurs were dissected and defleshed with care being taken not to damage the periosteum. Each femur was wrapped in saline soaked gauze, and frozen at -20°C until assayed. For analysis each bone was brought to room temperature. Bone morphology was determined by measuring length, width, and depth using a vernier caliper (Bel-Art Products, NJ). Bones were dried at 110°C for 48 h and dry weights measured using an analytical balance (Mettler Toledo, OH). The dried femurs were ashed at 600°C in a muffle furnace (Lindberg 515A2, WI) for 24 h and total bone mineral content (BMC) determined by the weight of the bone ash. Ashed femur samples were dissolved in 70% nitric acid, diluted to 1:50 ddH₂O and femoral Ca and P content measured by ICP.

Femoral strength was determined on a TA.HDi Texture Analyzer (Texture Technologies Corp, NY) outfitted with a three-point bending apparatus. The femur was placed on supports (1 mm width at tip) and bent until broken by lowering a centrally placed blade (1 mm width) at constant crosshead speed (0.1 mm/s). The load-deflection data collected by a PC interfaced with the TA.HDi Texture Analyzer was used to determine the bone strength measurement of peak force. Bone morphometry, BMC, Ca, P, and bone strength results of the bone pairs were averaged after no differences between right and left femurs were determined by paired t-test with significance level set at $P < 0.05$.

3.3.7 Statistical analysis

The animal feeding study was a completely randomized design, with n=10 rats per diet treatment. The t-test was used to compare differences between treatment groups, and Mann-Whitney Rank Sum test was performed on data not normally distributed. The t-test and the Mann-Whitney Rank test were both performed using Sigma Stat 3.1 (Systat Software Inc., San Jose, CA). Differences were considered significant at $P<0.05$.

3.4 Results

3.4.1 Kidney mineral content

Figure 1 shows the relative weights (g/g body weight*100) of the kidneys, kidney total mineral (ash), Ca, and P content of rats fed KPC relative to rats fed casein. Kidney weights of rats fed KPC were lower ($P<0.001$) compared to the kidney weights of rats fed casein. Rats fed KPC had decreased ($P<0.001$) kidney total mineral content compared to rats fed casein. Measurements of specific minerals showed that rats fed KPC also had lower ($P=0.002$) kidney Ca concentrations, and lower ($P<0.001$) kidney P concentrations compared to rats fed casein.

3.4.2 Kidney histology

H&E and PAS stains were used to detect renal calcium deposits, with more than 115 renal tissue sections of n=4 rats / diet used. All slides containing the cortico-medullary junction from rats fed casein displayed extensive

microtubular calcification (Figures A and B, top). Interestingly, only one (Figure C) of the four rats fed KPC (Figures C and D) used for histological evaluation showed renal calcification in the same region of renal tissue and this was comparatively mild compared to casein fed rats. Focal areas of tubular atrophy were also observed, however no distinction between the diets could be made. There were no indications of fibrosis or other glomerular abnormalities in rats fed casein or KPC.

3.4.3 Urinary assays of renal function and damage

To determine kidney damage and function, various biomarkers were determined in urine collected during the final week of the 4 week study. Table 2 shows NAG concentrations were lower ($P=0.03$) in rats fed KPC compared to casein. Although not significant, rats fed KPC tended to have higher CrC ($P=0.055$) and lower total urinary protein ($P=0.057$) than rats fed casein. Rats fed KPC had a more basic ($P=0.004$) urinary pH values compared to rats fed casein. The urine of rats fed KPC also had higher urinary Na ($P<0.001$) concentrations compared to rats fed casein (Table 2).

3.4.4 Mineral Balance

Table 3 shows that rats fed KPC had higher a ($P=0.02$) urinary output after the initiation of the diet. Despite the higher urinary output, there was no difference in urinary Ca excretion between rats fed casein and KPC. There was no effect on Ca homeostasis indicated by no significant differences in Ca intake,

absorption, retention, or fecal excretion between rats fed KPC or casein at the initiation of the diet. During the final week, rats fed KPC had greater ($P<0.001$) Ca intake than the rats fed casein. However, there were no differences in measures of total Ca intake, absorption, retention, or fecal Ca concentrations between rats fed KPC and rats fed casein (Table 3).

Table 4 shows that consumption of KPC had no effect on P homeostasis at the initiation of the diet as indicated by no differences in P intake, absorption, retention, urinary, or fecal output between rats fed KPC and rats fed casein. During the final week, there was no effect on P homeostasis as indicated by no differences in P intake, absorption, retention, urinary or fecal P concentrations between rats fed KPC and rats fed casein.

3.4.5 Bone measurements

Table 5 shows there were no significant differences in femur morphometry measurements of weight, length, width or depth between rats fed KPC and rats fed casein. There were no significant differences in femur total BMC of rats fed KPC compared to rats fed casein. Similarly, measurements of the major bone minerals indicated no significant difference in either the femoral Ca or P content of rats fed KPC compared to rats fed casein. Measurement of bone strength as determined by peak force in the femur showed no significant difference between rats fed KPC and rats fed to casein.

3.5 Discussion

Protein quality of KPC is similar to that of casein, a standard protein source, used in commercial semi-purified rodent diets [1]. However, female rats fed KPC had lower ($P<0.001$) kidney weights compared to rats fed casein. To investigate the effect of KPC compared to casein on kidney health, NAG was measured as an early marker of renal injury. Despite being provided a low (10% g/kg diet) protein diet, urinary NAG concentrations were higher ($P=0.03$) in rats fed casein compared to KPC, indicating that protein source is an important factor to consider in renal health. Similarly, NAG excretion was greater in male Munich-Wistar rats consuming casein compared to a soy protein based diet. The authors suggested that casein induced renal injury through increased acidification of the kidney filtrate [27]. Ritskes-Hoitinga et al., [28] reported that high urinary pH values were associated with low degree of nephrocalcinosis indicated by a negative correlation ($r=0.68$, $p<0.05$) between urinary pH and the severity of nephrocalcinosis. Therefore, in our study higher ($P=0.004$) urinary pH in rats fed KPC compared to rat fed casein may be responsible for preventing early renal injury.

The pathogenesis of nephrolithiasis has been proposed to involve renal injury followed by deposition of calcium crystals to the site of injury [29]. In our study, kidney mineral content ($P<0.01$), Ca ($P<0.001$), and P ($P<0.001$) concentrations were lowered in rats fed KPC compared to casein. Furthermore, histological evaluation of the kidneys showed that rats fed casein had extensive renal calcification, whereas mild to absent renal calcification was observed in the

kidneys of rats fed KPC. Similarly, Zhang and Benyen [13] reported that female Wistar rats fed cod meal had lower kidney Ca content compared to rats fed casein or soybean protein isolate. Together, the study results suggest that pelagic protein sources may provide protection against mineral deposition in the kidneys.

After an initial renal injury, kidney function often declines [30].

Determination of urinary total protein excretion provides an estimation of kidney filtrating ability with proteinuria considered a risk factor for progressive loss of kidney function. CrC provides an estimation of glomerular filtration rate with reduced CrC used as a clinical diagnosis for renal disease [31]. Although not significant, rats fed KPC for 4 weeks tended to have lower ($P=0.057$) urinary total protein excretion and higher ($P=0.055$) CrC compared to rats fed casein.

Similarly, male Wistar rats fed soy protein had lower urinary total protein and higher CrC compared to rat fed casein [32]. These study results indicate that the protein source influences renal function. In the current study, urinary output was higher ($P=0.02$) in rats fed KPC compared to casein-fed rats. This may have been the result of a tendency ($P=0.10$) for rats fed KPC to consume more ddH₂O (970.3 ml/28d) than rats fed casein (620 ml/28d). Increased urinary output in rats fed KPC may have contributed to the observed decreased kidney mineral content and deposition. Promoting increased urinary output is a common recommendation for individuals suffering from kidney mineralization [33]. However, in a study comparing different dietary proteins and development of nephrocalcinosis, Zhang and Benyen [13] reported that the rats with the lowest kidney mineral content did not have the highest urinary outputs. Therefore, decreased mineralization of the

kidneys in rats fed KPC in the current study was likely not the result of an increased urinary output alone.

The tendency for higher glomerular filtration rates and lower urinary total protein excretion, accompanied by lower kidney calcification, in KPC compared to casein fed rats suggested that consumption of KPC had beneficial effects on kidney function. Furthermore, lower urinary NAG concentration and renal tissue Ca deposition suggested that KPC consumption prevented kidney injury. However, these beneficial effects may not be due to the protein alone, since KPC also contains minerals and lipids that may affect the kidneys.

KPC is composed of 4.4% minerals by weight [1]. Alterations in dietary Ca:P ratio has been reported to play an important role in the development of nephrocalcinosis [7]. Increasing the Ca:P ratio to greater than 1.0 has been reported to prevent nephrocalcinosis [8]. In our study the KPC and casein diets were formulated to have matching Ca and P concentrations which resulted in a Ca:P ratio of 1.2. Therefore, it appears that decreased kidney mineralization in rats fed KPC was not due to differences in dietary Ca and P content.

Common urinary abnormalities associated with nephrocalcinosis and nephrolithiasis are hypercalciuria and/or hyperphosphouria. In our study there were no differences in urinary Ca and P concentrations between rats fed KPC and casein. Unexpectedly, rats fed KPC had a 4 fold increase in urinary Na compared to rats fed casein. Subsequent analysis of diets showed that KPC had twice the Na content of the casein diet. Increased Na was likely due to the processing method used to obtain KPC. As a result rats fed KPC consumed twice the amount of Na

compared to rats fed casein. Higher Na consumption in rats fed KPC may explain their tendency ($P=0.10$) to consume more ddH₂O, and in turn, their increased urinary output. High urinary Na has been suggested to interfere with tubular Ca reabsorption leading to hypercalciuria, a risk factor for nephrocalcinosis and nephrolithiasis [34, 35]. In the current study, the increased urinary Na in rats fed KPC did not appear to affect urinary Ca excretion compared to rats fed casein. Furthermore, Mattson et al., [36] concluded that dietary composition significantly influenced the development of renal disease in salt sensitive rodent models independent of the NaCl content. Taken together these data suggest that the mineral content of the diet is not the sole determinant of urinary mineral excretion, a risk factor for developing nephrocalcinosis. This finding is supported by Meyer et al., [11] who reported that changing the source of protein in semi-synthetic diets reduced mineral deposits, and this exceeded the effects of changing the mineral composition of the diet.

KPC also contains a lipid component that may benefit renal health. Gigliotti et al. [1] reported that KPC consists of 8.1% lipid by weight. The lipids in KPC are rich (~27%) in the ω -3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). EPA and DHA have anti-inflammatory properties that potentially contributed to renal health. Rats fed cod meal, a potential source of EPA and DHA, were reported to have reduced kidney Ca concentrations compared to rats fed casein or soy isolate [13]. Furthermore, Schlemmer et al. [37] reported that rats supplemented with ω -3 PUFAs had decreased calcification of the renal tubules. Consuming KPC as a source of protein provides a high

quality protein with the added benefit of ω -3 PUFAs, whereas other high quality protein foods tend to be high in saturated fatty acids.

In addition to renal injury, changes in renal function may negatively affect Ca and P balance, which can lead to compromised bone health [38]. Protein source appears to affect Ca and P absorption and retention. Van Dael et al. [39] reported that replacing casein with whey protein diets resulted in higher absorption of Ca and higher absorption and retention of P. In our study, there were no differences between Ca and P absorption and retention in rats fed KPC compared to casein. Furthermore, bone measurements showed no differences in femoral total bone mineral content, Ca, P or bone strength between rats fed KPC and casein. Similarly, the lower urinary pH observed in rats fed casein had no effect on bone mass or strength measurements. It is likely that extracellular pH was not lowered sufficiently to activate bone resorption in rats fed casein, a proposed mechanism for protein induced bone loss [40]. Studies addressing bone loss due to lower pH commonly use high protein diets of up to 50% protein. It was unlikely that our diet consisting of 10% protein would promote the metabolic acid load required to affect bone health. Recent evidence suggests that dietary protein may be as important as dietary Ca in preventing bone loss [41, 42]. Bone tissue is formed by the mineralization of the collagen, a protein-derived connective tissue. Thus, dietary protein may contribute to bone density and strength by promoting complete bone matrix formation.

In conclusion, early renal injury and Ca deposits were influenced by the protein source fed to young female Sprague-Dawley rats, even with a low protein

diet (10% g/kg diet). Therefore, it is important not only to consider the amount, but also the source of dietary protein. Compared to rats fed casein, the consumption of KPC prevented early renal injury. This was indicated by the lower urinary NAG, reduced kidney mineralization, and a tendency for higher glomerular filtration rates and lower proteinuria. Other nutrients associated with KPC, particularly the ω -3 PUFA rich lipids, may also have contributed to reduced kidney injury and Ca deposition. In addition to the beneficial effect on the kidney, KPC consumption had no effect on bone mass or strength. Based on the study results, KPC is a novel and sustainable protein source which appears to attenuate the development of diet-induced alterations in renal health and associated bone loss in rats.

3.6 References

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Table 1. Diet Composition

Ingredients (g/kg diet)	10% Casein	10% KPC^b
Casein	115	0
DL-Methionine	1.5	0
KPC	0	128
Sucrose	531.8	609.8
Corn Starch	200	229.4
Corn Oil	53.5	49.9
Cellulose	52	59.6
Vitamin Mix ^a	10	11.5
Ethoxyquin	0.01	0.01
Mineral Mix ^a	13.4	15.3
Calcium Phosphate	20.2	20.9
Calcium Carbonate	2.6	3.6
Gross Energy (kcal/g)	4.3	4.3
KPC, krill protein concentrate		

^a Based on the AIN-93G vitamin and mineral mixes [7].

^b Diet formulated for 872 g of diet + 128 g addition of KPC

Table 2. Urinary assays for kidney damage and function in female Sprague-Dawley rats fed different dietary sources of protein.

Biomarkers^a	Casein^b	KPC^b	<i>P</i> value
Urinary NAG (IU/L)	22.9 ± 6.9	3.9 ± 1.0	0.005
CrC (ml/min)	1.7 ± 0.7	5.5 ± 2.1	0.055
Total Urinary Protein (µg/ml)	0.6 ± 0.02	0.5 ± 0.03	0.057
Urinary pH	7.5 ± 0.5	9.0 ± 0.02	0.004
Urinary Na (mg/7d)	81.3 ± 10.4	246.2 ± 40.1	<0.001

CrC - creatinine clearance; NAG - n-acetyl glucosaminidase; Na – sodium

^a Rats were individually housing in metabolic cages. Urine samples consisted of pooled samples collected during the final 7 d of the 4 week feeding study.

^b Values are expressed as the mean ± SEM of n=10 rats/group. The t-test was used to compare differences between treatment groups, Differences were considered significant at $P<0.05$.

Table 3. Calcium balance of female Sprague-Dawley rats fed different dietary sources of protein

Measurements ^a	Casein	KPC	P value
Total Ca Intake	3.16 ± 0.08	3.23 ± 0.08	0.5
First Week			
Urinary Output (ml/d)	6.05 ± 3.5	13.9 ± 3.4	0.02
Fecal Output (mg/d)	1.9 ± 0.1	2.03 ± 0.1	0.7
Ca Intake (mg/d)	82.0 ± 2.7	82.6 ± 3.7	0.9
Ca Apparent Absorption (%)	40.2 ± 4.4	46.6 ± 3.7	0.6
Fecal Ca Excretion (mg/d)	43.0 ± 5.6	44.1 ± 3.7	0.7
Urinary Ca Excretion (mg/d)	0.67 ± 0.2	1.01 ± 0.3	0.4
Ca Retention (mg/d)	22.3 ± 2.8	26.3 ± 2.4	0.6
Final Week			
Urinary Output (ml/d)	6.7 ± 2.9	13.6 ± 3.6	0.03
Fecal Output (mg/d)	2.2 ± 0.134	2.2 ± 0.113	0.9
Ca Intake (mg/d)	111.0 ± 4.4	133.0 ± 2.7	<0.001
Ca Apparent Absorption (%)	23.8 ± 5.1	31.1 ± 6.4	0.7
Fecal Ca Excretion (mg/d)	83.7 ± 4.3	79.9 ± 11.4	0.7
Urinary Ca Excretion (mg/d)	3.3 ± 0.7	3.6 ± 1.7	0.3
Ca Retention (mg/d)	19.1 ± 4.1	31.4 ± 6.6	0.2
Serum Ca (mg/dl)	11.5 ± 0.4	11.4 ± 0.8	0.6

^aValues are expressed as the mean ± SEM of n=10 rats/group. The t-test was used to compare differences between treatment groups, Differences were considered significant at $P<0.05$.

Table 4. Phosphorus balance of female Sprague-Dawley rats fed different dietary sources of protein

Measurements ^a	Casein	KPC	<i>P</i> value
Total P Intake (mg/28d)	2.44 ± 0.06	2.50 ± 0.06	0.5
First Week			
P Intake (mg/d)	89.7 ± 3.0	90.9 ± 4.1	0.8
P Apparent Absorption (%)	71.3 ± 2.4	74.8 ± 1.7	0.5
Fecal P Excretion (mg/d)	25.1 ± 1.9	23.1 ± 2.0	0.8
Urinary P Excretion (mg/d)	18.2 ± 3.7	13.7 ± 1.1	0.3
P Retention (mg/d)	30.3 ± 3.4	37.9 ± 2.5	0.3
Final Week			
P Intake (mg/d)	87.6 ± 3.4	91.3 ± 1.9	0.4
P Apparent Absorption (%)	47.4 ± 3.9	51.2 ± 4.6	0.8
Fecal P Excretion (mg/d)	45.1 ± 2.3	42.2 ± 3.3	0.4
Urinary P Excretion (mg/d)	15.6 ± 1.4	13.5 ± 0.8	0.2
P Retention (mg/d)	19.9 ± 3.6	25.5 ± 3.2	0.4
Serum P (mg/dl)	11.3 ± 0.4	10.7 ± 0.5	0.4

^aValues are expressed as the mean ± SEM of n=10 rats/group. The t-test was used to compare differences between treatment groups, Differences were considered significant at $P < 0.05$.

Table 5. Bone measures in female Sprague-Dawley rats fed different dietary sources of protein

Measurements^a	Casein	KPC	<i>P</i> value
Femoral Weight (g)	0.53 ± 0.02	0.55 ± 0.02	0.4
Femoral Length (mm)	27.2 ± 0.22	27.3 ± 0.33	0.9
Femoral Width (mm)	3.8 ± 0.09	3.7 ± 0.14	0.5
Femoral Depth (mm)	3.00 ± 0.03	2.98 ± 0.04	0.7
Femoral BMC (g)	0.54 ± 0.03	0.52 ± 0.01	0.2
Femoral Ca (mg/g)	41.4 ± 4.6	39.0 ± 1.7	0.7
Femoral P (mg/g)	55.0 ± 8.8	67.5 ± 7.9	0.5
Femoral Strength (N)	159.0 ± 6.5	174.6 ± 9.1	0.2

^aValues are expressed as the mean ± SEM of n=10 rats/group. The t-test was used to compare differences between treatment groups. Differences were considered significant at $P < 0.05$.

Figure 1.

Figure 2.

A

B

C

D

Figure 1. Weights and total mineral, Ca, and P content of kidneys from rats fed 10% protein diets consisting of either casein or KPC for 4 weeks. Data presented as percent relative to casein. Symbols indicate significant difference of $P=0.002$ (*) or $P<0.001$ (**) by t-test.

KPC, krill protein concentrate.

Figure 2. Hematoxylin and eosin (H&E) stained renal tissue from rats fed 10% protein diets consisting of either casein (A & B) or KPC (C & D) for 4 weeks. Arrows highlight mineral deposition observed at 4X magnification.

KPC, krill protein concentrate.

Gigliotti, J.C., Davenport, M., Beamer, S., Tou, J.C., & Jaczynski, J. (2011)

**Extraction and characterization of lipids from
Antarctic krill (*Euphausia superba*)**

Food Chemistry, (2011), 125, 1028-1036

4.1 Background

Results from Chapter 1 (Section 3) indicated that nephrocalcinosis and associated kidney injury was lower in rats fed KPC as compared to casein. Similar results have been reported elsewhere. Replacing casein with soybean, flaxseed, or codmeal also resulted in decreased kidney Ca content, suggesting that casein is nephrocalcigenic [1]. However, why different proteins have varying influence on kidney health remains unclear.

It is understood that changing the source of dietary protein also changes the dietary lipid content. In some animal studies, the protein sources used have commonly contained residual lipid content [1, 2]. This could explain how different protein sources differentially influence kidney health, since soybean, flaxseed, codmeal, and KPC are known sources of ω -3 PUFAs. Rodent models of kidney disease have benefitted from ω -3 PUFAs supplementation [3, 4]. Therefore, the residual ω -3 PUFA content of KPC may be responsible for the decreased nephrocalcinosis.

Consumption of ω -3 PUFAs has been associated with multiple health benefits, and therefore has become a well-recognized nutritional supplement. Krill oil is an available ω -3 PUFA supplement and it is advertised as being a superior ω -3 supplement due to its high phospholipid content [5]. Health claims state that there are nutritional benefits of consuming phospholipids as opposed to triglycerides; however little compelling evidence exists supporting these claims.

The unique composition of krill oil also has manufacturing implications. This unique lipid composition can pose difficulties during the processing of krill

oil, resulting in inefficient usage of the nutritional properties of krill. Given the vital role of krill in its ecosystem, and its valuable nutritional potential, efficient usage of this resource is vital. Therefore, the objective of the next study is to determine the composition of krill oil isolated using different processing methods.

4.2 Introduction

Antarctic krill (*Euphausia superba*) are small, shrimp-like planktonic crustaceans found predominately in the Southern ocean. Despite their small size, the Antarctic krill is estimated to be one of the most numerous multicellular organisms on the planet [6]. A sustainable harvest of krill has been estimated at 70-200 million tons (MT) [7]), whereas the total annual capture of all fisheries has been approximately 130 MT since 2000 [8]. Although it is difficult to accurately determine the fluctuating krill biomass [9], the harvest of krill alone could be comparable to the biomass of all other aquatic species currently harvested by humans. Nicol and Foster [10] estimated a potential annual krill capture to be 0.1 MT, making krill an underutilized species given proper harvest management [11, 12, 13].

Despite this large biomass, the use of krill as a human food source has been limited [7]. The interest in using krill as a food source stems from its nutrient composition, where krill was reported to contain 77.9-83.1% moisture, 11.9-15.4% protein, 0.4-3.6% lipids, and ~2% chitin and glucides [14]. Krill protein has been shown to be high quality, making krill a large potential source of valuable dietary protein [15]. In addition, krill oil is currently marketed as a

valuable dietary supplement, due to its unique lipid composition. Traditionally, triglycerides (TRIGs) are the largest component of dietary oils and can represent over 60% of the lipid by TLC and densitometry analysis (unpublished data). However, krill oil has been cited as having around 40% phospholipid by weight [5]. In addition, Kolakowska et al. [16] reported that krill oil is rich in the omega-3 polyunsaturated fatty acids (ω -3 PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Intakes of EPA and DHA are associated with decreased risk of cardiovascular disease [17]. Furthermore, krill oil is also rich in the carotenoid astaxanthin, which is a potent antioxidant and provides additional nutraceutical value [18]. Together, these multiple bioactive components have resulted in various clinical benefits associated with krill oil consumption [19, 20, 21].

Given their role in Antarctic ecosystems, it is crucial to develop ways to efficiently utilize harvested krill. Currently, krill oil is extracted by a two-step solvent extraction using acetone and then ethanol in the first and second step, respectively [21, 22]. However this extraction requires two separate processing steps and is preferably conducted overnight. This long incubation time could potentially result in the degradation of lipids and proteins due to the rapid autolysis that occurs in krill post-mortem [23]. Additionally, there is no mention about water removal from krill prior to oil extraction. Research has shown an improved extraction efficiency is obtained with krill dehydration prior to oil extraction [24]. Other popular processing methods use supercritical- CO_2 entrained with up to 20% ethanol, and uses heat to inactivate endogenous krill

lipases [25]. The heating process ($>50^{\circ}\text{C}$) is efficient at inactivating the endogenous lipases; however heating may also denature heat-labile krill proteins [26]. Denaturation of these proteins likely result in a low recovery, and the recovered proteins would unlikely be functional (i.e., gel-forming ability, water-holding-capacity, emulsification, etc). This process also does not require water removal prior to processing, despite lyophilized krill having a threefold increase in lipid extraction efficiency using the supercritical- CO_2 method.

Therefore, we hypothesize that one-step extraction with acetone:ethanol mixture for 2 h from freeze-dried whole krill will result in high extraction efficiency. The study objectives were to determine: (1) oil extraction efficiency from whole krill using different solvent systems, (2) lipid classes of extracted oils, (3) FA profile of the lipid classes, (4) antioxidant capacity and cholesterol content of extracted oils, and (5) composition of the residual spent krill following oil extraction. The residual protein in the spent krill could be recovered with established techniques, such as isoelectric solubilization/precipitation. If protein functionalities are retained, this protein could be used in human food products and would contribute to the efficient utilization of this tremendous resource [27, 28, 29].

4.3 Materials and methods

4.3.1 Sample preparation and oil extraction

Whole frozen Antarctic krill (*Euphausia superba*) was obtained from Krill Canada (Langley, BC, Canada). The krill blocks were transported overnight to the West Virginia University food science laboratory in heavily insulated industrial strength boxes filled with dry ice. Upon arrival the boxes were immediately stored at -80°C until use. Whole frozen krill was freeze-dried without thawing (VirTis Genesis 35SQ Super XL freeze-dryer, Virtis, Gardiner, NY, USA), vacuum-packed and stored at -80°C until processed. A flow diagram of oil extraction from krill is shown in Figure 1.

In the one-step extraction oil was extracted from freeze-dried krill using 1:1 acetone:ethanol (v:v) solvent mixture (ACS grade acetone, Fisher Scientific, Fairlawn, NJ, USA; ACS grade 95% ethanol, Pharmco, Brookfield, CT, USA). The following krill:solvent ratios were tested 1:6, 1:9, 1:12, and 1:30 (w:v). The weight of the initial freeze-dried krill was recorded in order to determine extraction efficiency (see below). The 1:30 ratio was used in the present study to mimic the 1:6 ratio of whole fresh krill as described by [21] and [22]. Fresh whole krill is currently used in commercial oil extraction [21, 22, 25]. However, in the present study freeze-dried krill was used [24]. Therefore, 1:30 ratio (freeze-dried krill) used in the present study approximately corresponded to 1:6 ratio (fresh whole krill) during commercial krill oil extraction with respect to lipid content in relation to the solvent volume. Freeze-dried krill was dispersed in the solvent mixture (acetone:ethanol) by homogenization for 30 s using a laboratory

blender (model 51BL31, Waring Commercial, Torrington, CT, USA). Oil extraction was conducted for 2 h at 4°C using a continuous shaker (model Excella E25R, New Brunswick Scientific, Edison, NJ, USA) followed by centrifugation at 10,000 x *g* for 20 min and 4°C (model Sorvall RC-5B Refrigerated Superspeed, Kendro Laboratory Products, Newtown, CT, USA). The supernatant (i.e., extracted krill oil) was decanted and dried under air at atmospheric pressure. The sediment (residual spent krill including protein, shell, etc.) was also dried under air at atmospheric pressure and analyzed for crude protein (Kjeldahl), total fat (Soxhlet), and ash content (see below).

In the two-step extraction oil was extracted from freeze-dried krill using two separate extractions. The weight of the initial freeze-dried krill was recorded in order to determine extraction efficiency (see below). The freeze-dried krill was first mixed with acetone at a 1:6 ratio (krill:acetone, w:v), centrifuged, and then the sediment was mixed with ethanol at a 1:6 ratio (krill:ethanol, w:v), followed by final centrifugation. Therefore, two separate extracts were obtained. Acetone extract was obtained in step 1 and ethanol extract in step 2. In step 1 freeze-dried krill was dispersed in acetone by homogenization for 30 s using the laboratory blender. Oil extraction in step 1 was conducted for 2 h at 4°C using the continuous shaker followed by centrifugation at 10,000 x *g* for 20 min and 4°C. The supernatant (i.e., acetone extract) was decanted and dried under air at atmospheric pressure; while the sediment was subjected to step 2 of the extraction. Step 2 was conducted in the same manner as step 1 except ethanol was used instead of acetone. Therefore, total extraction time (i.e., step 1 and 2)

was 4 h. Following step 2, final centrifugation at 10,000 x g for 20 min and 4°C was applied. The supernatant (i.e., ethanol extract) was decanted and dried under air at atmospheric pressure. The sediment (residual spent krill including protein, shell, etc.) was analyzed for crude protein (Kjeldahl), total fat (Soxhlet), and ash content (see below).

Dry krill oils (i.e., krill oil from one-step extraction, acetone extract from two-step extraction, and ethanol extract from two-step extraction) were clarified in 2:1 chloroform:methanol (v:v) mixture (ACS grade chloroform, Fisher Scientific, Fairlawn, NJ, USA; HPLC grade methanol, Fisher Scientific, Fairlawn, NJ, USA) with 20 ml of 10% NaCl in water added to a separation funnel [30]. This clarification removed any residual water in the krill oil samples. Sufficient volume of the chloroform:methanol mixture was added until there was no visible separate layers. Following clarification, the krill oil samples were re-dried under air at atmospheric pressure. Dry clarified krill oils were weighed and compared to the initial weight of the freeze-dried krill subjected to extraction in order to determine extraction efficiency (see below). The lipid classes of the oils were determined by thin layer chromatography (TLC). Fatty acid profile (FAP) of the lipid classes was determined with gas chromatography (GC). In addition, cholesterol content and antioxidant capacity of the oils were determined. These analyses are described in the subsequent sections.

For comparison, oil was also extracted from freeze-dried krill using the Folch method and Soxhlet extraction [31]. In Folch method, 3g of freeze-dried krill were dispersed in 60 ml of the 2:1 chloroform:methanol mixture by

homogenization for 30 s using the laboratory blender, followed by filtration through Whatmann nr 40 paper (Whatman International, Maidstone, UK) in the separation funnel. An aliquot of 20 ml of 10% NaCl in water was added to the separation funnel and the mixture was manually shaken and allowed to stand until the phases had completely separated. The bottom organic solvent phase was removed and dried under air at atmospheric pressure. The drying resulted in solvent evaporation yielding krill oil. The weight of krill oil was recorded in order to determine extraction efficiency. In Soxhlet extraction, 3 g of freeze-dried krill were dispersed in petroleum ether (ACS grade petroleum ether, VWR International, Bridgeport, NJ, USA) [31]. The extraction was carried out in a standard Soxhlet apparatus for 18 h [32, 33], followed by drying under air at atmospheric pressure. The drying resulted in evaporation of petroleum ether yielding krill oil. The weight of krill oil was recorded in order to determine extraction efficiency. The same set of analyses was performed for krill oil extracted with Folch method and Soxhlet extraction as described above for oils extracted with one- and two-step extractions (i.e., extraction efficiency, TLC, GC, cholesterol content, and antioxidant capacity).

In preliminary experiments, the above solvent systems were applied to extract oil from fresh krill and freeze-dried krill. The extraction efficiency was significantly lower ($P < 0.05$) for all solvent systems using fresh krill when compared to freeze-dried krill. These preliminary experiments confirmed earlier reports [24]. Therefore, the data from preliminary experiments is not shown and oil extracted from fresh krill was not used for analyses described in the

subsequent sections (i.e., TLC, GC, cholesterol content, and antioxidant capacity).

This is also why only freeze-dried krill was used in the present study. All oil extractions were performed in triplicate ($n = 3$) using the same batch of fresh and freeze-dried krill.

4.3.2. Extraction efficiency

The weight of the initial freeze-dried krill prior to the extraction with different solvent systems was recorded. Following extraction, the weight of extracted oils was also recorded. The weight of acetone extract and ethanol extract were combined in order to determine an overall extraction efficiency of the two-step extraction. The extraction efficiency was determined according to the following equation:

$$\text{Extraction Efficiency} = \frac{\text{weight of extracted krill oil (g)}}{\text{weight of freeze - dried krill subjected to extraction (g)}} * 100$$

Extraction efficiency is expressed as grams of extracted oil per 100 grams of freeze-dried krill subjected to the extraction.

4.3.3. Determination of lipid classes with thin layer chromatography (TLC) and densitometry analysis

Thin layer chromatography (TLC) was applied to resolve lipid classes of the oils extracted with all extraction methods tested in the present study (i.e., one-step, two-step, Folch, and Soxhlet). For krill oil extracted with the two-step extraction, TLC and subsequent densitometry analysis were applied to acetone and ethanol extracts (Figure 4). An aliquot of 10 mg of the oil extracted from krill was dissolved in 1:1 chloroform:methanol (v:v) and loaded onto TLC plates (Whatman K6F silica plates with 60 Å pore sizes, P.J. Cobert Associates, St. Louis, MO). The TLC plates were developed using a 80:20:1.5 hexane:ether:acetic acid solution (v:v:v) as a mobile phase. Once developed, plates were air-dried for 5 min.

Plate images were captured using a digital camera interfaced with a PC and spot densitometer using transilluminating white light (Alpha Innotech Corp., San Leandro, CA). The images were analyzed using the Fluorchem software (ver. 1.0). Phospholipid (PL) and TRIG classes were identified using R_F values obtained from triolein (Sigma-Aldrich, St. Louis, MO) and soybean lecithin (Fisher Scientific, Fairlawn, NJ) standards. Once identified, the bands corresponding to PL, TRIG, and polar non-PL class were extracted (i.e., scraped) from the TLC plates and suspended in 1:1 chloroform:methanol (v:v) for determination of fatty acid profile (FAP) by gas chromatography (GC).

Although at least three replicate TLC plates were run per each extraction method (one-step, two-step, Folch, and Soxhlet), only a representative image

(three replicates) of the krill oil extracted with one-step extraction using 1:12 krill:solvent ratio (w:v) is shown in Figure 3. However, Figure 4 shows densitometry data for all extraction methods (one-step, two-step, Folch, and Soxhlet). The densitometry data was analyzed statistically; and therefore, the densitometry data in Figure 4 is quantitative; while the sample TLC image shown in Figure 3 is qualitative. The densitometry data are reported as mean values ($\pm$ standard deviation) of at least three replicates and the mean values are expressed as percent of lipid class in total krill oil.

4.3.4. Determination of fatty acid profile (FAP) of phospholipids (PL) and triglycerides (TRIG) with gas chromatography (GC)

TLC was applied to resolve lipid classes of the oils extracted with all extraction methods tested in the present study (i.e., one-step, two-step, Folch, and Soxhlet) (Figure 3 and 4). However, only phospholipids (PL) and triglycerides (TRIG) had detectable levels of FA (data not shown). Since PL was the major lipid class containing FA (PL – 20-33% vs. TRIG – 1-3%; Figure 4), preliminary experiments were conducted in order to determine if extraction methods (i.e., one-step, two-step, Folch, and Soxhlet) had an effect ($P < 0.05$) on composition of FA (i.e., FAP). FAP for PL extracted with all extraction methods was determined (data not shown). However, the contents of major FA were not ($P > 0.05$) different regardless of the extraction method. Therefore, extraction methods tested in the present study did not ($P > 0.05$) affect FAP of the extracted oils. In addition, Figure 2 indicates that one-step extraction resulted in the highest ($P < 0.05$)

extraction efficiency for 1:12 and 1:30 krill:solvent ratio (w:v); while the extraction efficiency at 1:12 and 1:30 was similar ($P>0.05$). Therefore, only FAP for PL and TRIG extracted with one-step extraction using 1:12 krill:solvent ratio (w:v) is reported (Figure 5).

Lipid classes (i.e., PL and TRIG) were extracted (i.e., scraped) from the TLC plates. The PL and TRIG were separately extracted from the TLC scrapings according to [30] and FAP was determined as described elsewhere [33, 34, 35, 36]. FA were transmethylated by the addition of 4 ml of 4% (w:v) methanolic H_2SO_4 and heated in a water bath set at 90°C for 60 min. The mixture was saponified by transferring through a Na_2SO_4 -filled glass Pasteur pipette and subsequently dried under N_2 in a water bath set at 60°C for 60 min. The FA methyl esters (FAME) were re-suspended in filtered iso-octane (HPLC grade iso-octane, Fisher Scientific, Fairlawn, NJ, USA). The FAME were analyzed by a GC (model CP-3800, Varian Analytical Instruments, Walnut Creek, CA, USA) using flame ionization detector fitted with a WCOT-fused silica capillary column (50 m length, 0.25 mm inside diameter; Varian Analytical Instruments, Walnut Creek, CA, USA). Injection and detection temperatures were maintained at 220°C and column temperature was 190°C . The stationary phase was CP-Silica 88 (Varian Analytical Instruments, Walnut Creek, CA, USA). Nitrogen was the carrier gas and a split ratio of 10 to 1 was used. The FA were identified by comparing their retention times with those of known standards. Peak area and the amount of each FA were computed by an integrator using the Star GC workstation version 6 software (Varian Analytical Instruments, Walnut Creek,

CA, USA). The data are reported as mean values ($\pm$ standard deviation) of at least three replicates and the mean values are expressed as percent of a fatty acid in total fatty acids.

4.3.5. Cholesterol content in extracted krill oils

Due to the prevalence of polar, non-PL class (Figure 3), the TLC and densitometry analysis did not allow specific determination of cholesterol content. Cholesterol was probably contained within the polar, non-PL class. However, krill is a shellfish; and therefore, determination of cholesterol content in the oil extracted from krill in this study was of interest. However, two-thirds of the sterols in shellfish such as krill are non-cholesterol sterols interfering with cholesterol absorption [37, 38]. Therefore, cholesterol content of oil extracted from krill with all extraction methods (i.e., one-step, two-step, Folch, and Soxhlet) was determined using a colorimetric assay (catalog number 428901, EMD Chemicals Inc., an affiliate of Merck, Darmstadt, Germany). For krill oil extracted with the two-step extraction, cholesterol content was separately determined for acetone and ethanol extracts (Figure 6). This cholesterol assay relies on the oxidation of cholesterol by added cholesterol oxidase and yielding H_2O_2 . The resulting H_2O_2 interacts with a cholesterol probe to produce resorufin that is detected spectrophotometrically. The results are compared to a standard curve constructed with cholesterol standards.

Krill oil samples were prepared in a cholesterol reaction buffer. In a 96 well microplate, 50 μ L of the buffered krill oil samples were mixed with 50 μ L of

reaction mix (containing cholesterol oxidase and cholesterol probe). Samples were covered and incubated at 37°C for 1h. Absorbance was read at 570 nm using a Spectramax Plus microplate reader (Molecular Devices, Sunnyvale, CA). In order to determine cholesterol content in krill oil, standard curve was constructed using cholesterol standards mixed with 50 μ L of the reaction mix. The cholesterol content is reported as mean values ($\pm$ standard deviation) of at least three replicates and the mean values are expressed as grams of cholesterol per 100 grams of krill oil.

4.3.6. Antioxidant capacity (trolox equivalents – TE) of extracted krill oils

The oil extracted from krill in a present study had a deep maroon red color. It has been reported that krill oil is rich in antioxidants, in particular astaxanthin [7, 16, 39, 40]. These antioxidants were likely included in the polar non-PL class. Therefore, total antioxidant capacity for the oil extracted from krill with all extraction methods (i.e., one-step, two-step, Folch, and Soxhlet) was determined and compared to trolox equivalents (TE) according to a colorimetric antioxidant assay (catalog number 709001, Cayman Chemical Company, Ann Arbor, MI). For krill oil extracted with the two-step extraction, total antioxidant capacity was separately determined for acetone and ethanol extracts (Figure 7). This antioxidant assay relies on the ability of endogenous antioxidants in krill oil to inhibit the metmyoglobin-mediated oxidation of ABTS (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS⁺. ABTS and metmyoglobin were added to krill oil samples as reagents and the amount of ABTS⁺ was determined

spectrophotometrically. The capacity of the endogenous antioxidants in krill oil to prevent ABTS oxidation was compared with that of a water-soluble tocopherol analogue, trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).

In a 96 well microplate, 10 μL of krill oil samples were mixed with 10 μL of metmyoglobin and 150 μL of ABTS. To initiate the reaction, 40 μL of H_2O_2 was added to each well. Samples were covered and incubated at room temperature on a shaker for 5 min. Absorbance was read at 750 nm using a Spectramax Plus microplate reader (Molecular Devices, Sunnyvale, CA). In order to determine TE for krill oil, standard curve was constructed using trolox standards mixed with 10 μL of metmyoglobin and 150 μL of ABTS. The antioxidant capacity is reported as mean values ($\pm$ standard deviation) of at least three replicates and the mean values are expressed as micromols of TE per milliliter of krill oil.

4.3.7. Crude protein (Kjeldahl), total fat (Soxhlet), and ash content of the residual spent krill following oil extraction

The crude protein, ash content, and total fat were determined in the residual spent krill following oil extraction with one- and two-step extraction only (i.e., sediment in Figure 1). Crude protein was determined by Kjeldahl assay (AOAC, 1995). Ash content was performed by incinerating samples in a muffle furnace at 550°C for 24 h [31]. Residual fat content was determined according to the Soxhlet extraction method [31]. To determine proximate composition (i.e., crude protein, total fat, and ash content) on dry basis, the residual spent krill was

analyzed for moisture content. A sample (2 g) was placed on an aluminum dish (Fisher Scientific, Fairlawn, NJ, USA), spread evenly across the dish and oven-dried (105°C for 24 h) [31]. The proximate data are reported as mean values ($\pm$ standard deviation) of at least three replicates and the mean values are expressed as grams per 100 grams of the residual spent krill following oil extraction (dry weight basis).

4.3.8. Statistical analysis

The oil extraction experiments were triplicated ($n = 3$). For each triplicate, at least three measurements were performed. One-way independent measures analyses of variance (ANOVA) were used to examine the effects of all methods used. Post-hoc analysis was conducted only in the presence of significant population differences ($P < 0.05$). ANOVA statistical comparisons were conducted using KYPlot (KyensLab, Tokyo, Japan) with a significance level of $P < 0.05$. Tukey's test ($P < 0.05$) was used to determine individual differences between treatments except for the differences of FA content between TRIG and PL where Student's t-test was used (Figure 5).

4.4 Results and Discussion

4.4.1. Extraction efficiency

Extraction of krill oil using the 2-step procedure resulted in similar ($P > 0.05$) efficiency as the 1-step procedure using a krill:solvent ratio of 1:6 (Figure 2). The extraction efficiency for the 1-step procedure increased ($P < 0.05$)

as the krill:solvent ratio increased, with 1:12 and 1:30 ratios having the greatest efficiencies. Although these two krill:solvent ratios resulted in the highest ($P<0.05$) efficiencies, they were similar ($P>0.05$). Therefore, krill:solvent ratio is a critical parameter for extraction efficiency. Likely, if the krill:solvent ratio were increased in the 2-step extraction, the extraction efficiency would similarly increase. However, 1-step extraction is simpler than the 2-step procedure; and therefore, 1-step extraction with acetone:ethanol (1:1, v:v) using 1:12 krill:solvent ratio is recommended.

4.4.2. Determination of lipid classes with thin layer chromatography (TLC) and densitometry analysis

Major lipid classes (triglycerides – TRIG, phospholipids – PL, and polar non-PL) of the extracted krill oils were identified using TLC (Figure 3), quantified by densitometry and presented as % of lipid class in total krill oil extracted (Figure 4). The extraction procedure did not ($P>0.05$) affect TRIG content in the extracted oils, which ranged 1.0-3.2% (Figure 4). However, extraction procedure had an effect ($P<0.05$) on PL content, with Folch, Soxhlet, and 1-step (1:6 krill:solvent ratio) extractions having the highest ($P<0.05$) content of PL. The PL content ranged from 20.4-32.7% (Figure 4). A high PL content in krill oil has been described previously, with total PL at approximately 40% [5]. The high PL content makes krill oil unique as compared to other dietary lipids. The TRIG content in fish oils is approximately 60% [39].

The major lipid class in all of the oils extracted from krill was the polar non-PL class (>60%). This class consists of the following lipid classes: cholesterol, mono- and di-glycerides, and the red pigment associated with krill oil (primarily astaxanthin). The association of the red pigment with the polar non-PL class on the TLC plates made quantifying each class individually difficult, thus these classes were combined and accounted for as the polar non-PL class. The 1-step extraction with krill:solvent ratios greater than 1:6 and the acetone fraction from the 2-step extraction had the highest ($P<0.05$) content of the polar non-PL class. The densitometry results of the TLC plates coincided with the visual appearance of the oils. The extractions that resulted in the highest content of the polar non-PL also had a deeper red color. The differences in the polar non-PL class content should be of commercial interest. It is possible that the extraction procedure could yield oil with lower cholesterol content, but higher antioxidant capacity. Therefore, cholesterol content and total antioxidant capacity (as an indirect measure of astaxanthin content) were determined (see section 3.4 and 3.5, respectively).

4.4.3. Determination of fatty acid profile (FAP) of phospholipids (PL) and triglycerides (TRIG) with gas chromatography (GC)

Following TLC analysis, TRIG and PL spots were scraped from the TLC plates and methylated for FAP by GC (Figure 5). The TRIG had a higher ($P<0.05$) content of myristic, palmitoleic, and oleic acid than PL (Figure 5A). Conversely, EPA and DHA were predominant ($P<0.05$) in PL, contributing to

much higher ($P<0.05$) content of total n-3 FA and unsaturated FA in PL than in TRIG (Figure 5B). Consequently, the ratios of n6/n3 and saturated FA/unsaturated FA were lower ($P<0.05$) in PL (Figure 5C).

Consumption of ω -3 FA in a form of PL or TRIG has different health and physiological effects [41, 42]. Therefore, having EPA and DHA esterified as PL in krill oil has significant implications for human health. The preferential esterification of EPA and DHA into PL is intriguing and has been noted previously [43]. However, available data comparing the benefits of consuming ω -3 FA as krill oil compared to other sources is scarce. Therefore, further studies are needed to compare and understand the nutritional value and health effects of krill oil versus other sources of n-3 FA.

4.4.4. Cholesterol content in extracted krill oils

The extraction procedures yielding oil with the highest ($P<0.05$) content of polar non-PL (one-step extractions in Figure 4) did not have high cholesterol content (Figure 6). The one-step procedures with krill:solvent ratios $\geq 1:9$ had lower ($P<0.05$) cholesterol content than acetone extract of the 2-step procedure. Cholesterol content of the 2-step, Folch, and Soxhlet extractions mimicked content of the polar non-PL (Figure 4), suggesting that the cholesterol content contributed more to the content of polar non-PL in these procedures than the one-step procedures.

In general, cholesterol content of krill is lower than shrimp, and slightly higher than fish [39]. Importantly, not only are sterols in krill composed of

cholesterol, but they also contain ~30% non-cholesterol sterols [44]. Non-cholesterol sterols from both shellfish and plant sources have been shown to reduce cholesterol absorption in experimental studies [38]. Krill oil resulted in 18% reduction in total serum cholesterol compared to 6% with fish oil in hyperlipidemic patients [19]. The hypolipidemic effects of seafood oils are not fully understood, but are significantly influenced by ω -3 PUFA. Since krill oil is rich in ω -3 PUFA and contains non-cholesterol sterols, the hypolipidemic effects of krill oil could be additive.

4.4.5. Antioxidant capacity (trolox equivalents – TE) of extracted krill oils

The red color of krill oil is due to the carotenoid astaxanthin, a potent antioxidant. Frozen krill contains 3-4 mg of carotenoids/100 g and astaxanthin is >80% of the total carotenoids [18]. Aside from measuring the total antioxidant capacity of each extraction procedure as an indirect indicator of astaxanthin content, it is also useful information in terms of human health benefits. It has been shown that astaxanthin decreases induced membrane oxidative injury to a greater degree than α -tocopherol, an antioxidant commonly used in food products.

Oils extracted with the one-step procedure using 1:9 and 1:30 krill:solvent ratios as well as the ethanol extract of the two-step procedure had the highest ($P<0.05$) antioxidant capacity (Figure 7). Despite the highest ($P<0.05$) content of polar non-PL (Figure 4), the acetone extract of the 2-step procedure had the lowest ($P<0.05$) antioxidant capacity. Therefore, the polar non-PL of the acetone extract may be predominately cholesterol. The Folch extraction also yielded oil

with the lowest ($P<0.05$) antioxidant capacity. The antioxidant capacities of oils extracted with one-step procedures mimicked their respective contents of polar non-PL (Figure 4), suggesting that astaxanthin was the primary component of the polar non-PL in the oils extracted with one-step procedures.

4.4.6. Crude protein, total fat, and ash content of the residual spent krill following oil extraction

Proximate analysis performed on the residual spent krill that was recovered from the sediment following oil extraction and centrifugation (Figure 1) shows that the sediment was primarily composed of protein (~74 g/100 g, dry weight, Table 1). The amount of protein in the residual spent krill was not ($P>0.05$) affected by oil extraction procedure. The lipid content decreased ($P<0.05$) as the krill:solvent ratio increased from 1:9 to 1:30 in the one-step extraction. The two-step extraction and one-step extraction using 1:12 and 1:30 krill:solvent ratios resulted in the lowest ($P<0.05$) lipid content in the residual spent krill. Likewise, the one-step extraction using 1:30 krill:solvent ratio and two-step procedure also resulted in the lowest ($P<0.05$) mineral content. However, the ash content in the residual spent krill was comparable to the ash content in whole krill prior to extraction (ash 17.4 g/100 g, dry basis, [45]). Therefore, the fluoride (F) content was likely high and would need to be reduced if the protein in the residual spent krill were to be recovered and used for human/animal consumption.

The proximate data clearly shows that there is high protein content in the residual spent krill; though ash (and likely F) is also high. The protein could be recovered with the isoelectric solubilization/precipitation (ISP), while ISP also significantly reduces ash content [27, 45]. It has been shown that the proteins recovered from whole krill using ISP result in good quality heat-set gels [27]. Therefore, a fuller utilization of krill as a human food would be possible by extracting oil as shown in the present study, followed by protein recovery by ISP for subsequent inclusion in human food products.

4.5 References

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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Figure 1. A flow diagram for oil extraction from krill and subsequent analyses of the extracted oil and residual spent krill.

Figure 2. Extraction efficiency* of different solvent systems for extraction of oil from freeze-dried whole krill.

* Different letters on the top of data bars indicate significant differences (Tukey's test, $P < 0.05$) between mean values ($\pm$ SD, $n = 3$).

Figure 3. An example plate showing lipid classes resolved with thin layer chromatography (TLC) for krill oil extracted with one-step extraction using 1:12 krill:solvent ratio (w:v) (triplicate). TLC plates for krill oils extracted with different solvent systems were analyzed quantitatively with densitometry (Figure 4). Lipid classes were isolated from TLC plates and analyzed for fatty acid profile (FAP) using gas chromatography (GC) shown in Figure 5.

Figure 4. Densitometry analysis* of TLC plates (an example of TLC plate is shown in Figure 3). For two-step extraction, TLC plates with acetone and ethanol extracts were analyzed separately.

* Different letters on the top of data bars indicate significant differences (Tukey's test, $P < 0.05$) between mean values ($\pm$ SD, $n = 3$) within a lipid class.

Figure 5. Major FA* in phospholipids (PL) and triglycerides (TRIG) of oil extracted from krill with one-step extraction using 1:12 krill:solvent ratio (w:v). The PL, TRIG, and polar non-PL class were resolved with TLC (Figures 3 and 4). The polar non-PL

class showed no detectable FA. This class likely contained antioxidants including asthaxantin. EPA – eicosapentaenoic acid (20:5n3), DHA – docosahexaenoic acid (22:6n3), n3 – ω -3 FA, n6 – ω -6 FA, UFA – total unsaturated FA, and SFA – total saturated FA.

* Different letters on the top of data bars indicate significant differences (Student's t-test, $P < 0.05$) between mean values ($\pm$ SD, $n = 3$) within the FA.

Figure 6. Cholesterol content in oil extracted from freeze-dried whole krill using different solvent systems.

* Different letters on the top of data bars indicate significant differences (Tukey's test, $P < 0.05$) between mean values ($\pm$ SD, $n = 3$).

Figure 7. Antioxidant capacity of oil extracted from freeze-dried whole krill using different solvent systems.

* Different letters on the top of data bars indicate significant differences (Tukey's test, $P < 0.05$) between mean values ($\pm$ SD, $n = 3$).

Table 1. Crude protein*, total fat*, and ash content* of the residual spent krill following oil extraction with one- and two-step extraction.

	Extraction method				
	1-step extraction with acetone:ethanol (1:1, v:v) at different krill:solvent ratio (w:v)				2-step extraction
	1:6	1:9	1:12	1:30	
Crude protein (g/100g, dry basis)	72.9±1.0 a	73.8±0.8 a	74.8±0.8 a	75.8±1.0 a	75.3±4.0 a
Ash content (g/100g, dry basis)	17.7±0.7 a	17.3±0.5 a	17.9±0.5 a	16.1±0.6 b	15.6±0.4 b
Total fat (g/100g, dry basis)	6.4±1.0 a	6.5±1.2 a	4.4±1.0 b	3.7±0.7 b	3.4±1.1 b

* Different letters indicate significant differences (Tukey's test, $P < 0.05$) between mean values ($\pm$ SD, $n = 3$) within the same row.

Gigliotti, J.C., Turk, P., Livengood, R., Benedito, V., & Tou, J.C. (2012)

**The Influence of Different Dietary Sources of Omega-3 Polyunsaturated Fatty Acids
on Renal Health in Female Rats**

In preparation for submission to Kidney International

5.1 Background

Results from Section 4 concluded that krill oil is rich in ω -3 PUFAs, and is a complex lipid composed of phospholipids and other lipid soluble pigments. This unique lipid content has been the focus of advertisement for krill oil supplementation. While research supports a benefit of krill oil supplementation in regards to various aspects of health, it is unclear how krill oil and other ω -PUFA sources may influence kidney health. In addition, residual ω -3 PUFA content may explain why certain protein sources (i.e soybean, KPC, codmeal, flaxseed, etc.) benefit renal health.

5.2 Introduction

Nephrocalcinosis (NC) is a form of renal disease characterized by increased deposition of Ca within kidney tissue. The progression of NC is associated with detrimental changes in renal morphology and subsequent renal failure [1]. While NC affects various animal species (including humans), it is a serious issue in rodents where dietary components and gender impact its occurrence [2, 3]. The occurrence of NC in rats was a major consideration during the formulation of the current American Institute of Nutrition (AIN) rodent diet (AIN-93). For the AIN-93 diet formulation, the occurrence of diet induced NC in rats was addressed through alterations in the dietary Ca and P content [3, 4]. While there is a clear relationship between the dietary mineral content and NC in rats [5, 6, 7], research suggests that changes in other dietary components can also influence NC. Mayer et al. [8] found that changing the dietary protein source has a greater influence on NC than the dietary mineral content. Further research has suggested that casein, which is commonly used in rodent diets, is more nephrocalcinogenic [9, 10].

Aside from NC, changing the dietary protein source has been associated with benefits in other forms of renal disease as well [11, 12].

The mechanism for how dietary protein can modulate renal health is still unclear. Some of the protein sources used in previous studies contained residual lipids from known sources of omega-3 polyunsaturated fatty acids (ω -3 PUFAs) [9, 10]. While the role of ω -3 PUFAs in human renal diseases is unclear [13], ω -3 PUFA supplementation has been reported to benefit rodent models of renal calcification [14, 15], 5/6 nephrectomy [16, 17], diabetic renal disease [18], and polycystic kidney disease [19]. Furthermore, corn oil was replaced with soybean in reformulation of the AIN-93 diet [20] and may also be responsible for the reduction in observed NC. Soybean oil contains both essential fatty acids, the omega-6 polyunsaturated fatty acid (ω -6 PUFA) LA and the omega-3 (ω -3) PUFA ALA. Corn oil is predominately the ω -6 PUFA LA, which provides a dietary ω -6 PUFA content suggested to be proinflammatory [21, 22]. Inflammation and cellular damage are involved in the etiology of several renal diseases including NC [23, 24]. Therefore, increasing the consumption of the anti-inflammatory ω -3 PUFAs may influence the development of NC.

In the current study, we determined the lipid class, and fatty acid composition of corn oil and different dietary sources of ω -3 PUFAs. The characterized oils were then fed to young (28d), healthy female rats. To our knowledge, this is the first study to compare the influence of different dietary sources of ω -3 PUFAs on renal health using non-diseased animals. A primary aim of this study was to determine if changing the PUFA content of a casein-based diet can influence the development of NC in female rats. In turn, changing the dietary lipid content can also influence prostaglandin production [25-28], the activity of the proinflammatory transcription factor NF κ B [21, 22, 28, 29],

and the expression of the proinflammatory, profibrotic chemokine transforming growth factor-beta 1 (TGF- β 1) [30]. Aside from NC, inflammation and fibrosis are key components to the etiology of several renal diseases. Therefore, the objectives of this study were:

1. to determine if diets differing in ω -3 PUFA content influence the development of NC in healthy female rats fed a casein-based diet,
2. to determine if diets differing in ω -3 PUFA content influence systemic prostaglandin production and the renal expression of COX-II,
3. to determine if providing ω -3 PUFAs can influence the activity of NF κ B and the expression of TGF- β in renal tissue.

5.3 Materials and methods

5.3.1 Selection of dietary oils

Dietary sources of ω -3 PUFAs used in this study were flaxseed (FO), menhaden (MO), krill (KO), salmon (SO), and tuna oils (TO). FO is considered the richest source of the essential ω -3 PUFA, ALA (Cunnane SC). MO and SO are common sources of EPA and DHA in commercial fish oils supplements. KO is a novel ω -3 PUFA source and has a similar fatty acid profile as MO. However, KO has been suggested to be a rich source of phospholipid [10] as compared to most dietary oils which are presumed to be predominately triglycerides. TO was chosen because it is considered a rich source of DHA, whereas other seafood ω -3 PUFA sources are generally higher in EPA. CO was included because it is rich in the essential ω -6 PUFA, linoleic acid (LA, 18:2 ω -3). Additionally, the inclusion of an oil rich in ω -6 PUFAs allows the comparison between the physiological effects of ω -6 and ω -3 PUFA rich oils. FO, SO, and TO were

generously provided by Jedwards International (Quincy, MA). MO was purchased from Omega Protein Inc. (Houston, TX), and the KO was purchased from Enzymotec USA Inc. (Morristown, NJ). All oils were of commercial feed grade, and were kept at 4°C in the dark.

5.3.2 Lipid analysis of dietary oils

Quantifying lipid classes

Thin-layer chromatography (TLC) was performed to identify and quantify the different lipid classes. Approximately 1mg of each oil was dissolved in chloroform:methanol (1:1; v:v) and spotted onto Whatman K6F Silica plates with 60Å pore sizes, (Cobert Associates, MO). Silica plates were developed in a hexane:ether:acetic acid (80:20:1.5; v:v:v) solvent. Following development, plates were air-dried for 5 min and visualized using a Fluorchem 8000 densitometer using transilluminating white light (Alpha Innotech Corp, CA). Plate images were captured using a camera interfaced to PC and the lipid classes were quantified using the spot densitometer tab in the Fluorchem (version 1.0) computer program. Total glycerophospholipid (PL) and triacylglyceride (TAG) lipid classes were identified using R_F values obtained by comparison to the standards soybean lecithin (Fisher Scientific, PA) and triolein (Sigma-Aldrich, MO), respectively. Due to the pigmentation of KO, which is presumably astaxanthin, lipid classes located between the PL and TAG spots were quantified and summed together collectively as polar, non-phospholipids (representative image in Figure 1). Data from the TLC plates are presented as a percentage of the total pixel count of the lane.

Determining fatty acid composition

Fatty acid methyl esters (FAMES) were prepared from the dietary oils using the procedure described by Fritsche and Johnston [31]. Briefly, 4 ml of a 4% H_2SO_4 /methanol solution was added to 20-30 mg of oil. The methylation reaction was carried out by heating at 90°C for 1 h and was terminated by the addition of water. FAMES were then isolated in chloroform, dried under nitrogen gas, and resuspended to a known concentration (2-5mg/ml) in isooctane. FAMES were analyzed by gas chromatography (GC, CP-3800 Varian), using nitrogen as the carrier gas. GC setting consisted of an initial temperature of 140°C for 5 min followed by an increase of 1°C per min to a final temperature of 220°C. Fatty acids were identified as FAMES by comparing retention times to a FAME 37 standard (Supelco, PA) and presented as percent of total peak area counts corrected for isooctane.

5.3.3 Diet formulation

Experimental diets (Table 1) were based on the standard semi-purified AIN-93G formulation. Diets were formulated to be 12% (by weight), high fat diets with the lipid source supplied as either CO, FO, MO, KO, SO, or TO. MO and KO diets consisted of a blend of 11.8% test oil + 0.2% CO in order to meet the recommendation for LA in growing rats [32]. The lipid dose (12% by wt) is based on levels of oil achievable in the human diet (approximately 27% caloric intake); whereas much higher lipid inclusions have been used previously. Diets were flushed with nitrogen gas, vacuum sealed in 1 kg packages, and stored in the dark at -20°C until fed.

5.3.4 Animal Study

All animal procedures were conducted in accordance with the guidelines set forth by the National Research Council for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee at West Virginia University.

Young (28 d), female Sprague-Dawley rats were obtained from Hilltop Lab Animals Inc. (Scottsdale, PA). Female Sprague-Dawley rats were chosen due to their susceptibility to diet induced renal damage [2]. Upon arrival, rats were individually housed in metabolic cages, and maintained in a room at 21°C with a 12 h light/dark cycle. During a 7 d acclimation period, animals were given *ad libitum* access to deionized distilled water (ddH₂O) and AIN-93G diet (Harklan Teklad, IN). Following the 7 d acclimation period, rats were then randomly assigned ($n = 10$ rats) to one of the six experimental diets. The rats were provided (14.75 ± 0.75 g), to prevent variation in total caloric intake, and fresh diets were supplied daily throughout the 8 week study. Body weights and diet and ddH₂O consumption was recorded throughout the study.

5.3.5 Determining kidney mineral content

At the end of the 8 week feeding study, rats were euthanized by CO₂ inhalation. Both kidneys were excised, trimmed, decapsulated, and weighed separately. After no bilateral difference in left and right kidney weights was determined, the left kidney was divided transversely and both halves were frozen at -80°C until analyzed.

To determine kidney mineral content, half of the left kidney was ashed in a muffle furnace (Lindberg 515A2, WI) at 550°C for 24 h. Ashed samples were dissolved in 2 mL of 70% nitric acid, filtered through Whatman no. 1 paper, and diluted to a final volume of 25 mL in ddH₂O. The Ca and P content of the kidney samples (mg/g kidney

tissue) were determined using inductively coupled plasma spectrometry (ICP) (model P400, Perkin Elmer, Shelton, CN).

5.3.6 Kidney histology

A center sagittal section was removed from the right kidney and immediately fixed in 10% formalin solution (VWR, PA) for histological evaluation. Tissues were dehydrated through a series of increasing ethanol concentrations in ddH₂O to xylene and embedded in paraffin. 3µm sections from each block were stained with hematoxylin and eosin with appropriate staining control tissue. Histologic parameters evaluated included glomerular hypercellularity and matrix deposition, arteriosclerosis, tubular dilation, tubulointerstitial fibrosis, interstitial inflammation, and interstitial matrix deposition or calcification. All slides were analyzed under a Nikon TE 2000-S light microscope (Nikon Instruments, NY) by two trained individuals who were blinded to the treatments. Representative images presented were captured using a PC interfaced with Q-Capture imaging software (Quantitative Imaging Corporation, BC, Canada).

5.3.7 Kidney fatty acid composition

To determine the fatty acid composition of the kidneys, total lipid was extracted from the remaining half of the left frozen kidney following a modified method of Bligh and Dyer [33]. Briefly, ~400 mg of frozen renal tissue was homogenized in Tris/EDTA buffer and mixed with a chloroform:methanol:acetic acid (2:1:0.15 v/v/v) solution. After centrifugation at 900 x g for 10 minutes at 10°C, the bottom chloroform layer was collected. The collected chloroform was filtered through 1-phase separation filters, dried

under N₂ gas, and the extracted lipid samples were then methylated and quantified as described above for the fatty acid analysis of dietary oils.

5.3.8 RNA isolation and real-time PCR analysis

A portion of the right kidney was frozen in liquid nitrogen and stored at -80°C until processed for RNA isolation. Total renal RNA was extracted from ~100 mg of frozen tissue following the Total RNA Isolation procedure of the mirVana miRNA Isolation Kit (Ambion, TX). Isolated RNA integrity was visualized on a 1.5% agarose gel, and isolate RNA concentration was quantified by spectrophotometry (Thermo Scientific, DE). After DNase treatment of the isolated RNA (Applied Biosystems, CA), total mRNA was amplified using the Superscript III First Strand Synthesis assay with oligo dT primers (Invitrogen, CA).

Primers were designed for rat COX-II (Ptgs2, NM_017232), TGF- β (Tgfb1, NM_021578), and the housekeeping gene GAPDH (Gapdh, NM_017008.3) using the Primer3 program [34] and respective mRNA sequences obtained from NCBI. Forward and reverse primers for COX-II (PTGS2) were 5' CGGAGGAGAAGTGGGTTTTAG 3', and 5' TGAAAGAGGCAAAGGGACAC 3' respectively. Forward and reverse primers for TGF- β (Tgfb1) were 5' GCCTGAGTGGCTGTCTTTTG 3' and 5' CCCTGTATTCCGTCTCCTTG 3' respectively. Primer efficiencies were determined on pooled RNA samples serially diluted in ddH₂O up to 1:10000.

PCRs consisted of 2.5 μ l of SYBR[®] Green Master Mix (Applied Biosystems, CA), 1 μ l of cDNA, 1 μ l of respective forward and reverse primers, and 0.5 μ l of ddH₂O for a total reaction volume of 5 μ l. The thermal profile consisted of 50°C for 2 minutes, 95°C for 10 minutes, and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Data

was analyzed as described by Pfaffl [35], and normalized to GAPDH expression. Data is presented as relative expression compared to rats fed (CO).

5.3.9 Isolation of nuclear proteins and measurement of NFκB activation

A portion of the right kidney (~300mg) was processed for isolation of nuclear proteins using a commercially available nuclear protein extraction kit (Active Motif, Ca). The protein content of the nuclear extract was determined using a commercially available protein determination assay based on the Bradford method (Cayman Chemical, MI). The activation of NFκB was determined using the TransAM NFκB p65 transcription factor assay kit (Active Motif, CA). Nuclear protein (10μg) was used for the assay, and results were calculated as a percentage of NFκB activity seen in rats fed CO.

5.3.10 Serum and urinary measures of renal function and health

At the end of the 8 week feeding study, rats were euthanized by CO₂ inhalation and trunk blood was collected. Blood samples were maintained at 4°C for 15 min, and then centrifuged for 10 min at 1500 X g for serum isolation. Serum measures of kidney function included: total protein, albumin, blood urea nitrogen (BUN), creatinine, Ca, and P. Serum measures of kidney function were determined by colorimetric assays using a commercially available Vet-16 rotor quantified by a Hemagen Analyst automated spectrophotometer (Hemagen Diagnostics Inc., MD).

Urinary volume was recorded and collected throughout the study. Urinary measures of kidney function were performed on pooled urine samples collected during the final week of the study and included urinary: Ca and P content, creatinine, albumin, PGE₂ and pH. Urinary Ca and P content was determined by diluting the urine 1:40 in

ddH₂O, and then analyzed by ICP. Urinary creatinine was determined using a colorimetric assay (Cayman Chemical, MI). Urinary albumin (Alpco, NH) and PGE₂ metabolite content (Cayman Chemical, MI) was determined using commercially available assays. Urinary pH was determined using a standard pH meter (Beckman, CA). Glomerular filtration rate (GFR) was estimated by measuring creatinine clearance (CrC) determined as: $CrC = (UC/SC) \times V$, where UC is urinary creatinine concentration, SC is serum creatinine concentration, and V is the volume of urine excreted per minute.

5.3.11 Statistical analysis

The animal feeding study was analyzed as a completely randomized design, with n=10 rats per diet treatment. One-way analysis of variance (ANOVA) was used to determine differences among rats fed the different diets, while a nonparametric rank analysis was performed on data not normally distributed. Post-hoc analysis was performed using Tukey's test, and all statistical analysis was performed using Sigma Stat 3.1 (Systat Software Inc., San Jose, CA). Differences were considered significant at $P < 0.05$.

5.4 Results

5.4.1 Lipid Analysis of Dietary Oils

All dietary oils used in the study were predominately TAG (>65%) with the exception of KO. The lipid content of KO was unique in that it was primarily composed of PLs and polar, non-phospholipid classes (Figure 1). The plant oils were abundant in the essential fatty acids LA and ALA, and devoid of the long chain PUFAs AA, EPA and DHA. The seafood sources of ω -3 PUFAs all contained EPA and DHA, and had ω -6: ω -3

of at least 1:10. However, absolute amounts and ratios of EPA and DHA varied among sources. KO and SO had the highest EPA content, while TO had the highest DHA content (Table 2).

5.4.2 Kidney mineral content

Rats fed KO had heavier ($P<0.001$) relative kidney weights compared to rats fed FO or MO (Table 3). Rats fed KO had the highest kidney Ca content ($P<0.001$) and higher kidney P content compared to rats fed MO ($P=0.03$).

5.4.3 Kidney histology

All rats fed KO displayed tubular calcification, dilation, tubulointerstitial changes (specifically fibrosis), and interstitial inflammation (Figure 2). Sparse fibrosis and interstitial inflammation was observed among all treatment groups. However, rats fed FO also displayed a higher occurrence of these two conditions compared to the other dietary groups. No differences in glomerular morphology or arteriosclerosis were observed among the treatment groups.

5.4.4 Kidney fatty acid composition

No differences were found in total lipid content among treatment groups ($P=0.4$). The fatty acid content of the kidneys was similar to the fatty acid composition of the diet. Rats fed CO had a tissue ω -6: ω -3 ratio greater than 30:1, the highest tissue ω -6 content, and the lowest EPA and DHA content. All rats provided ω -3 PUFA sources had an ω -6: ω -3 ratio less than 3:1. Among ω -3 PUFA sources, renal tissue from rats fed FO had a

similar LA content as found in rats fed CO, the lower renal EPA and DHA content, and the highest total ω -6 content as compared to rats fed the marine ω -3 PUFA oils (Table 4).

5.4.5 Real-time PCR analysis

COX-II expression was lower in all rats provided ω -3 PUFAs as compared to rats fed CO (Figure 3). The expression of COX-II was the lowest in rats fed SO or TO diets, which was 45 and 16% lower than in rats fed CO, respectively. COX-II expression in rats fed KO was approximately half (55%) of that of CO fed rats, while MO and FO fed rats expressed COX-II at 85 and 78% of that in rats fed CO, respectively.

Feeding ω -3 PUFAs also resulted in reduced expression of TGF- β (Figure 4). Rats fed KO, SO, and TO had the lowest TGF- β expression, which was approximately 45% of that in rats fed CO. Rats fed FO and MO had TGF- β expression at 84 and 80% compared to rats fed CO, respectively.

5.4.6 NF κ B activation

NF κ B activity in rats fed SO and TO was approximately 17 and 11% of that found in rats fed CO. The measured NF κ B activity appeared similar in rats fed CO, MO and KO. Rats fed FO had the highest apparent NF κ B activity, which was at 30% higher than all other groups (Figure 5).

5.4.7 Serum and urinary measures of renal function and health

There were no differences in serum measures of kidney function of creatinine, phosphorus, Ca, and total protein between rats fed the different diets. Rat fed SO had

higher ($P=0.04$) BUN content compared to rats fed CO, however this difference was absent when BUN was corrected for creatinine (Table 5).

There were significant differences in the urinary measures of kidney health and function. Rats fed MO and FO had greater ($P=0.009$) urinary excretion of Ca compared to rats fed TO, while rats fed KO had the highest ($P<0.001$) urinary excretion of P and greatest reduction in urinary pH ($P<0.001$). These results occurred in the absence of differences in urinary volume ($P=0.9$) and albuminuria ($P=0.4$) (Table 6).

There was also an apparent differences in urinary excretion of PGE_2 during the final week of the study (Figure 6). Rats provided seafood sources of ω -3 PUFAs (MO, KO, SO, and TO) had <50 % lower excretion of urinary PGE_2 compared to rats fed FO or CO.

5.5 Discussion

5.5.1 Nephrocalcinosis and associated damage

A major objective of this study was to determine if changing the PUFA content of casein-based diets influences the development of NC in female rats. Differences in kidney weights provided initial evidence for the presence of NC. Previous studies have found that heavier kidney weights were positively correlated with the kidney Ca content [5, 36]. Unexpectedly, the heaviest kidney weights were observed in rats fed a source of ω -3 PUFAs (KO), but not in rats fed the ω -6 PUFA rich CO. The presence of NC in rats fed KO was confirmed by elevated renal Ca content and histological identification of mineral deposits. In addition, histological evaluation showed tubular dilation, tubulointerstitial changes (namely fibrosis), and/or interstitial inflammation in all rats fed KO. These structural changes are common with advanced renal mineralization [37, 38].

Fibrosis and interstitial inflammation are common in a numerous forms of advanced renal diseases, and supports the involvement of NC in the initiation of renal failure [1].

NC, and its associated renal disease, can be generated experimentally by increasing dietary P content [5, 37, 38]. In the current study, the same basal diet was used for all diets fed and therefore contained equal amounts of inorganic P (0.03% by weight). However, rats fed KO had elevated urinary P content and acidity, a urinary composition typically associated with increased P intake [5, 6]. Further analysis revealed that the KO diets had an increased P content (0.46% vs 0.3%). Characterization of the oils showed that KO had a high PL content. Therefore, we concluded that the observed NC in rats fed KO was due to elevated P intake in the form of PLs. This conclusion was supported by a previous study, where rats fed diets high in P (0.4-0.6%) had similar renal Ca content [36].

It was unexpected that rats fed the EPA rich KO, and not the ω -6 PUFA rich CO, developed NC. This contradicts previous findings where EPA retarded calcium gluconate induced renal mineralization in both male and female rats [14, 15]. However, calcium gluconate induces NC through hypercalcemia and hypercalciuria; a form of NC that likely differs in etiology as compared to P induced NC [39]. Therefore, the results presented here suggest that ω -3 PUFA supplementation does not influence P induced NC.

5.5.2 Renal fatty acid composition and prostaglandin production

Another aim of this study was to determine if changing the PUFA content of the tissue can influence inflammatory gene expression in non-diseased induced animals. To our knowledge, this is the first study comparing the influence of multiple dietary sources of PUFAs on renal fatty acid composition and health in non-diseased animals. Our diets

were high fat (12% vs. 7%), but remained lower than high fat diets used in other studies. The increased lipid content was chosen to amplify effects while maintaining a physiologically relevant dose.

In general, the fatty acid composition of renal tissue was similar to the fatty acid composition of the diet. Rats fed ω -3 PUFAs had a renal ω -6: ω -3 ratio ten times lower than that found in rats fed CO. Among rats fed ω -3 PUFA rich oils, rats fed FO had the highest renal ω -6 PUFA content and renal ω -6: ω -3 ratio. Ogborn et al., [19] reported that rats fed a chow based diet with a 10% inclusion of whole flaxseed had reduced renal Ca content. In our study, rats fed FO had a higher renal EPA and DHA content than rats fed CO. However, these levels were less than half that found in rats fed seafood based ω -3 oils. Mammalian conversion of ALA to EPA and DHA is considered an inefficient process, and is the basis for recommendation for increased fatty fish consumption. Based on our results, providing ω -3 PUFAs as ALA was inefficient at saturating renal tissue EPA and DHA as compared to feeding rats seafood based oils rich in EPA and DHA.

The PUFA content of renal tissue is important given the role of PUFAs as substrates for prostaglandin production. PGE₂ is crucial for renal homeostasis [40] and therefore, the changes in tissue PUFA observed in our study may influence prostaglandin production and renal health. Rats provided the seafood sources of ω -3 PUFAs (MO, KO, SO, and TO) had over 50% reduction of urinary PGE₂ excretion compared to rats fed CO and FO. Increased tissue EPA content can compete with AA for membrane incorporation, and this can affect substrate availability for COX mediated prostaglandin production. This is supported by previous studies, where feeding rats a mixture of MO and CO led to a reduction in PGE₂ production [23] and increase in PGE₃ [41] production

by renal medullary tissue. Therefore, the decreased PGE₂ excretion in these rats could be the result of altered substrate availability and increased PGE₃ production.

5.5.3 Renal molecular analysis

The lower PGE₂ excretion in rats provided ω -3 PUFAs could also be a consequence of reduced renal COX-II expression. *In vitro* studies suggest that ω -3 PUFAs can influence COX-II expression, and this occurs through mediating the activity of the NF κ B transcription factor [29]. In the current study, rats provided ω -3 PUFAs had reduced renal COX-II expression and NF κ B activity as compared to rats fed CO. This difference was most pronounced in rats fed SO and TO. *In vitro* studies suggest that NF κ B activation is sensitive to ω -3 PUFA content, especially DHA [29, 42]. In our study, TO had the highest DHA content which may explain the reduced NF κ B activation. Rats fed SO also had lower renal NF κ B activity, despite having a lower oil and tissue DHA content. Preliminary studies suggest that DHA bioavailability is higher in rats fed SO, which may partly explain these results (unpublished data). We expected the NF κ B activity in rats fed FO and KO to be elevated, due to the occurrence of inflammation in these animals. In agreement, rats fed FO had greater than a 30% increase in NF κ B activity compared to all other rats. NF κ B is considered a master regulator of the inflammatory response [43, 44], and may explain the mild morphological changes observed in rats fed FO. However, there was no apparent increase in NF κ B activity in rats fed KO. As previously discussed, the damage observed in rats fed KO was likely due to the P induced NC. An *in vitro* study suggested that the inflammatory signaling induced by calcium-phosphate crystals is mediated by transcription factors other than NF κ B [39].

The renal expression of TGF- β was also of interest, since it is considered a key signaling molecule in renal fibrosis [45]. Furthermore, previous research has shown that renal TGF- β expression is decreased with ω -3 PUFA supplementation in rodent models of lupus [30]. In our study, rats fed KO, SO, and TO had a 45% reduction in TGF- β expression compared to rats fed CO. However, this reduction in TGF- β expression did not reflect the occurrence of observed renal tubulointerstitial fibrosis. Fibrotic processes are complex, and consist of various cell types and signaling molecules. Therefore, it is unlikely that the degree of interstitial fibrosis observed here is due to variations in TGF- β expression alone.

In summary, feeding high fat diets differing in PUFA content appears to influence renal composition and health in non-diseased female rats. All diets rich ω -3 PUFAs led to an increase in renal ω -3 PUFA content; however, the absolute amount varied among sources. Rats fed the seafood sources of ω -3 PUFAs excreted > 50% less PGE₂ than rats fed CO and FO, which could be related to the lower tissue ω -6: ω -3 ratio and suppressed renal COX-II expression in these animals. Additionally, altered NF κ B activity and renal expression of COX-II and TGF- β suggests that the potential anti-inflammatory activities differ between dietary ω -3 PUFA sources. However, increased tissue ω -3 PUFA content did not prevent the development of P induced NC and associated renal damage in rats fed KO. Therefore, we conclude that changing the dietary PUFA content can have profound influence on renal health in rats. This, and other data, suggests that the kidneys are heavily influenced by dietary composition, and that renal health could become compromised with the consumption of an improper diet.

5.6 References

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Figure 1. Lipid Class Diagram

Lipid Class	Corn	Flax	Menhaden	Krill	Salmon	Tuna
Phospholipid (%)	0.0	9.0 ± 0.3	0.0	16.4 ± 0.6	0.0	0.0
Polar non-phospholipid (%)	34.7 ± 0.8	9.0 ± 0.3	25.5 ± 1.0	60.0 ± 0.8	26.2 ± 0.7	33.7 ± 2.4
Triglyceride (%)	67.1 ± 1.9	81.6 ± 0.8	74.5 ± 1.0	24.0 ± 1.6	73.8 ± 0.7	82.4 ± 2.9

Figure 2. Histological Observations

	Corn	Flaxseed	Menhaden	Krill	Salmon	Tuna
Tubular Calcification (A)	0 / 10	1 / 10	1 / 10	10 / 10	0 / 10	1 / 10
Tubular Dilation (B)	0 / 10	0 / 10	0 / 10	10 / 10	0 / 10	1 / 10
Tubulointerstitial Changes (C)	1 / 10	6 / 10	2 / 10	8 / 10	2 / 10	1 / 10
Interstitial Inflammation (D)	4 / 10	5 / 10	1 / 10	10 / 10	4 / 10	2 / 10

Figure 3. Renal COX-II (PTGS-2) expression

Figure 4. Renal TGF- β expression

Figure 5. Renal NF κ B activity.

Figure 6. Urinary excretion of PGE₂

Figure 1. Representative image and quantification of lipid classes in PUFA oils of interest. Spots were quantified by densitometry and data expressed as % of total lipid detected on TLC plate. Data presented as mean $\pm$ SEM.

TRG = triglyceride, PL = phospholipid.

Figure 2. Representative images of identified changes in renal morphology. Data is represented as proportion of animals displaying morphological changes (n=10).

Figure 3. Expression of PTGS-2 (COX-II) in renal tissue of rats fed diets varying in PUFA content. Data is reported as a percentage of expression found in rats fed corn oil.

Figure 4. Expression of TGF- β in renal tissue of rats fed diets varying in PUFA content. Data is reported as a percentage of expression found in rats fed corn oil.

Figure 5. Activation of NF κ B in renal tissue nuclear extracts of rats fed diets varying in PUFA content. Data is reported as a percentage of expression found in rats fed corn oil.

Figure 6. Urinary excretion of PGE₂ in rats fed diets varying in PUFA content. Data is reported as a percentage of expression found in rats fed corn oil.

Table 1. Experimental Diet Compositions

Ingredients (g/kg diet)	AIN-93G	Corn	Flax	Menhaden^b	Krill^b	Salmon	Tuna
Soybean Oil	70.0	-	-	-	-	-	-
Corn Oil	-	120.0	-	2.0	2.0	-	-
Flaxseed Oil	-	-	120.0	-	-	-	-
Salmon Oil	-	-	-	-	-	120.0	-
Menhaden Oil	-	-	-	118.0	-	-	-
Tuna Oil	-	-	-	-	-	-	120.0
Krill Oil	-	-	-	-	118.0	-	-
Casein	200.0	200.0	200.0	200.0	200.0	200.0	200.0
L-Cysteine	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Corn Starch	397.5	347.5	347.5	347.5	347.5	347.5	347.5
Maltodextrin	132.0	132.0	132.0	132.0	132.0	132.0	132.0
Sucrose	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Cellulose	50	50	50	50	50	50	50
Mineral Mix ^a	35	35	35	35	35	35	35

Vitamin Mix ^a	10	10	10	10	10	10	10
Choline Bitartrate	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Antioxidant (TBHQ)	0.014	-	-	-	-	-	-

^a Based on the AIN-93G vitamin and mineral mixes [7].

^b Diets formulated for 118 g of test oil + 2 g corn oil to meet LA requirements.

Table 2. Fatty acid composition of experimental oils

Fatty Acid (%)	Corn	Flax	Menhaden	Krill	Salmon	Tuna
14:0	-	-	12.3	5.1	10.3	3.7
16:0	10.5	4.9	24.3	31.4	21.9	16.7
16:1	-	-	17.6	3.8	12.7	5.2
18:0	1.6	2.96	3.1	1.9	2.9	4.4
18:1	26.8	18.7	12.1	16.7	16.8	14.4
18:2 (ω6)	57.8	15.5	0.7	1.9	4.0	1.7
18:3 (ω3)	1.0	55.9	3.1	1.3	1.4	0.7
20:4 (ω6)	-	-	-	-	-	1.9
20:5 (ω3)	-	-	3.9	20.3	14.4	8.6
22:6 (ω3)	-	-	3.3	5.9	3.5	24.8
EPA:DHA	ND	ND	3:1	3:1	4:1	1:3
Total ω6	58	16	0.7	2	4	3.6
Total ω3	1	56	15	27	19	34
ω6:ω3	59:1	1:4	1:23	1:14	1:5	1:10

Table 3. Relative kidney weights, and renal Ca and P content

	Corn	Flaxseed	Menhaden	Krill	Salmon	Tuna	<i>P</i>
Kidney weight (mg/100g BW)	837.2 ± 24.5 ^{ab}	749.1 ± 16.6 ^b	785.3 ± 37.9 ^b	918.7 ± 18.3 ^a	823.7 ± 22.1 ^{ab}	830.7 ± 12.7 ^{ab}	<0.001
Kidney Ca content (mg/g)	0.21 ± 0.02 ^b	0.21 ± 0.02 ^b	0.21 ± 0.02 ^b	0.58 ± 0.12 ^a	0.18 ± 0.02 ^b	0.18 ± 0.02 ^b	<0.001
Kidney P content (mg/g)	2.40 ± 0.06 ^{ab}	2.43 ± 0.05 ^{ab}	2.31 ± 0.09 ^b	2.88 ± 0.27 ^a	2.47 ± 0.03 ^{ab}	2.49 ± 0.03 ^{ab}	0.03

Table 4. Total lipid and fatty acid content of kidney tissue

	Corn	Flax	Menhaden	Krill	Salmon	Tuna
Kidney lipid content (mg/g)	43.3 ± 8.8	44.6 ± 4.6	42.7 ± 2.7	36.2 ± 5.3	31.8 ± 3.5	35.0 ± 5.1
Fatty Acid (%)						
16:0	23.53 ± 0.24	22.42 ± 0.25	25.30 ± 0.44	25.05 ± 0.56	26.64 ± 0.57	28.06 ± 0.55
16:1	-	1.10 ± 0.07	2.23 ± 0.16	1.58 ± 0.10	2.16 ± 0.14	2.05 ± 0.21
18:0	13.51 ± 0.41	13.93 ± 0.33	13.81 ± 0.45	14.81 ± 0.20	15.80 ± 0.43	15.20 ± 0.57
18:1	11.93 ± 1.19	13.80 ± 0.54	11.86 ± 0.65	11.83 ± 0.45	13.39 ± 0.63	13.92 ± 0.80
18:2 (ω6)	11.85 ± 0.89	11.68 ± 0.28	3.18 ± 0.23	2.77 ± 0.12	2.39 ± 0.19	2.17 ± 0.13
18:3 (ω3)	-	4.01 ± 0.24	-	-	-	-
20:4 (ω6)	20.95 ± 1.04	10.83 ± 0.32	13.11 ± 0.36	9.90 ± 0.48	11.96 ± 0.58	14.57 ± 1.09
20:5 (ω3)	-	4.52 ± 0.26	6.73 ± 0.36	9.69 ± 0.54	8.86 ± 0.32	5.08 ± 0.27
22:6 (ω3)	1.05 ± 0.08	1.81 ± 0.06	6.54 ± 0.36	6.16 ± 0.37	4.71 ± 0.15	5.56 ± 0.15
EPA:DHA	0:1	3:1	1:1	2:1	2:1	1:1
Total ω6	33	23	16	13	14	17
Total ω3	1	10	13	16	13	11

ω6:ω3

33:1

2:1

1:1

1:1

1:1

2:1

Table 5. Serum measures of kidney function

Measurement	Corn	Flax	Menhaden	Krill	Salmon	Tuna	<i>P</i>
BUN (mg/dL)	10.7 ± 1.3 ^b	10.7 ± 1.3 ^{ab}	13.6 ± 0.9 ^{ab}	12.2 ± 0.7 ^{ab}	15.2 ± 0.9 ^a	13.1 ± 0.6 ^{ab}	0.04
Serum creatinine (mg/dL)	0.5 ± 0.04	0.5 ± 0.02	0.5 ± 0.04	0.4 ± 0.05	0.7 ± 0.09	0.6 ± 0.08	0.2
BUN/Creatinine	22.7 ± 1.7	26.1 ± 2.6	33.5 ± 5.4	30.6 ± 3.8	23.4 ± 4.3	27.7 ± 4.7	0.5
Serum phosphorus(mg/dL)	9.4 ± 0.7	10.1 ± 0.3	10.4 ± 0.6	9.7 ± 0.4	9.8 ± 0.5	10.1 ± 0.2	0.8
Serum calcium (mg/dL)	13.4 ± 1.0	13.6 ± 0.5	13.9 ± 1.0	14.6 ± 1.3	13.0 ± 0.5	12.6 ± 0.3	0.7
Serum protein (g/dL)	5.8 ± 0.4	6.1 ± 0.3	6.3 ± 0.1	6.2 ± 0.3	6.3 ± 0.2	6.0 ± 0.2	0.7
CrC (ml/min)	0.65 ± 0.1	0.59 ± 0.1	0.71 ± 0.08	0.80 ± 0.2	0.48 ± 0.2	0.99 ± 0.2	0.3

Table 6. Urinary measures of kidney health and function during the final week of the study

Measurement	Corn	Flax	Menhaden	Krill	Salmon	Tuna	<i>P</i>
Urinary output (ml/d)	4.3 ± 0.7	4.0 ± 0.6	4.2 ± 0.6	3.5 ± 0.7	4.0 ± 0.6	4.6 ± 1.0	0.9
Urinary albumin (mg/7d)	3.3 ± 1.4	4.3 ± 1.2	2.1 ± 0.5	6.6 ± 3.1	3.0 ± 1.0	2.5 ± 0.7	0.4
Urinary Ca (final) (mg/7d)	4.7 ± 2.1 ^{ab}	4.0 ± 0.9 ^a	4.7 ± 1.5 ^a	3.6 ± 1.5 ^{ab}	1.5 ± 0.3 ^{ab}	0.9 ± 0.2 ^b	0.009
Urinary P (final) (mg/7d)	30.3 ± 13.05 ^b	52.7 ± 12.2 ^b	29.2 ± 9.2 ^b	149.2 ± 14.5 ^a	18.5 ± 2.3 ^b	22.38 ± 4.5 ^b	<0.001
Change in urinary pH (%)	-1.28 ± 0.7 ^b	0.48 ± 0.4 ^b	-4.07 ± 4.3 ^b	-9.79 ± 4.7 ^a	-0.06 ± 0.07 ^b	-0.29 ± 0.33 ^b	<0.001

6.0 Results and conclusion

Results presented here suggest that dietary components can have profound influence on renal health in female rats. We found that changing the protein source (from casein to KPC) led to reduced NC and associated renal damage. KPC contained residual lipid, which we described as being a unique ω -3 PUFA source in regards to lipid content. To determine if the residual ω -3 PUFA content was responsible for the observed benefits of KPC, we formulated casein-based diets that contained variable PUFA content. To our surprise, the diet rich in the “pro-inflammatory” ω -6 PUFAs did not result in NC. Female rats fed krill oil developed NC, and this appeared to be due to the high PL (and subsequent high dietary P) content of the krill oil. Aside from NC, changing the dietary PUFA content resulted in extreme variations in renal tissue PUFA composition. *In vitro* studies have suggested that manipulating the PUFA content can modulate the activity of proinflammatory mediators such as PGE₂, as well as proinflammatory signaling. Providing ω -3 PUFAs lead to reduced excretion of PGE₂ and reduced expression of proinflammatory mediators. Differences were also found among the dietary sources of ω -3 PUFAs, in regards to both tissue PUFA content and elicited physiological responses.

Therefore, we conclude that:

- 1.) diet has a profound influence on renal health in non-diseased female rats,
- 2.) changing the source of protein from casein to KPC influenced the development of NC in female rats,
- 3.) krill oil has a unique lipid composition that is sensitive to processing methods,
- 4.) PUFAs do not appear to modulate diet induced NC in female rats,
- 5.) consumption of ω -3 PUFAs influences inflammatory signaling in non-diseased female rats, and

- 6.) lipid content and physiological responses varies among dietary ω -3 PUFA sources.

The results presented here provide valuable information to the fields of nutrition, agriculture, physiology, and cell biology. By understanding the interaction of diet and physiology, we can develop preventative measures to help decrease the prevalence of morbid diseases.