EVALUATION OF MOLLUSCS AS DIETARY SOURCES OF IRON: HEME AND NON-HEME IRON CONTENT OF CLAMS AND OYSTERS CONSUMED IN THE ASIA-PACIFIC REGION

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ABSTRACT

Iron deficiency is the most prevalent nutrient deficiency in the world. Limited bivalve data indicates discrepancies in iron and heme iron concentration. Therefore, fresh Manila clams, Pacific oysters, canned blood clams (A. granosa), canned akagai (A. broughtonii), and frozen blood clams were assayed and compared to beef liver as a reference food rich in heme iron. Heme iron ranged from 0.53 ± 0.08 to 5.77 ± 0.34 mg/100 g edible portion in Manila clams and blanched frozen blood clams, respectively. Non-heme iron ranged from 4.47 ± 0.44 to 9.81 ± 0.58 and total iron ranged from 4.35 ± 0.85 to 12.28 ± 0.44 mg/100 g EP in Manila clams and canned blood clams, respectively. Frozen blood clams had significantly more heme and total iron compared to the liver reference. Although these data indicate that clams could be a reasonable iron source, some samples contained potentially toxic amounts of cadmium.

EXECUTIVE SUMMARY

Iron deficiency is the most prevalent nutrient deficiency in the world, afflicting more than 2 billion people. Iron deficiency is characterized by low levels of iron in the body resulting in physiological dysfunction and numerous deficiency signs and symptoms, including iron deficiency anemia. Dietary iron exists in two forms, heme iron and non-heme iron. Incorporating foods high in heme iron can increase iron absorption since heme iron is more bioavailable than non-heme iron and is less susceptible to absorptive inhibitors. Bivalve molluscs have been purported to be excellent sources of heme iron from sources ranging from Web MD to the New York Times. However, there is limited data on iron content in molluscs and it is not certain if all bivalves have the same heme iron content. The objective of this study was to perform heme, non-heme, total iron and mineral analysis on a selection of bivalves. Fresh Manila clams, Pacific oysters, canned blood clams (A. *granosa*), canned akagai (A. *broughtonii*), and frozen blood clams were assayed. Beef liver was also assayed as a reference food rich in heme iron. Results showed that heme iron ranged from 0.53 ± 0.08 to 5.77 ± 0.34 mg/100 g edible portion in Manila clams and blanched frozen bloods clams, respectively. Non-heme iron ranged from 4.47 ± 0.44 to 9.81 ± 0.58 and total iron ranged from 4.35 ± 0.85 to 12.28 ± 0.44 mg/100 g EP in Manila clams and canned blood clams, respectively. Frozen blood clams had significantly more heme and total iron compared to the liver reference. These findings demonstrate that blood clams can be an alternative heme iron source. Additionally, most bivalves showed nutritionally significant levels of zinc, manganese, selenium, and copper. However, some samples contained potentially toxic amounts of cadmium. A more in depth analysis of other species and growing environments is needed to strengthen food composition databases and dietary recommendations for iron in shellfish.

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LIST OF ABBREVIATIONS

AI	Adequate Intake
ATP	Adenosine Triphosphate
CDC	Center for Disease Control and Prevention
DI	Deionized
DMT1	Divalent Metal Transporter 1
DRI	Dietary Reference Intake
DV	Daily Value
EP	Edible Portion
FDA	Food and Drug Administration
Fe	Iron
Hgb	Hemoglobin
IOM	Institute of Medicine
ND	Not Detected
nHb	Nerve Hemoglobin
NLEA	Nutritional Labeling and Education Act of 1990
P	Purged
PTDI	Provisional Tolerable Daily Intake
PTMI	Provisional Tolerable Monthly Intake
PTWI	Provisional Tolerable Weekly Intakes
RACC	Reference Amount Customarily Consumed
RDA	Recommended Dietary Allowance
SEM	Standard Error of the Mean
Srv	Serving
UL	Upper Tolerable Limit
UP	Un-purged
USDA	United States Department of Agriculture
WHO	World Health Organization

CHAPTER 1 - INTRODUCTION

Iron Deficiency

What is it?

According to the World Health Organization, iron deficiency is the most prevalent nutrient deficiency and affects more than 2 billion people worldwide (WHO, 2001). Iron deficiency is characterized by low levels of iron in the body resulting in physiological dysfunction that can result in numerous deficiency symptoms including general fatigue; impaired immunity, mental function, and thermoregulation; glossitis; pregnancy complications; pica; brittle nails; and ultimately iron deficiency anemia (Zimmermann et al., 2005). However, the impact of iron deficiency also goes beyond these symptoms. Iron deficiency affects productivity in the work place where treatment could result in a 20% increase in national productivity as well as improve quality of life (WHO, 2001).

Causes

Causes of iron deficiency can be nutritional, environmental, physiological, or a combination of these factors. Those most at risk are infants and young children, women of childbearing age, and pregnant women. Infants and young children are prone to iron deficiency due to rapid growth which depletes iron stores. As children get older and growth slows, their risk of iron deficiency decreases. Women of childbearing age have increased needs due to blood losses during menses where1 mL of blood loss equates to a loss of 0.5 mg of iron. Those with heavy menses pose a higher risk for iron loss and iron deficiency (Harvey et al., 2005). Pregnant women have a higher requirement for iron due to expanding blood volume and erythropoiesis. Nutritional iron deficiency arises when there is inadequate iron or low iron bioavailability in the diet. Vegetarians for example require more iron in their diet due to decreased bioavailability (Hurrell et al., 2010). Vegetarians require twice the amount of iron compared to those on a mixed diet due to the low bioavailability of the iron in plant sources (Food and Nutrition Board, 2001). Additionally, environmental factors such as gastrointestinal parasites can cause internal

bleeding which leads to blood loss and increased iron needs. Physiological conditions can also impact iron requirements. Celiac and other inflammatory bowel diseases decrease the body's ability to absorb nutrients such as iron and can result in iron deficiency if not kept in check (Rashid et al., 2005). Chronic inflammation can also result in iron deficiency because in the inflammatory state, iron absorption from the enterocytes into the blood stream is inhibited and causes a decrease in available iron (Glinz et al., 2015).

What can we do?

Addressing iron deficiency from a nutritional standpoint requires a few things. The public needs accurate information about foods with available iron and sound recommendations to help them make educated decisions about their diet. This will allow the public to consume foods nutritious in iron and could help curb the prevalence of iron deficiency.

Iron metabolism

In the diet iron comes in two forms, heme iron which is ferrous iron bound to a porphyrin ring, and all other dietary iron referred to as non-heme iron which can be either ferric or ferrous iron. Both forms of iron are primarily absorbed in the proximal intestine but can have very different bioavailabilities due to their absorption pathways and chemical properties. Heme iron is found in animal tissues such as beef, poultry, pork and fish while non-heme iron is found in both animal and plant sources. In a typical American diet, 10-15% of dietary iron is found as heme iron and 85-90% as non-heme iron. Although non-heme iron dominates most western diets, it is estimated to be about 5% bioavailable compared to heme iron, which is approximately 25% bioavailable (Food and Nutrition Board, 2001). Additionally, non-heme iron absorption has numerous known inhibitors. These inhibitors are primarily found in plant sources and include phytochemicals such as polyphenols, phytates, and oxalates. Calcium is also an inhibitor of both heme and non-heme absorption. Conversely, ascorbic and other organic acids have been shown to increase non-heme iron bioavailability and can even dull the effects of these inhibitors. Additionally, iron status can greatly affect iron absorption where those with low iron stores can

have a six-fold increase in absorption compared to those with healthy iron stores (Food and Nutrition Board, 2001).

Non-heme absorption

One of the main factors affecting non-heme iron absorption is intestinal solubility. At the near neutral pH of the intestines ferric iron is mostly insoluble, as it chelates to form insoluble iron hydroxide complexes, which makes the iron unavailable for iron absorption. For non-heme iron to be absorbed, it must be in the ferrous state. Ferroreductases such as duodenal cytochrome b are found on the apical membrane of the enterocytes and reduce soluble forms of ferric iron to ferrous iron. Additionally, other dietary compounds such as ascorbic acid aid in reduction of ferric iron. Once in the reduced state, non-heme iron is transported into the cell via divalent metal transporter 1 (DMT1). DMT1 is fueled by a proton gradient and is maintained by sodium/hydrogen exchangers and sodium/potassium pumps. Once in the enterocyte iron then enters the labile iron pool, which consists of iron atoms weakly chelated with organic acids, amino acids or other proteins. It is a transition state between iron storage in the enterocyte or iron transport to the blood (Food and Nutrition Board, 2001) .

Heme-iron absorption

Heme-iron is primarily found in hemoglobin and myoglobin and is freed from these proteins during the digestive process and does not need to undergo any modifications for absorption. Heme-iron is taken in by the enterocytes via heme carrier protein 1. Once in the enterocyte, the iron is freed from its porphyrin ring via heme oxygenase, releasing the free ferrous iron. Once the iron is freed, it enters the same labile iron pool as the non-heme iron (Raffin et al., 1974).

Iron transport by enterocytes and distribution to tissues

When iron enters the labile iron pool in the enterocyte, it can migrate from the apical to the basolateral membrane. It is unknown if there are chaperones facilitating this movement. At the basolateral membrane, ferrous iron is transported out of the enterocyte via ferroportin 1. The amount of ferroportin on the basolateral membrane is regulated by iron stores. The liver is responsible for recognizing iron status and controlling iron absorption. In response to high iron levels, the liver releases hepcidin and targets the ferroportin on the enterocytes by binding to it, resulting in internalization and degradation. This decreases the amount of iron able to be exported from the enterocytes for use. Inversely, with low iron stores, less hepcidin is released by the liver to increase the amount of ferroportin on the basolateral membrane for increased absorption. Upon exiting the enterocyte, ferrous iron is oxidized to ferric iron via hephaestin or other ferroxidases. Iron then readily binds to transferrin for transport throughout the body (Conrad and Umbreit, 2006).

Transferrin has two binding sites and under normal conditions, the plasma transferrin is approximately one third saturated with iron. It is also able to bind to other metal cations such as chromium, copper or manganese. After binding to transferrin, iron is transported to peripheral tissues where the iron-transferrin complex is recognized by transferrin receptors on the cellular membrane of cells, resulting in endocytosis. Iron status also affects the number of theses cellular receptors. Next, an ATP dependent proton pumps creates an influx of protons into the endosome to decrease pH from about 7.4 to 5.5. This results in the release of iron from transferrin. Still in the endosome, the ferric iron is reduced to ferrous iron via Steap3 and is then exported out of the endosome and into the cytoplasm via DMT1 for use in the cell (Conrad and Umbreit, 2000).

Iron storage

Iron that is not needed immediately is stored primarily in the liver, bone marrow and spleen. Ferritin is the primary storage molecule for intracellular storage and is found in all cells. It is a large, spherical, globular protein and acts not only as a storage site but also as a barrier to keep the highly reactive iron species from undergoing unwanted chemical reactions. For storage to take place, ferrous iron must be oxidized to ferric iron. Normally, a single ferritin molecule can

store up to 4500 atoms of iron (Ponka et al., 1998). The amount of ferritin in cells is regulated by cellular iron status, where more iron signals for more ferritin synthesis.

Most of the ferritin in the body is found in the cytosol. To access and utilize the iron, ferritin must first be degraded. There are two main pathways in which iron is released from ferritin. Firstly is lysosomal ferritin degradation. Lysosomes are vesicles found in the cell and are mainly responsible for recycling cellular waste for reuse. Lysosomes have a pH ranging from 5 -5.5 and is optimal for cysteine, serine and aspartate protease function. Ferritin is incorporated into the lysosome via microautophagy and chaperone mediated autophagy. Once in the lysosome, the low pH and proteases degrade the ferritin and release the stored iron (Linder, 2013). The second pathway in which iron is released from ferritin is by proteasomal degradation. Proteasome are large hollow structures found in the cytosol and degrade short-lived, damaged or misfiled proteins using an array or proteases. Proteins such as ferritin must first be tagged with ubiquitin for degradation to occur. Once tagged, the proteasomes recognize the protein for degradation, bind to it, remove the ubiquitin tag, and degrade the protein via ATP hydrolysis. Once ferritin is degraded by one of these two pathways, the free iron is transported back in to the cytosol via DMT1 or other metal transporters and joins the labile pool, although the exact mechanism has yet to be elucidated (Linder, 2013). Following release, iron can be utilized, or exported out of the cell via ferroportin and is reoxidized by the copper containing protein ceruloplasmin before binding to transferrin.

Functions of iron in the body

Iron is a transitional metal and can exist in a number of oxidation states. However, in living organisms these are often limited to the 2+ and 3+ oxidation states. In the body, iron is utilized in iron-containing proteins. There are four major classes of these proteins: iron-containing heme proteins, iron-sulfur enzymes, transport and storage proteins, and other iron containing enzymes (Food and Nutrition Board, 2001).

Dietary recommendations and recommended sources of iron

In the United States, the Institute of Medicine (IOM) created the Dietary Reference Intakes (DRIs) that serve as a scientifically based nutrition guide. Iron requirements are based on daily iron losses of those who are not iron deficient. Their nutritional recommendations for iron are summarized in the table below.

Table 1-1. Recommended Dietary Allowance (RDA) for Iron [A]

	Males	Females	Pregnancy	Lactation
Age (years)	(mg/day)	(mg/day)	(mg/day)	(mg/day)
0.5 to 1	11	11	-	-
1 to 3	7	7	-	-
4 to 8	10	10	-	-
9 to 13	8	8	-	-
14 to 18	11	15	27	10
19 to 50	8	18	27	9
51+	8	8	-	-

[A] Adapted from the DRIs (Food and Nutrition Board, 2001)

Even with these recommendations, iron deficiency is still a problem. Replenishing iron stores will require additional iron intake above the DRI recommendations for normal healthy individuals. Therefore iron rich foods are important for recovering and preventing iron deficiency.

Recommended sources of iron

Animal sources are recommended because of their high heme iron content, which has higher bioavailability and fewer dietary inhibitors. The table below summarizes common heme iron sources and their iron composition. Values represent a meta-analysis of different literature values (Schönfeldt and Hall, 2011).

Table 1-2. Average iron content of common heme iron sources[A]

	Heme Iron	Non-Heme Iron	Total Iron
	mg iron per 100 g edible portion		
Beef	1.64	1.19	2.83
Pork	0.45	0.67	1.12
Chicken	0.31	0.72	1.03
Fish	0.46	1.29	1.75

[A] Adapted from Schönfeldt and Hall, 2011.

Red meats such as beef have been recommended as good sources of heme iron and have considerably more than other meats such as pork, chicken and fish. However, recently the Dietary Guidelines for Americans is making a shift and recommends limiting red meat consumption because of its association with conditions such as cancer and heart disease. Therefore we need to find other heme iron sources to supplement beef as a heme iron source.

Clams

Clams and other molluscs have been purported to be excellent sources of total iron. The Center for Disease Control puts canned clams at the top of the list of iron rich foods, containing 23.8 mg/serving. Even wide reaching sources such as the New York Times recommends clams as a top source for both total and heme iron. However, there is limited data on iron composition between species, and it is not certain if all clams have the same heme iron content even though they are lumped together on food composition databases and recommendations. Recently there are two papers showing varying ranges of iron composition in bivalves, both in total iron as well as percent heme iron (Lai et al., 2012; Kongkachuichai et al., 2002).

Iron values for clams found in literature

Two major papers concerning iron composition in clams show differences in iron values (Lai et al., 2012; Kongkachuichai et al., 2002). The table below summarizes these key findings.

Table 1-3 Literature evaluation of iron content of bivalves per 100 g edible portion

Sample	Total Fe (mg/srv)	Heme Fe (mg/srv)	Heme Fe %	
Baby Canned	30.7	0.64	1.9	Lai et al., 2012
Minced Clam	1.02	0.07	8.1	
V. philippinarum				
Unpurged	3.93	0.34	9.5	
Purged	3.93	0.34	9.5	
P. undulata				Kongkachuichai et al., 2002
Steamed	15	2.5	16.7	
A. granosa				
Blanched	17.7	9.1	51	
Blanched	17.6	5.3	30.1	
P. viridis				
Raw	14.7	4	27.3	
Steamed	12.3	5	40.7	

% heme iron is calculated as Heme Fe/Total Fe x 100%.
UP = Unpurged P = Purged

The data above shows that there is quite a varying range of total iron composition between these samples. There is wide disagreement with respects to their heme iron content with even the highest levels found by Lai et al. (2012) being markedly lower than those found by Kongkachuichai et al. (2002). These differences could be due to factors such as species, preparation, developmental stage and environmental factors. This results in very different %

heme iron compositions in theses samples and would therefore make some of these excellent sources of iron and some not.

Where is iron found in clams

Table 1-4 illustrates the structures and anatomy of clams. This vocabulary will be used throughout the thesis.

Table 1-4 Clam structures and anatomy

Structure	Description
Valve	Made from calcium carbonate and covers the left and right sides of the soft body. The two valves are connected dorsally by a hinge and ligament. There are numerous sub divisions of valve types.
Ctenidium/Gill	Serves as both a respiratory organ and can have some feeding function as well. Water enters the gills anteriorly and passes through posteriorly.
Abductor Muscle	A large muscle typically found anteriorly and posteriorly. Its function is to draw the two valves together.
Hemolymph	A fluid found in invertebrates and is analogous to blood.
Hemocyte	A phagocytotic immune cell found in the hemolymph.
Foot	A large muscle found in the mantle and protrudes from the shell to aid in burrowing.
Mantle	Forms the body wall of the entire clam and encloses its visceral mass. Secretes calcium bicarbonate to form the two valves.

Iron is a crucial transition metal for the growth and maintenance of marine life, ranging from processes such as photosynthesis and nitrogen fixation in phytoplankton to oxygen transport in the largest of sea creatures. In marine life, iron has a high cellular requirement when

compared to the low solubility and concentrations found in our oceans. Recent work using isotopic comparison has shown that marine sources of iron come from three primary sources, mineral dust, mineral dissolution and hydrothermal vents with latter being the largest contributor to new iron (Horner et al., 2015).

Since iron is a crucial element for marine life, it follows that clams and other bivalves should contain iron in their tissues. In fact, bivalves are starting to be used as environmental indicators for marine pollution of metals because of their ability to bioaccumulate and concentrate metals to several orders of magnitude higher than what is found in the environment. In the shortneck clam, *Paphia malabarica*, iron values in specific tissues were analyzed using atomic absorption spectroscopy (Kumari et al., 2006). Based on the tissue type, there were differences in where the iron was bioaccumulated in these clams. The order of tissues from highest to lowest iron concentrations was: gills, mantle and abductor muscles, digestive gland and gonad, and foot. It should be noted, however, that their findings only depict the concentrations found and do not determine the total amounts of iron found in these various tissues and does not provide heme and non-heme iron composition breakdown. Table 1-5 is adapted from their data, showing the average iron concentrations based on tissues.

Table 1-5 Iron concentrations found in tissues of *P. malabarica*

Fe Content	Gills	Mantle and abductor muscle	Digestive gland and gonads	Foot
Fe (μg/g dry weight)	183 ± 86	94 ± 24	89 ± 39	55 ± 29

The table above summarizes the findings of Krishna Kumari et al. (2006) for the total iron concentrations found in the shortneck clam, *P. malabarica*, based on tissue location. Values are expressed as means ± SD.

Heme iron in clams

Heme iron in clams is thought to primarily be associated with heme proteins and possibly hematin (Vismann, 1993). Hemoglobins are amongst the most ancient genes and are found in all kingdoms of organisms (Weber and Vinogradov, 2001). Hemoglobins were first discovered because of their high abundance in human blood, but since then hemoglobins have been found throughout various tissue types. From phylogentic analysis, the hemoglobin gene is thought to have originated from a common ancestor dating back beyond invertebrate and vertebrate divergence, over 670 million years ago, due to the alignment of their amino acid sequences. It could even possibly predate prokaryote and eukaryote divergence based on intron/exon arrangements. During evolution, these heme-binding proteins have adapted to perform various functions and roles. Electron transfer, cytochromes, intra and extracellular peroxidases, detoxification, immunity, and oxygen binding are a few of these roles that heme-binding proteins are used for (Hardison, 1998).

Recent studies have been looking into and finding new hemoglobins in various species of molluscs. In this new line of research, the terms hemoglobin and heme proteins are synonymous to one another due to the novelty of the heme proteins they are discovering and researching.

One of these new proteins is referred to as nerve hemoglobins (nHgb). nHgb was initially discovered in invertebrates by Lankaster in 1872 in the annelid *Aphrodite aculeata* and recently more research has been done looking into these proteins across a wide range of species. nHgb exhibits a bright red color consistent with other heme containing proteins. Vertebrate nHgb is found to be expressed at lower concentrations (μM), when compared to invertebrates, which express them at higher concentrations (Zimmermann et al., 2005). The higher concentrations found in invertebrates is thought to have evolved to extend oxygen delivery to nerve cells during times of hypoxic and anoxic conditions. In many cases, nHgb is specifically expressed in glial cells but can sometime be expressed in neurons themselves (Dewilde et al., 2006). It has been shown that oxygen delivery can be extended for periods as long as 30 minutes when comparing *Tellina alternata*, a clam species containing nHgb, to a related species, *Tagelis plebeius, which* did not contain nHgb (Kraus and Colacino, 1986). nHgb do not cooperatively bind oxygen and have P50 values ranging from 1.1 – 4 torr in invertebrates. This is similar to the values seen in

vertebrate myoglobin which further strengthens the idea that these nHgb are used to aid in supplying oxygen to nervous tissues (Wittenberg et al., 1965).

Closely related to nHgb is overall oxygen transport in clams and other bivalves. Typically, clams do not utilize hemoglobin for oxygen transport like mammals do. Molluscs in general have hemolymph (analogous to blood) as a major component of their circulatory system, but they utilize hemocyanin, a monomeric, copper-containing protein dissolved in the hemolymph to transport oxygen. The hemolymph is often a blue color because of the copper compound. However, there is a family of bivalves, arcidae or ark clam, which differ from the norm. They are distinguished by their red-pigmented flesh and the use of hemoglobin as a primary oxygen transport mechanism. Unlike most molluscs, these blood clams contain erythrocytes, which harbor the hemoglobin and function as both an oxygen transport and storage site (de Zwaan et al., 1995). The erythrocyte volume is about 10% of the hemolymph and has 3.5 mg of cells per 100 mL. Therefore, oxygen capacity is about 5 mL/100 mL of hemolymph compared to 0.5 mL/100 mL for seawater. This means that they can concentrate oxygen ten-fold of what is found in their environment (Weber and Vinogradov, 2001). Additionally, compared to vertebrates where their blood volume accounts for less than 10% of their bodyweight, the hemolymph of blood clams is about 60% of their body weight (Deaton and Mangum, 1976). This increases the oxygen capacity per unit bodyweight of invertebrates compared to vertebrates. These blood cells, however, are different than what we typically find in the mammalian circulatory system and are specially adapted to native environmental conditions of blood clams.

Blood clams are commonly found in estuaries, lagoons and mudflats with muddy or sandy substrates; and prefer intertidal or subtidal elevations, especially in the Indo-pacific region. Blood clams such as Anadarae have varying preferences for salinity and can adapt to yearly environmental changes. For example, *A. granosa* are found to live in environments where salinity ranges from 28-31ppm in the dry season and as low as 5-10ppm in the rainy season. It has been reported that some species such as A. senilis can survive in salinities of 50 ppm in the dry season. However, their ability to adapt to such varying salinities is not yet fully known (Broom, 1985).

Nearly 50 years ago the blood clam Scapharca inaequivalis was introduced to a non-native habitat in the Mediterranean. Since the 1970s this area has been subjected to strong

eutrophication. Eutrophication is enrichment of waters either artificially or naturally and leads to large algae blooms and hypoxic conditions. This has resulted in high mortality rates of the native marine population, but the blood clams have been thriving and out competing two native bivalve species, *Mytilus galloprovincialis* and *Venus gallina* (de Zwaan et al., 1991). Studies have shown that *S. inaequivalis* is capable of surviving in anoxic conditions for over a month which makes its oxygen binding and transport mechanism particularly interesting. Previously, erythrocytes in other invertebrates have been discovered, but they lyse under anoxic conditions after a few minutes (Mangum and Mauro, 1985). However, the erythrocytes in *S. inaequivalis* show greater stability under anoxic conditions (de Zwaan et al., 1991). This is thought to be in part due to high-energy conservation leading to low metabolite accumulation in anaerobic metabolism that increases osmotic stability of the erythrocyte. This increases the survivability of this species in low oxygen environments. When these erythrocytes were examined via light and electron microscope they were found to be nucleated and contain granules, a Golgi apparatus, rough endoplasmic reticulum, mitochondria and cytoplasmic microtubules. They were relatively large and spherical or ovoid in shape (Holden et al., 2009).

The key to the blood clam's survivability in hypoxic conditions may be in their ability and mechanism for anaerobic metabolism. They are able to sustain aerobic metabolism at much lower pO2 than other non-erythrocyte containing bivalves and only shift to anaerobic metabolism when levels reach between 1.5 and 2.5 mg O2/L. The fermentation pathway seems to initiate between 3 and 6 hours of anoxia. During this process there is a marked reduction in metabolic rate, which may be crucial to their survivability in low oxygen environments. For *S. inaequivalis,* ATP turnover rate during anaerobic metabolism drops to less than 5% of its rate during aerobic metabolism (de Zwaan et al., 1995).

Besides hemoglobin in the erythrocytes, there are also freely dissolved heme-containing compounds in the hemolymph that are used to deal with other problems besides low oxygen. Hydrogen sulfide is an extremely toxic substance to aerobes and often accompanies low oxygen environments. Hydrogen sulfide is toxic because it inhibits ATP production along the electron transport chain at cytochrome C oxidase. Various mechanisms have evolved in the animal kingdom to deal with this issue, such as sulfide trapping in mucus layers, precipitation with iron compounds, detoxifying pigments and detoxification in the mitochondria. For marine

invertebrates, the mechanism seems to be heme containing compounds in the hemolymph that aid in hydrogen sulfide detoxification. *S. inaequivalis* was analyzed for heme compounds in the hemolymph because of its sulfide resistance. The hemolymph was found to contain both hemoglobin as well as hematin (heme iron coordinated with a hydroxyl group). The hemolymph contained 3.89 ± 1.34 mM total heme, 2.23 ± 0.95 mM as hemoglobin and 1.66 ± 1.25 mM as hematin. They believe that the heme found in the hemolymph came from both hemoglobin and hematin. Because of the experimental conditions, they do not believe that the hematin dissociated from hemoglobin, but rather, held in granules (Vismann, 1993). Additionally the hemoglobin in the hemolymph showed reactivity to hydrogen sulfide and nitric oxide (Ramos-Alvarez et al., 2013)

Besides oxygen transport and detoxification, Hgb has been shown to play a role in many other functions in molluscs such as antioxidation, molting, and immunity, with variations in the structure of Hgb structures depending on the species as well as function. Recently a study out of China demonstrated the use of Hgb in the antibacterial immune response in *A. granosa*. Live samples were introduced to *V. parahaemolyticus*, a common bacteria to most molluscs, and DNA expression of Hgb was monitored in the hepatopancreas, abductor muscle, foot, gill, mantle and hemocyte. They found that after introducing the bacteria to the live clams, there was a marked increase in expression of the Hgb. The Hgb gene expression was found to increase in the mantle and gills with the hemocytes having the greatest expression. Hemocytes are over 90% Hgb and after 1.5 hours post challenge there was a 3.4 fold increase in expression, and after 12 hours expression peaked at 600 fold. This demonstrates that Hgb plays some role in the antibacterial immune response, although the mechanism has not been fully elucidated. If hemoglobin is truly involved in the immune response, it makes physiological sense that we see an increase of Hgb in the mantle and gills because they are exposed to the environment where the clam would be introduced to the bacteria and the hemocyte is an immune cell meant to deal with bacterial invaders. Additionally, they found minor increases in expression in other tissues, but this is probably explained by the fact that hemocytes are in the clam's circulatory system and can be found in all tissue types (Bao et al., 2011, Bao et al., 2013).

In summary, clams have a physiological reason for containing heme iron for their use in oxygen storage and transport, detoxification and immune functions. However, we still do not

have the complete picture of the content of heme proteins across the vast majority of clams. Therefore, different species may have adapted to express more or less of these heme-containing proteins. Blood clams seem to have the highest potential as a heme iron source for humans mainly due to the large amounts of hemoglobin and other heme compounds found in erythrocytes and other hemolymph structures. One goal of the current study is to evaluate the heme content of several species of bivalves common to the Asia-pacific region for heme, non-heme, and total iron content. We expect to find varying levels of iron and heme iron between species, and higher levels in blood clams than other species.

Chapter 2: Heme iron, non-heme iron, nutrient and toxic mineral content of bivalve molluscs: Manila clams, blood clams (Arcidae species) and Pacific oysters.

Introduction

Iron deficiency is the most prevalent nutrient deficiency, affecting more than 2 billion people worldwide (WHO, 2002). The causes of iron deficiency can be nutritional, environmental, physiological or a combination of these factors. Those at most risk of developing iron deficiency are young children, women of childbearing age and pregnant women. This demographic is susceptible to iron deficiency because of factors such as rapid growth, menstruation and expanding blood volume that increases their need for iron.

Nutritional iron deficiencies arise when there is inadequate iron or low iron bioavailability in the diet. To protect against nutritional iron deficiency, people need to consume enough bioavailable iron to meet daily losses. The Institute of Medicine has come up with specific guidelines called the dietary reference intakes (DRIs) which provide the public with guidance on how much iron they should be consuming on a daily basis in order to prevent deficiency. Those, however, with pre-existing iron deficiency will require intakes above the DRIs in order to resolve their deficiency.

Nutritionally, there are two major forms of iron in the diet, heme and non-heme iron. Heme iron is ferrous iron bound to a porphyrin ring. All other dietary iron is referred to as non-heme iron, which can occur as various organic and inorganic compounds containing either ferric or ferrous iron. It is important to distinguish between the two forms of iron because they have different nutritional significance.

Heme iron has a bioavailability of approximately 25%, however it makes up only 10 - 15% of the typical American diet and is less subject to dietary inhibitors than non-heme iron (Food and Nutrition Board, 2001). Non-heme iron has a lower bioavailability. Restricted vegetarian diets were judged to be about 5 percent bioavailable. The overall iron bioavailability of a typical mixed American diet is estimated to be about 16.8% bioavailable, but this value can be much lower if certain dietary inhibitors (such as polyphenols, oxalates and phytates) are present in the diet (Food and Nutrition Board, 2001). In setting the DRI, the IOM assumes consumers are getting 10-15% of their dietary iron as the more bioavailable heme iron. Heme iron is obtained from animal-based foods, with red meats like beef containing high amounts. However, the

Dietary Guidelines for Americans is beginning to recommend less intake of red meat and it is important to find other food sources of heme iron. Clams may provide an alternative source for consumers (USDA, 2010).

Recently, bivalves such as clams have been purported to be excellent sources of iron. Organizations such as the FDA list clams at the top of the best foods for iron, having 23.8 mg of iron per serving, more than enough to meet the daily recommendations. This high value for the iron content for clams is riddled throughout the Internet and media sources as well, with some also stating that clams are a good source of heme iron (McKinley Health Center - University of Illinois at Urbana-Champaign, 2010; WebMD, 2015). Currently, there is little published research regarding iron content and composition in clams. The works by Lai et al., (2012) and Kongkachuichai et al., (2002) have shown values ranging from 0.57–17.2 mg of iron per serving depending on species and processing. Additionally, Kongkachuichai et al., (2002) found significant levels of heme iron in the clam species they studied, with heme iron comprising over 50% of the total iron. In contrast, Lai et al., (2012), found that less than 10% of total iron came from heme iron in the species they sampled.

The clam that Kongkachuichai et al., (2002) examined comes from an unusual family of bivalves, Arcidae, commonly referred to as ark clams or blood clams. These bivalves are unlike most species of clams because of the presence of erythrocytes in their circulatory system. Blood clams are thought to have evolved this trait in order to survive in low oxygen environments and have demonstrated an ability to thrive in hypoxic conditions, outcompeting other species (de Zwaan et al., 1995). Heme iron has also been found to have other functions within blood clams such as sulfide detoxification, immune response and increased oxygen supply to nerves (Bao et al., 2011, Vismann, 1993, Wittenberg et al., 1965).

The objectives of this study were to examine heme, non-heme and total iron content of selected bivalves common to the Asia-Pacific region and begin to make comparisons between species. Curbing the prevalence of nutritional iron deficiency requires adequate intake of iron in the diet, but in order to do so, the public needs accurate information about foods that contain bioavailable iron and sound recommendations to help them make educated decisions about their diet. We hope to increase the information in the literature regarding heme iron in clams in order

to help health professionals make reliable recommendations for dietary sources of heme iron in order to help curb the prevalence of iron deficiency.

Materials and methods

Chemicals and glassware

All water used throughout the experiment was distilled deionized (DI) water. All chemicals used in this study were purchased from Sigma-Aldrich unless otherwise noted. All glassware was acid washed in a 20% nitric acid solution (v/v) for 24 h and rinsed with water before a final 24 h soak in a water bath to remove any trace iron and contaminants.

Sample preparation and homogenization

All meat and shellfish samples were purchased from local markets in Honolulu, Hawaii, except for the canned blood clams, which were purchased in Bangkok, Thailand. Descriptions of the food samples as purchased are presented in Table 2-1.

Beef livers

Frozen packaged beef livers were thawed in the refrigerator overnight before the start of the experiment. The liver samples were trimmed of all visible fat and oxidized sections, and cut into 2cm cubes. Approximately 200 g of sample was homogenized in a stainless steel blender for one minute on high.

Table 2-1.

Bivalve molluscs analyzed for iron, mineral and moisture content

Common name	n	Sample state at purchase	Scientific name	Geographic origin
Manila clam	3	Live	*Venerupis philippinarum*	Washington
Pacific oyster	5	Live	*Crassostrea gigas*	Washington
Canned akagai, drained	3	Seasoned and canned	*Anadara broughtnonii*	Japan
Canned blood clam	3	Seasoned, fried and canned	*Anadara granosa*	Thailand
Frozen blood clam	2	Blanched and frozen	*Anadara granosa*	Vietnam

Fresh shellfish: Manilla clams (v. philippinarum) and Pacific oysters (c. gigas)

The shellfish were rinsed with DI water to remove dirt, sand, vegetation, etc. that may be on the shells. The rinsed shellfish were shucked with a stainless steel knife and the flesh was placed in a stainless steel strainer. The flesh was then rinsed with DI water once for five seconds, agitated manually for three seconds, and allowed to drip dry for thirty seconds. This was repeated three times. Approximately 200 g of the sample (30-40 clams or 8-12 oysters) was homogenized in a stainless steel blender for one minute on high.

Frozen blood clams (a. granosa)

The blanched frozen clams were thawed at room temperature and then shucked with a stainless steel knife making sure to get the dark red flesh that may stick to the shells. Additionally, the flesh was squeezed as little as possible to minimize fluid loss. Approximately 200 g of the sample (60-70 clams) was placed directly into a plastic food processor with a stainless steel blade and homogenized for one minute on high. A food processor was used instead of a blender because the blender did not adequately homogenize the sample due to its spongy texture.

Canned blood clams (a. granosa)

The seasoned, fried blood clams were used as is out of the can. Ingredients included: 90% blood clam, 4.5% soy sauce, 4% sugar, and 1% salt. A small number of clams were used to determine their approximate moisture content by drying to a constant weight at 60 °C. The canned blood clams had a moisture content of only 38%; therefore, enough water was added during the homogenization step to increase the moisture content to 80%. Approximately 100 g of clams and 210 mL of deionized water were homogenized in a stainless steel blender one minute on high. The homogenized sample was refrigerated for 30 minutes to hydrate, and re-homogenized for one minute on high. Note: less than 200 g of sample is needed since water was added. This dilution factor was accounted for in the final calculations.

Canned akagai (a. broughtonii)

The canned akagai had been cooked and seasoned with a sweet soy sauce. The contents of the can (clams and sauce) were put into a stainless steel strainer for five minutes to allow the sauce to drain. Every minute the clams were agitated manually for three seconds. A small aliquot of the drained clams was used to determine the approximate moisture content by drying to a constant weight at 60 °C. The drained, canned akagai had a moisture content of only 64%, therefore, water was added during the homogenization step to increase the moisture content to 80%. Approximately 100 g of the clams and 80 mL of deionized water were homogenized in a stainless steel blender for one minute on high. The sample was refrigerated for 30 minutes to hydrate and re-homogenized for one minute on high. Note: less than 200 g of sample is needed since water was added. This dilution was be accounted for in the final calculations.

Moisture content analysis

Triplicate samples (30 g each) of the homogenized meat or shellfish were weighed into 500 mL glass beakers. The samples were dried at 60 ºC in a Fisher-Scientific Isotemp stainless steel oven containing Dririte until a constant weight was achieved (approximately 48 h depending on moisture content) and weighed. Moisture content was determined using the following equation:

Change in mass/Mass of homogenate used = Moisture content (%)

After moisture content was determined, the dried samples were combined and ground into a fine powder using a stainless steel coffee grinder. Three aliquots of the powder (2 g each) were placed into plastic sample bags and stored in a desiccator until processed for total iron analysis.

Total iron analysis and mineral

Total iron and mineral contents in the dried ground samples was analyzed at the Louisiana State University AgCenter using inductively coupled plasma emission spectroscopy (ICP-ES). The procedure can be found on their website under Plant Tissue Tests (LSU, 2015).

Briefly, 0.5 g of the dried sample was digested in concentrated HPLC grade nitric acid and hydrogen peroxide on a hot plate and then allowed to cool. Samples were then diluted and read using ICP-ES. Values were reported in ppm (ug/g or % dry mass). Upon receiving the report, values were converted into ug/g edible portion using the moisture content of the original fresh, frozen or canned sample. National Institute of Standards and Technology peach and spinach leave reference material (SRM 1547 and SRM 1570a, respectively) were analyzed every 20 samples for quality control. The mean concentration (n=5) for each mineral fell within the uncertainty limits of their certified value except for the following: Fe was 91 ± 2%, Al was 80 ± 3%, and Na was 128 ± 28% of the certified values. SRM 1570a (spinach leaves) was used to verify the accuracy of the cadmium assays since cadmium was below the detection limit in the peach leaves. The limit of detection for the mineral assays was taken as the mineral concentrations of the lowest ICP-ES calibration standard.

Non-heme iron assay

The non-heme iron content was determined using the modified Schricker procedure (Rhee and Ziprin, 1987) with slight modifications, as described below. Briefly, seven aliquots (1.5 g each) of freshly homogenized liver or shellfish were weighed into 40 mL glass centrifuge tubes with silicone-lined lids, then 0.06 mL of a 0.39% (w/v) sodium nitrite solution was added to each sample and mixed using a glass stir rod. Next, 18 mL of the acid mixture (3N HCl, 20% trichloroacetic acid) was added, the samples were tightly capped, vortexed for five seconds and placed into a 65 ºC water bath for 20 h. After incubating, the samples were vortexed again for five seconds and allowed to cool to room temperature. After cooling, 0.2 mL of the liquid phase above the sample and 1mL of the bathophenanthroline disulfonate color reagent were added to 1.5 mL plastic microfuge tubes and vortexed to mix. A second set of samples was created using 0.2 mL of the liquid phase and 1 mL of the sodium acetate solution instead of the bathophenanthroline disulfonate color reagent to create a correction blank that accounts for the sample pigments. The liquid phase samples were then centrifuged at 3500 x g for 10 min and the absorbance measured at 540 nm against a reagent blank.

A standard curve (1 ug-5 ug Fe/mL) was created using a 1000 ppm iron metal ICP standard solution in 3% nitric acid (Ricca Chemical Company) to determine non-heme iron

concentrations in the liquid phase. If the absorbance of the liquid phase sample was above the standard curve, then smaller aliquots of the samples were diluted with the acid mixture to a volume of 0.200 mL prior to the addition of the bathophenanthroline disulfonate color reagent (for example, mixing 0.050 mL of the liquid phase and 0.150 mL of the acid mixture to achieve a four-fold dilution). This dilution must be accounted for in the final calculation. Non-heme iron content in the sample was determined using the following equations and conditions:

Non-heme Fe (ug/g EP) = [dilution factor x non-heme Fe in liquid phase (ug/mL)] x Total volume of liquid phase (mL) / EP (g)

Total volume of liquid phase (mL) = 18 mL acid mixture + 0.06 mL sodium nitrite + water in EP (mL)

Dilution factor = 0.200 mL / Liquid phase added to microfuge (mL)

Non-heme iron spike recovery assay

The methods for the non-heme iron spike recovery assay were identical to the methods explained above except for the preparation of the acid mixture for the spiked samples. Ten aliquots (1.5 g each) of homogenized samples were used. Half were extracted in the normal acid mixture while the other half were extracted in the acid mixture spiked with non-heme iron. The spike was made by diluting a small amount of the 1000 ppm iron standard solution with the acid mixture in a 250 mL volumetric flask to achieve a solution that would add approximately 100% more non-heme iron during the extraction than is naturally present in the 1.5 g samples. For example, the clam samples were extracted in 18 mL of the spiked acid mixture containing 2 ug iron/mL which increased the total non-heme iron content of the samples by 36 ug. Recovery of the non-heme iron spike was determined using the equations below:

%Recovery = [mean (n=5) non-heme Fe content of spiked samples (FDA) - mean (n=5) non-heme Fe content of control samples (FDA)] / amount of non-heme Fe in spike (FDA) x 100%

Heme iron assay

Heme iron was determined using the Hornsey method (Hornsey, 1956) with slight modifications. Briefly, seven aliquots (4.0 g each) of freshly homogenized meat or shellfish were placed into 40 mL amber jars with silicone-lined lids. Using the sample moisture content, an acetone:water:HCl solution was made up to produce an 80:18:2 (v/v/v) ratio in a total liquid volume of 20 mL after being mixed with 4.0 g of the homogenized meat or shellfish. The 20 mL volume and 80:18:2 ratio are the final volume and acetone:water:HCl ratio which includes the water content of the meat or shellfish sample. The acidified acetone solution was made using HPLC grade acetone, trace metal grade HCl, and DI water in a 250 mL volumetric flask. After the acidified acetone solution was added to each sample they were tightly capped, vortexed for five seconds, and incubated in the dark at room temperature for 1 h. After incubation, the samples were vortexed for five seconds. The liquid phase was vacuum filtered through ashless Whatman 42 filter paper (moistened with acetone to reduce filtration time and evaporation) being careful not to cause any boiling. The filtrate was then placed into 40 mL glass centrifuged tubes with teflon screw caps and centrifuged at 10,000 x g for 10 min at 24 ºC. The absorbance of the supernatant was measured at 640 nm against a reagent blank using glass cuvettes. Heme iron content in the meat or shellfish was determined using the following equations:

$$\text{Hemin (ppm)} = Abs_{640} \times 652 \text{ mg hemin/mmol} \times \text{total liquid in solution (L)} / \text{mass of EP (g)} / 4.8 \text{ Lmmol}^{-1} \times 1000 \text{ ug/mg}$$

Heme iron concentrations can be determined knowing that hemin is 8.54% iron.

$$\text{Heme Fe (ug/g)} = \text{Hemin (ug/g)} \times 8.54\%$$

Heme iron spike recovery assay

The methods for the heme iron spike recovery assay were identical to the methods for the heme assay explained above except for the preparation of the acidified acetone solution for the spiked samples. Ten aliquots (4 g each) of the homogenized samples were used. Half were extracted in the normal acidified acetone solution while the other half were extracted in acidified

acetone spiked with heme iron as hematin (Alfa Aesar). The spike was made by mixing the hematin and acidified acetone in a 250 mL volumetric flask to achieve a solution that would add approximately 100% more heme iron than what is naturally present in the 4 g samples. For example, for the Manila clam sample a 1 ug heme iron/mL acidified acetone solution was used to increase the total heme iron by 16 ug when extracted in 16 mL. Percent recovery was determined using the equations below:

%Recovery = [mean (n=5) heme Fe content of spiked samples (ug) - mean (n=5) heme Fe content of control samples (ug)] / amount of heme iron in spike (ug) x 100%

Purged vs. un-purged assays

To determine if purging has an effect on heme and non-heme iron content, a comparison was done between purged and un-purged fresh Manila clams and Pacific oysters. One batch of shellfish was purchased and individuals were randomly allocated into two separate groups, purged and un-purged. In order to purge the Manila clams and Pacific oysters, enough brine (640 mmol/L NaCl) was added to cover the them in a plastic container, then 50 g of cornmeal was added and the shellfish were left to purge in an air-conditioned room at 20 ºC for 12 h. Meanwhile the un-purged shellfish were analyzed for heme and non-heme iron content using the methods described previously. After 12 h, the purged shellfish were rinsed with deionized water to remove any brine and cornmeal, and analyzed for heme and non-heme iron.

Statistical analysis

Data were expressed as means ± SEM (n=2-5 as shown in Table 2-2). All analyses were performed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA). Comparisons between means for heme, non-heme, and total iron measurements were done using one-way ANOVA followed by Tukey's post hoc test. When two means were compared to assess the effects of purging, a paired t-test was used. Differences were considered significant at $p<0.05$.

Results

Heme, non-heme and total iron content

Heme, non-heme and total iron content in the samples were expressed as mg of iron per 100 g edible portion (Table 2-2). Although 100 g is not the serving size for all samples, we chose this value in order to make direct comparisons between samples. The reference amount customarily consumed (RACC) for Manila clams, Pacific oysters and beef liver is 110 g; the RACC for canned clams is 55 g and for frozen blood clams it is 85 g (FDA, 2015). For heme iron content, the samples fell into four different groups with Manila clams having the lowest and blanched, frozen, blood clams having the highest values (0.53±0.08 and 5.77±0.34 mg/100 g EP, respectively). This demonstrates there was greater than a 10-fold difference in heme iron content between the lowest and highest samples. For non-heme iron, samples fell into two groups with Manila clams having the lowest and canned blood clams having the highest values (4.47±0.44 and 9.81±0.58 mg/100 g EP, respectively). Measured total iron fell into three statistically different groups with Manila clams having the lowest and canned blood clams having the highest values (4.35±0.85 and 12.28±0.44 mg/100 g EP, respectively). Total iron recovery ([heme + non-heme] / total measured) is also presented and in general showed greater than 100% apparent recovery, with the average recovery being 126% (Table 2-2).

The effects of purging Manila clams and Pacific oysters on heme, non-heme and total iron content are shown in Figure 2-1. There were no significant effects on heme and total iron by ICP for both Manila clams and Pacific oysters. However, purging did decrease non-heme iron values for both Pacific oysters only ($p < 0.05$).

Table 2-2

Heme and non-heme iron, total iron and moisture content of bivalve molluscs and beef liver[A]

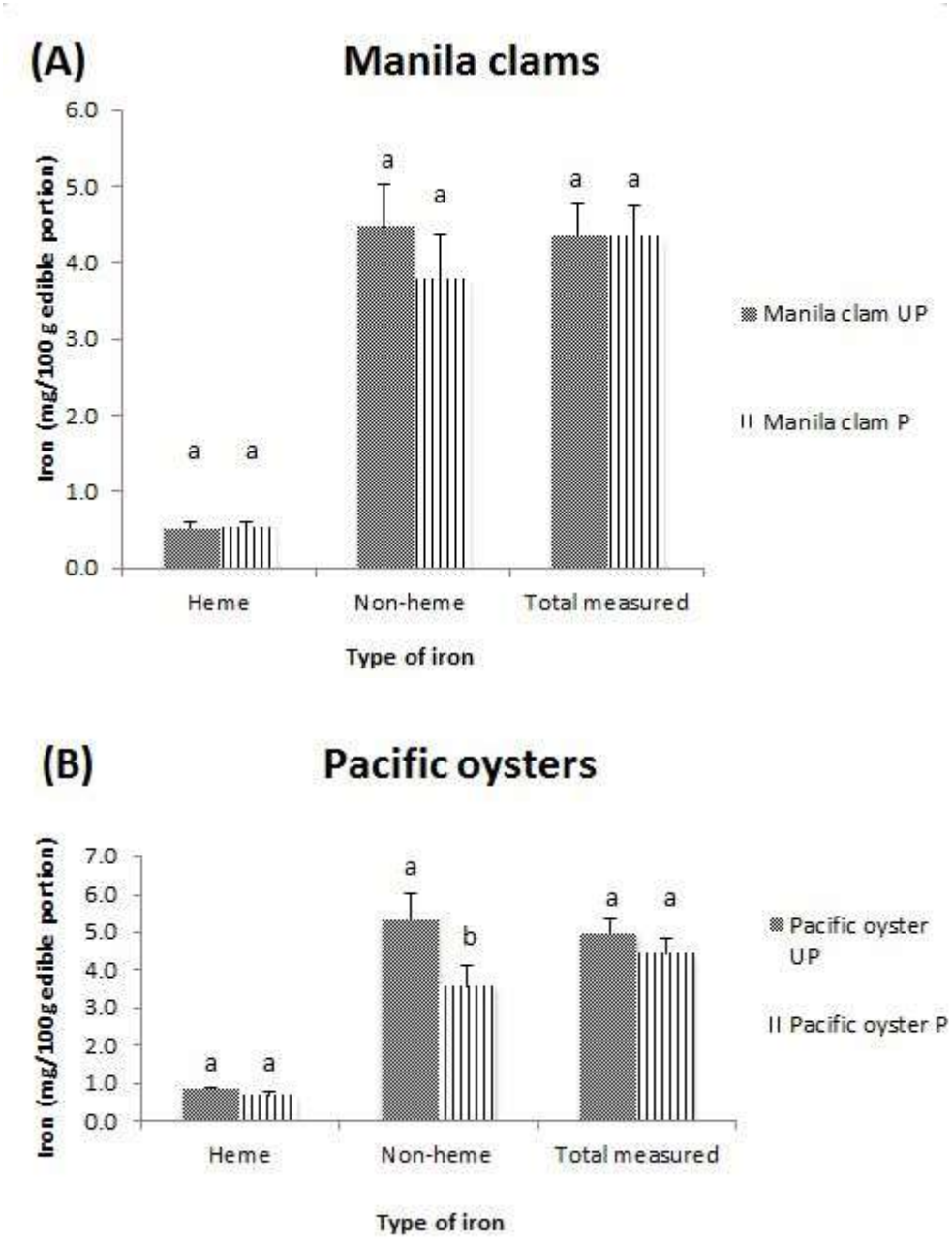
Common name	n	Moisture (%)	Heme iron (mg/100 g EP[B])	Non-heme iron (mg/100 g EP[B])	Total iron (mg/100 g EP[B])	Apparent recovery[C] (%)
Manila clam	3	80.2±1.5	0.53±0.08[a]	4.47±0.44[a]	4.35±0.85[a]	130±40
Pacific oyster	5	76.8±1.1	0.85±0.03[a]	5.30±0.7[a]	4.93±0.41[a]	129±26
Canned akagai, drained	3	62.1±0.3	2.35±0.07[b]	5.64±0.36[a]	7.16±0.17[b]	112±3
Canned blood clam	2	36.7±0.9	2.01±0.04[b]	9.81±0.58[b]	12.28±0.44[c]	96±8
Frozen blood clam	3	82.1±0.4	5.77±0.34[d]	6.60±0.26[a]	9.85±0.17[c]	126±5
Frozen beef liver	2	76.6±0.2	3.58±0.18[c]	5.80±0.01[a]	5.81±0.06[a,b]	161±1

[A] Values are means ± SEM. Means in a column with different superscripts are statistically different ($p<0.05$) using Tukey's post-hoc test.

[B]EP = edible portion, the part of the food product that can be eaten after trimming and removing non-edible components.

[C] Apparent recovery is defined as [(measured heme + non-heme iron) / (measured total iron)] x 100%.

Figure 2-1. Effects of purging on heme, non-heme and total iron content of live Manila clams (A) and Pacific oysters (B)



Figures 2-1: Effects of purging on heme, non-heme total iron content of live Manila clams (A) and Pacific oysters (B). Values are means ± SEM (n=3 for unpurged (UP) Manila clams, n=3 for purged (P) Manila clams, n=5 for unpurged Pacific oysters, n=5 for purged Pacific oysters). Paired columns with different superscripts are statistically different using a paired t-test ($P<0.05$).

Mineral nutrient content

Calcium, copper, magnesium, manganese, molybdenum, phosphorus, potassium, selenium, sodium and zinc content were obtained using ICP-ES and were expressed as mg per 100 g edible portion (Table 2-3). Samples with mineral contents below the limits of detection were labeled as not detected. Notably, there was a greater than 10-fold variation among samples for copper, manganese, molybdenum and zinc content with no one sample type consistently having the highest or lowest values.

Further analysis was performed on the total nutrient mineral contents to express them as percent of the daily value (DV) in a 100 g edible portion (Table 2-4). Shellfish are typically recognized as sources of minerals such as iron, selenium, zinc, copper, and manganese. All samples were found to be rich sources of iron (>24% DV). Additionally, all samples were rich sources of selenium (>34% DV) with the exception of the canned akagai clams which did not have any detectable selenium. All samples were also rich sources of zinc (<12% DV) with the exception of Manila clams**.** Both Manila clams and Pacific oysters were rich sources of copper, containing well above 100% of the DV whereas the canned blood clams contained between 7 – 14% DV. All shellfish samples, were rich sources of manganese with the exception Manila clams (purged or unpurged) which did not have any detectable manganese. Overall there was not a significant effect of purging on the mineral contents of either Manila clams or Pacific oysters.

Non-nutrient mineral content

The non-nutrient mineral content of purged and unpurged bivalves and beef liver were expressed as mg per 100 g edible portion (Table 2-5). Samples with mineral contents below the limits of detection were labeled as not detected. No lead was detected in the samples with the exception of a small amount in the canned blood clams. All samples, however, showed some level of non-nutrient mineral content, but the only mineral with levels high enough to pose a potential health risk was cadmium. Therefore, further analysis was performed on the cadmium

content in the samples. Cadmium content was reported as the percent of the provisional tolerable monthly intake (PTMI) in 100 g edible portion (Table 2-6). Because the PTMI is dependent on the body weight of the individual, we reported values for body weights ranging from 40–90 kg. For smaller individuals, as few as three to four 100 g servings of Manila clams or canned blood clams could put them at risk for potential cadmium-related health issues. Larger individuals have the capacity to handle a greater number of servings.

The Pacific oysters had the highest cadmium content of all the samples. Two servings and four 100 g servings a month for smaller and larger individuals may pose a potential health risk. The frozen blood clams and canned akagai clams showed lower levels of cadmium when compared to the other samples, containing only 2-4% of the PTMI, even for the smaller individuals. The beef liver reference sample did not contain detectable cadmium and was not shown in the table.

Table 2-3.

Nutrient mineral content of bivalve molluscs and beef liver expressed as mg/100 g edible portion (EP)[A]

Sample	n	Ca	Cu	Fe	Mg	Mn	Mo	P	K	Na	Se	Zn
Manila clam UP[B]	3	70±13	4.3±0.8	4.4±0.9	42.8±9.9	nd	nd	188±34	251±24	287±16	0.04±0.01	1.2±0.1
Manila clam P[C]	3	67±24	4.4±0.4	4.3±0.4	34.3±6.4	nd	nd	210±43	237±20	301±65	0.04±0.00	1.3±0.1
Pacific oyster UP	5	127±29	3.4±0.9	4.9±0.4	43.3±3.3	1.03±0.11	nd	205±25	240±4	356±18	0.03±0.00	12.9±0.9
Pacific oyster P	5	100±23	3.2±0.9	4.4±0.3	33.7±2.7	1.02±0.06	nd	204±14	226±11	392±51	0.02±0.01	12.2±1.2
Canned akagai, drained	3	35.9±0.2	0.19±0.01	7.2±0.2	26.0±0.5	0.99±0.02	nd	155±1	94.7±2.3	877±9	nd	3.10±0.02
Canned blood clam	2	90.1±0.2	0.28±0.02	12.3±0.4	35.4±0.7	2.02±0.12	0.13±0.02	149±13	48.8±2.52	1242±52	nd	3.47±0.04
Frozen blood clam	3	40.6±1.5	0.14±0.00	9.8±0.2	28.0±0.6	0.94±0.07	nd	133±3	153±4	122±9	0.03±0.00	1.81±0.03
Frozen beef liver	2	nd[D]	3.7±1.0	5.8±0.1	13.4±0.3	nd	0.09±0.00	244±34	216±9	44.4±1.6	0.03±0.01	2.67±0.00

[A]Values are means±SEM and n=number of samples. Each sample was analyzed in triplicate. EP = edible portion, the part of the food product that can be eaten after trimming and removing non-edible components.

[B]UP=unpurged

[C]P=purged

[D]nd=not detected. Limits of detection in mg/100 g EP were as follows: Manila clam, 0.4 for Mn and 0.04 for Mo; Pacific oyster, 0.05 for Mo; canned akagai, 0.08 for Mo and 0.04 for Se; canned blood clam, 0.06 for Se; frozen blood clam, 0.04 for Mo; and frozen beef liver, 9.3 for Ca and 0.5 for Mn.

Table 2-4.

Nutrient mineral content of bivalve molluscs and beef liver expressed as % of daily value (DV) per 100 g edible portion (EP)[A]

	Ca	Cu	Fe	Mg	Mn	Mo	P	K	Se	Zn
Daily Value (mg):	1000	2	18	400	2	0.075	1000	3500	0.07	15
Sample	% DV per 100 g EP									
Manila clam UP[B]	7.0	214.8	24.2	10.7	nd	nd	18.8	7.2	54.3	8.2
Manila clam P[C]	6.7	218.1	24.1	8.6	nd	nd	21.0	6.8	60.0	8.7
Pacific oyster UP	12.7	169.6	27.4	10.8	51.6	nd	20.5	6.9	44.3	85.8
Pacific oyster P	10.0	160.3	24.7	8.7	51.0	nd	20.4	6.5	34.3	81.2
Canned akagai, drained	3.6	9.4	79.3	6.5	49.6	nd	15.5	2.7	nd	20.6
Canned blood clam	9.0	13.8	68.2	8.8	101.0	170.7	14.9	1.4	nd	23.1
Frozen blood clam	4.1	7.0	54.7	7.0	46.8	nd	13.3	4.4	48.6	12.0
Beef liver	nd[D]	187.1	32.3	3.4	nd	118.7	24.4	6.2	48.6	17.8

[A]Percent daily value is calculated as [average mineral content (mg/100 g EP) divided by DV (mg)] x 100%. DVs are daily recommendations for key nutrients and are based on a 2000 kcal diet for adults and children four years of age and up (FDA, 2013). However, this is not the reference amount customarily consumed for all samples. For comparison, the reference amount customarily consumed for Manila clams, Pacific oysters and beef liver is 110 g; for canned clam samples it is 55 g; and for the frozen blood clams it is 85 g (FDA, 2015). EP = edible portion, the part of the food product that can be eaten after trimming and removing non-edible components.

[B]UP=unpurged

[C]P=purged

[D]nd=not detected, therefore, no % DV could be calculated

Table 2-5.

Non-nutrient mineral content of bivalve molluscs and beef liver expressed as mg/100 g edible portion (EP)[A]

Sample	n	Al	As	B	Cd	Pb	Ni
Manila clam UP[B]	3	nd[D]	0.21±0.07	0.04±0.01	0.33±0.12	nd	0.09±0.03
Manila clam P[C]	3	nd	0.15±0.03	0.04±0.01	0.30±0.13	nd	0.13±0.07
Pacific oyster UP	5	0.75±0.41	0.23±0.06	0.21±0.07	0.49±0.16	nd	0.04±0.01
Pacific oyster P	5	0.47±0.20	0.17±0.05	0.17±0.05	0.50±0.17	nd	nd
Canned akagai, drained	3	3.2±0.1	0.05±0.01	0.09±0.00	0.03±0.00	nd	0.06±0.00
Canned blood clam	2	7.42±0.04	0.08±0.00	nd	0.26±0.02	0.08±0.03	0.09±0.01
Frozen blood clam	3	2.3±0.4	0.04±0.00	0.09±0.01	0.04±0.00	nd	0.04±0.00
Frozen beef liver	2	nd	nd	0.05±0.00	nd	nd	nd

[A]Values are means ± SEM and n=number of samples. Each sample was analyzed in triplicate. EP = edible portion, the part of the food product that can be eaten after trimming and removing non-edible components.

[B]UP=unpurged

[C]P=purged

[D]nd=not detected. Limits of detection in mg/100 g EP were as follows: Manila clam, 0.4 for Al and 0.02 for Pb; Pacific oyster, 0.02 for Pb and 0.04 for Ni; canned akagai, 0.04 for Cd and 0.04 for Pb; canned blood clam, 0.08 for B; frozen blood clam, 0.02 for Pb; frozen beef liver, 0.02 for Al, 0.01 for As, 0.02 for Cd, 0.02 for Pb and 0.02 for Ni.

Table 2-6.
Cadmium content in in bivalve molluscs and beef liver per 100 g edible potion (EP) expressed as a percent of the provisional tolerable monthly intake (PTMI) for persons with body weights (BW) ranging from 40 to 90 kg[A]

Body weight (kg):	40	50	60	70	80	90
Sample	% PTMI per 100 g EP					
Manila clam UP[B]	33	26	22	19	17	15
Manila clam P[C]	30	24	20	17	15	13
Pacific oyster UP	49	39	33	28	25	22
Pacific oyster P	50	40	33	29	25	22
Canned akagai, drained	3	2	2	2	2	1
Canned blood clam	26	21	17	15	13	12
Frozen blood clam	4	3	3	2	2	2

[A] Body weight specific %PTMIs in 100 g EP were calculated as: [mean Cd content (mg/100 g EP)] / [PTMI (mg/kg BW) x BW (kg)] x 100%. The PTMI for Cd is 25 ug/kg BW/month (FAO, 2011). 100 g EP were used for all samples in order to make direct comparisons. However, this is not the reference amount customarily consumed for all samples. The reference amount customarily consumed for Manila clams and Pacific Oysters is 110 g; for canned samples it is 55 g; and for the frozen blood clams it is 85 g (FDA, 2015). Beef liver was excluded because there was no detectable amount of cadmium in the samples.

Discussion

The major finding of this study was that heme, non-heme and total iron differed significantly amongst the bivalve species assayed. Most notably, frozen blood clams contained the highest amount of heme iron, over 60% more than beef liver and over 6-fold more than Manila clams and Pacific oysters. Blood clams (Anadara species) may be uniquely rich in heme iron because, unlike other bivalves, blood clams contain hemoglobin and hematin in their circulatory system (de Zwaan et al., 1995; Terwilliger and Terwilliger, 1978) . Canned blood clams contained less heme iron than the frozen ones as a result of the cooking process where heme iron is converted to non-heme iron. Total iron also varied amongst the samples, with Manila clams and canned blood clams having the lowest and highest content, respectively. The higher total iron in the canned blood clams could be related to the lower moisture content of these samples. On a dry weight basis, total iron contents in all shellfish samples were similar ranging from 19.3-24.9 mg Fe/100 g DM, with the exception of the frozen blood clams which contained 55.3 mg Fe/100 g DM (Appendix Table 2-2a). These data show that bivalve molluscs cannot be placed into a single group in food databases. A key reason for this is the differences in heme iron content which can drastically affect iron bioavailability. Most databases like the USDA nutrient database do not contain a value for heme iron (USDA 2015). Currently however, the Dutch nutrient database contains heme iron values for its foods and could be an example for updating current nutrient databases (Dutch Food Composition Database, 2013).

Our data on the iron composition of bivalves is consistent with what little data exists in the literature. The relatively low levels of heme iron in Manila clams and Pacific oysters is consistent with Lai et al. (2012), who found heme iron values ranging from 0.07 to 0.38 mg /100 g EP in minced canned clams and Manila clams, respectively. The high heme iron content we found in the frozen blood clams is also consistent with the values found by Kongkachuichai et al., (2002) where blanched (3 min) blood clams (A. granosa) contained 5.3 mg heme Fe/100 g EP. Our non-heme and total iron values for the blood clam species were somewhat lower than findings by Kongkachuichai et al. (2002) but, non-heme and total iron in Manila clams were similar to that found by Lai et al. (2012). In addition, total iron values for Manila clams, Pacific oysters, frozen blood clams and beef liver showed general similarities to the USDA and Pacific Islands nutrient databases (Dignan et al., 1994; USDA, 2015). The differences found among

databases can be attributed to numerous factors such as environment, processing, age and time of year (Krishna Kumari et al., 2006; Abbas Alkarkhi et al., 2008).

In comparison to the heme iron content of beef liver, which is considered a good source of heme iron, both Manila clams and Pacific oysters contained significantly lower amounts, indicating they should not be considered good sources of heme iron. Although the total iron content of Manila clams and Pacific oysters meet the NLEA legal definition of a good iron source, neither shellfish species contained any significant amount of heme iron when compared to beef liver. In contrast, the heme iron content of frozen blood clams was over 60% higher than what was found in beef liver. The RDA for iron for premenopausal women is 18 mg/day and is based on the assumption that 10-15% comes from heme iron in order to get adequate absorption. Therefore, premenopausal women need 1.8 mg of heme Fe/day at a minimum. Blood clams provided more than 1.8 mg heme Fe/100 g EP compared to Manila clams and Pacific oysters which provided less than half this amount. Blood clams analyzed contained higher heme iron levels than various cuts of beef analyzed by Shoenfeldt and Hall (2012), who found beef to have 1.64 mg heme Fe/100 g EP on average.

These findings confirm that not all bivalves can be a good source of heme iron. The data also show that blood clams are an especially good source of total and heme iron.

There were some limitations in our assays of heme, non-heme and total iron. The average total iron recovery was 126%, where the sum of heme and non-heme iron was typically greater than the measured total iron value. This is consistent with (Carpenter and Clark, 1995) who found that non-heme iron tends to be overestimated due to the dissociation of heme iron from the porphyrin ring during the assay, especially for samples with higher heme iron contents. Additionally, they found that total iron measured by ICP-ES is underestimated and is supported by the 91% iron recovery of the NIST certified reference sample in our study. These factors together can explain the greater than 100% recovery we found. Another limitation was the small sample sizes (n = 2-5). Nevertheless, our findings were consistent with other literature values (Kongkachuichai et al., 2002; Lai et. al, 2012).

In addition to heme and non-heme iron, we measured the content of 10 other nutrient minerals in our samples. In general, our findings agree with the USDA and Pacific Island

nutrient databases (Dignan et al., 1994, USDA, 2015). Differences again may be due to water quality, age and time of year as discussed before with respect to total iron. One notable exception was with sodium. The canned akagai and canned blood clams contained significant amounts of sodium which is likely attributed to the seasoning and lower moisture content.

Some trace mineral nutrients typically found in high amounts in shellfish are copper, iron, manganese, selenium and zinc. For copper, this is supported by our data for the Manila clam and Pacific oyster samples as they contained over 150 %DV in 100 g EP. However, copper was much lower in the blood clam species (<14 %DV in 100g EP). This may be partly to do with the bivalve circulatory system. Blood clams contain hemoglobin as an oxygen transport molecule (de Zwaan et al., 1995), while Manila clams and Pacific oysters use the copper-containing protein hemocyanin (Terwilliger and Terwilliger, 1978). Zinc was detected in all of the samples, with Pacific oysters standing out because they contained more than 80 %DV in 100 g EP. As for selenium and manganese, most of the bivalves contained considerable amounts of these nutrients, more than 30 %DV in 100 g EP. However, the few samples that contained levels below the detection limit could still have nutritionally significant amounts of these nutrients because the limits of detection were not low enough to be specific for these nutrients. With respect to iron, all samples contained more than 24 %DV in 100 g EP, showing that bivalves have the potential as a total iron source.

Six non-nutrient minerals were also measured in our samples. Aluminum was detected in all the bivalve samples except for Manila clams. All levels found, however, are well below the provisional tolerable weekly intake (PTWI) for aluminum (1 mg/kg BW/week) (FAO, 2011). Canned blood clams contained the highest levels of aluminum, but 100 g EP only accounts for 19 and 8% of the PTWI for those with body weights of 40 and 90 kg respectively. In general, arsenic values were also below previous the provisional tolerable daily intake (PTDI) levels (3 µg/kg BW/day) (FAO, 2010), but this value has since been removed because intake below the PTDI did not have a protective effect. However, the arsenic accumulated in shellfish is organic arsenic and is not toxic like the inorganic form (Washington State Department of Health, 2014). The levels of boron were also unlikely to cause adverse health effects. The minimal risk level established by the CDC is 0.2 mg/kg BW/day. For lead, no values were detected except for the

canned blood clam which was still relatively low. Values for nickel were also low throughout all the samples.

One non-nutrient mineral of some concern was cadmium. Manila clams, Pacific oysters, and canned blood clams contained considerable amounts of cadmium. For a 40 kg individual, 100 g EP of these samples contain more than 30% of the PTMI (25 μg/kg BW/month) and, therefore, could pose health risks if eaten on a consistent basis. The high cadmium content of Manila clams and Pacific oysters was consistent with findings by (Bendell, 2009), who found high cadmium levels in shellfish raised in the Pacific North West region. Shellfish raised in this region have higher cadmium content than most shellfish raised in other locations and may explain the high levels found in our samples. On the other hand, the canned akagai and frozen blood clams contained considerably less cadmium (<4% PTMI for 40kg individual). These findings show that when consuming shellfish, cadmium may be a potential toxicant and consumers should be mindful of where their shellfish originates and potential health risks. Additionally, these data show that bivalves can be produced with low cadmium levels as well.

Lastly, purging is a common practice used on clams in the food industry. The purpose is to remove any grit. Manila clams and Pacific oysters were analyzed for the effects of purging on heme, non-heme and total iron. Because there was no statistical change in heme iron content for both clams and oysters, this gives evidence that the heme iron we found is intrinsic to the clam and oyster. There was, however, a significant decrease in non-heme iron in Pacific oysters after purging. There was also a slight decrease in non-heme iron in Manila clams, but this change was not statistically significant. The decrease in non-heme iron content may be due to removal of undigested food with purging or contaminants in the gastrointestinal tract. However, as a whole, total iron was not statistically significant after purging for both clams and oysters.

Conclusions

In conclusion, our data show that heme, non-heme and total iron can differ significantly amongst bivalve species and, therefore, food database should reflect these differences. Only blood clams were found to contain high amounts of heme iron. Additionally, purging had no effect on heme iron, demonstrating that heme iron is intrinsic to the animal. Our results were

also consistent with claims of shellfish being rich sources of copper, iron, manganese, selenium and zinc. However, when eating shellfish, consumers should be aware of potentially high levels of metals such as cadmium. Although some samples were high in cadmium, frozen blood clams had much lower levels. These data show that blood clams have the potential to be an alternative dietary heme iron source as well as provide other essential nutrients.

Future Research

This study only looked at a few different species of bivalves and showed great differences in their iron compositions. Therefore, to update nutrient databases and increase knowledge about iron in bivalves, more species should be analyzed for their nutrient contents. Nutrient analysis should also be sensitive enough to detect nutritionally significant mineral levels. Additionally, samples from different regions and times should be analyzed to investigate the effects of environment, water conditions, time of year and growth stage on nutrient and non-nutrient content of bivalves in order to make better health recommendations to the public. Another important concept when considering dietary iron is bioavailability. Iron bioavailability of bivalves should be assayed to determine if the iron found in bivalves is being absorbed in the small intestines, if heme iron is better absorbed than non-heme iron and if there are any inherent inhibitors for nutrient absorption.

Appendix

Table 2-2a.

Heme, non-heme and total iron content of bivalve molluscs and beef liver expressed as mg per 100 g dry matter[A]

Common name	n	Heme iron	Non-heme iron	Total iron	Apparent recovery[C]
		(mg/100 g DM[B])			(%)
Manila clam	3	2.67±.44	22.98±3.35	21.88±3.71	130±40
Pacific oyster	5	3.74±0.30	23.66±4.12	21.63±2.47	129±26
Canned akagai, drained	3	6.33±0.07	15.17±0.72	19.25±0.17	112±3
Canned blood clam	2	3.17±0.10	15.48±0.68	19.42±0.98	96±8
Frozen blood clam	3	32.21±1.23	36.94±1.74	55.09±1.74	126±5
Frozen beef liver	2	15.31±0.92	24.84±0.20	24.88±0.49	161±1

[A] Values are means ± SEM. Means in a column with different superscripts are statistically different ($p<0.05$) using Tukey's post-hoc test.

[B]DM = dry matter

[C]Apparent recovery is defined as [(measured heme + non-heme iron) / (measured total iron)] x 100%.

Table 2-3a.

Nutrient mineral content of bivalve molluscs and beef liver expressed as mg/100 g dry matter (DM)[A]

Sample	n	Ca	Cu	Fe	Mg	Mn	Mo	P	K	Na	Se	Zn
Manila clam UP[B]	3	353±63	22±4	22±4	215±45	nd	nd	937±118	1270±97	1454±63	0.19±0.03	6.2±0.5
Manila clam P[C]	3	330±113	22±3	22±3	167±27	nd	nd	1020±164	1162±36	1461±242	0.20±0.01	6.4±0.4
Pacific oyster UP	5	551±132	16±5	22±2	192±24	4.44±0.35	nd	905±133	1047±48	1134±137	0.14±0.02	56.6±6.0
Pacific oyster P	5	424±67	15±5	20±2	160±22	4.56±0.23	nd	930±108	1011±38	1348±281	0.11±0.03	55.7±7.7
Canned akagai, drained	3	96±2	0.5±0.0	19±0	70±2	2.67±0.10	nd	417±11	225±8	2361±42	nd	8.3±0.2
Canned blood clam	2	142±2	0.4±0.0	10±1	56±2	3.19±0.24	0.20±0.03	236±17	77±3	1962±53	nd	5.5±0.0
Frozen blood clam	3	228±13	0.8±0.0	55±2	157±6	5.25±0.48	nd	745±5	854±36	680±40	0.19±0.01	10.1±0.2
Beef liver	2	Nd	16±4	25±0	57±1	nd	0.38±0.01	1045±155	923±31	190±5	0.14±0.03	11.4±0.1

[A]Values are means±SEM and n=number of samples. Each sample was analyzed in triplicate. DM = dry matter.

[B]UP=unpurged

[C]P=purged

[D]nd=not detected. Limits of detection in mg/100 g DM were: 40 for Ca, 2.0 for Mn, 0.2 for Mo, and 0.1 for Se.

Table 2-4a.

Nutrient mineral content of bivalve molluscs and beef liver expressed as percent of the dietary reference intakes (DRIs) per 100 g edible portion (EP)[A]

	Ca	Cu	Fe	Mg	Mn	Mo	P	K	Na	Se	Zn
Daily Value (mg):	1000	0.9	18	320	1.8	0.045	700	4700	1500	0.055	8
Sample	% RDA or AI per 100 g EP										
Manila clam UP[B]	7.0	477.2	24.2	13.4	nd	nd	26.8	5.3	19.2	69.1	15.4
Manila clam P[C]	6.7	484.7	24.1	10.7	nd	nd	29.9	5.1	20.1	76.4	16.4
Pacific oyster UP	12.7	376.8	27.4	13.5	57.3	nd	29.3	5.1	17.1	56.4	160.9
Pacific oyster P	10.0	356.1	24.7	10.9	56.7	nd	29.1	4.8	19.5	43.6	152.2
Canned akagai, drained	3.6	9.4	79.3	8.1	55.1	nd	22.1	2.0	58.5	nd	38.7
Canned blood clam	9.0	30.6	68.2	11.1	112.2	284.4	21.3	1.0	82.8	nd	43.4
Frozen blood clam	4.1	15.6	54.7	8.8	52.0	nd	19.0	3.2	8.1	61.8	22.6
Beef liver	nd[D]	415.7	32.3	4.2	nd	197.8	34.8	4.6	3.0	58.2	33.4

[A]Percent RDA or AI is calculated as [average mineral content (mg/100 g EP) divided by RDA or AI (mg)] x 100%. RDAs and AIs are daily recommendations for key nutrients and are based on requirements for females ages 31-50 (IOM, 2001). For comparison, the reference amount customarily consumed for Manila clams, Pacific oysters and beef liver is 110 g; for canned clam samples it is 55 g; and for the frozen blood clams it is 85 g (FDA, 2015). EP = edible portion, the part of the food product that can be eaten after trimming and removing non-edible components.

[B]UP=unpurged

[C]P=purged

[D]nd=not detected, therefore no % DRI could be calculated.

Table 2-5a.

Non-nutrient mineral content of bivalve molluscs and beef liver expressed as mg/100 g dry matter (DM)[A]

Sample	n	Al	As	B	Cd	Pb	Ni
Manila clam UP[B]	3	nd[D]	1.1±0.3	0.2±0.0	1.7±0.7	nd	0.5±0.2
Manila clam P[C]	3	nd	0.7±0.1	0.2±0.0	1.4±0.5	nd	0.6±0.3
Pacific oyster UP	5	3.0±1.6	1.1±0.3	0.9±0.3	2.2±0.8	nd	0.2±0.1
Pacific oyster P	5	1.9±0.7	0.8±0.3	0.7±0.2	2.4±0.9	nd	nd
Canned akagai, drained	3	8.6±0.5	0.1±0.0	0.2±0.0	0.1±0.0	nd	0.1±0.0
Canned blood clam	2	11.7±0.2	0.1±0.0	nd	0.4±0.0	0.1±0.4	0.1±0.0
Frozen blood clam	3	12.8±2.6	0.2±0.0	0.5±0.0	0.2±0.0	nd	0.2±0.0
Beef liver	2	nd	nd	0.2±0.0	nd	nd	nd

[A]Values are means±SEM and n=number of samples. Each sample was analyzed in triplicate.

[B]UP=unpurged

[C]P=purged

[D]nd=not detected. Limits of detection in mg/100 g DM were: 2.0 for Al; 0.2 for B; and 0.1 for As, Cd, Pb and Ni.

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