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Effect of Feed Additives on Amino Acid and Dipeptide Transport by Intestines of American Lobster and Atlantic White Shrimp

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Effect of Feed Additives on Amino Acid and Dipeptide Transport by Intestines of American Lobster and Atlantic White Shrimp

by

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ABSTRACT

Previous nutritional physiology research using L-histidine and zinc in American lobster intestine (*Homarus americanus*) has suggested that these solutes can be co-transported as complexes (Histidine-Zinc-Histidine) across the intestine using a peptide transporter. Furthermore, transport of L-leucine was shown to be inhibited by high calcium concentrations. Dipeptide and bis-complex transport and the role of calcium were investigated in the perfused intestines of lobster and Atlantic white shrimp (*Litopenaeus setiferus*). Following trans-intestinal transport, serosal medium was analyzed for amino acid composition by gas chromatography. In lobster, the transport of glycylsarcosine (Gly-Sar) from mucosa to serosa was stimulated two-fold with luminal pH 8.5, compared to the pH 5.5 control. Mucosa to serosa and serosa to mucosa fluxes of Gly-Sar were measured; the dipeptide was transported intact in both directions, but the net flux was from mucosa to serosa. The use of 0.5mM calcium chloride stimulated Gly-Sar transport two-fold, compared to 25 mM. In shrimp, the addition of 50 µM zinc chloride increased the rate of L-histidine transport, while Gly-Sar inhibited histidine transport in the presence of zinc. The rate of histidine transport was significantly higher with 1mM calcium chloride than with 25mM. These results suggest that shrimp transport bis-complexes in a manner similar to lobster. High calcium concentration had an inhibitory effect on both amino acid and dipeptide transport. Proposed mechanisms accounting for the effects of metals and calcium on trans-intestinal transports of both amino acids and dipeptides by lobster and shrimp digestive tracts are discussed.

INTRODUCTION

Aquaculture Practices

Aquaculture is expected to play an important role in the near future as the global population expands and the demand for high quality, rapidly produced protein increases. In fact, the production of domestic seafood has increased steadily since the 1980s, with a current estimated output of 500,000 metric tons at harvest per year, valued close to $1 billion (Nash, 2004). Aquaculture is considered one of the fastest-growing global food industries; accordingly, the US Department of Commerce has an updated aquaculture policy which includes the intent to increase domestic production by a factor of five by the year 2025 (Nash, 2004). Farmed shrimp are of particular interest because they can be grown and harvested within a year and the majority of the animal is edible. However, the profit margin for farming shrimp can be low due largely to costs from loss of animals to disease, the low efficiency of weight gain from feed and environmental regulation of tank wastewater (Martínez-Porchas et al., 2010). Feed production and formulation is an important area of research in the aquaculture industry in order to improve the efficiency of nutrient conversion to body mass.

The production of farmed shrimp, as well as other fish and crustacean species such as lobster, has relied on the use of commercially produced feed that is formulated mainly from fish meal and fish oil (Rust et al., 2011). This type of feeding method is convenient because fish meal contains essential nutrients in optimal proportions and is easily digestible (Rust et al., 2011). However, it is becoming progressively more

expensive to continue to catch and process fish for fish meal because population sizes are decreasing and the cost of fuel for fishing boats is increasing (Rust et al., 2011). Several US organizations, including the National Oceanic and Atmospheric Administration and the US Department of Agriculture, are promoting research into alternate sources of protein for use in feed, such as soybeans, green peas, or algae (Terova et al., 2013). While many plants contain enough protein for sustaining shrimp and other species, they do not contain all of the essential micronutrients needed and may not have specific essential amino acids in the appropriate concentrations (Rust et al., 2011). The information learned from the present study will be used to help elucidate which additives could be used in commercial feed to increase amino acid and peptide uptake, thereby increasing nutrient absorption and the efficiency of body mass gain. Incorporating this knowledge into feed formulation could significantly reduce the overhead costs of aquaculture, which would aid in expanding the domestic production of farmed shrimp and encourage the limited lobster aquaculture business (Factor, 1995).

In addition to feed, crustacean growth is also affected by water quality. It has been reported that shrimp can survive in near freshwater conditions; however, salinities that are at least 50% of that of seawater are preferred by juveniles as this approximates natural estuarine conditions (Jacoby, 2012). Lobsters do not have such a wide range of water quality tolerance and must be maintained in oxygenated, 32-35 ppt saltwater (Factor, 1995). The water quality for farmed species depends on the water source, as well as any treatment processes used by the facility. Current aquaculture practices, for

shrimp in particular, make use of large raceways that provide a constant flow of water from nearby sources (Martínez-Porchas et al., 2010). By determining the ideal water quality for growth, farms can be established where existing well or surface water meets these requirements, or can be easily treated to create ideal water conditions.

Calcium mainly functions as a structural component in crustacean exoskeletons, and is necessary for nerve impulse transmission and osmoregulation (National Research Council, 1993). Earlier studies have determined the optimal levels of particular dietary minerals and established the requirements for calcium to be 1-2 g per 100 g diet for *Penaeid* shrimp. However, most calcium can be absorbed through drinking, as long as mineral sources are soluble at the same pH as that of the intestinal lumen, or by direct absorption through gills or skin (Davis and Gatlin, 1996). Studies in lobster (*Homarus americanus*) have found that a small calcium to phosphorous ratio (0.56 : 1.10) is ideal during the juvenile stage, while a ratio above 1:1 is desirable for adults (Factor, 1995). It has been recommended that calcium in particular should be limited in prepared feeds, due to its deleterious effects when interacting with other nutrients, such as phosphorus and magnesium (Davis and Gatlin, 1996). In addition, recent work in the Department of Biology at the University of North Florida has shown that calcium has an inhibitory effect on amino acid transport in lobster intestine, though the exact mechanism is unknown (Abdel-Malak and Ahearn, 2014). The effects of calcium, sulfate, and zinc on nutrient transport will be investigated here to provide further information about optimizing crustacean growth.

Nutrient Transport

Amino acids are vital nutrients, some of which are only acquired in the diet. They are typically absorbed by cells and used to manufacture new proteins, but they also have several non-protein functions. For instance, certain amino acids can be used in gluconeogenesis as a source of energy, while other amino acids function as neurotransmitters. Therefore, the study of amino acid absorption by the gastrointestinal tract is necessary for ensuring the health of many farm-raised animals. In both shrimp and lobster, the hepatopancreas is the major organ of digestion, where enzymes are produced and absorption of sugars and amino acids occurs after passage of digested food materials from the cardiac and pyloric stomachs (Mantel, 1983). However, the anterior part of the intestine also contains a dense lining of columnar epithelial cells with microvilli borders, suggesting absorptive functions (Mantel, 1983). This histological relevance to nutrient absorption has been confirmed by many studies examining the kinetics of nutrient transport in lobster and shrimp intestines (Ahearn 1976, Ahearn and Maginniss, 1976; Brick and Ahearn, 1978; Conrad and Ahearn, 2005, Abdel-Malak and Ahearn, 2014).

As large molecules that may either be charged or neutral, amino acids must be transported across the plasma membrane of intestinal epithelial cells via transport proteins (Figure 1). These proteins typically co-transport amino acids and sodium ions into the cell using the ATP-powered sodium-potassium pump located on the basolateral side which results in the movement of sodium down its concentration gradient, thus

pulling the amino acid into the cell (Bröer, 2008). The amino acids can then exit the cell on the basolateral side by facilitated diffusion through another transmembrane protein, since the concentrations of amino acids are greater inside the cell than in the blood (Bröer, 2008). The absorption of amino acids has been extensively studied in mammals and the pathways for their transport from the lumen to the blood have been characterized (Bröer, 2008). However, amino acid transport in crustaceans has yet to be fully understood. Previous studies have looked at the mechanisms involving one or two amino acids and many ions, including H^{+}, Na^{+}, K^{+}, Ca^{+2}, Cu^{+2}, and Zn^{+2} (Conrad and Ahearn, 2005; Glover and Wood, 2008; Daniel and Kottra, 2004).

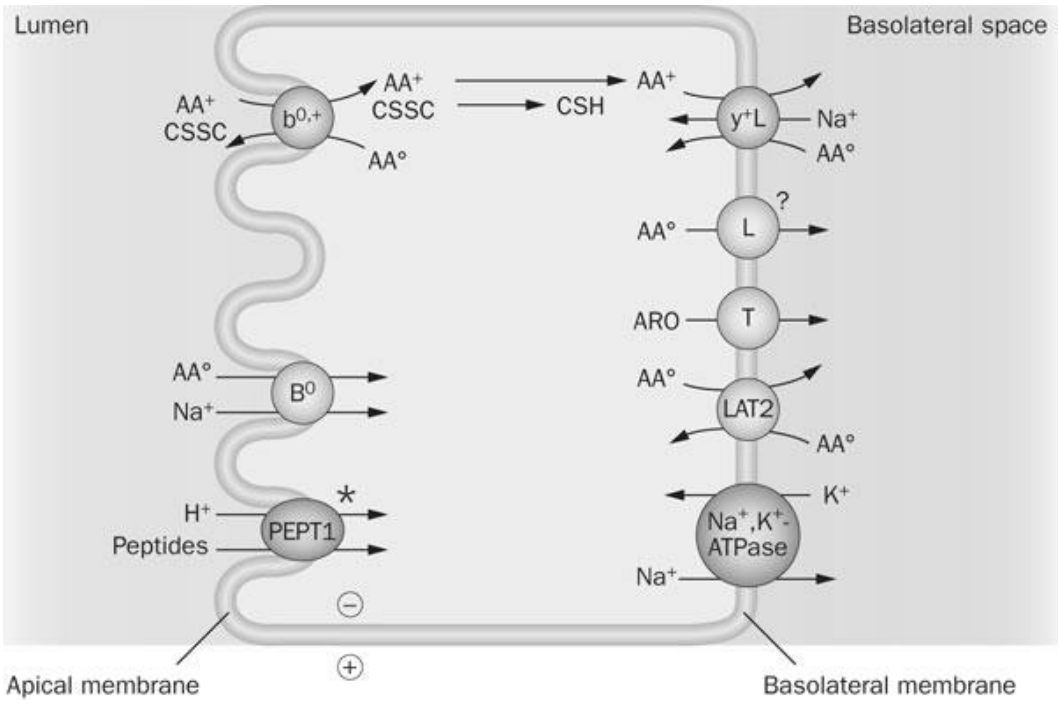

Figure 1. Transport of amino acids and peptides in small intestine epithelial cells. On the apical membrane, peptides are cotransported with protons into the cell via PepT1. These protons are supplied by a sodium-proton exchanger (NHE) on the apical membrane, not pictured here. Amino acids are cotransported with sodium by transporters on the apical membrane (B^{o}) and by antiport with sodium-independent processes ($b^{o,+}$). They exit the cell by antiport with sodium ions ($y^{+}L$) or by facilitated diffusion down their concentration gradients (L,T, LAT2). The amount of sodium in the cell is maintained by the sodium-potassium ATPase pump, which provides the driving force for apical transport. Source: Chillarón et al., 2010.

It has been recently found that some amino acids can be moved across intestinal epithelia using metals as co-transporters (Conrad and Ahearn, 2005; Glover and Wood, 2008; Obi et al., 2011; Abdel-Malak and Ahearn, 2014). It is thought that two amino acids combine with a metal to form a bis-complex (e.g., Histidine-Zinc-Histidine) which is then taken up by a dipeptide transporter similar to PepT-1 (Conrad and Ahearn, 2005). This proton-oligopeptide transport system (PepT-like) requires a proton gradient to

function (Figure 1). In mammals, protons are supplied by the apical sodium-proton exchanger (NHE), which are cotransported across the apical membrane with di- and tri-peptides (Watanabe et al., 2005). Researchers proposed that the dipeptide transporter was in use because transport was increased when a proton gradient was present and when zinc and L-histidine were present together in the lumen, but decreased when a competing dipeptide was added (Conrad and Ahearn, 2005). Analysis with other amino acid-ion combinations, including L-histidine and copper, showed a similar pattern (Glover and Wood, 2008). Their research suggested that under conditions where an excess of histidine is available for uptake, as is common in commercial fish pellets, more histidine could be transported when chelated with copper since this would increase the number of pathways for histidine to be absorbed (Glover and Wood, 2008).

The goals of the present study will be to determine if a similar transport pathway is present in shrimp, and if they also have the ability to transport amino acids in a bis-complex formation through potential PepT-like transporters, as has been suggested in lobster and trout (Conrad and Ahearn, 2005; Glover and Wood, 2008; Abdel-Malak and Ahearn, 2014). This will be accomplished by analyzing the effect of zinc on amino acid transport. Since shrimp are crustaceans and have similar digestive physiology, it is thought that their nutrient uptake pathways will be comparable to those found in lobster, and therefore results found in this study may be applicable to both species. One key difference in this study will be the use of a neutral dipeptide, glycylsarcosine. This dipeptide has been used extensively to characterize transporters through competitive interactions along the luminal brush border membrane (Daniel and Kottra,

2004). However, few studies have examined transport through the basolateral membrane. One recent study analyzed the uptake of glycylsarcosine from the basolateral side of the epithelium in human Caco-2 cells, and found it to be pH-dependent (Bethelsen et al., 2013). This is significant because it shows the ability of epithelia to transport small peptides from the blood to epithelial cytosol. It also gives a cellular mechanism whereby small peptides can be transported intact across an epithelium without first being broken down into their constituent amino acids. Transepithelial transport of glycylsarcosine will be investigated here to give further evidence for the basolateral transport of small peptides, which has had limited study thus far. This information could be used in feed formulations, since small peptides could be incorporated instead of pure amino acids, which may result in more efficient nutrient uptake and lower cost feed.

Hypotheses

Hypothesis 1: Amino acids will form a bis-complex with zinc ions and will be taken up by a putative dipeptide-like transporter.

The ability of amino acids, specifically L-histidine and L-leucine, to combine with metal ions, including zinc and copper, has been previously tested by sequentially perfusing a series of treatments through the intestine (Conrad and Ahearn, 2005; Glover and Wood, 2008; Obi et al., 2011). The first treatment had amino acids, the second treatment had the same concentration of amino acids plus a metal, and the third treatment had amino acids, metal, and a potential competitive inhibitor, such as a dipeptide. It has been

shown in lobster and trout (Conrad and Ahearn, 2005; Glover and Wood, 2008; Abdel-Malak and Ahearn, 2014) that L-histidine and L-leucine are transported at a higher rate when metal is added, and are inhibited by the addition of a dipeptide. These experiments will be duplicated with shrimp intestine to give evidence for the existence of the same transport system in these closely related crustaceans.

Hypothesis 2: Glycylsarcosine will be transported across the basolateral membrane intact.

Much previous work has focused on the apical, or brush-border, transport of peptides and amino acids, and the transporters have been well characterized (Bröer, 2002; Daniel and Kottra, 2004). Less emphasis has been placed on transporters on the basolateral side, where these nutrients exit the cell and enter the blood stream. Additional experiments will be performed to demonstrate the movement of a neutral dipeptide, glycylsarcosine, across the basolateral membrane. It is as yet unclear in invertebrates whether dipeptides are completely broken down enzymatically within the cell prior to transport, though there is evidence for basolateral transporters of small peptides in vertebrates (Dyer et al., 1990; Shepherd et al., 2002; Rønnestad et al., 2010; Bethelsen et al., 2013).

Hypothesis 3: Amino acid and dipeptide transport will be stimulated or inhibited by changes in luminal pH.

Many previous experiments have shown that a luminal pH lower than that of the bath, or outer pH, stimulates the transport of L-histidine or L-leucine (Conrad and Ahearn,

2005; Simmons et al., 2012; Watanabe et al., 2005). This effect will be studied by using saline with the same pH on both side of the intestine and by altering the pH of the luminal saline to reflect physiological conditions. Recent work has shown zebrafish (*Danio rerio*) to have more efficient transport at higher pH, and enzymes such as trypsin from lobster function best at either pH 6 or 8 (Factor, 1995; Romano et al., 2013). It is possible that lobster and shrimp, which live in an alkaline environment, may transport amino acids and dipeptides more effectively at a higher pH than previously thought.

Hypothesis 4: Water quality will stimulate or inhibit amino acid and dipeptide transport.

Information about the dietary mineral requirements of crustaceans shows that calcium is unnecessary for supplementation, since it can be absorbed from the water (Davis and Gatlin, 1996). Calcium concentration will be examined by testing higher and lower concentrations and observing their effect on amino acid, bis-complex, and dipeptide transport. Previous work has shown that calcium may have an inhibitory effect on transport, when present in sufficient amounts to block bis-complex formation with dietary metals, such as manganese (Abdel-Malak and Ahearn, 2014). In certain shrimp farms, high levels of calcium and sulfate are found in the water due to their well source in limestone bedrock. Experiments using varying levels of calcium and sulfate in the perfusate and bath saline will investigate the role of these minerals in amino acid and dipeptide transport, and a potential optimum balance will be suggested.

MATERIALS AND METHODS

Materials

Live male and female Atlantic white shrimp (*Litopenaeus setiferus)* of approximately 15 g (± 5 g) each and live male lobsters (*Homarus americanus*) were purchased from local sellers. Shrimp were wild-caught in the St. Johns River near Mayport, FL, while lobsters were wild-caught off the Atlantic coast near Massachusetts. Shrimp were maintained in fresh, oxygenated river water at 20°C for up to three days until used for experimental purposes. Since they were maintained without feeding, to lessen waste accumulation, one to two shrimp were lost daily prior to experimental use. Lobsters were maintained in a saltwater aquarium at 10°C with a pH of 8.2. Lobsters were kept up to four days until used in experiments. Both shrimp and lobster were dissected by cutting the ventral nerve cord, then cutting the carapace to expose the intestine once the animals' heart had stopped (Figure 2).

Physiological saline was prepared to match the osmolarity and make-up of crustacean hemolymph. The salt concentrations used in the buffer were as follows: 420 mM sodium chloride, 25 mM calcium chloride, 10 mM potassium chloride, 8.4 mM sodium sulfate, and 30 mM HEPES for a final adjusted pH of 7.0 or 8.5. When the adjusted pH was 5.5, 30 mM MES was used instead of HEPES. In all preliminary experiments, the perfusion chamber buffer was pH 7.0 and the perfusate buffer was pH 5.5, resulting in a proton gradient from lumen to bath. Adjustments to the physiological saline were made in later experiments, with lower concentrations of calcium chloride,

higher concentrations of sodium sulfate, and a higher pH perfusate, as designated in the results section.

Intestinal Perfusion Technique

In vitro transmural mucosal to serosal transport of L-histidine, L-leucine, L-isoleucine, L-methionine, L-phenylalanine, L-threonine, L-tryptophan, L-lysine, L-valine, and glycylsarcosine were studied using a perfusion apparatus as described previously (Ahearn and Maginniss, 1977). Isolated, intact intestine was flushed with buffered saline solution and mounted onto 18 or 22 gauge blunted stainless steel needles, for lobster and shrimp respectively. Surgical thread was used to secure both ends to the needles (Figure 3).

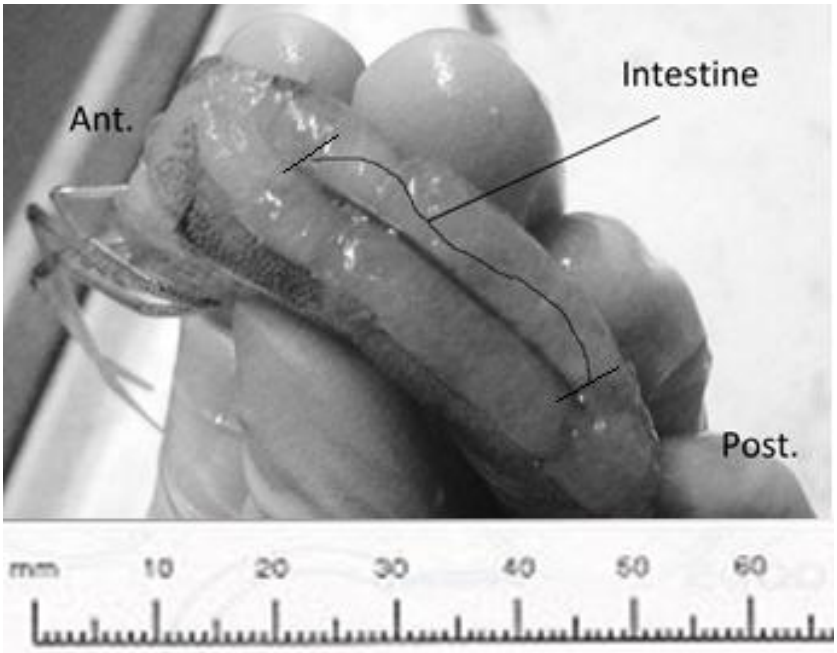

Figure 2. A dissected shrimp with intestine exposed on the dorsal side, shown between bracketed lines. Anterior (Ant.) and posterior (Post.) ends of shrimp identified. Photo taken at the University of North Florida, M. Peterson, 2012.

The perfusion chamber was filled with 35 mL of physiological saline, which served as the serosal medium for the duration of the experiment. The diameter and length of the intestine was measured and used in calculating its surface area with the equation $SA=\pi ld$, where "SA" equals the calculated surface area in cm^2, "l" equals the length and "d" equals the diameter of the intestine in cm. Experimental perfusate made with physiological saline was perfused through the intestine as the mucosal medium using a peristaltic pump (Insteach Laboratories, Inc., Plymouth Meeting, PA, US) at a rate of 0.30 mL min^{-1} for shrimp or 0.38 ml min^{-1} for lobster for up to 240 minutes. Various concentrations of amino acids, zinc chloride, calcium chloride, sodium sulfate, and glycylsarcosine were added to the saline perfusate as needed for each experiment. All experiments were conducted at room temperature with the bath saline mixed by pipette every 15 minutes. Triplicate 200 µL samples were removed from the bath at set time points relative to the starting time, such that each experimental treatment was perfused for 60 minutes prior to samples being removed. For instance, samples would be taken 60 minutes after the start of the first treatment perfusion; the second treatment would begin and samples would be taken after 60 additional minutes, and so forth. Samples were placed in labeled microcentrifuge tubes, capped, and stored at 0°C until analysis by gas chromatography (GC). An equal amount of physiological saline was added to the bath after removing samples to maintain a constant bath volume of 35 mL.

A similar procedure was followed when measuring the *in vitro* transmural serosal to mucosal transport of glycylsarcosine in lobster intestine. Experiments were conducted by adding glycylsarcosine and zinc chloride and/or L-leucine to the bath

saline. Physiological saline was perfused through the intestine and various experimental treatments were added to the bath after each hour. Triplicate 200 µL samples were removed from the collected perfusate exiting the intestine after each experimental treatment. Since the perfusate effluent accumulated over the duration of the experiment, the total volume of the effluent was measured when samples were taken and used in calculations of glycylsarcosine concentrations during sample analysis.

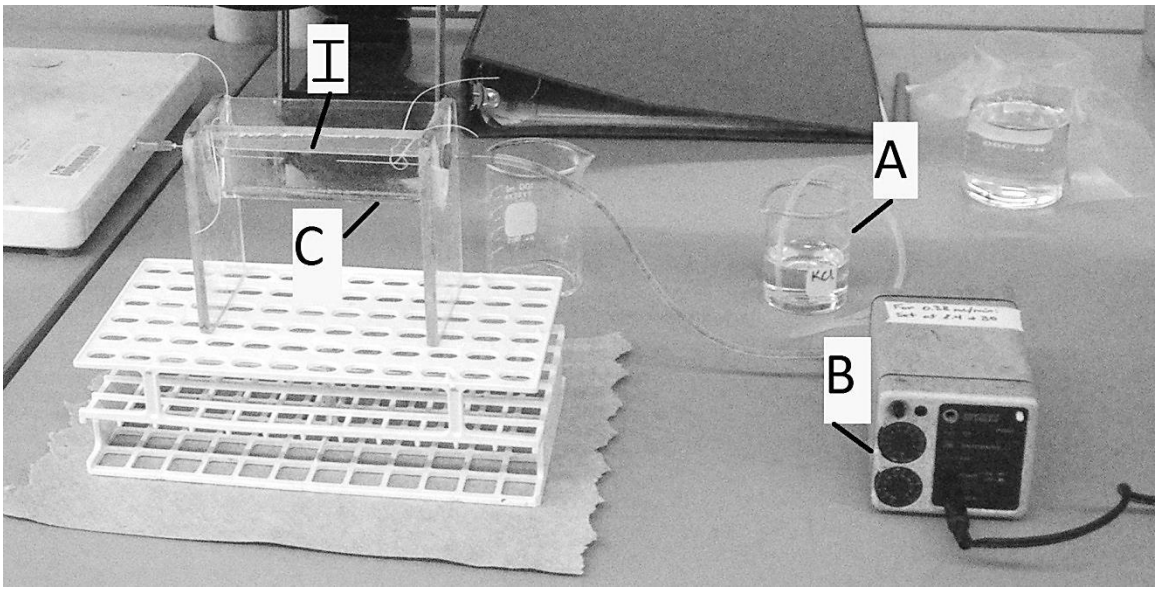

Figure 3. Perfusion apparatus used to measure transepithelial transport of amino acids and dipeptides. The perfusate solution (A) represents the mucosal medium inside the intestine, which was pumped via the peristaltic pump (B) through the shrimp intestine (I). The intestine was suspended within the perfusion bath chamber (C) containing serosal medium. Samples of the bath saline were removed and analyzed by gas chromatography. Photo taken at the University of North Florida, M. Peterson, 2012.

Data Analysis – Gas Chromatography

Once perfusion was complete, samples were prepared for analysis using the EZ:faast ™ GC-FID Free (Physiological) Amino Acid Analysis Kit (Phenomenex Inc., Torrance, CA, US). Briefly, this kit allows the user to extract amino acids from biological samples, including serum, plasma, and urine. The amino acids are separated from interfering compounds, which are discarded, then derivatized using propyl chloroformate. This reaction yields volatile derivatives of the amino acids, which may then be vaporized in the GC and separated based on the molecule's size and reaction with the inside of the column. As the molecules pass out of the column and into the flame ionization detector (FID), the hydrogen and air flame combusts the sample and produces positive and negative ions. The negative ions are attracted by a collector electrode, which produces an analog signal, called a chromatogram, which displays ion abundance (pA) versus retention time (Figure 4).

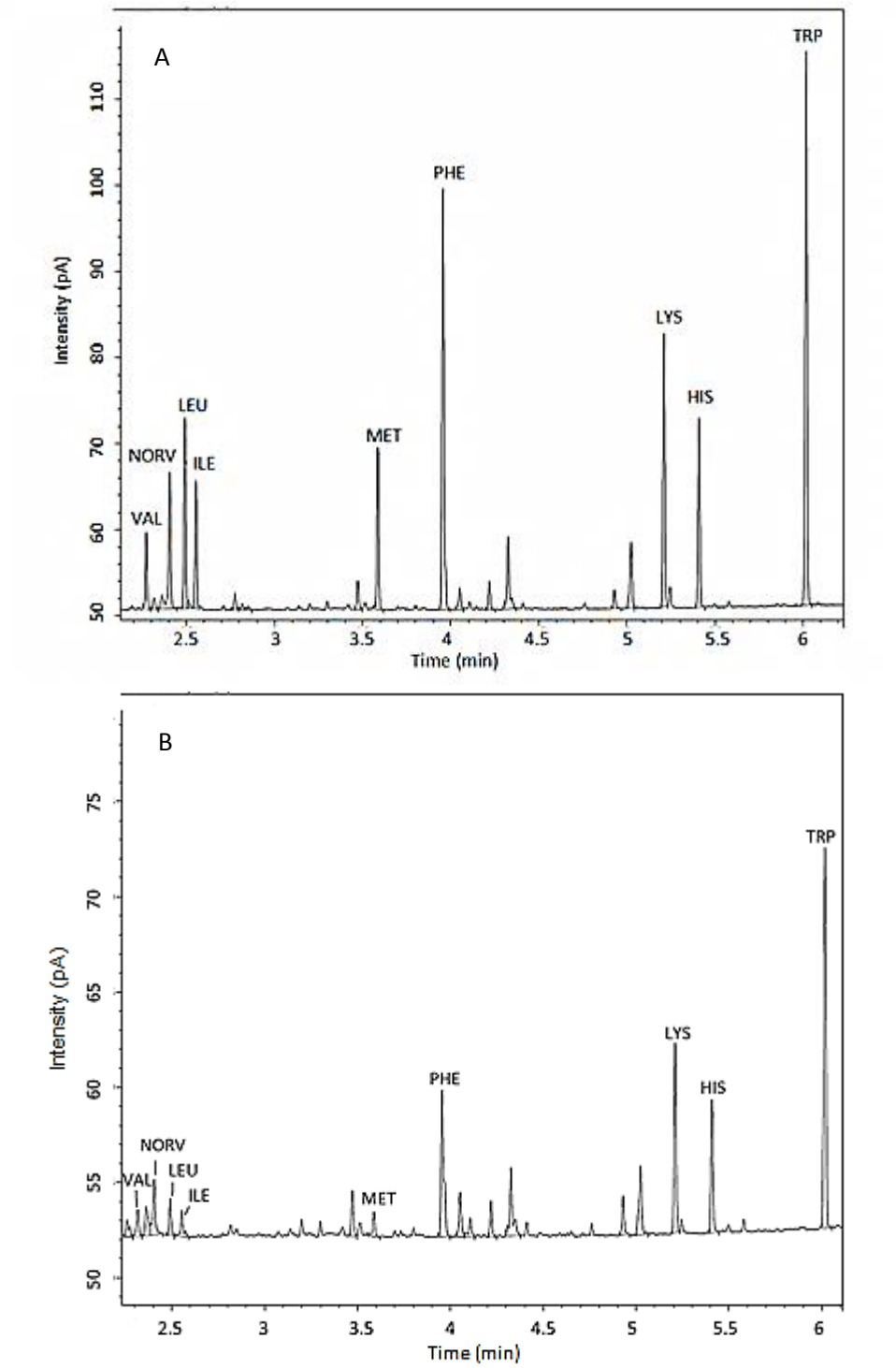

Figure 4. Separation of amino acid mixture (A) before transport and (B) after transport through the shrimp intestine. The vertical scale for (B) is decreased by ¼ for clarity. Eight amino acids are shown clearly separated by GC-FID with retention times conserved from sample to sample.

Amino acid standards dissolved in saline were prepared for each experiment by diluting the experimental perfusate to the desired concentration in fresh saline. A typical experimental perfusate contained three amino acids, each at 10 mM. The most often used standard concentrations were 50 µM, 200 µM, and 500 µM because bath sample concentrations regularly fell within this range. Samples of the experimental perfusate (200 µL) and each of the three standards were placed in labeled microcentrifuge tubes, capped, and stored at 0°C until analysis. These samples were derivatized and analyzed by an HP Agilent 6890 series GC-FID system at the same time as the experimental samples (Agilent Technologies, Inc., Santa Clara, CA, US). The standard curve preparation allowed for "known" and "unknown" samples to be run concurrently and was used in determining amino acid concentrations in the "unknown" samples, to ensure the derivatization procedure was successful and to account for any minor alterations in experimental analysis.

Analyte signals were identified through the derivatization of pure standards provided with the EZ:faast™ kit as well as sample chromatograms that showed relative retention times for many amino acids (Phenomenex Inc., Torrance, CA, US). The numerical output from the GC was used to determine the bath sample concentrations using a standard curve generated by the analysis of saline standards prepared during every perfusion experiment. To calculate the sample concentrations, the area under the signal for the analyte of interest was divided by the area under the signal for the provided internal standard, norvaline. These ratios were plotted versus the known concentrations of the standard solutions. By performing a linear regression between

the three known concentrations, the given linear equation was used to calculate the concentration of the "unknown" bath samples. The bath volume and intestine surface area were used in further calculations to quantify the amount of transport of each amino acid, measured in nmol cm^{-2}.

The transport rate of amino acids and glycylsarcosine were calculated by linear regression by plotting the amount of analyte in nmol cm^{-2} versus time. The slope of the linear regression line was equal to the transport rate of that analyte over the treatment period. These rates were displayed as histograms by plotting transport rate in nmolcm^{-2} min^{-1} versus experimental treatment. Each experiment was conducted at least three times using intestines from different animals and freshly prepared experimental treatments. All values shown are means ± SEM. Statistical differences between means were calculated using two-tailed Student's t-test and paired Student's t-test with Bonferroni correction factor using Microsoft Excel and SPSS (IBM Corporation, Armonk, NY). Family-wise significant difference was established at $p < 0.05$. With the correction factor, p (or α) was divided by the number of treatments (3) to reach a corrected $p < 0.02$ for significant difference.

Data Analysis – Liquid Scintillation Counting

To assess the kinetics of leucine transmural mucosal to serosal transport in shrimp intestine, radiolabeled ^{3}H- leucine (American Radiolabeled Chemicals, Inc., Saint Louis, MO) was used in conjunction with experimental treatments and measured by a Beckman LS6500 scintillation counter. For these experiments, 5 µL of ^{3}H-leucine was

added to 25 mL perfusate solutions containing varying concentrations of leucine, from 2.5 µM up to 600 µM. Triplicate 200 µL initial samples of the bath were taken to measure initial radioactivity levels, given as counts per minute (cpm). The total amount of radioactivity in the perfusate treatment was determined by taking a 200 µL positive control sample from each prepared treatment prior to perfusion through the intestine. Triplicate 200 µL samples from the bath were taken every 5 minutes and were replaced by an equal volume of unlabeled bath saline to maintain constant volume. All samples were placed in a 7 mL tube containing 3 mL of scintillation cocktail.

To calculate radioactivity, the average of three initial background counts were subtracted from each sample. The concentration of leucine in picomoles was determined by dividing the counts per minute by the specific activity for each experimental treatment as generated by the positive control samples. The bath volume and surface area of the intestine were taken into account to give pmol cm^{-2}. The averages of the triplicate samples for each time point were plotted, and the slope generated by linear regression was used to find the transmural flux rate expressed in pmol cm^{-2} min^{-1}. These slopes were plotted versus treatment leucine concentration in µM to determine the kinetic constants using Sigma Plot 10.0 software (Systat Software, Inc., Point Richmond, CA, US). Each experiment was conducted at least three times using intestines from different shrimp and freshly prepared experimental treatments. All values shown are means ± SEM.

Method Development

Analysis by gas chromatography was a novel technique used here to quantify amino acid concentrations for many amino acids in the same sample. Previous work has made use of radiolabeled amino acids and sugars; the major limitation of this method is the inability to analyze more than one or two amino acids in the sample. Multiple initial experiments were conducted to optimize this new technique. Additionally, the intestinal perfusion of shrimp had only been previously attempted in *Melicertus* (previously *Penaeus*) *marginatus* and *Macrobrachium rosenbergii*, both much larger species on average than the Atlantic white shrimp used in this study (Ahearn and Maginniss, 1977). The flow rate of the experimental perfusate through the intestine needed to be modified for the shrimp intestine, which has a mean surface area ten times smaller than that of the lobster. It was found that a flow rate of 0.3 mL min^{-1} produced a linear accumulation of the three amino acids tested, L-leucine, L-methionine, and L-histidine, in the bath over a period of 3.5 hours, whereas a higher flow rate (0.38 mL min^{-1}) resulted in an exponential increase, signifying possible tissue damage and paracellular amino acid movement (Figures 5 and 6). In addition, the perfusion chamber required modifications from previous use with lobster that included changing the needle size to a much smaller bore (22 gauge instead of 18 gauge) to accommodate the smaller intestinal diameter of the shrimp, which was 0.15 mm on average.

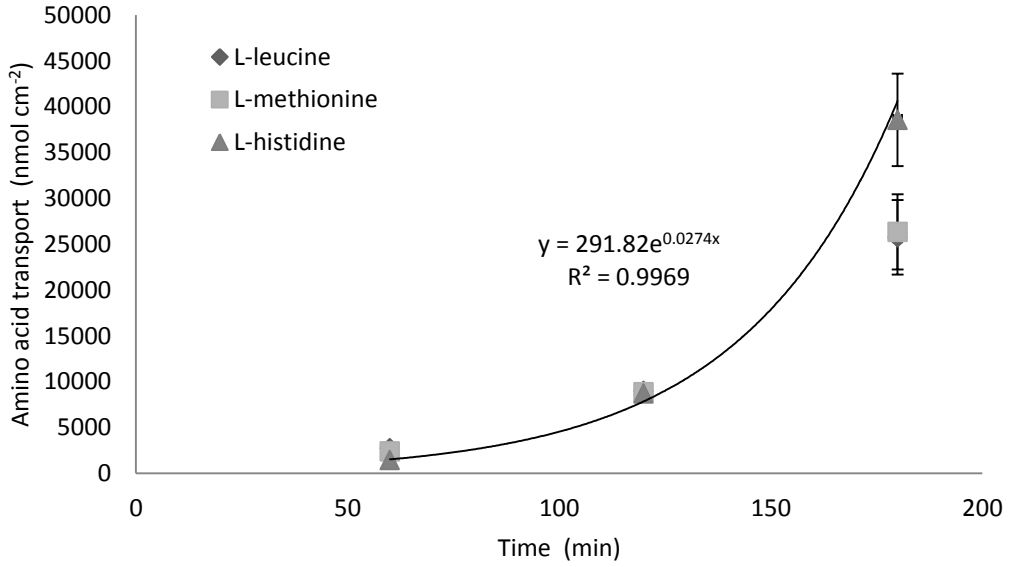

Figure 5. Accumulation of three amino acids, each 10 mM, with perfusate flow rate changed each hour from 0.25 mL min^{-1} after the first 60 min to 0.3 mL min^{-1} for 60 to 120 min, to 0.38 mL min^{-1} for 120 to 180 min. The last hour of perfusion demonstrates the exponential increase in amino acid concentration, possibly due to tissue damage and paracellular trasport. A flow rate of 0.3 mL min^{-1} was used in all subsequent experiments due to its linearity. Data represents average of two shrimp intestines with error bars representing ± SEM.

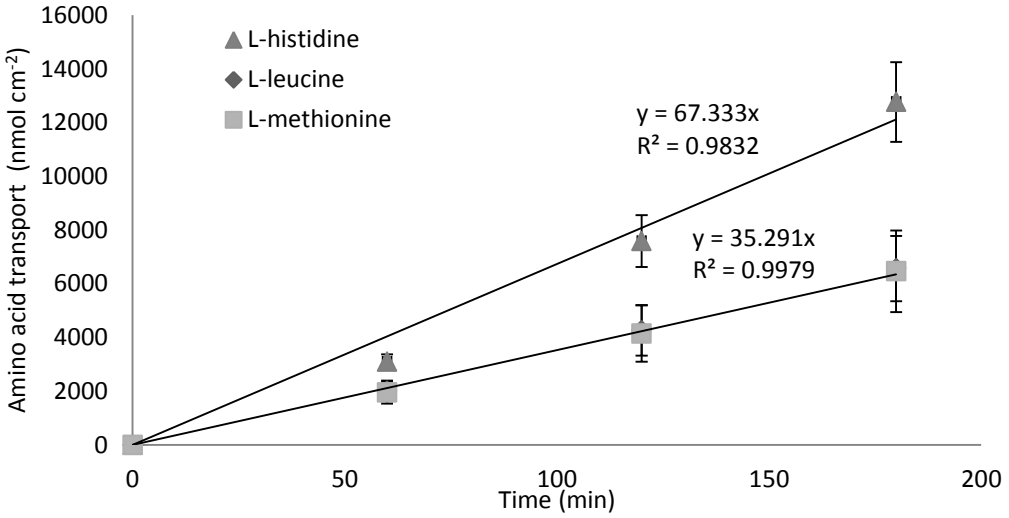

Figure 6. Transport of three amino acids at a perfusate flow rate of 0.3 mL min^{-1} for three hours, showing linear transport when using the lower flow rate as compared to 0.38 mL min^{-1} as seen in Figure 5. Data points represent the average from three shrimp intestines ± SEM. Linear regression equations shown are for L-histidine (upper) and L-methionine (lower). The slope of these lines gives the rate of amino acid transport, in nmol cm^{-2} min^{-1}. L-methionine and L-leucine data points are similar enough that they overlap.

Preliminary experiments also included a constant perfusion of a mixture of 9 amino acids, each at 10 mM concentration, over a 3.5 hour period (Figure 7). This not only demonstrated the consistent measurement of a complex mixture by GC-FID, it also gave an expected amount of accumulation over time for each amino acid. The amino acids chosen were essential for shrimp, meaning they cannot be synthesized *de novo*. It was found that the GC-FID EZ:faast kit (Phenomenex, Inc., Torrance, CA, US) could not measure L-arginine, the tenth essential amino acid, so L-arginine was excluded from further experimentation. It was also found that L-threonine does not always appear in the bath in concentrations sufficient for measurement by GC-FID.

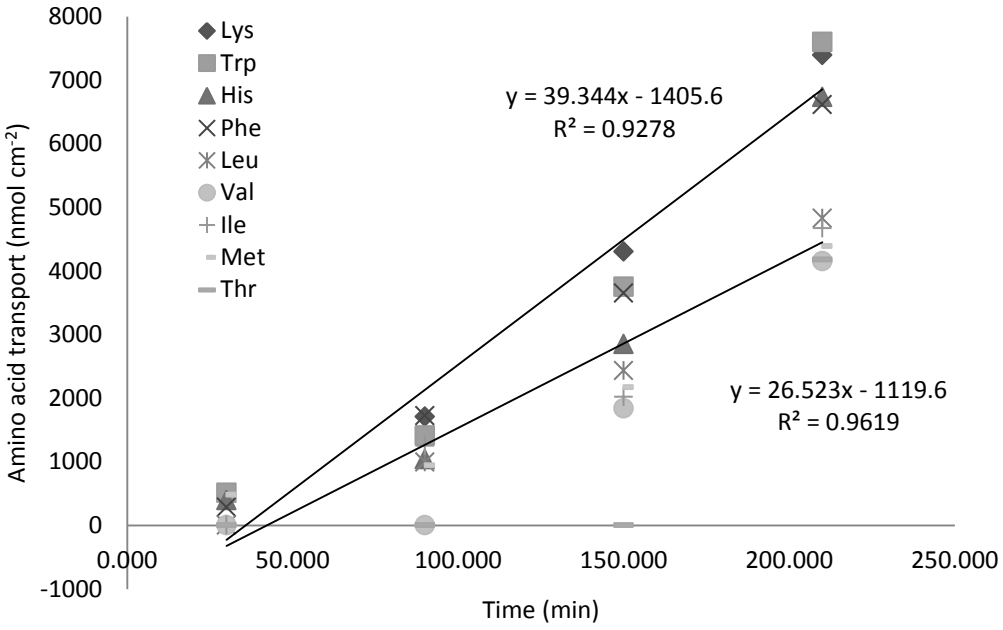

Figure 7. The accumulation of 9 essential amino acids over time. Shown is the transport (nmol cm^{-2}) of nine amino acids as measured in the perfusion bath chamber: 30 minutes, 90 minutes, 150 minutes, and 210 minutes after the start of perfusing a 10 mM mixture at 0.3 mL min^{-1} through the shrimp intestine.

It is of interest to note that there appears to be a distinct grouping of amino acid transport (Figure 7). Lysine, tryptophan, histidine, and phenylalanine are consistently transported by the shrimp intestine at a higher rate than leucine, valine, isoleucine, methionine, and threonine. There are a few possible explanations for this behavior. Firstly, these four amino acids could be more completely ionized by GC-FID and therefore would appear to have a higher final concentration at all time points. GC-FID gives an output based on how well different molecules burn; the larger amino acids and those with more nitrogen could be ionizing better than the smaller amino acids.

In addition, it was found that perfusate concentrations of 10 mM were necessary to ensure adequate concentrations of amino acids in the bath. This is problematic for future work if kinetic constants are being determined, since these values are typically found in the micromolar range. Testing was done to see if large bath samples could be taken and concentrated in the event that micromolar perfusate treatments were desired. However, sample concentration using solid phase extraction of amino acids in conjunction with lyophilization of saline samples and reconstitution in smaller volumes of distilled water proved to be inadequate for detection by GC-FID and highly variable (Figure 8). The tested samples had an initial volume of 1 mL, 2 mL, or 3.5 mL and either 50% or 100% salinity. Lyophilization and reconstitution in 100 µL deionized water should have given 1 mM concentrations for each amino acid, as the same moles of amino acids were added to each initial saline sample. Future work using this method will either need to use high micromolar or low millimolar concentrations of amino acids or an alternative technique for sample concentration prior to analysis. As shown, only 3

samples that were extracted via solid phase extraction, lyophilized, and reconstituted had higher than 50% recovery of amino acids (Figure 8). In addition to low recovery, there was a high degree of variation between samples.

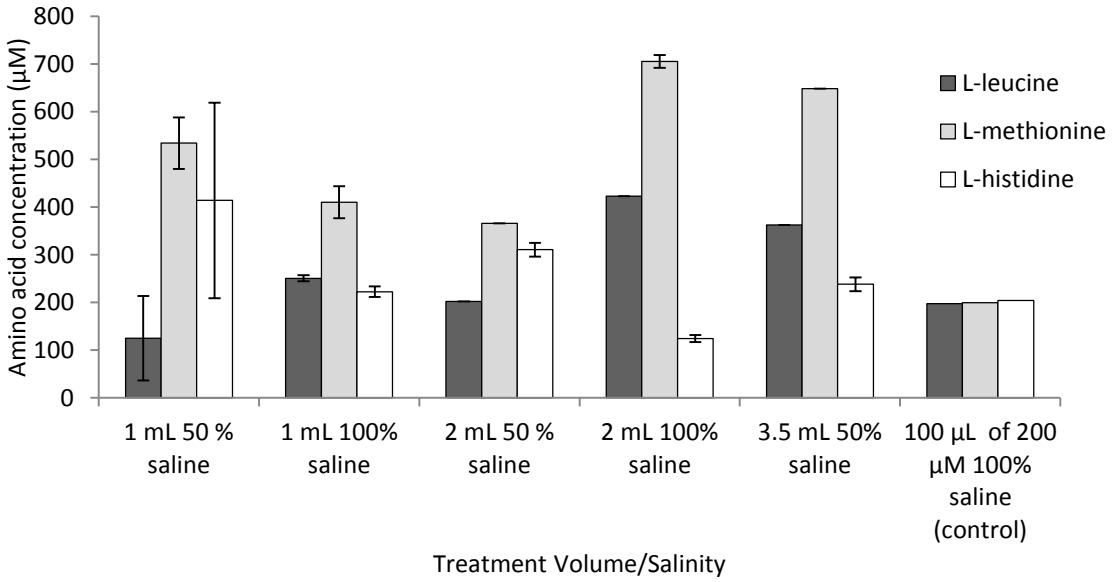

Figure 8. Amino acid recovery using various initial samples with solid-phase extraction and lyophilization. All solutions began with 200 µmoles L-leucine, L-methionine, and L-histidine. After sample concentration by lyophylization and reconstitution in 200 µL distilled water, estimated amino acid concentration was 1000 µM for all samples with 100% recovery, except the control, which used normal sample preparation methods not involving lyophylization. As seen, recovery was much lower than expected, typically 50% or less for all volumes and salinity concentrations for all three amino acids. Bars represent the mean of two pseudoreplicate samples with error bars representing ± SEM. Means without error bars indicate that one sample was below the limit of detection for gas chromatography.

RESULTS

Transport Experiments Using Lobster Intestine

Initial testing was performed using lobster intestine in order to verify the new GC-FID technique and to assess how it could be further used with shrimp intestine. An experiment similar to those already attempted with lobster intestine was designed that would analyze the amount of amino acids transported across the lobster intestine alone and in the presence of a stimulatory metal and a competing dipeptide. Since the GC allows for the analysis of a complex mixture, three amino acids were used. Previously only one or two amino acids could be tested at a time using the radioisotope technique.

A pH 5.5 saline solution containing 1 mM each of L-histidine, L-methionine, and L-leucine was perfused through lobster intestine for one hour. These amino acids were chosen due to their abundant use in previous studies as well as their distinctly different retention times (typically 2.501 min for leucine, 3.596 min for methionine, and 5.407 min for histidine). At the start of the second hour, a new solution containing the same concentration of amino acids plus 25 μM zinc chloride was perfused. At the start of the third hour, a new solution containing the same concentration of amino acids, zinc chloride, and 2 mM glycylsarcosine was perfused. There was no significant difference between the transport rates of amino acids when perfused by themselves versus when zinc was added to the perfusate (Figure 9). However, there was significant inhibition of transport for histidine when the competing dipeptide glycylsarcosine was added (paired Student's t-test, Bonferroni correction, $p < 0.02$). This suggests that bis-complexes may

have formed with the amino acid, since the transport rates decreased when the competitive inhibitor was added. It was also found in previous experiments that leucine could be used to inhibit histidine movement across the intestine, when the amount of leucine added to the perfusate was five times higher that of the histidine (Conrad and Ahearn, 2005; Mullins and Ahearn, 2008). The current results suggest that leucine does not necessarily inhibit histidine when present in the same concentrations.

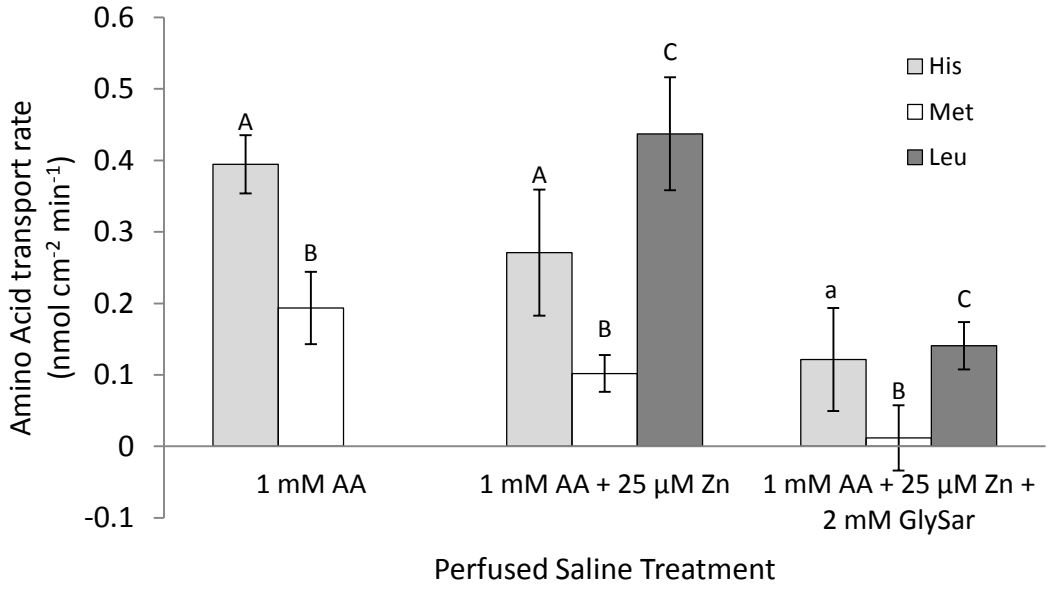

Figure 9. Effect of 25 µM Zn^{+2} and 2 mM Gly-Sar on 1 mM L-histidine, 1 mM L-methionine, and 1 mM L-leucine transport in lobster intestine. Bars represent the mean rate of amino acid transport in nmol cm^{-2} min^{-1} (N=3). Error bars represent ± SEM. Significant difference was analyzed pair-wise for each amino acid separately, across the range of three treatments. Significance for each amino acid is denoted using different letters for each amino acid (A for L-histidine, B for L-methionine or C for L-leucine). Means with lower-case letters denote significant difference from means with capital letters between treatments for each amino acid (paired Student's t-test, Bonferroni correction, $p < 0.02$). L-leucine was not detected during the first treatment; means are given for the second and third treatments only.

Since it was found that the dipeptide can be analyzed with the same derivatization procedure used to analyze amino acid content, further testing with lobster intestine investigated the transport of the neutral dipeptide glycylsarcosine. It has been used extensively as a competitive inhibitor, but transepithelial dipeptide transport has not been as extensively studied as amino acid transport (Conrad and Ahearn, 2005; Mullins and Ahearn, 2008; Obi et al., 2011; Berthelsen et al., 2013). One major question was if the dipeptide would be transported across the intestine intact, or if it would be broken into its constituent amino acids, which were also detected by GC-FID. It was found that glycylsarcosine was transported intact across the membrane, with little to no breakdown to glycine and sarcosine (Figure 10). When 1 mM glycylsarcosine was perfused, with the additions of 25 µM zinc chloride and 2 mM L-leucine during the second and third hours of perfusion, respectively, there was a significant decrease in glycylsarcosine transport during the third hour (paired Student's t-test, Bonferroni correction, $p < 0.02$, Figure 11). This was expected, since zinc should not affect glycylsarcosine transport unless an amino acid was also present to form bis-complexes and thus compete for the same peptide transporter. There was no significant difference between glycylsarcosine transport with and without zinc chloride.

A similar experiment was conducted to ensure L-leucine alone did not affect glycylsarcosine transport. Glycylsarcosine (10 mM) was perfused through the lobster intestine, followed by the addition of 20 mM L-leucine during the second hour and 50 µM zinc chloride during the third hour (Figure 12). There was no significant difference when L-leucine was added to the perfusate. However, when zinc chloride and leucine

were present together, glycylsarcosine transport was significantly inhibited (paired Student's t-test, Bonferroni correction, $p < 0.02$). The results of this test show that a single amino acid has no effect on dipeptide transport, unless it is combined with a metal, which is in agreement with the previous experiment.

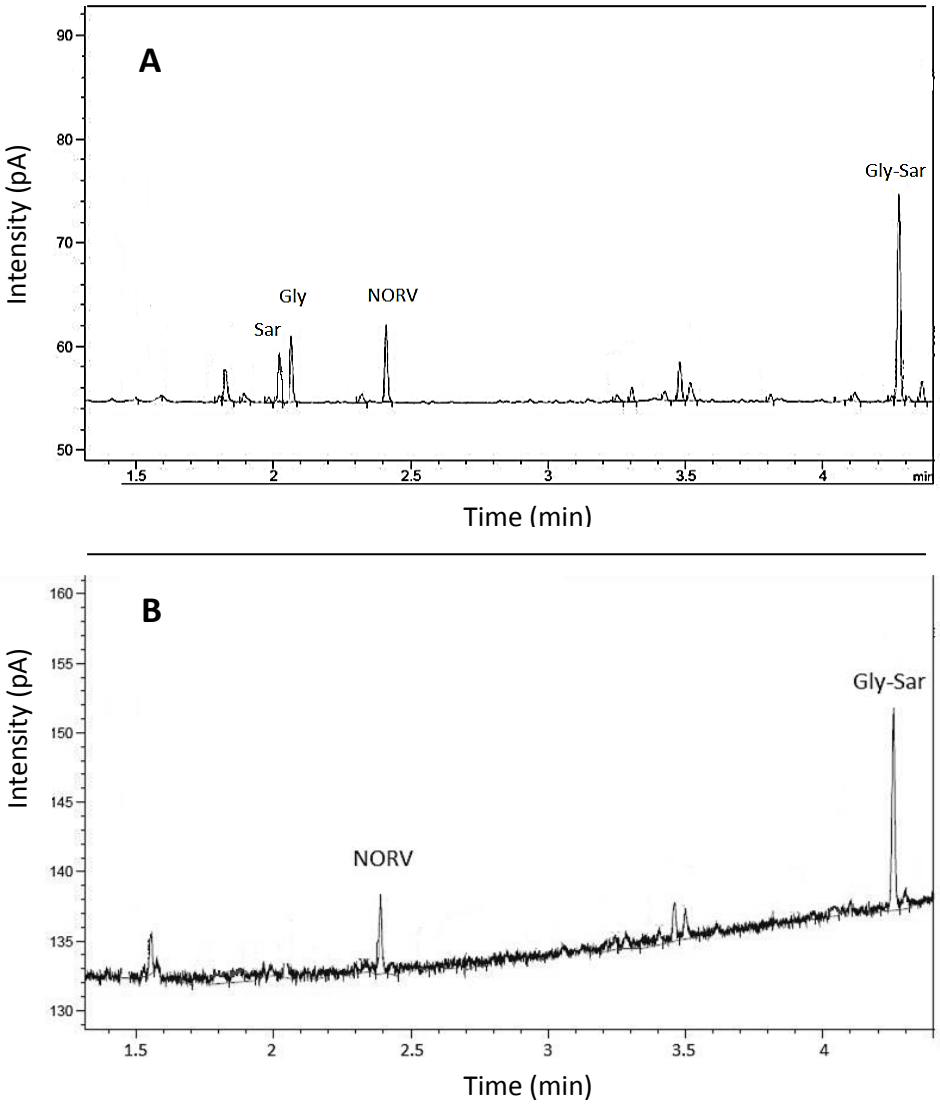

Figure 10. Gas chromatograms of glycylsarcosine, glycine, and sarcosine saline standard mixture (A) and of a saline bath sample after 3 hours of 10 mM glycylsarcosine perfusion through the lobster intestine (B). Glycylsarcosine is transported intact through the lobster intestine and is minimally broken down into glycine and sarcosine.

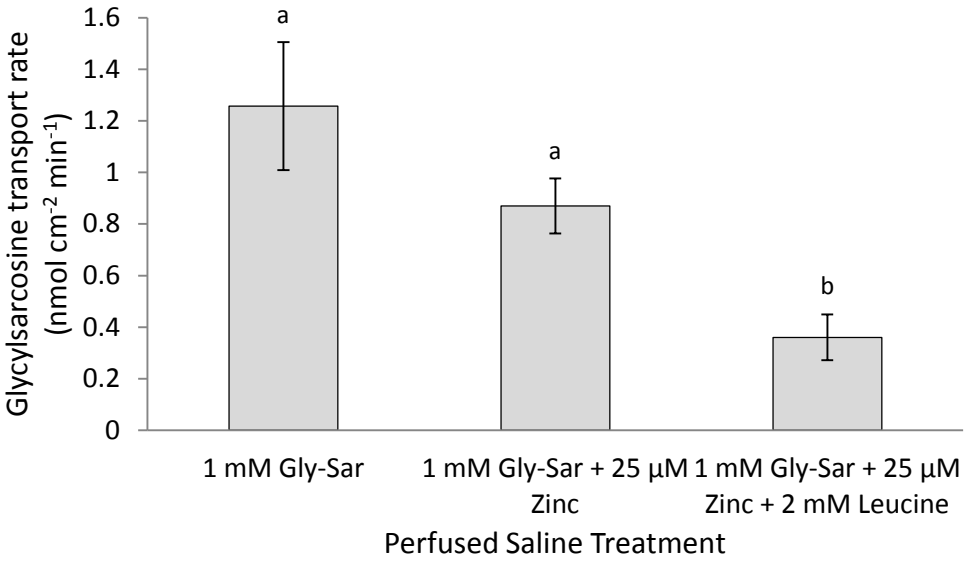

Figure 11. Effect of 25 µM Zn^{+2} and 2 mM L-leucine on 1 mM glycylsarcosine transport. Bars represent the average rate of amino acid transport in nmol cm^{-2} min^{-1} for three lobster intestines. Error bars represent ± SEM for the three replicates. Means with different letters denote significant difference between treatments (paired Student's t-test, Bonferroni correction, $p < 0.02$).

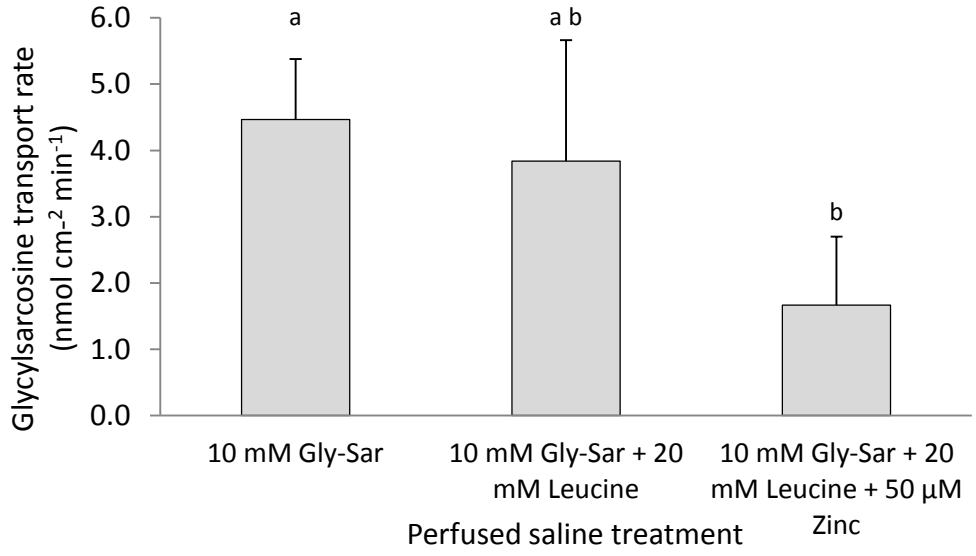

Figure 12. Effect of 20 mM L-leucine and 50 µM Zn^{+2} on 10 mM glycylsarcosine transport. Bars represent the average rate of amino acid transport in nmol cm^{-2} min^{-1} for three lobster intestines. Error bars represent ± SEM for the three replicates. Means with different letters denote significant difference between treatments (paired Student's t-test, Bonferroni correction, $p < 0.02$).

An analysis of the mucosal to serosal (M to S) glycylsarcosine transport was conducted and compared to the serosal to mucosal (S to M) transport of the same concentration of dipeptide. The net flux was determined by subtracting the rate of glycylsarcosine transport S to M from the transport rate for M to S (Figure 13). As shown, the net flux was approximately 3 $nmolcm^{-2}min^{-1}$, indicating that under the experimental conditions, glycylsarcosine was transported from mucosa to serosa even with equal concentrations of glycylsarcosine on either side of the epithelium. It also suggests the presence of a basolateral peptide transporter for glycylsarcosine. Since the rates of transport are different from M to S and S to M, it may be inferred that the movement of glycylsarcosine is not due to paracellular flow, but rather may be attributed to either facilitated diffusion or active transport. While the addition of leucine and zinc to perfused glycylsarcosine had a significantly inhibitory effect on the glycylsarcosine transport rate (Figures 11 and 12), this was not the case when the S to M transport was investigated and no difference between the three bath treatments was found.

To positively identify the presence of a peptide transport protein, the transmural transport rate of glycylsarcosine was measured for a range of glycylsarcosine concentrations from 1 mM to 15 mM. As the luminal concentration of glycylsarcosine increased, the transport rate increased in a hyberbolic manner consistent with Michaelis-Menten kinetics (Figure 14). The maximum transport rate is 4.11 ± 1.05 nmol cm^{-2} min^{-1} (J_{max}) and the transporter is working at half of its maximum velocity at 6.93 ± 3.83 mM glycylsarcosine(K_m). A high K_m is indicative of a low-affinity system, which is

not consistent with the previously defined 'scavenger' nature of the intestinal epithelium (Ceccaldi, 1989).

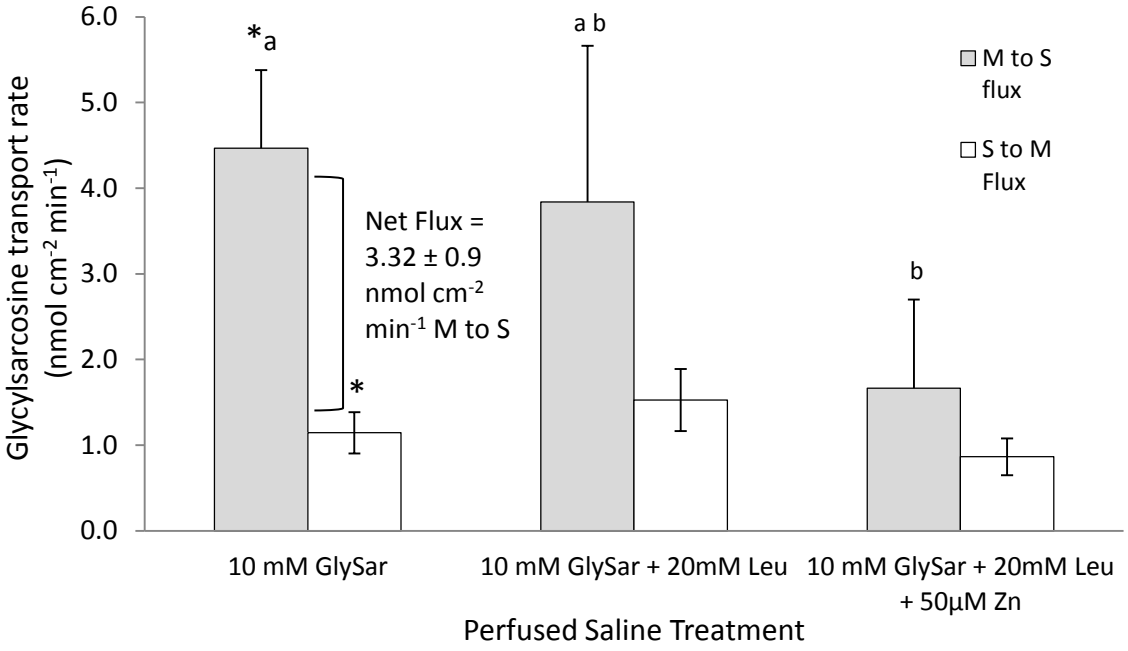

Figure 13. The net flux of glycylsarcosine was calculated from mucosal to serosal (M to S) transport rates and serosal to mucosal (S to M) transport rates under the same treatment conditions. Bars represent the average rate of amino acid transport in nmol cm^{-2} min^{-1} for lobster intestines (N=3). Error bars represent ± SEM. For M to S replicates, the saline treatment was perfused through the intestine and measurements of the normal bath saline were taken. For S to M replicates, the saline treatment was in the bath chamber and normal saline was perfused through the intestine. Measurements were taken from the perfusate effluent that was collected for the duration of the experiment. Means with different letters denote significant difference between treatments (paired Student's t-test, Bonferroni correction, $p < 0.02$). Asterisk represents significant difference between M to S and S to M transport rates with 10 mM glycylsarcosine (Student's t-test, $p < 0.05$).

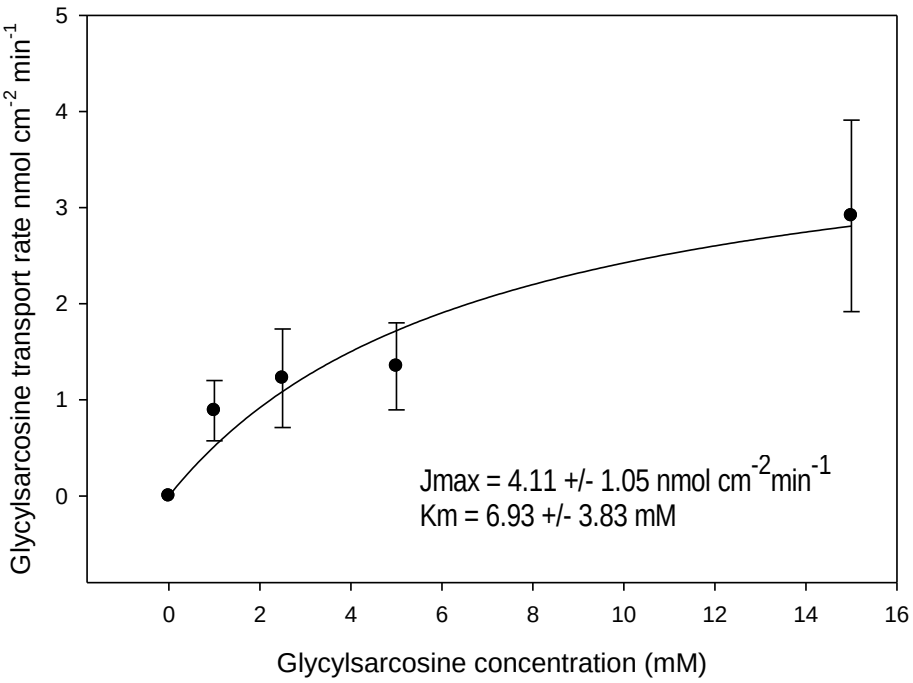

Figure 14. The effect of increasing glycylsarcosine concentration on the mucosal to serosal transmural transport of glycylsarcosine. Data points represent the transport rate nmol cm^{-2} min^{-1} with error bars ± SEM from three lobster intestines (N=3). The kinetic constants found by fitting a hyperbolic curve were J_{max} = 4.11 ± 1.05 nmol $cm^{-2}min^{-1}$ and K_m = 6.93 ± 3.83 mM glycylsarcosine.

In addition to the effect of metals and amino acids, the amount of calcium and pH of the perfusate were examined for their effects on dipeptide transport. First, the effect of pH was tested by perfusing saline containing 10 mM glycylsarcosine through the lobster intestine at a range of pH from 5.5 to 8.5, with the bath saline maintained at pH 7.0. The initial experiment contained 25 mM calcium chloride in both perfused and bath saline solutions. It was found that there was no difference between glycylsarcosine transport rates when the perfused saline was at pH 5.5, 6.5, or 7.5. However, at pH 8.5 the transport rate was significantly increased (Figure 15). Since a higher transport rate at a high pH was unexpected, the experiment was adjusted and

attempted with three more lobster intestines, this time with a lower calcium chloride concentration of 0.5 mM (Figure 15). This change in calcium concentration was found to significantly increase the rate of glycylsarcosine transport at both pH 7.5 and 8.5. At pH 5.5 and 6.5 there was a larger amount of standard error between the treatments, so any observed difference was not statistically significant. It was also seen that the transport rate at pH 8.5 was significantly different than the rate at pH 5.5, for intestines perfused with a lower concentration of calcium (paired Student's t-test, Bonferroni correction, $p < 0.02$).

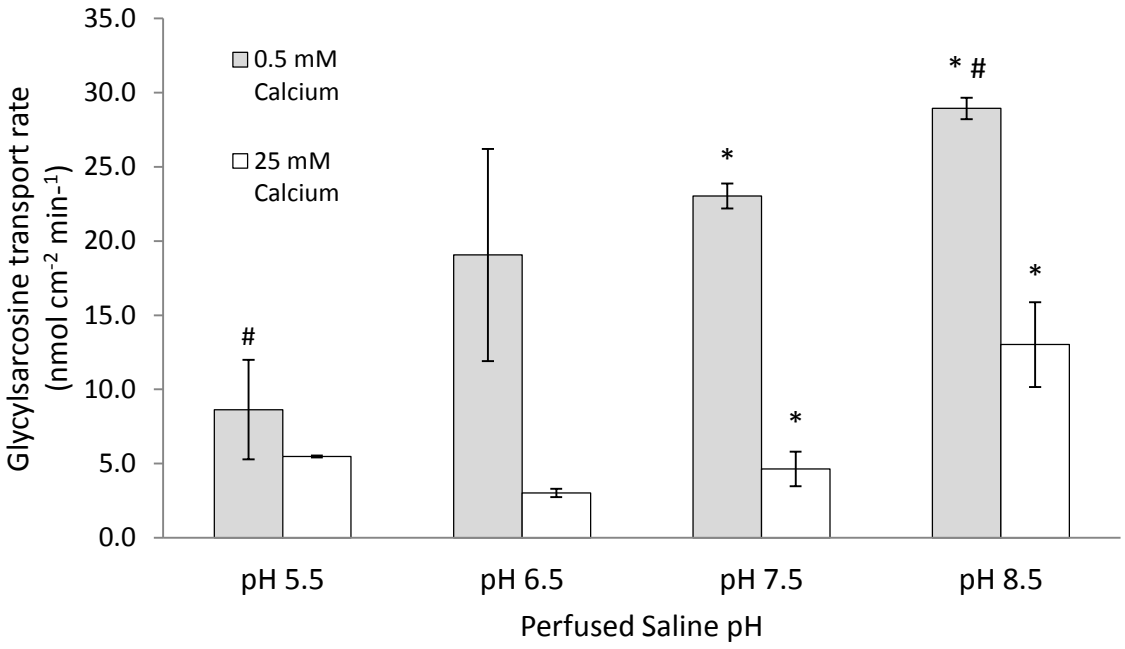

Figure 15. Effect of calcium concentration and perfused saline pH on transport rate of 10 mM glycylsarcosine. Bath saline was maintained at pH 7.0 for all treatments. Data represent means from three lobster intestines for both 0.5 mM and 25 mM calcium levels (total N = 6) and error bars represent ± SEM. Asterisks represent significantly different transport rates between calcium treatments at the same saline pH (two-tailed Student's t-test, $p < 0.01$). Number signs represent significantly different transport rates between pH treatments for 0.5 mM calcium (paired Student's t-test, Bonferroni correction, $p < 0.02$).

Since the combination of higher perfused pH and lower calcium chloride concentration appeared to optimize the transport of glycylsarcosine, this experimental set-up was tested for linearity by perfusing 10 mM glycylsarcosine through the lobster intestine for 3 hours with a perfusate pH of 8.5, a bath pH of 7.0, and a 1 mM calcium chloride concentration (Figure 16). The calcium was increased slightly from 0.5 mM to 1 mM due to a slight swelling observed in the tissue during perfusion at the lower calcium concentration, as well as nutritional data confirming the use of 1g calcium/100g pelleted food (Davis and Gatlin, 1996) and lobster physiological data about the concentration of calcium in the hemolymph (Factor, 1995). There was no significant difference in the transport rates of glycylsarcosine over the three hour experimental period (Figure 16).

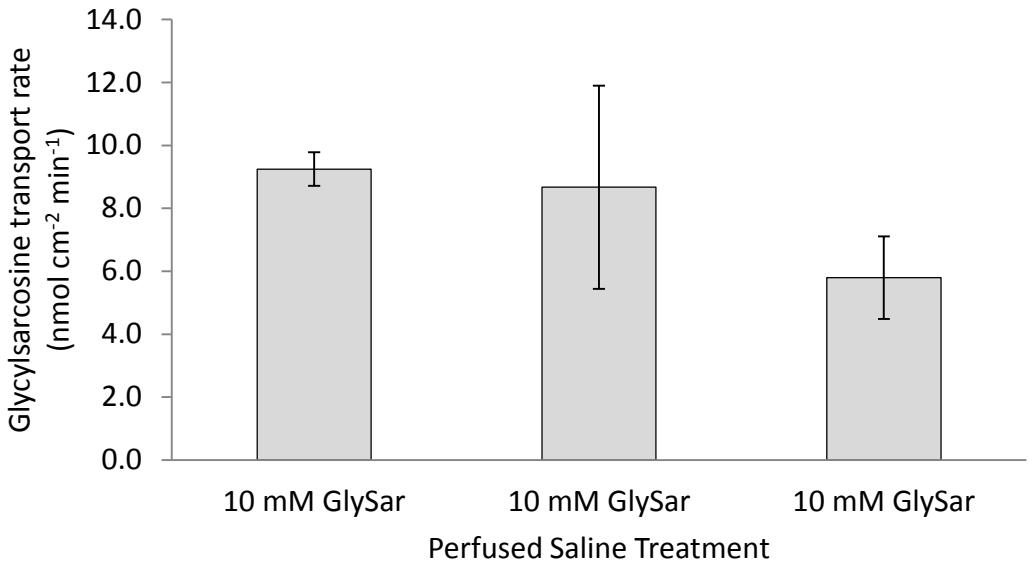

Figure 16. Transport rates of 10 mM glycylsarcosine perfused with 1 mM calcium chloride at pH 8.5 through lobster intestine. Each treatment was perfused for 1 hour, with a total experiment time of 3 hours. Bath saline was maintained at pH 7.0 with the same concentration of calcium chloride. Data represent means from three lobster intestines and error bars represent ± SEM. No significant difference was found between the transport rates during the three hours of perfusion (paired Student's t-test, Bonferroni correction, $p < 0.02$).

Transport Experiments Using Shrimp Intestine

Initial experiments with shrimp intestine began with the radioisotopic technique, as this had been used previously for the study of lobster intestine and shrimp intestine and was well documented as a functional technique (Ahearn and Maginnis, 1976; Conrad and Ahearn, 2005). H^3-L-leucine was used in conjunction with a range of concentrations of L-leucine from 5 µM to 50 µM to determine the transepithelial transport kinetics of this amino acid. As the luminal concentration of L-leucine increased, the transport rate increased in a hyperbolic manner (Figure 17). The maximum transport rate is 416 ± 58.9 pmol cm^{-2} min^{-1} (J_{max}) and the transporter is working at half of its maximum velocity at 76.2 ± 16 µM leucine (K_m). A low K_m is indicative of a high-affinity system, which is consistent with the previously work using L-leucine in the lobster intestine (Obi et al., 2011). These data show that the shrimp intestine was functioning properly and did not have paracellular transport of amino acids, as would be expected if the tissue was stretched or otherwise compromised using the modified perfusion apparatus for the shrimp intestine, which is considerably smaller than the previously used lobster intestine.

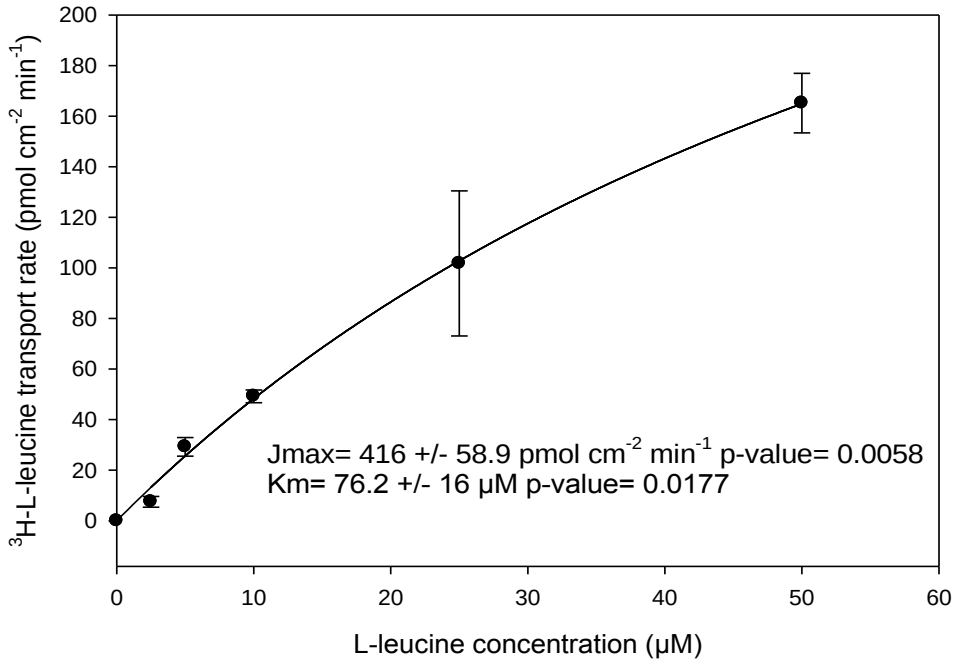

Figure 17. The effect of increasing ^{3}H-L-leucine concentration on the mucosal to serosal transmural transport of L-leucine. Data points represent the transport rate pmol cm^{-2} min^{-1} with error bars ± SEM from three shrimp intestines (N=3). The kinetic constants found by fitting a hyperbolic curve were J_{max} = 416 ± 58.9 pmol cm^{-2}min^{-1} and K_m = 76.2 ± 16 µM L-leucine.

After using the new GC-FID method with lobster intestine, testing was conducted to see if previous experimental procedures (Conrad and Ahearn, 2005; Conrad and Ahearn, 2007) would give similar results using shrimp intestine. An experimental perfusate of 10 mM L-leucine, L-methionine, and L-histidine was perfused through the shrimp intestine at 0.30 mL/min for 3.5 hours to show a linear increase in transport over time (Figure 5).

When this was established in three trials using different shrimp intestines, a perfusate containing 10 mM L-histidine was used with the addition of 50 μM zinc during the second hour and 20 mM glycylsarcosine during the third hour. This amount of zinc was used in order to achieve a measurable effect on histidine transport. The level of zinc present in formulated shrimp diets is typically 100 mg/kg (Davis and Gatlin, 1996). As shown below, the addition of zinc to the perfusate caused a significant increase in transport and a decrease in transport when the dipeptide inhibitor was added (paired Student's t-test, Bonferroni correction, $p < 0.01$, Figure 18). This finding is consistent with previous studies on the stimulatory effect of zinc on histidine transport in the lobster (Conrad and Ahearn, 2005; Conrad and Ahearn, 2007).

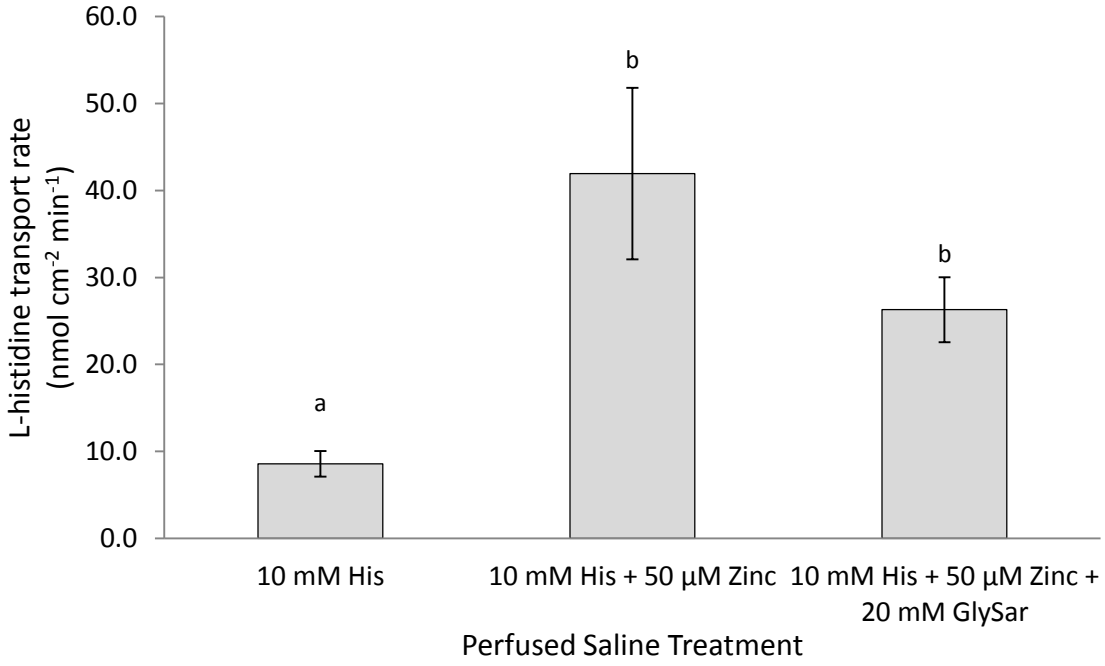

Figure 18. Effect of 50 µM zinc chloride and 20 mM glycylsarcosine on the transport rate of 10 mM L-histidine through shrimp intestine. Perfused saline contained 1 mM calcium chloride and 8.4 mM sodium sulfate and was at pH 8.5, while the bath saline was maintained at pH 7.0. Data represent means (N = 4) and error bars represent ± SEM. Means with different letters denote significant difference between treatments (paired Student's t-test, Bonferroni correction, $p < 0.01$).

The effects of calcium chloride and sodium sulfate were examined using the perfusion of 10 mM L-histidine alone and with the addition of 50 µM zinc during the second hour and 20 mM glycylsarcosine during the third hour. However, the amount of calcium chloride and sodium sulfate in both the perfused saline and bath saline were altered over the course of four experiments, each with at least three replicates of shrimp intestines. There remained a significant increase in L-histidine transport with the addition of zinc to the perfusate when the calcium chloride concentration was raised to 25 mM (paired Student's t-test, Bonferroni correction, $p < 0.02$, Figure 19). There was

no significant difference found between the transport rates of L-histidine in the presence of zinc or both zinc and glycylsarcosine. The concentration of calcium had an impact on the transport rate of L-histidine alone, but not in the presence of zinc or when both zinc and glycylsarcosine were added. It was found that histidine transport was significantly higher with 1 mM calcium chloride than with 25mM calcium chloride when histidine was perfused by itself (two-tailed Student's t-test, $p < 0.05$, Figure 19).

The data from an earlier experiment with 1 mM calcium chloride and 8.4 mM sodium sulfate in the physiological saline was used to analyze the effect of increasing sodium sulfate concentration to 26mM (Figure 20). It was seen that the use of 26 mM sodium sulfate had a slightly inhibitory effect on L-histidine transport in the presence of zinc that was not significant ($p < 0.1$). The higher concentration of sulfate did not affect L-histidine transport during the other treatments in the experiment. The transport rate of L-histidine was not significantly increased with the addition of zinc to the perfusate ($p < 0.1$), and the further addition of glycylsarcosine had no effect on transport rate (Figure 20).

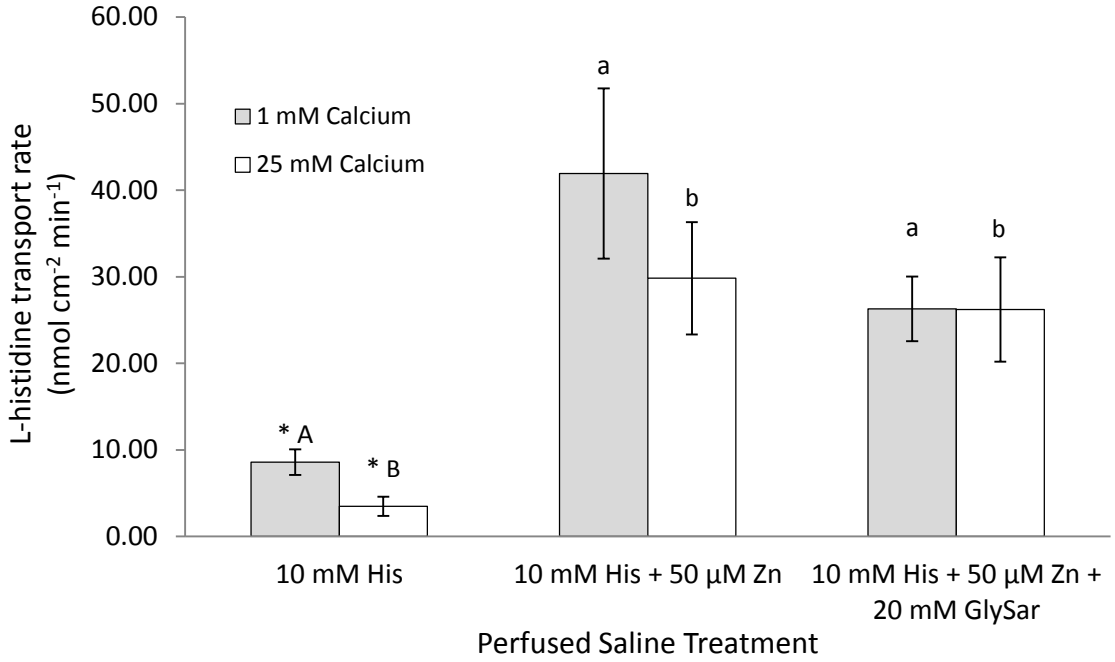

Figure 19. Effect of calcium chloride on the transport rate of 10 mM L-histidine through shrimp intestine. Perfused saline contained 8.4 mM sodium sulfate and either 1 mM or 25 mM calcium chloride and was at pH 8.5, while the bath saline was maintained at pH 7.0. Data represent means and error bars represent ± SEM (N=4 for 1mM, N=3 for 25mM calcium). Asterisk represents significant difference between 1 mM and 25 mM calcium treatments ($p < 0.05$) using two-tailed Student's t-test. Significance for each concentration of calcium is denoted using different letters (A for 1 mM, B for 25 mM). Means with lower-case letters denote significant difference from means with capital letters between treatments for L-histidine (paired Student's t-test, Bonferroni correction, $p < 0.02$).

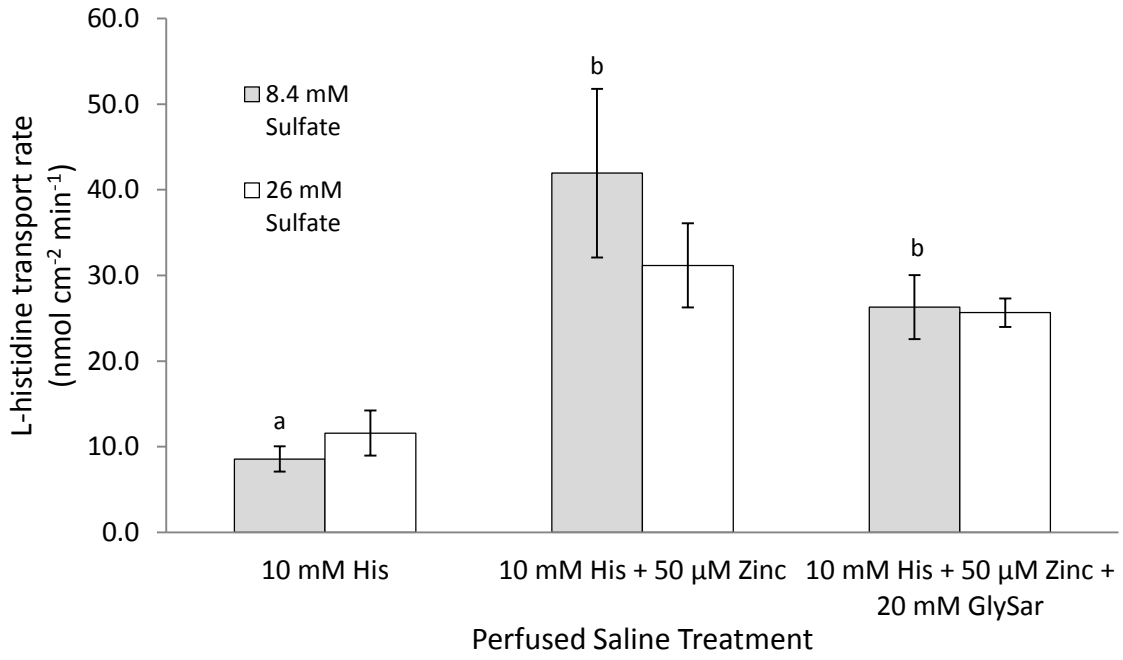

Figure 20. Effect of sodium sulfate on the transport rate of 10 mM L-Histidine through shrimp intestine. Perfused saline contained 1 mM calcium chloride and either 8.4 mM or 26 mM sodium sulfate and was at pH 8.5, while the bath saline was maintained at pH 7.0. Bars represent means and error bars represent ± SEM (N=4 for 8.4mM, N=3 for 26mM sodium sulfate). No significant difference was found between perfused treatments for 26 mM sodium sulfate ($p > 0.05$) or between each treatment at either 8.4 mM or 26 mM sodium sulfate. Means with different letters denote significant difference between treatments using 8.4mM sodium sulfate (paired Student's t-test, Bonferroni correction, $p < 0.02$).

DISCUSSION

Gas Chromatography Technique

The present study investigated numerous lines of research that both extended previous physiological knowledge of crustacean transport mechanisms as well as provided a physiological basis for improving crustacean nutrition. Valuable insight was gained in the novel use of gas chromatography for amino acid analysis, which can be applied to future studies examining complex mixtures that would otherwise not be feasible using methods such as radioisotopic labeling of single amino acids or sugars. The results from the investigation of transport mechanisms in shrimp intestine can be used to provide suggestions for changes in water quality and feed composition. This could ultimately improve shrimp nutrition, allowing for faster growth and quicker maturation to the desired size. In addition, these data can be used to optimize feed composition. Knowledge of the cellular processes of nutrient uptake in shrimp can ensure that feed does not contain excessive amounts of unnecessary supplements, and adequate amounts of others.

The preliminary experiments in this study verified that gas chromatography could be used in subsequent studies of amino acids. It was found that a complex mixture of amino acids as well as certain dipeptides can be analyzed effectively and relatively quickly. Several drawbacks to this method were discovered through trial-and-error. For instance, it is necessary to have a fairly large concentration of amino acids present in the solution for analysis by GC-FID. This is a hindrance for kinetic studies due

to the typically low K_m and J_{max} kinetic constants that are useful in cellular transport research, which often occur in the picomolar or nanomolar range. The current study found that analysis by GC-FID is only effective for saline sample solutions containing at least 50 µM concentrations of amino acids. It was often necessary to perfuse at least 10 mM concentrations of amino acids through the lumen of the intestine in order to have sufficient accumulation of amino acids in the bath saline. It may be possible through further optimization of the perfusate saline to use lower concentrations in the perfusate, but this study recommends that no lower than 1 mM be used, as it was found that this concentration would only provide measureable results half of the time. However, many transporters in the intestine of crustaceans are saturable in the micromolar range. This is due to the use of the intestine as a 'scavenger' organ, as most of the nutrient uptake occurs in the hepatopancreas (Factor, 1995). While it may be difficult to generate kinetic constants, the method used in this study is still applicable for intestine research due to several low-affinity systems present within the epithelium.

Metal Co-Transporter Experiments

Multiple experiments using lobster intestine were carried on concurrently with shrimp studies. Initially, the research focused on bis-complex transport in both animals, using zinc ions to form complexes with amino acids that result in molecules that resemble dipeptides and can be transported through a PepT-like dipeptide transporter (Conrad and Ahearn, 2005; Conrad and Ahearn 2007; Obi et al., 2011). Bis-complexes have been investigated in teleost fish as well as lobster, and their apparent formation in

shrimp was confirmed in this study (Glover and Wood, 2008). Even though zinc was used at a concentration that was 200 times lower than the concentration of L-histidine in the saline perfusate, it was still found to significantly increase the rate of histidine transport by enabling the transepithelial transport of histidine by multiple routes (Figures 18-20). While a similar result has been seen in lobster intestine and interpreted as molecular mimicry of dipeptides, this information was novel for shrimp intestine (Obi et al., 2011). Since the production of farmed shrimp is a much larger industry than farmed lobster, data that directly supports the use of supplemental metals in the shrimp diet is preferred to analogous data from a different crustacean species.

Glycylsarcosine Experiments

During the course of the bis-complex investigation, it was found that the dipeptide glycylsarcosine could be analyzed by GC-FID, though its visualization proved more difficult than for the three amino acids that were measured most often in this study (L-leucine, L-methionine, and L-histidine). Even at the same millimolar concentrations, the relative signal area shown on the chromatogram was much smaller for glycylsarcosine. This necessitated the use of a 10 mM perfusate, although a few experiments were conducted successfully using a 1mM concentration (Figure 11).

Multiple conclusions can be drawn from the analysis of glycylsarcosine. First, it was shown that the dipeptide is transported intact in both directions across the intestinal epithelium. Although dipeptide transport has been investigated for many years, early researchers came to the conclusion that any small peptides that entered

cells would be enzymatically degraded into their amino acid components prior to exit from the cell (Rubino, Field, and Shwachman, 1971). Transporters on the apical side of the membrane have been identified and extensively studied, including members of the PepT and PHT families (Daniel and Kottra, 2004). These transporters have been characterized in vertebrates and invertebrates and appear to be highly conserved across evolutionary history (Bröer, 2008). More recent studies have shown that peptides are not necessarily degraded within the epithelium, and that small peptides can be transported to the blood (Dyer et al., 1990; Shepherd et al., 2002; Daniel and Kottra, 2004). The results seen with glycylsarcosine in this study demonstrate that crustaceans can also absorb small peptides from the lumen and transport them directly to the blood (Figures 10-16). It also gives rise to the assumption that since glycylsarcosine is not being broken down within the cell, there must be a basolateral transporter for peptides. Basolateral transport mechanisms have not been as widely investigated as apical transporters and warrant further research to enhance knowledge of nutrient transport across epithelia (Shepherd et al., 2002). A recent study investigated the pH-dependence of a basolateral transporter in human Caco-2 cells using glycylsarcosine (Bethelson et al., 2013). The results from the present investigation support the findings from this previous study, and also indicate that basolateral transport proteins may be as conserved as apical transport proteins, due to their existence in invertebrates as well as vertebrates.

The results from the glycylsarcosine data also showed the net movement of this dipeptide from the lumen to the blood, indicating its transport is absorptive (Figure 12).

These data, as well as those from the kinetic study (Figure 14), confirm that much of the transport of glycylsarcosine as seen in this investigation is due to the presence of a transport protein, and does not occur via paracellular transport. The existence of a PepT-like transporter in crustaceans has been previously suggested as a mechanism of transport for amino acid and metal complexes (Conrad and Ahearn, 2005). The use of glycylsarcosine in this study confirms the existence of a peptide transporter in crustaceans. This is of interest both physiologically as well as for use in feed formulation. By adding small peptides to shrimp feed, it may be possible to increase their growth rates. It has been shown that pure amino acids are present in shrimp feed in excess, and that a large proportion of nutrients from feed are dissolved in the water and lost before consumption (Martínez-Porchas et al., 2010). The addition of small peptides to feed will mimic natural foods more closely and could enhance the uptake of other components of the feed, which could reduce waste.

Water Quality Experiments – pH, Calcium and Sulfate Effects

During the course of experiments using dipeptides and amino acids, it was found that their transport can be enhanced at a high luminal pH (Figure 15). This finding was not expected; previous studies investigating amino acid transport have found that a luminal pH range from 5 to 6 and a blood pH of 7 results in optimal transport (Conrad and Ahearn, 2005; Obi et al., 2011). This is the accepted model in vertebrates, and the uptake of peptides has been shown to be proton-dependent, such that peptides enter the cell via antiport with protons, while the uptake of amino acids is sodium-dependent

(Watanabe et al., 2005; Bröer, 2002). However, another study using zebrafish (*Danio rerio)* also found transport of glycylsarcosine to be more effective at an alkaline pH (Romano et al., 2013). In that study, it was posited that since the zebrafish does not possess an acidic stomach, the luminal pH may be naturally much more alkaline that what is seen in other fish and mammals. The stomach of the shrimp is known to be slightly acidic. It is possible that shrimp produce enzymes or buffers to regulate the pH of the intestine so that it is closer to neutral or slightly basic. This idea is supported by the findings that an alkaline pH optimizes the enzymatic processes for proteinases such as trypsin and peptidase in shrimp (Mantel, 1983; Jones et al., 1997). Future studies could further examine this aspect of their physiology. The results from this experiment imply that shrimp raised in near freshwater conditions may not be absorbing nutrients as effectively as shrimp raised in brackish water, as freshwater conditions would have a more acidic pH than brackish or saltwater. Water quality on shrimp farms could be monitored to maintain brackish or saltwater conditions, rather than raising shrimp in sub-optimal freshwater conditions.

Under typical conditions, the crustacean epithelium is electronegative inside the cell (Ahearn, 1982). This electrical gradient is used along with the concentration gradient to drive nutrient uptake from the lumen to the cell and then to the blood. Low amounts of sodium within the cell also help power their transport, and the low intracellular concentration of sodium is maintained by Na^+/K^+ -ATPase pumps. The findings from this study suggest that calcium may also play a role in nutrient uptake. It was seen that high luminal concentrations of calcium result in a significantly lower rate

of transport for both sodium-dependent amino acid transport and dipeptide transport (Figures 15 and 19). This may be explained by the presence of calcium channels along the apical membrane (Abdel-Malak and Ahearn, 2014; Ahearn and Zhuang, 1995). When the luminal concentration of calcium is high, these channels may open and allow the flow of calcium from the lumen to inside the cell. This movement may depolarize the cell, which may inhibit the uptake through other electrogenic systems. This mechanism could explain why higher concentrations of calcium negatively affect the rate of nutrient uptake. Since this was also seen in a recent study in the Ahearn laboratory using lobster intestine, it is likely that the same process is occurring in both lobster and shrimp intestine when exposed to high levels of calcium (Abdel-Malak and Ahearn, 2014). Additionally, excess calcium in the diet can react with metals such as zinc and magnesium, and consequently form chelates, which could inhibit the stimulatory effect of zinc on amino acid transport (Davis and Gatlin, 1996). This finding is supported by field observations on a Florida shrimp farm, where a deep well provides water for 25 acres of shrimp ponds (Wood's Fisheries, Inc., Port St. Joe, FL). At about five parts per thousand salinity, the well water is composed primarily of calcium and sulfate salts, and shrimp grown for a period of 165 days only reach a total weight of about 30 grams, as compared to a typical adult weight of 30-45 grams (Rosenberry, 2007; Godwin and Ahearn, personal communication, 2013; FAO, 2006). In this type of situation, shrimp growth may be occurring at a less than optimal rate due to the proportionately increased calcium content of the water when compared to natural conditions.

As compared to the effect of calcium, sulfate was seen to have little to no effect on L-histidine transport in shrimp (Figure 19). It has been discovered previously that fish ponds built on acid sulfate soils, such as those found in mangroves, will have diminished growth of cultured species (Golez, 1995). By conversion of the sulfate in the soil to an acidic form by anaerobic bacteria, pond water above the soil will become acidified (Golez, 1995). The data generated from the current research indicates that sulfate itself, in the form of sodium sulfate in saline, does not have a negative effect on amino acid transport. It is possible, however, that ponds containing an excess of sulfate in the water may become acidified more quickly than those with less of a sulfate load. This acidification could lead to a reduction in growth through other deleterious effects than by inhibition of amino acid transport.

Future work will needed to identify the particular calcium concentrations in water that promote the highest growth rate in shrimp, preferably with live shrimp reared in an easily controlled environment. A simple experimental design would involve multiple aquarium tanks with juvenile shrimp exposed to low, medium, and high calcium chloride conditions. Results from this study indicate that the use of aquaculture ponds containing 25 mM calcium salts could be reducing the growth rate of their larvae. It would also be of use to test feed formulations with a reduction in calcium, as it has been found that shrimp can acquire sufficient calcium from their ingestion of water and through their gills (Davis and Gatlin, 1996). Feed formulations with supplemented metals, such as zinc and manganese, or small peptides could also be analyzed for higher growth rates in shrimp.

Aside from rearing juvenile shrimp under various conditions, further work using both shrimp intestine and shrimp hepatopancreas could continue to improve the research community's knowledge of the physiological processes of digestion in crustaceans. Future experiments utilizing gas chromatography would be of particular use since various mixtures of amino acids and dipeptides could be analyzed at the same time. While this would not elucidate the transport mechanisms responsible for each amino acid or dipeptide, it would provide data that would better represent the natural state of digestion, in which concentrations and types of peptides could vary greatly. New research has focused on the role of PepT-1 transporters in teleost fish and other aquaculture species, and it has been found that feed formulations using plant proteins rather than fish meal can have a negative impact on digestion and cause enteritis in carnivorous fish as well as lowered expression of PepT-1 mRNA (Rønnestad et al., 2010; Lui et al., 2013). The use of gas chromatography to analyze perfused mixtures of amino acids and peptides would give more information about competitive inhibition within the intestine, and could aid in future aquaculture studies of PepT-1 and basolateral transporters.

CONCLUSIONS

The following information was concluded from this study:

1. Gas chromatography can be used in subsequent studies of amino acids. A complex mixture of amino acids as well as certain dipeptides can be analyzed effectively and quickly, but only at high concentrations.
2. Zinc significantly increased the rate of L-histidine transport in the shrimp intestine. Transport was reduced with the addition of a competing dipeptide, indicating that bis-complex formation and a PepT-like transporter may be present in shrimp intestine.
3. Glycylsarcosine is transported intact in both directions across the intestinal epithelium.
4. Net flux of glycylsarcosine is from mucosa to serosa; movement of glycylsarcosine from serosa to mucosa is not affected by the presence of bis-complexes, while in the opposite direction, glycylsarcosine transport is significantly inhibited by bis-complexes.
5. An alkaline pH significantly increased the transepithelial transport of glycylsarsocine and L-histidine in lobster and shrimp, respectively.
6. Excess calcium significantly inhibited the transport of L-histidine across shrimp intestine, and glycylsarcosine across lobster intestine.

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