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## Nutrient Transport by Shrimp Hepatopancreas

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Nutrient Transport by Shrimp Hepatopancreas

by

Tamla Simmons

A thesis submitted to the Department of Biology

in partial fulfillment of the requirements for the degree of

Master of Science in Biology

UNIVERSITY OF NORTH FLORIDA

COLLEGE OF ARTS AND SCIENCES

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## Signature Page

The thesis "Nutrient Transport by Shrimp Hepatopancreas" submitted by Tamla Simmons in partial fulfillment of the requirements for the degree of Master of Science in Biology has been

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## **Abstract**

Purified brush border membrane vesicles (BBMV) were isolated to characterize primary cellular transport mechanisms for white shrimp. The ultimate goal is to determine the effective components of a shrimp's diet, thereby enhancing growth, as well as nutrient content. Juvenile shrimp are dependent on plant material as a food source. Potassium is a key component of plants, thus it may play a role in nutrient transport. In addition, divalent metals have been shown to act as co-transporters in several other organisms, thus they may serve as a transport mechanism for shrimp.

Fresh, live, white or brown shrimp were obtained, and from them 15-30 hepatopancreases were dissected to prepare the BBMV. Methods for preparing BBMV were based on the  $Mg^{2+}$  precipitation technique developed by Kessler et al., (1978) and Biber et al. (1981) for mammalian epithelia and applied to crustaceans.

The results suggest that there is a sodium/potassium-dependent glucose transport system that resembles the SGLT1 system of vertebrates, except the shrimp transporter can accept both sodium and potassium as cofactors, while the vertebrate system is restricted to sodium stimulation. Potassium showed strong stimulation of L-histidine uptake by shrimp BBMV, suggesting that a crustacean isoform of the insect potassium-dependent carrier protein (KAAT1) might be present in shrimp, and contribute to amino acid uptake.

Amino acids also appear to form bis-complexes with divalent metals, that are transported by an analog of the dipeptide transporter (PEPT1). The metals appear to be accommodated, with varying affinities. PEPT1 has been described as a very non-specific carrier process because it transports such a wide range of di- and tripeptide combinations.



## **Introduction**

*Litopenaeus setiferus*, Atlantic white shrimp, are large, economically important, decapod crustaceans distributed throughout tropical and subtropical regions of the world. They are arguably “America’s most valuable and probably most popular seafood”, according to the South Carolina Department of Natural Resources. Shrimp are subjected to intense fishing efforts as fisheries work to meet the demand for human consumption. Shrimp aquaculture in many countries has developed as an increasingly important source of crustacean protein and revenue. In 2007, aquaculture income in the United States was \$1.4 billion. Crustacean production (i.e. shrimp, crawfish, prawn) from 17 farms reported \$2.5 million in sales in Florida (Florida Department of Agriculture, 2009). Despite successes in shrimp farming throughout the world, remarkably little is known about many basic biological aspects of this important human food source. In particular, the cell transport mechanisms of shrimp have not been well characterized. Determining the best combinations for the formulation of a balanced diet for cultured species would help improve the productivity of shrimp farms (Vilella et al., 2003).

In addition to white shrimp, three other types of shrimp live off the coast of Jacksonville, FL: *Penaeus aztecus* (brown shrimp), *Penaeus duorarum* (pink shrimp), and *Sicyonia* sp. (rock shrimp). Brown and white shrimp comprise the majority of shrimp caught in Northeast Florida, thus both represent valuable commercial revenue. White shrimp appear lighter in body compared to brown and pink, and typically the base of their tail is black with bright yellow and green margins. Their antennae and rostrum is longer (Figure 1) (Whitaker, 2010). Generally, they are found in shallow, brackish water

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**Figure 1**

Basic anatomy of a shrimp.

From “Shrimp News  
International”

[www.shrimpnews.com](http://www.shrimpnews.com)

Illustration by Molly Kelly  
Ryan.

habitats that are 180 feet deep. They prefer muddy, sandy bottoms, rich in organic matter, because they are omnivorous, opportunistic, nocturnal, bottom feeders (Whitaker, 2010). They eat most animal or plant material found on the ocean floor, and they are also cannibalistic. Their feeding patterns change over the course of their lives; as juveniles, the diet is more plant-based, and becomes more predatory as they age.

White shrimp spawn usually in spring, seemingly due to water temperature. Shrimp populations fluctuate as a result of near total losses of spawning stocks (Whitaker, 2010). Dramatic decreases in roe stem from: cold temperatures, since shrimp are subtropical; water salinity which changes due to amount of rainfall and/or river discharge; predators; availability of food; and/or winds that may carry away larvae (Whitaker, 2010).

All of these attributes must be analyzed for potential growth enhancement of individual shrimp, and shrimp populations, in aquaculture. Practical farming considerations include: the type of holding facility; feed preparation and technique; water quality; photoperiod; and crustacean stocking density (Tacon et al., 2004). The current farming conditions include the utilization of high-density ponds. In some cultures shrimp

are fed dried pellets, but shrimp feed comprises a wide array of types, and a variety of feeding strategies are used throughout their lifecycle (Tacon et al., 2004).

Since the human population is doubling in size, to say the demand for food resources is increasing is an understatement. Protein is an essential component of the human diet, thus the focus on aquaculture research. As with other industries, the goal is to grow shrimp for less than the selling price, while preventing extinction and depletion of environmental resources. The current emphasis for aquaculture investigations include: genetic engineering; growth hormones; methods for decreasing bacteria, viral, and fungal disease; adapting polyculture to local conditions; decreasing mortality during larval growth; and nutritional physiology (Ahearn, 2011).

The hepatopancreas, a complex midgut diverticulum (Vilella et al., 2003), is the primary digestive organ of shrimp (see Figure 2). It has the dual role of secreting digestive enzymes and absorbing nutrients (Rosas et. al, 1995). It is considered a major organ for decapods, because it is used for: the synthesis and secretion of enzymes; to absorb digested material; as a site for mineral reserves and organic products; for the metabolism of lipids and carbohydrates; and to distribute stored reserves during the molting cycle (Ceccaldi et al, 1989). It is a gland that ends in ducts that open into the stomach. The hepatopancreas is comprised of four types of epithelial cells: embryonic cells (E-cells), fibrillar cells (F-cells), resorptive cells (R-cells), and blister cells (B-cells). Each plays a different role in hepatopancreatic function.

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**Figure 2**

Internal and external anatomy of *Litopenaeus setiferus*. From Food and Agriculture Organization of the United Nations document “Internal and External Anatomy of Penaeid Shrimp”; <ftp://ftp.fao.org/docrep/fao/005/y1679e/y1679e04.pdf>; Web; July 19, 2012.

The E-cells seem to be undifferentiated, embryonic cells. They are the least abundant hepatopancreatic cells, and do not seem to have a role in digestion. The R-cells are the most abundant cell type, and seem to have a role in calcium and sugar exchange with the hemolymph during molting. Due to the extensive Golgi body and rough endoplasmic reticulum network, F-cells seem to have a principal role in protein synthesis and nutrient absorption. The B-cells seem to be responsible for digesting nutrients from the lumen, due to their structure and ability to form digestive enzymes. Their structures make it very plausible that the B and F cells work in tandem with one another.

As stated previously, a primary function of the crustacean hepatopancreas is to secrete digestive enzymes and emulsifiers (Verri et al., 2001). The secondary role of the hepatopancreas is absorption and this function is not as well characterized as secretion. It

can also function in the storage of calcium and phosphorus in the exoskeleton during the molting process, and packaging glycogen and lipids as a reservoir when there is plenty of food available (Verri et al., 2001).

The intestine, in particular the midgut where the hepatopancreas is located, has a secondary role in digestion. In addition to the midgut, the intestine is composed of two other regions: the fore and hindgut. The midgut, composed of a “single, tightly packed, luminal layer of columnar epithelium attached to a thin basal lamina” (Ahearn, 1974), is where major nutrient absorption occurs. Brush border membrane vesicles (BBMV) (Figure 3) are made from microvilli that line the columnar epithelium. Microvilli constitute the last stage in carbohydrate digestion and absorption (Ahearn et al., 1985). This site is the focus of this investigation as it seeks to characterize the specific mechanisms that allow for nutrient uptake.

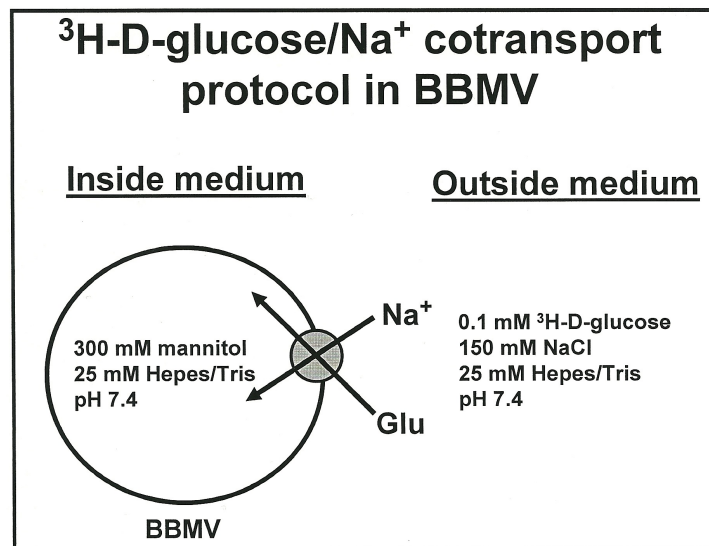
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**Figure 3**

This figure is an example of basolateral membrane vesicles from hepatopancreas, which shows their round shape. From Ahearn, et al. “Proton-stimulated  $\text{Cl}/\text{HCO}_3$  antiport by purified lobster hepatopancreatic basolateral membrane vesicles.” 1987.

In the gastrointestinal lumen, transport of most hexoses is dependent on sodium ion ( $\text{Na}^+$ ).  $\text{Na}^+$  and glucose share the same symport (see Figure 4), sodium-dependent glucose transporter (SGLT). SGLT-1 is responsible for the uptake of dietary glucose from

the gut, and glucose transporter (GLUT-2) is responsible for glucose transport out of gut cells to the hemolymph.  $\text{Na}^+$  concentration is low intracellularly so it moves down its concentration gradient. Glucose moves with  $\text{Na}^+$  and is released in the cell, and from there GLUT-2 carries it to the capillaries.  $\text{Na}^+$  is actively transported out of the cell by a sodium-potassium pump (Na/K pump), therefore, the  $\text{Na}^+$  gradient is maintained, and more  $\text{Na}^+$  can be carried in, which in turn enables more glucose to be digested. The  $\text{Na}^+$  requirement has been characterized in a diverse array of animal groups. Historically, in most animals, there is an “absolute  $\text{Na}^+$  requirement for glucose transport on the mucosal side of the intestine” (Ahearn et al., 1985).



**Figure 4**

SGLT-1 is responsible for the uptake of dietary glucose from the gut. BBMVs are loaded with inside medium during vesicle preparation. During the experiment the vesicles are washed with outside medium then filtered using the Millipore filtration technique developed by Hopfer et al. (1973), finally uptake values were assessed using a Beckman LS-6500 scintillation counter.

Previous studies of penaeid nutritional physiology have focused on amino acid and sugar absorption processes of the intestine and hepatopancreas. Detailed characterization of glycine and sodium uptake by epithelial cells of pink shrimp, *Penaeus marginatus*, indicated that amino acid transport had an absolute requirement for sodium ions and energy, and its absorption was competitively inhibited by L-proline and non-competitively inhibited by L-alanine (Ahearn, 1974). The coupling coefficient for Na<sup>+</sup>/glycine co-transport across the mucosal epithelial membrane was 2:1 at physiological substrate concentrations and potassium was a competitive inhibitor of sodium binding to the co-transport carrier (Ahearn, 1976). Amino acid absorption is less complicated in decapods, when compared to mammals, because they do not have capillaries. Hemolymph simply bathes the entire intestine; thus, organs with an extraordinary requirement for the energy high nutrient content provides are located close to the intestines (Ahearn, 1974).

### **Materials & Methods**

Fresh, live, white or brown shrimp were obtained from one of two commercial dealers near the mouth of the St. Johns River at Jacksonville, FL. Shrimp were maintained in seawater during transport. Depending upon the size, up to 100 specimens were purchased each time. The shrimp were housed in an oxygenized tank with shallow, artificial seawater with a sandy bottom. Shrimp were not fed while in captivity. For this, and other reasons including, but not limited to, waste accumulation, shrimp were lost daily. Several were lost in transport, and within the first two days in captivity several more were lost. The losses diminished as the week continued, though it remained

difficult to keep most of them for more than one week. Therefore, shrimp were sacrificed immediately for experimentation.

Depending on the size of the shrimp, 15-30 hepatopancreases were needed to prepare the round, BBMV with up to 5 mg/mL of protein. (Figure 5 displays hepatopancreatic cells, from which BBMV are made.) Shrimp were dissected by cutting the ventral nerve cord to paralyze their tail. The carapace was cut in a rectangular fashion to expose the hepatopancreas, which was then lifted and placed in a combination of ice cold distilled water and buffer 1 (Figure 6).

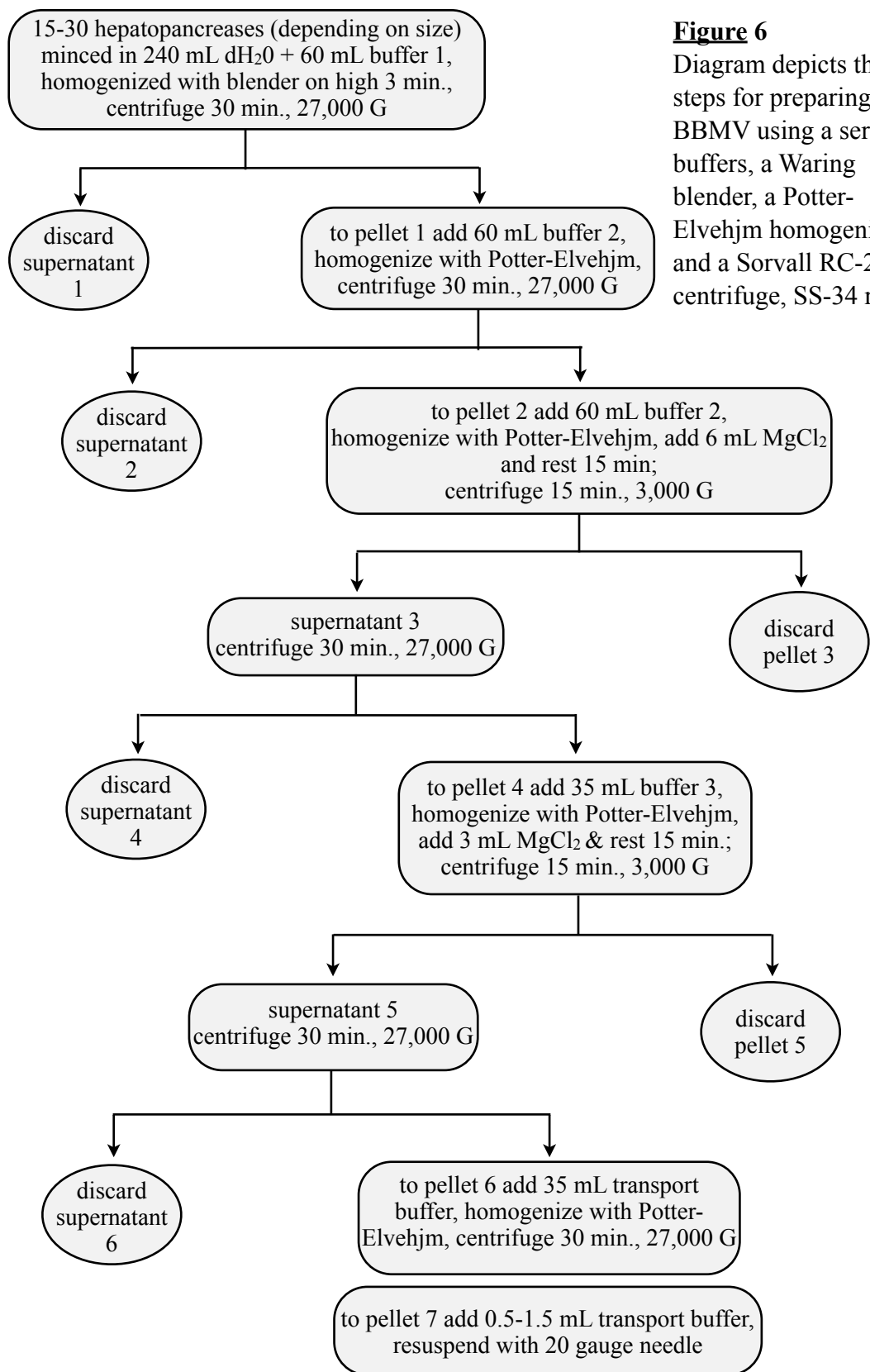
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#### **Figure 5**

Histological section of a lobster hepatopancreas showing the cells and lumens. Purified BBMV of hepatopancreatic epithelia from these organs were made by following the methods based on the  $Mg^{2+}$  precipitation technique developed by Kessler et al., (1978) and Biber et al. (1981) for mammalian epithelia and applied to lobster (*H. americanus*) hepatopancreas (Ahearn et al., 1985). From Factor, Biology of the Lobster: *Homarus americanus*. 1995. 413

White shrimp hepatopancreatic brush border membrane vesicles (BBMV) were prepared by combining a distinct series of homogenizing steps, centrifugation, and buffers (Figure 6). Purified BBMV of hepatopancreatic epithelia from these organs were





**Figure 6**

Diagram depicts the steps for preparing BBMVs using a series of buffers, a Waring blender, a Potter-Elvehjm homogenizer, and a Sorvall RC-2B centrifuge, SS-34 rotor.

made by following the methods based on the  $Mg^{2+}$  precipitation technique developed by Kessler et al., (1978) and Biber et al. (1981) for mammalian eipithelia and applied to lobster (*H. americanus*) hepatopancreas (Ahearn et al., 1985), and to the analogous organs from Japanese penaeid shrimp (Vilella et al., 2003), digestive glands from starfish (Ahearn and Behnke, 1991), and teleost fish pyloric ceca and intestine (Ahearn et al., 1992). The purpose of the preparation was to prepare purified, osmotically-reactive, vesicles of the hepatopancreatic luminal membrane (BBMV) through which nutrients would be transported during absorption of dietary elements.

Pooled hepatopancreases from several animals were minced and made hypotonic by the addition of 240 mL of water and 60 mL of buffer 1. Buffer 1 consisted of 300 mM mannitol, 5 mM ethyleneglycol-bis( $\beta$ -aminoethylether)-N, N' -tetraacetic acid (EGTA), 0.3 mM phenylmethylsulfonylflouride (PMSF), 12 mM tris (hydroxymethyl) aminomethane-HCl, at pH 7.4. The ice-cold mixture was homogenized in a Waring blender, on high, for 3 minutes. Then it was centrifuged with a Sorvall RC-2B centrifuge, SS-34 rotor, for 30 minutes at 27,000 G. The resulting second pellet was resuspended in 30 ml of Buffer 2 (60 mL 300 mM mannitol, 12 mM tris-HCl, 240 mL distilled water, at pH 7.4) and homogenized with a Potter-Elvehjm homogenizer, using ten up and down strokes, while on ice. It was centrifuged again for 30 minutes at 27,000 G. To this second pellet was added 30 mL Buffer 2, followed by homogenization. After this homogenization step, 3 mL of 300 mM  $MgCl_2$ , was added to the slurry which was placed on ice for 15 minutes. Centrifugation followed for 15 minutes at 3000 G. Pellet 3 was discarded, and the supernatant was retrieved and centrifuged for 30 minutes at

27,000 G. Pellet 4 was resuspended in 35 mL of Buffer 3 (60 mM mannitol, 5 mM EGTA, 12 mM tris-HCl, at pH 7.4), then homogenized. The homogenization was followed by the addition of 3 mL of 300 mM  $\text{MgCl}_2$ , and placed on ice for 15 minutes. It was centrifuged for 15 minutes at 3000 G, and the recovered supernatant was centrifuged for 30 minutes at 27,000 G, and pellet 5 was discarded. The resulting pellet 6 was resuspended in 35 mL of Transport Buffer (usually 300 mM mannitol, 12 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), at pH 7.4, but this varied for some experiments) and homogenized. This was followed by centrifugation for 30 minutes at 27,000 G. Pellet 7 was resuspended in enough transport buffer for experimentation, usually 500 - 15000  $\mu\text{L}$ . The vesicles were resuspended using a 20 gauge needle and kept on ice during experimentation. The vesicles were vortexed between treatments during the experiment to keep them suspended at a uniform density.

Vesicles were tested to determine the amount of protein present. A standard curve was prepared using different concentrations of bovine serum albumin (BSA). Then 5 mL of BioRad dye was added to each sample and vortexed. The standards were tested using the Bradford-Stahl test, in a spectrophotometer, which yielded absorbances in the 595 nm range. Three 10  $\mu\text{L}$  samples of the vesicles were tested against the standards (i.e. 10  $\mu\text{L}$  of vesicles, 90  $\mu\text{L}$  transport buffer, 5 mL of BioRad dye). The spectrophotometer assigned absorbance and concentration values to the vesicle samples. The absorbance values of the vesicles fell on the standard curve and were used to estimate protein concentrations of the vesicles. The vesicles used in these experiments were standardized to no more than 5 mg/mL of protein. Standardizing the protein amount for each

experiment was important for comparison. The concentration of protein the vesicles yielded by the vesicles was a critical portion of the calculations done to determine the rate of transport in the vesicles (Ahearn et al., 1985).

Ideally the preparation takes about 4 hours. The experiment takes up to four hours as well.

White shrimp hepatopancreatic BBMV were used in transport experiments conducted at 23°C, using the Millipore filtration technique developed by Hopfer et al. (1973). In these experiments 20 µL of BBMV were added to 180 µL of radio-labeled external medium containing <sup>3</sup>H-D-glucose, <sup>3</sup>H-L-leucine (Amer. Radiochemical Corp.) and other chemical constituents specific to each experiment, which are detailed in the discussion of each experiment. Incubation of vesicles with the selected radio-labeled nutrient were continued for time periods from 10 seconds to 60 minutes after which time 3 mL of ice-cold stop solution (generally the same as the inside medium) was added to stop the uptake process. The chemical composition and pH of the stop solution varied according to experimental conditions. The resulting suspensions were rapidly filtered through Millipore filter paper (0.45 µm) to retain the vesicles and washed with another 2 mL of stop solution. Filters were then added to a Beckman scintillation cocktail and counted for radioactivity in a Beckman LS-6500 scintillation counter. Uptake values were expressed as picomoles/mg of membrane protein per filter. Each experiment was conducted at least 3 times using membranes from different animals and the data were pooled for subsequent analysis. Values are presented as means ± 1 SEM. Statistical differences between means were determined using the non-parametric Kruskal-Wallis H test and significant

differences between treatment means were established at  $p < 0.05$ . Curve-fitting software from Systat Software Inc., Point Richmond, CA, USA (Sigma Plot 10.0) was used to obtain carrier-mediated influx kinetic constants.

### **Questions & Results**

The present investigation extends the work of previous studies and seeks to characterize primary cellular transport mechanisms for white shrimp. The goal is to demonstrate transport mechanisms that will help determine the effective components of a shrimp's diet, thereby enhancing growth, as well as nutrient content.

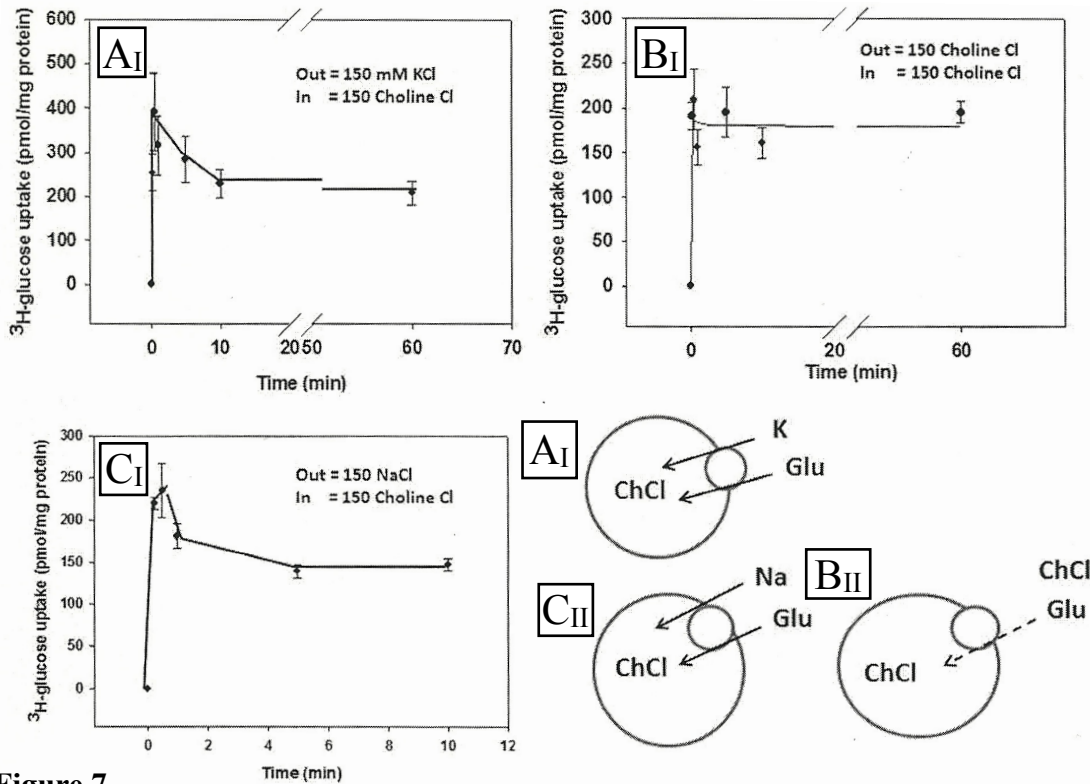
#### **Question 1: Is sodium or potassium a more effective co-transport cation for sugar and amino acid uptake by white shrimp hepatopancreas BBMV?**

Glucose transport has been shown to be stimulated by a  $\text{Na}^+$  gradient in lobster BBMV (Ahearn et. al., 1985). While there is a great deal of similarity in the anatomy of decapods, cellular transport systems for specific nutrients have not been studied in detail. Lobsters are carnivores, consuming little potassium in the foods they eat. Shrimp, on the other hand, eat a great deal of plant material, rich in potassium, as juveniles. These dietary differences suggest a change in ion transport systems, from a potassium co-transporter as juveniles, to a sodium co-transporter as adults. This experiment is designed to demonstrate whether or not sodium or potassium is a more effective symporter for glucose transport.

BBMV were infused with 150 mM of ChCl at pH 7.0 during preparation. The experiment was a time course from 0 to 60 minutes, during which vesicles were incubated with 150 mM of either choline chloride (ChCl), sodium chloride (NaCl), or

potassium chloride (KCl), at pH 7.0 (Figure 7). Figures 7-B<sub>I</sub> & II demonstrate the control for the experiment, thus no catalyst was added for this treatment, only choline chloride. In this instance, there was an influx of <sup>3</sup>H-glucose at 15 seconds, but there is no significant change in the amount of transport for the remainder of the time course. For NaCl (Figures 7-C<sub>I</sub> & II) there is an overshoot at 15 seconds, which implicates SGLT-1

### Fresh White Shrimp Vesicle Transport of glucose with ChCl, NaCl, or KCl pH 7.0 inside & outside

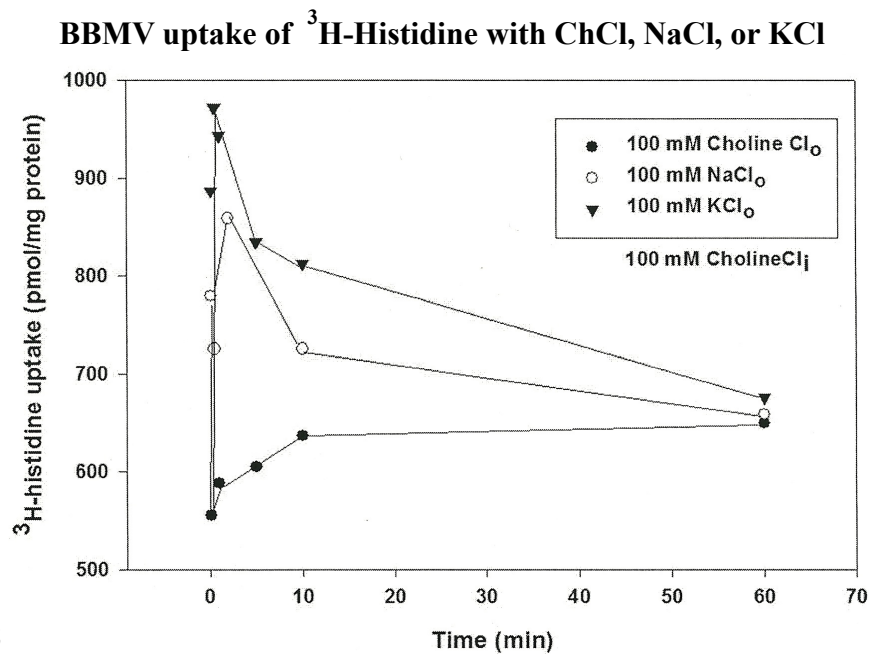


**Figure 7**

This experiment demonstrates the transport of glucose with either ChCl, NaCl, or KCl. A<sub>I</sub> illustrates the transport of glucose with KCl, showing an overshoot at 15 seconds. This is a mechanism that has not been seen much in animals, but it may be attributed to the plant-based diet of the juvenile shrimp. A<sub>II</sub> depicts a BBMV with a potassium-glucose symporter. B<sub>I</sub> is the control for the experiment, with only ChCl on either side of the membrane, B<sub>II</sub> is the BBMV illustration. C<sub>I</sub> is the co-transport of glucose with NaCl, a well-characterized mechanism in a variety of animals. C<sub>II</sub> is the BBMV illustration of this mechanism. Plotted points represent the means of each time period, and were measured in triplicate.

symport. The overshoot for KCl is almost twice that of NaCl, at 15 seconds (Figures 7-AI & II). This transport mechanism substantiates the role of potassium in glucose transport. Potassium-dependent sugar transport has been less-reported in physiological literature than sodium-coupled movements across membranes.

Another time-course experiment seems to bolster the fact that both potassium and sodium are effective co-transporters for nutrients. The vesicles were loaded with 100 mM ChCl during preparation, then washed with 100 mM of either ChCl, NaCl or KCl to aid in the transport of the amino acid  $^3\text{H}$ -histidine (Figure 8). Histidine is an important physiological agent, “[with] vital catalytic roles in proteins” (Glover and Wood, 2007). It



**Figure 8**

This is a graphical depiction of BBMV uptake of histidine using either ChCl, NaCl, or KCl as co-transport. The experiment was a time-course from 0 to 60 minutes. There is a clear overshoot at 15 seconds using KCl and NaCl, with KCl showing the greater effectiveness. The plotted points represent the means of each time period measured in triplicate.

is an essential amino acid, thus it must be obtained from food. Several transporters have been identified as histidine carriers in mammals (Glover and Wood, 2007).

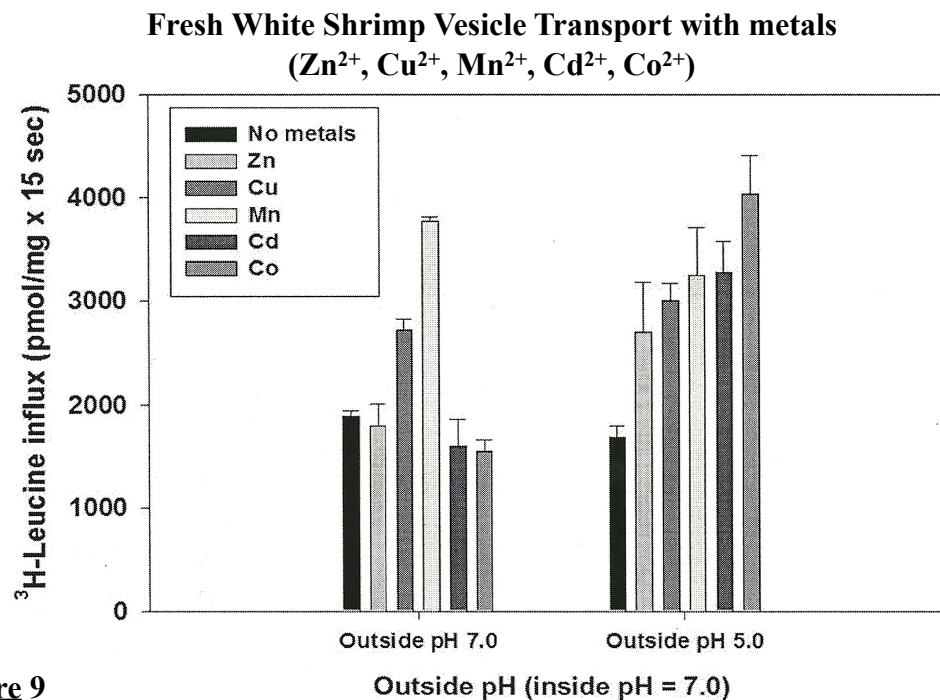
**Question 2: Do metals, such as  $Zn^{2+}$  or  $Mn^{2+}$ , stimulate sugar and amino acid uptake in shrimp hepatopancreatic BBMV?**

Beginning in the 1990s, it was established - via red blood cells and the pig intestine - that amino acid uptake in mammals was stimulated when zinc was present (Tacnet et al., 1990; Horn and Thomas, 1996). Experiments with *Salmo trutta*, brown trout, found zinc as well as copper was important to amino acid transport (Glover et al., 2003; Glover and Wood, 2008). Experiments with lobster intestine showed that zinc highly stimulated the transport of  $^3H$ -L-histidine across the gut when compared to the control condition lacking the metal (Conrad and Ahearn, 2004). Recently zinc was also shown to be effective in the movement of the amino acid L-leucine across the lobster gut (Obi et al., 2011). These comparisons suggest that metals appear to be effective as a co-transport stimulant of nutrient transport in both vertebrates and crustaceans.

BBMV were loaded with 300 mM mannitol during preparation, then incubated with 20  $\mu M$   $^3H$ -leucine, 300 mM mannitol and 20  $\mu M$  of either  $Zn^{2+}$ ,  $Cu^{2+}$ ,  $Mn^{2+}$ ,  $Cd^{2+}$ ,  $Co^{2+}$ , or no metal during the experiment. The pH was 7.0 both inside and outside the membrane. Another experiment was completed using the same parameters, but with pH 7.0 inside and pH 5.0 outside. Each set of experiments, was conducted three times, using five replicates for each treatment (Figure 9). At pH 7.0 inside and outside there is a clear stimulation of leucine when  $Mn^{2+}$  was the catalyst. For pH 5.0 there was increased



transport of leucine with  $\text{Co}^{2+}$ ; and there was also stimulation with  $\text{Mn}^{2+}$ ,  $\text{Cd}^{2+}$ , and to a lesser degree  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$ .



**Figure 9**

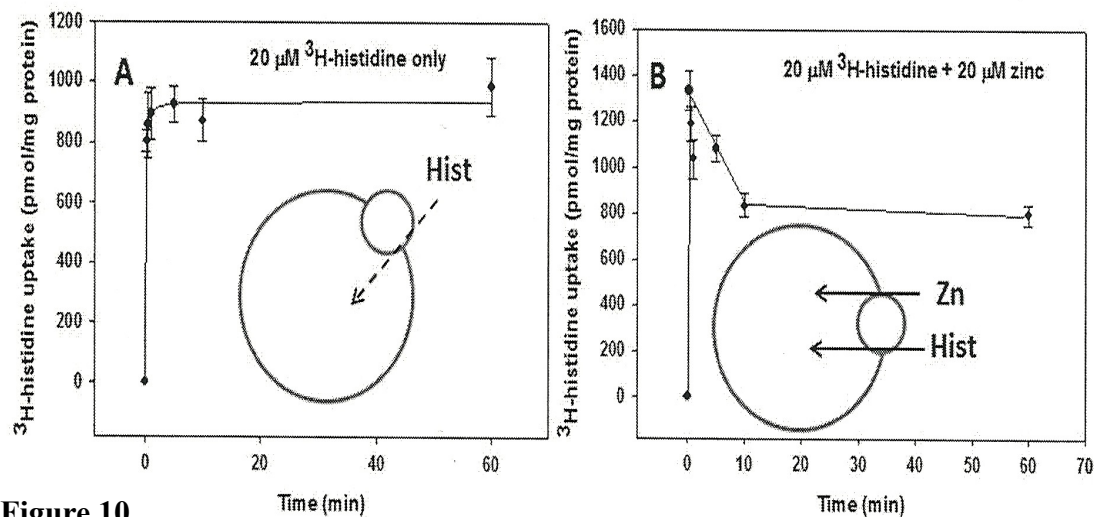
Graph of leucine influx with the aid of metals. BBMV were incubated with 300 mM mannitol during preparation, then washed with 20  $\mu\text{M}$   $^3\text{H}$ -leucine, 300 mM mannitol and 20  $\mu\text{M}$  of either  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Co}^{2+}$ , or no metal during the experiment. In one experiment the pH was 7.0 on both sides of the membrane. In another experiment the pH was 7.0 on the inside and 5.0 outside. At pH 7.0 there was a clear stimulation by  $\text{Mn}^{2+}$ . At pH 5.0  $\text{Co}^{2+}$  increased the transport of leucine, but  $\text{Mn}^{2+}$  and  $\text{Cd}^{2+}$  were also stimulatory.

An additional experiment reinforced the fact that metals stimulate nutrient uptake.

Histidine influx was measured without the benefit of a catalyst, then with the aid of zinc as a stimulant (Figure 10). Transport of the amino acid was measured from 0 to 60 minutes, and each treatment was done in triplicate. Vesicles were loaded with 300 mM

mannitol during preparation, then bathed in 300 mM mannitol, 20  $\mu\text{M}$   $^3\text{H}$ -histidine for the control (Figure 10A). For the variable all of the previous parameters were in place

### Time Course of $^3\text{H}$ -Histidine Transport using Zinc



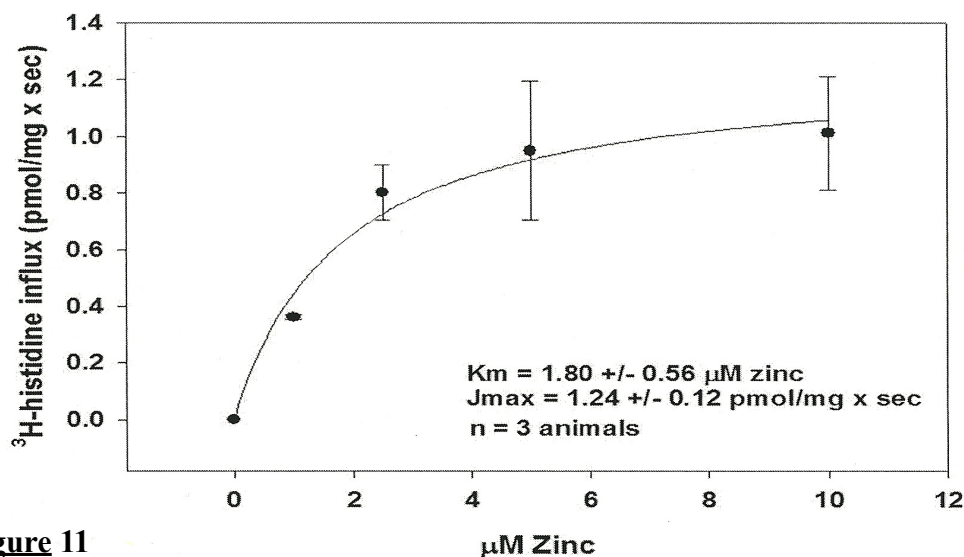
**Figure 10**

Histidine influx was measured without the benefit of a catalyst, then with the aid of zinc as a stimulant. Vesicles were infused with 300 mM mannitol during preparation, then bathed in 300 mM mannitol, 20  $\mu\text{M}$   $^3\text{H}$ -histidine for the control. For the variable all of the previous parameters were in place and 20  $\mu\text{M}$  zinc was added. Zinc helps to aid histidine across the membrane at a greater rate than when no catalyst is present.

and 20  $\mu\text{M}$  zinc was added (Figure 10B). Zinc helped to aid histidine across the membrane at a greater rate than when no catalyst was present.

To further explore the histidine transporter's affinity for  $\text{Zn}^{2+}$  and/or  $\text{Mn}^{2+}$  two separate experiments were designed using Michaelis-Menten parameters. In both experiments, BBMV were loaded with 300 mM mannitol at pH 7.0 during preparation, then treated with 300 mM mannitol, variable concentrations of  $\text{Zn}^{2+}$  (0 - 10  $\mu\text{M}$ ) (Figure 11) or  $\text{Mn}^{2+}$  (0.01 - 1.5  $\mu\text{M}$ ) (Figure 12), and 20  $\mu\text{M}$   $^3\text{H}$ -histidine. The treatments were

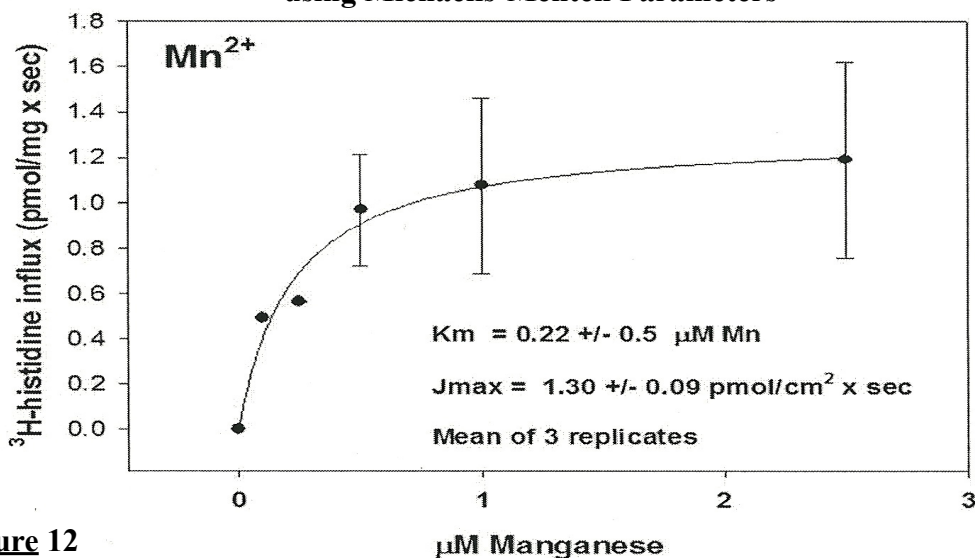
### Effect of External $[Zn^{2+}]$ on Histidine Uptake using Michaelis-Menten Parameters



**Figure 11**

This graph displays histidine uptake using  $Zn^{2+}$  as a catalyst. BBMV were loaded with 300 mM mannitol. During the experiment they were incubated with 300 mM mannitol,  $[Zn^{2+}]$  within the range 0 - 10  $\mu\text{M}$ , and 20  $\mu\text{M}$   $^3\text{H}$ -histidine for five replicates per treatment. The pH was 7.0 on both sides of the membrane.

### Effect of External $[Mn^{2+}]$ on Histidine Uptake using Michaelis-Menten Parameters



**Figure 12**

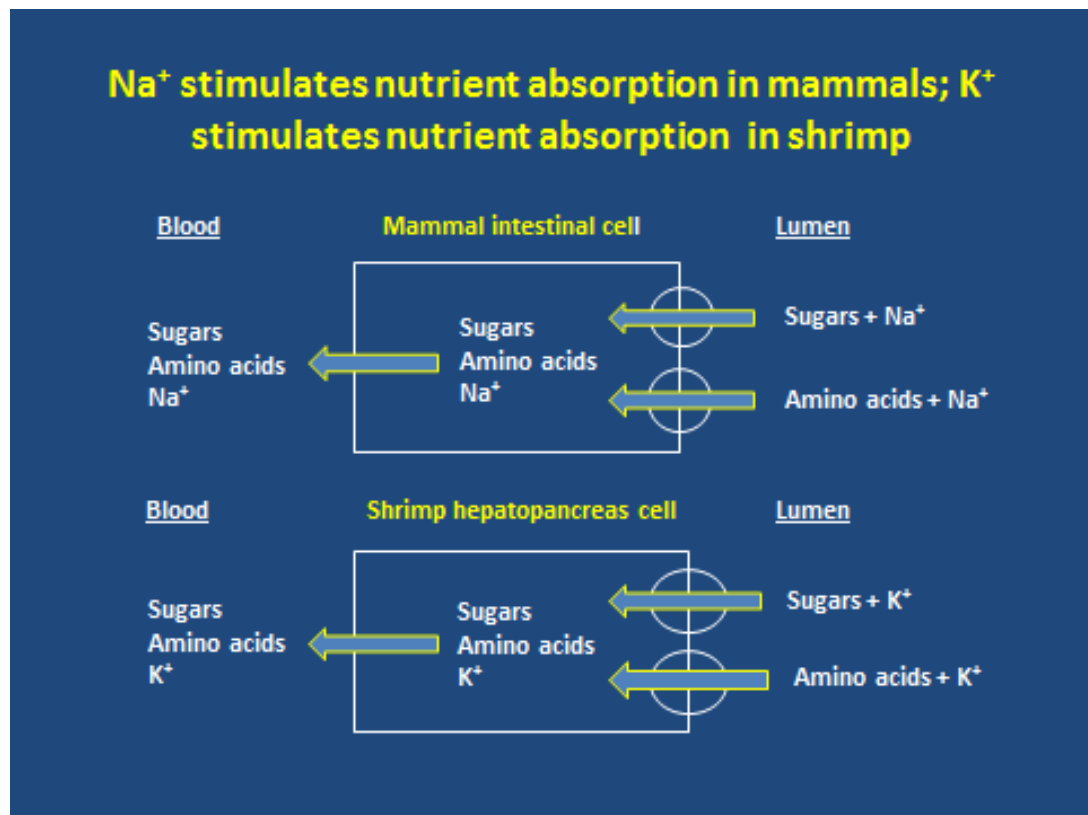
This graph displays histidine uptakes using  $Mn^{2+}$  as a catalyst. BBMV were loaded with 300 mM mannitol. During the experiment they were incubated with 300 mM mannitol,  $[Mn^{2+}]$  within the range 0.01 - 1.5  $\mu\text{M}$ , and 20  $\mu\text{M}$   $^3\text{H}$ -histidine for five replicates per treatment. The pH was 7.0 on both sides of the membrane.

done in triplicate, 15 second uptakes. The histidine transporter appeared to have an almost ten-fold greater affinity for manganese, (e.g. lower  $K_m$  value) when compared to zinc.

## **Discussion**

The present investigation seeks to characterize the use of  $\text{Na}^+$ ,  $\text{K}^+$ , and metals in sugar and amino acid transport across BBMV in the shrimp. The results suggest that there is a sodium/potassium-dependent glucose transport system that resembles the SGLT1 system of vertebrates, except the shrimp transport system can accept both sodium and potassium as cofactors, while the vertebrate system is restricted to sodium stimulation (Fig. 7). This co-transport system works by making use of the  $\text{Na}^+$  gradient. Since  $\text{Na}^+$  has a low intracellular concentration, it moves along its concentration gradient into the cell, and glucose moves with it and is released in the cell. From there the basolateral,  $\text{Na}^+$ -independent, GLUT-2 system carries the sugar to the hemolymph. The  $\text{Na}^+/\text{K}^+$  pump is used to maintain the low intracellular concentration of  $\text{Na}^+$ , and thus glucose can be continuously transported. Young shrimp eat a considerable amount of marine algae which contains high concentrations of potassium. Therefore, it is conceivable, that for shrimp at this early life history stage there may also be a potassium gradient across the hepatopancreatic luminal membrane which may also serve as a driving force, like sodium, for nutrient uptake (Figure 13).

It has been previously established that most solutes are transported into cells by the downhill movement of specific ions (Parenti et al., 1992). In other transport systems  $\text{K}^+$  has been shown to be a competitive inhibitor of  $\text{Na}^+$  and stimulates the uptake of amino



**Figure 13** This is a model of how potassium-dependent nutrient transport in shrimp is different than sodium-dependent nutrient transport in mammals. It seems that potassium can use the same co-transport mechanism as sodium to transport sugars and amino acids into the cell. In both cases the ion diffuses down its electrochemical gradient into the cell via facilitated diffusion, and the sugar or amino acid are co-transported up their respective concentration gradients. Cation-independent transport for both sugars and amino acids occurs across the basolateral cell membrane to the blood as in mammals.

acids. In the lepidopteran larvae midgut the co-transport mechanism for L- leucine, a nonpolar, essential amino acid, was strongly dependent on the concentration of potassium on both sides of the membrane. The transport was enhanced by both a negative intracellular membrane potential and a pH gradient (Parenti et al., 1992). The results of the experiments in this thesis make it clear that both Na and K are stimulatory for glucose

in shrimp, without a pH gradient (Figure 7). The overshoot at 15 seconds (Figures 7-A<sub>1</sub> & II) demonstrates the fact that transient transport of glucose occurs against a concentration gradient using sodium or potassium as alternative co-transport stimulators. Following transient transport against a concentration gradient at 15 seconds intracellular radiolabelled sugar and amino acid concentrations reach equilibrium with their respective concentrations in the external media as shown by the constant rate of uptake by 5 to 10 minutes of incubation.

As with the uptake of <sup>3</sup>H-D-glucose by shrimp BBMV, the uptake of the amino acid <sup>3</sup>H-L-histidine was also transiently stimulated by the presence of either sodium or potassium in the incubation medium (Figure 8). In this instance a greater stimulatory effect on the amino acid accumulation occurred in the presence of potassium than sodium, suggesting that the former was a preferred co-transport substrate for the transport event. It is well-known in the insect transport literature that herbivorous insects display potassium-stimulated transport systems for several amino acids (Parenti et al., 1992; Parenti et al, 2001; Forcella et al., 2006), and this relates to the presence of a specific potassium-dependent carrier protein, KAAT1, present in their digestive tract absorptive epithelial cells (Castagna et al., 1998). The strong potassium stimulation of L-histidine uptake by shrimp BBMV suggests that a crustacean isoform of the insect KAAT1 might be present in shrimp, and contribute to amino acid uptake.

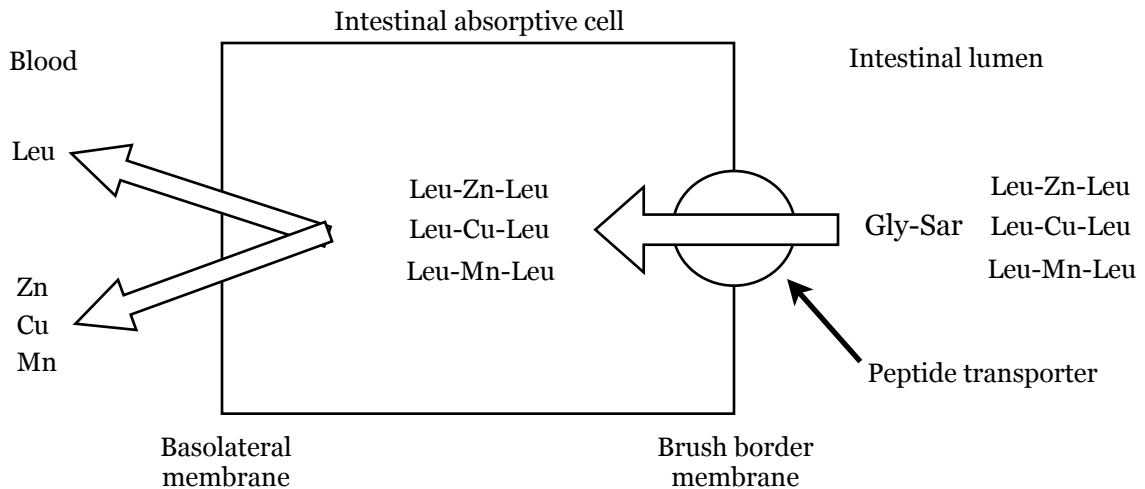
In addition to amino acid transport occurring by way of several distinct amino acid transport carrier systems, with various substrate specificities, recent work has suggested that amino acids may also be transported across vertebrate and invertebrate biological

membranes complexed with metallic cations (Tacnet et al., 1990; Horn and Thomas, 1996; Glover et al., 2003; Conrad and Ahearn, 2004, 2007; Glover and Wood, 2007, 2008a,b). This metal-dependent transport appeared to occur by bis-complexes formed between two amino acids and a single divalent cation. Such a molecular configuration would resemble a dipeptide where two amino acids are linked by a peptide bond. It was therefore suggested that these bis-complexes might be transported by molecular mimicry using a brush border dipeptide transport protein such as PEPT1, which has been thoroughly characterized in mammals and other vertebrates (Adibi, 1997).

Figure 14 shows that a variety of different amino acid configurations can be associated with metals in solution forming many bis-complexes that appear to be accommodated, with varying affinities, to the dipeptide transporter. The PEPT1 has been described as a promiscuous carrier process, because it transports such a wide range of di- and tripeptide combinations. In fact, its specificity is so broad that drug companies now use this carrier mechanism for the absorption of several different drugs that otherwise have difficulty passing across the absorptive human intestine.

Recent work in the Department of Biology at the University of North Florida has suggested that amino acid transport by bis-complex formation with zinc and other metals may be a means by which these nutrients are transported in crustacean digestive tract (Conrad and Ahearn, 2004, 2007; Mullins and Ahearn, 2008; Obi et al., 2011). In lobster

### Molecular mimicry increases absorption of both amino acids and metals



**Figure 14**

This diagram illustrates a possible mechanism for the movement of amino acids across the intestinal brush border membrane by mimicry. Metals form a bis complex with two amino acids, and the combination structurally resembles a dipeptide with the metal ion substituting for a peptide bond.

(*Homarus americanus*) intestine transmural mucosal to serosal transport of both L-histidine and L-leucine were stimulated by luminal zinc and these transports were inhibited by the presence of glycylsarcosine, a normal dipeptide substrate for the PEPT1 transport protein (Conrad and Ahearn, 2004; Mullins and Ahearn, 2008). In the present study,  $^3\text{H}$ -L-leucine transport across shrimp hepatopancreatic BBMV was stimulated by several different metals ( $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Cd}^{2+}$ , and  $\text{Co}^{2+}$ ) and the stimulation appeared greater when the luminal pH was acidic (e.g., pH5.0) (Figure 9). The PEPT1 transporter is proton-dependent in vertebrate cells, and the data in Figure 9 suggests that bis-complex



transport, presumably by a crustacean analog of the PEPT1, may also be proton-dependent as well.

Metals in a bis-complex appear to be able to drive their associated amino acids against a transient concentration gradient (Figure 10) as was previously seen with sodium and potassium transport enhancement of D-glucose (Figure 7) and L-histidine (Figure 8). In this instance 20  $\mu$ M zinc led to a temporary two-fold accumulation of 20  $^3$ H-L-histidine against its equilibrium concentrations in shrimp BBMV (Figure 10).

In the present work, Figures 11 and 12 show that L-histidine transport across shrimp hepatopancreatic BBMV were hyperbolic functions of external zinc or manganese concentrations. Similar results were found for zinc stimulation of  $^3$ H-L-leucine transport across the intestine of the lobster (Mullins and Ahearn, 2008; Obi et al., 2011). In both cases the presence of saturation kinetics of each amino acid by increasing metal concentrations clearly implies that the respective amino acids were co-transported and activated with either zinc or manganese. It is likely that the same carrier type was present in both organs (hepatopancreas and intestine) and that it performs the same basic bis-complex transport, involving the amino acids and metals, by way of an apparent PEPT1 carrier protein. Because the  $K_m$  for bis-complex transport of L-histidine involving manganese by hepatopancreatic BBMV was lower (0.22  $\pm$  0.05 mM  $Mn^{2+}$ ; Figure 11) than that displayed by zinc (1.80  $\pm$  0.58 mM  $Zn^{2+}$ ; Figure 10), these results suggest that the shrimp bis-complex transporter exhibits a greater apparent affinity for manganese than for zinc. In fact, the recent work by Obi et al. (2012) shows that for the lobster intestine there is a bis-complex transport hierarchy involving the stimulatory capacity of

all divalent cations tested ( $Mn^{2+}$ ,  $Cu^{2+}$ ,  $Zn^{2+}$ ,  $Ca^{2+}$ ) with manganese enhancing the transport of L-leucine the greatest and calcium the least.

The feeding strategies for shrimp aquaculture include: 1) no fertilizer or feed input; 2) a diet supplemented with fertilizer; 3) a natural diet supplemented with live organisms; or 4) a diet composed completely of external nutrient sources. Choice #1, no fertilizer or feed input, involves simply an extensive pond system totally dependent on live food organisms in the pond. Choice #2, a diet supplemented with fertilizer added to the cultured pond, consists of chemical or organic compounds such as animal manure, plant cuttings, fresh agricultural compost, and microbial ingredients. Choice #3 is a diet that includes “low-cost agricultural or animal byproducts....which may involve mill sweepings, beer waste, or rice bran” in the form of “feed mash or pellet” (Tacon, 1988). Choice #4, the complete diet, is a “nutritionally complete high quality food” (Tacon, 1988), the contents of which have been pre-determined to maximize growth. This diet comes in the form of a moist or dry pellet. The diets could also be “trash fish, cultured live organisms such as *Artemia nauplii*, or a combination of both (Food and Agriculture Organization of the United Nations, 2012). The choice of feeding methods involves many factors and varies from farmer to farmer, country to country. The deciding factors include: whether or not its for local or home consumption; whether or not it is a cash crop or export; the product’s true market value; the financial resources of the farmer; and the availability of fertilizer and feed.

Pond culture of aquatic organisms, such as penaeid shrimp, has been occurring for more than 30 years, but a sense of urgency is upon us as the demand for more effective

food production becomes greater with explosive human population growth. The experiments presented here represent the most successful attempts from a larger group of investigations performed by this researcher. They verify that the hepatopancreas is a complex organ, with multifaceted roles. The concern for the composition of aquaculture feed is a complicated issue. It is an urgent concern as the demand for seafood increases more rapidly than commercial fishing can supply. The Food and Agriculture Organization in its 2012 report states that “considering past trends and current predictions, the sustainability of the aquaculture sector is more likely to be closely linked to the sustained supply of terrestrial animal and plant proteins, oils and carbohydrate sources for aquafeeds. Therefore, the aquaculture sector should strive to ensure sustainable supplies of terrestrial and plant feed ingredients”. One goal is to “[ensure] national quality standards for feed raw materials, feed additives, and “[improve] feed formulation and production” (Food and Agriculture Organization of the United Nations, 2012). This current study contributes to that goal by illustrating the role of potassium and divalent metals as co-transporters and stimulators of sugar and amino acid absorption, an understanding of which may prove essential to increasing shrimp production and nutritive quality.

The goal is to create a nutritionally complete high quality food, and there are some components that are clear from the literature. The protein requirement is 28-32% for *Penaeus setiferus* (Shiau, 1998). According to Shiau, “simple sugars are poorly utilized by shrimp”. Starch, at 25%, is the better option for growth (Shiau, 1998). Chitin, as a major structural component of the shrimp exoskeleton, should be included in the diet at a

minimum level of 0.5% (Shiau, 1998). Cholesterol has been shown to increase growth rates at values from 0.5 to 2.1% for various species of shrimp, and phospholipids from 0.84 to 1.25%. Vitamin requirements have not been reported for *Penaeus setiferus*, but a range of requirements have been characterized for *Penaeus monodon* and *Penaeus japonicus* (Shiau, 1998). In addition, minimum and maximum tolerance of mineral content has not been characterized for *Penaeus setiferus*, but this dietary source is necessary due to “repeated losses of certain minerals during molting” (Shiau, 1998). The current set of experiments suggests that potassium should be included as a vitamin, and there should be a ratio of minerals in the form of divalent metals. Further experimentation is required to determine the exact potassium content and mineral ratio. Previous studies by S. Ahamad Ali on *Penaeus indicus* suggest that 13.6 to 22.7 mg of copper be added per 100 g of feed. The growth of this species was not increased by manganese supplementation, but the survival rate of the shrimp improved, thus the level of this metal “may be very low”. It was also suggested that zinc be added to the *Penaeus indicus* diet at 23.6 to 38.6 mg%. (Ali, 2000)

“Feeds represent over 50% of the production costs on intensive shrimp farms..., and they [contribute] to the sludge at the bottom of the ponds” (Rosenberry, 2009). Thus it is imperative that the composition of that feed be as effective at increasing shrimp growth and production in the shortest time period possible, while not playing a part in polluting the water, to ensure profitability. Current feeds include trash fish, organic waste, and fishmeals that are greatly wasted due to their breakdown in the water. This is a highly

wasteful and expensive method. To be more strategic and deliberate in creating the feed would be much more cost effective.

Future studies could explore the role of these transport mechanisms with other essential amino acids, including leucine. It is also necessary to determine the appropriate concentration and combination of metals, potassium, and other components to maintain the standard of “nutritionally complete high quality food” product (Tacon, 1988). Furthermore, it is essential that the additives do not cause extra contamination in pond cultures. The ultimate goal is to inform farmers of the appropriate micronutrient composition for their cultures and feed.

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## Vita

Tamla Simmons has a Bachelor of Science degree from Hampton University in Biology, and expects to receive a Master of Science in Biology from the University of North Florida in December 2012. Dr. Gregory Ahearn, of the University of North Florida, serves as her thesis advisor. Tamla is currently employed as a high school Biology teacher at Stanton College Preparatory. She teaches Biology I Honors to ninth graders, and AP Biology to twelfth graders. Prior to teaching, she did Alzheimer's research at Mayo Clinic Jacksonville. Tamla has a great interest in nutrition and health, and plans to continue studies in this area.

She is the oldest of three children, a brother Timothy, and sister Shayla. They are founding members of her "trustee board". She also has two unofficial sisters, Janae' Ostolaza Sepulveda and Altoria White, and very close friends Mary Garrison and Tia Thomas. They all held her hand, and were her confidants throughout this process. She is a proud aunt to two nieces and one nephew. She has an incredible circle of doting friends and family.

### Publications

with Dr. Floyd B. Willis, MD, "*Learning About Dementia in Blacks*" brochure,  
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with O'Rourke, Shelia D., Graff-Radford, Neill R. MD, Lucas, John A. PhD, Willis, Floyd B. MD, "*Attitudes of Elderly African American Dementia Research Participants Toward Research and Autopsy*", © copyright 2003 Mayo Foundation

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