2016

Egesta Of The Sea Urchin Lytechinus Variegatus Promote Weight Gain In The Shrimp Litopenaeus Vannamei

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A THESIS

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Master of Science

BIRMINGHAM, ALABAMA

2016

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BIOLOGY

ABSTRACT

Long-term economic and environmental sustainability of aquaculture will be dependent on utilization of novel nutrient sources and remediation of effluent streams. Integrated Multi-Trophic Aquaculture (IMTA) is a method intended to maximize productivity and minimize waste by strategically reusing effluent from a primary, fed species in the production of one or more secondary, extractive species**.** Such relationships are based on the trophic role of the fed and extractive species in question. Sea urchin egesta are thought to play a role in nutrient cycling in natural habitats by providing an energy source for many coprophagous deposit feeders, suspension feeders, and microbes. The shrimp *Litopenaeus vannamei* is a generalist scavenger and has been observed previously to readily ingest the egesta produced by cultured specimens of the sea urchin *Lytechinus variegatus.* This work investigates the potential value of egesta from the sea urchin *Lytechinus variegatus* as a sole nutrient source or supplement for the shrimp *Litopenaeus vannamei*.

Chapter 1 compares several different feeds for shrimp held individually. Urchin feed allowed for weight gain in shrimp, but those shrimp gained significantly less weight than those proffered commercial shrimp feed. When urchin egesta were processed and proffered to shrimp as wet or dry rations, shrimp gained relatively little weight. However, when shrimp were held in sequential co-culture with sea urchins, they had the highest

weight gain of any other treatment. With proper handling, some nutritional benefit can be conferred to shrimp via the urchin egesta.

Chapter 2 explores sea urchin egesta as an exclusive diet at varying culture densities, as well as urchin egesta proffered with varying commercial shrimp feed rations. Shrimp proffered urchin egesta exclusively were not significantly different in weight gain or body composition from shrimp proffered a full ration of shrimp feed, despite lower estimated nutrient density of urchin egesta. When shrimp feed was proffered in addition to urchin egesta, shrimp weight gain was increased beyond what was achieved with shrimp feed alone. Sea urchin egesta appears to provide some growth enhancement factor that confers highly efficient nutrient utilization or protein retention, allowing shrimp to realize increased genetic potential for weight gain.

Keywords: shrimp, sea urchin, Integrated Multi-Trophic Aquaculture, egesta, polyculture, nutrition

ACKNOWLEDGEMENTS

I want to thank my mentor, Dr. Stephen A. Watts, and my committee members Dr. Louis D'Abramo and Dr. Allen Davis for their support, guidance, and encouragement through this process. I also want to thank members of the Watts lab, past and present, for sharing their knowledge, advice, and time to contribute to this research effort. I also want to thank the Department of Biology and the Graduate School at the University of Alabama at Birmingham for financial support and professional development opportunities during my graduate education.

This research was supported in part by the Aquatic Animal Research Core within the Animal Models Core at the University of Alabama at Birmingham, NIH NORC P30DK056, and by a student research grant from the Alabama Academy of Science.

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LIST OF ABBREVIATIONS

BFT	bio-floc technology
FCR	food conversion ratio
IMTA	integrated multi-trophic aquaculture
R/O	reverse osmosis
SEM	standard error of the mean
SD	standard deviation
SF	shrimp feed
UE	urchin egesta

CHAPTER 1

INTRODUCTION

Many studies have demonstrated the significance of natural productivity as a source of nutrients in shrimp culture. In extensive ponds with high natural productivity, *Litopenaeus vannamei* ingest a wide variety of phytoplankton, zooplankton, and small benthic invertebrates as well as detritus, mud, and other miscellaneous material (Moss and Pruder 1995; Varadharajan and Pushparajan 2013). Pond water containing organic particles larger than 0.5 μm has been shown to enhance shrimp growth and metabolic enzyme activity beyond what is attained in clear well water (Moss and Pruder 1995; Moss et al. 2001) and spare the adverse effects of a vitamin-deficient feed (Moss et al. 2006). Shrimp are also commonly grown in Biofloc technology (BFT) systems, which offer an aggregate of heterotrophic microbial biomass and organic and inorganic material as an *in situ* feed and biofilter (Burford et al. 2004; De Schryver et al. 2008; Crab et al. 2012). Floc aggregates contain microbial exopolymers which are highly absorptive and help to sequester organic matter, thereby providing recycled nutrients for shrimp while maintaining water quality (Decho 1990; Moss et al. 1995).

Due to the natural feeding behavior in these shrimp, it is likely that environmental- or diet-derived microbiota are frequently ingested. Populations of bacteria are found within the gut of *L. vannamei* at various life stages (Huang et al. 2016). The role of bacteria in supporting shrimp health has been suggested, as the addition of different probiotics to the water of shrimp culture systems has been shown to enhance or

contribute to existing digestive enzyme activity (Moss et al. 2001; Liu et al. 2009) and aid in disease control by outcompeting and displacing pathogenic organisms in the gut (Li et al. 2009; Thompson et al. 2010). The resident microbiota of the shrimp gut can be altered or enhanced by feeding different diets (Moss et al. 2001; Liu et al. 2009), suggesting that the extensive range of digestive capacity may lie in the ability of shrimp to utilize exogenously derived microbiota. Such an ability would prove invaluable to producers looking for alternatives to increasingly finite feed ingredients like fish meal and oil (Naylor et al. 2000; Tacon and Metian 2008; Ottinger et al. 2016).

L. vannamei have previously been observed to rapidly consume egesta of the sea urchin *Lytechinus variegatus* when held in co-culture (Siccardi et al. 2005). *Lytechinus variegatus* is an edible sea urchin found in seagrass beds along the east coast of the United States and the Gulf of Mexico. In natural systems, sea urchins can have a significant role in nutrient cycling through the contribution of large amounts of nutrient-rich fecal material to detrital food webs (Mamelona and Pelletier 2005; Sauchyn and Scheibling 2009). Adult *Lytechinus variegatus* often produce negatively buoyant, mucous-covered egesta pellets (approximately 1 mm diameter) composed of undigested feed material packed with an actively dividing microbial community, principally consisting of *Vibrio* and *Arcobacter spp.* among many others (Dennis 2014; Hakim et al. 2015). The microbial community associated with the egesta has been suggested to aid the urchin in the digestion of certain complex carbohydrates, proteins and lipids (Holland et al. 2013; Lawrence et al. 2013; Hakim et al. 2016). In addition to any direct nutritional benefit that the shrimp might gain from it, consumption of urchin egesta may also have the potential to provide beneficial probiotic material.

This study was designed to evaluate the possible use of egesta produced by the sea urchin *Lytechinus variegatus* as a supplement or feed for *Litopenaeus vannamei*. This study compared several feed management practices based on different feeds and the physical properties of urchin egesta to determine any effects on survival and growth of juvenile *L. vannamei*. We hypothesize that urchin egesta could serve some nutritional or probiotic function for the shrimp, thereby increasing feed digestibility leading to enhanced growth.

METHODS

Culture Conditions and Water Quality

Litopenaeus vannamei stock were initially acquired from Shrimp Improvement Systems (FL, USA) and transported to the E.W. Shell Fisheries Center in Auburn, AL. They were then transported to the University of Alabama at Birmingham in Birmingham, AL and held in aerated holding tanks and offered a commercial shrimp feed until stocking.

Postlarval *Litopenaeus vannamei* (n=10-12 individuals per treatment) were stocked at ca. 0.49 g ±0.06 g initial body weight and housed individually in self-cleaning 2.8 L water volume polycarbonate tanks in a commercial zebrafish housing system (Aquaneering, Inc.). Flow rates were adjusted to provide at least six water changes per hour within each tank. Municipal tap water was filtered through 5 μm sediment filter, followed by charcoal, reverse osmosis (R/O), and a cation/anion exchange resin (Kent Marine, Franklin, WI) prior to the addition of synthetic sea salts (Instant Ocean) to obtain a final salinity of 32 ppt for the system water source. This system contained 84 housing

tanks connected to a central sump with a resulting total water volume of approximately 470 L. Filtration was achieved via a particulate filter pad for initial solids removal, followed in series by a fluidized glass bead filter, dual carbon finish filters and a high output UV sterilizer. Water temperature was maintained at 28 °C with a 1000-watt digitally controlled heater.

Total ammonia nitrogen, nitrite, nitrate, pH and alkalinity levels were checked weekly using saltwater test kits from Aqua Pharmaceuticals, LLC (Malvern, PA, USA) for ammonia and nitrogen and La Motte Company (Chestertown, MD, USA) for alkalinity. Photo-period, water temperature, and salinity were held constant (12:12 light: dark, 28±1 °C SD, 32.0±1.0 ppt SD).

Diets and diet preparation

Individually held *L. vannamei* were fed one of six dietary treatments. Two treatments consisted of a 2.4 mm commercial shrimp feed (Rangen Shrimp Production 35/2.5) fed exclusively (control), or a sea urchin feed (Hammer et al. 2012). Additional experimental diets included wet, rinsed sea urchin egesta, rinsed and dried sea urchin egesta (described below), a diet proffered as ½ shrimp feed and ½ rinsed wet sea urchin egesta (each contributing the ½ dry weight equivalent of the total amount fed), and natural fresh egesta produced in sequential polyculture with sea urchins. Proximate composition of these diets can be found in Table 1.

Table 1: Proximate composition (dry weight) of select diets. Shrimp feed values were provided by the manufacturer (Rangen). Urchin feed was formulated to meet listed values. Composition of urchin egesta was determined via colorimetric and gravimetric analysis (Chapter 2). Fiber in urchin egesta includes both insoluble protein and fiber and was calculated by difference. n=3 replicates of egesta combined from 40 sea urchins.

FEED TYPE	PROTEIN (%)	CARBOHYDRATE (%)	LIPID (%)	FIBER (%)	ASH (%)	MOISTURE (%)
SHRIMP FEED	39	40	9	3	9	10
URCHIN FEED	29	32	7	6	26	10
URCHIN EGESTA	16.67±0.37	1.74±0.10	3.99±0.21	30%	47.61±0.57	85.55±0.00

Collection and preparation of sea urchin egesta

Egesta for the collected wet and dry egesta dietary treatments were produced by urchins located in separate group housing and fed the previously-described extruded urchin feed *ad libitum*. The egesta, consisting primarily of small mucus-covered spherical pellets (Dennis 2014), were manually siphoned from urchin housing daily, placed on a 100µm sieve, and rinsed with reverse osmosis (RO) water to remove excess surface salt. Aliquots of wet egesta were collected by spatula and weighed. To determine moisture content of the egesta, excess moisture was removed by gentle blotting and egesta were then dried to constant weight at 30℃ in a mechanical convection oven (Economy Model 18EM, Precision Scientific) for 72 hours. Moisture content was determined *{[initial weight (g) - dry weight (g) / initial weight (g)] *100}*. Wet egesta were proffered in dry matter equivalents to the commercial diet, sea urchin diet, and the dry egesta. Dry egesta were broken into flakes and fed to the shrimp.

Natural fresh egesta in polyculture represented the collective egesta provided by two urchins per 2.8 L tank (sea urchin wet weight ca. 13.2±0.8 g SD each) housed above shrimp in segregated co-culture. Species were separated by the inner tank portion of The

Aquaneering Zebrafish Crossing Tank (Aquaneering, Inc.) nested inside the polycarbonate housing tank. This tank was designed with a slatted bottom which allowed urchins and their feed to be physically separated above the shrimp while allowing any negatively-buoyant sea urchin egesta to fall below for consumption by shrimp. Shrimp were not fed directly, but obtained food as the egesta were expelled from the urchins (egesta are produced transiently over time within a 24-hour period). Shrimp had full access to the egesta produced from two urchins fed the extruded urchin feed at 2% body weight once daily. Each tank of urchins received 14.28±0.06 g SD urchin feed over the course of the study.

Urchin Egesta Proximate Analysis

Dry egesta were manually ground to a powder using a ceramic mortar and pestle. Protein and carbohydrate levels were determined via colorimetric analysis (Lowry et al., 1951, and Dubois et al., 1956, respecively), lipid levels were determined by the method described by Folch (1957), and ash levels were determined by combustion in a muffle furnace at 50˚C for 4 h. Insoluble protein and fiber were also present in the sample, and combined levels were estimated by difference.

Feeding Rate

Shrimp were assigned randomly to one of the six dietary treatments and fed twice daily an equivalent ration (as feed weight/shrimp/day) except for those fed natural fresh egesta in polyculture. For the duration of the experiment, a total of 4.13±0.03 g SD dry matter was fed per shrimp (approximately 0.16 g per day per shrimp). Shrimp feed,

urchin feed, and collected dry egesta were weighed as fed. Collected wet egesta was provided as dry matter equivalent rations to the shrimp and urchin diets. Estimated dry material available to shrimp in polyculture was calculated as *{urchin feed proffered (g)*[1-absorption efficiency]}.*

Termination of Experiment

The trial was terminated after 27 days. Before being weighed, urchins were placed on paper towels outside of the water for at least 30 seconds to remove excess water. Before being weighed individually, shrimp were blotted with paper towels to remove surface and interstitial water. Both shrimp and urchins were weighed to the nearest 0.001 g and weight gain was calculated *[final weight (g) – initial weight (g)]*. Shrimp growth was expressed as percent weight gain *{[weight gain (g) / initial weight (g)] *100}.* Food conversion ratio (FCR) was calculated as *[feed proffered(g)/weight gain(g)]* in all groups except the polyculture. FCR for the shrimp in polyculture was calculated as *{urchin feed proffered (g)*[1-expected urchin absorption efficiency]}/shrimp weight gain(g).*

Statistics

Means were compared among treatments using SPSS Statistics 23 (IBM Corp.). Shrimp that died prior to termination of the experiment were not included in the analysis. Means were compared using one-way ANOVA followed by a Tukey HSD test for *post-hoc* analysis. A p-value ≤ 0.05 was considered significant. Mean urchin weight gain was determined and graphs were generated using Microsoft Excel.

RESULTS

Shrimp Survival and Growth

Table 2: Mean growth parameters (±SEM) of shrimp (*Litopenaeus vannamei*) juveniles after 27 days in culture under different feed regimes. Differing superscripts within a column indicate significant differences determined with one-way ANOVA and a Tukey HSD test for *post-hoc* analysis ($p \leq 0.05$; n=9-12).

FEED TREATMENT	INITIAL WEIGHT (G)	WET WEIGHT GAIN (G)	WEIGHT GAIN (%)	FCR
SHRIMP FEED	0.49±0.02	1.45±0.08[a]	300.87±17.55[a]	2.96±0.18
WET EGESTA	0.50±0.02	0.05±0.01[b]	10.70±3.63[b]	0
DRY EGESTA	0.48±0.02	0.19±0.03[b]	42.08±6.83[b]	27.20±3.91
½ SHRIMP FEED ½ WET EGESTA	0.49±0.02	1.37±0.09[a]	280.31±16.68[a]	3.20±0.23
URCHIN FEED	0.50±0.02	0.71±0.08[c]	147.34±17.50[c]	9.96±4.04
NATURAL EGESTA IN POLYCULTURE	0.51±0.02	2.30±0.13[d]	469.80±30.35[d]	1.81±0.10

Shrimp survival was > 90% in all treatments. Weight gain recorded for those fed either the shrimp feed treatment or the half commercial shrimp feed and half wet egesta treatment were not significantly different ($p>0.05$, Table 2). Shrimp proffered urchin feed achieved weight gain, but less than those proffered shrimp feed, shrimp feed with wet egesta, and natural fresh egesta in polyculture. Weight gain of shrimp proffered either dry or wet egesta did not differ significantly and both were significantly less than that of each of the other treatments. Weight loss in four of the shrimp proffered wet egesta resulted in a negative mean FCR in that treatment, which was simplified to 0 in Table 2. Highest growth rates and lowest estimated FCR were seen in those consuming natural fresh egesta in polyculture (Table 2).

Sea Urchin Survival and Growth

Survival was 95±5% SEM for all urchins. The single mortality was due to a culture system blockage that temporarily slowed or stopped water flow into the tank. This urchin was replaced immediately. Urchin pairs in each tank gained ca. 11.07± 0.74g SEM (40.20±3.31% weight gain) during the experiment, with an average FCR of 1.3.

Urchin Egesta Proximate Composition

Based on data presented in Chapter 2, moisture content of urchin egesta was determined to be to be 77.9 ± 0.2% SD. Proximate composition of urchin egesta can be found in Table 2. Expected urchin absorption efficiency was estimated at 71.59% based on data from Chapter 2. Expected dry material from the natural egesta available to shrimp in polyculture was 4.06±0.01 g.

DISCUSSION

Shrimp consuming natural fresh egesta in polyculture with sea urchins had the highest weight gain among all treatments. In contrast, the minimal weight gain in shrimp fed processed (wet or dry) egesta suggests that this form of egesta does not provide adequate nutrients to promote weight gain, or it does not have the physical properties necessary to promote adequate intake. We hypothesize this difference was due in part to the processing and/or the physical, chemical, and biological properties of the egesta. Collected wet egesta was rinsed with RO water before being proffered and many pellets of the egesta, normally held together by mucus, appeared to physically break apart

resulting in a small particle size. In this flocculent form, these small pieces could be flushed from the holding tanks too quickly for the shrimp to have full access to them. Dry egesta may have been more accessible to shrimp as the pieces remained demersal, however, the material was brittle and could have broken up into very small particulates during the process of feeding. In addition, the smell of ammonia from the dry egesta was noted and possibly could have acted as a feeding suppressant (Lee and Meyers 1997). Additionally, any biological advantage (live bacteria) may have been lost in the drying process. It is possible this collected material might be better utilized as alternative feed ingredient within a complete diet, as Kuhn et al. (2009) suggested with dried bioflocs.

Increased weight gain and decreased FCR observed in shrimp fed natural fresh egesta in polyculture when compared to shrimp fed urchin food suggest that some benefit is produced via the process of urchin digestion. Probiotic enhancements may explain the significantly higher growth observed for shrimp in the polyculture treatment. The addition of certain probiotics has been reported to alter the microbial community in the shrimp gut (Liu et al. 2009; Thompson et al. 2010) with benefits in both disease control (Li et al. 2009; Thompson et al. 2010) and digestive enzyme activity (De Schrijver and Ollevier 2000; Moss et al. 2001; Liu et al. 2009). With regular ingestion of natural fresh urchin egesta containing readily available, live microorganisms, it is possible that the shrimp gut was colonized with a microbial community already customized to utilize the remaining nutrients found in urchin egesta, Support for this explanation can be found in the results of Liu et al. (2009). They isolated a strain of *Bacillus subilis* with high protease activity from fermented soybeans and added it to shrimp feed containing soybean meal. The bacteria, found in high numbers in the gut of the shrimp, enhanced

enzymatic activity, resulting in greater shrimp weight gain in comparison to equivalent diets without the probiotic supplement. Even if urchin egesta microbes did not colonize the shrimp gut, shrimp may be able to utilize microbes as a direct nutrient source or utilize the enzymes produced by lysed microbes or other prey items they ingest (Harris 1993; Moss et al. 2001; Kuhn et al. 2009)

Further consideration should be made for investigating the use of certain species of microbes from sea urchin egesta as probiotics or single cell nutrient sources for shrimp culture. Special attention should be paid to those microbes with metabolic profiles suggesting specificity for digestion of certain compounds (Hakim et al. 2016), especially when shrimp do not produce related enzymes endogenously. Importantly, these data suggest that *Litopenaeus vannamei* has the potential to serve as an extractive species in an Integrated Multi-Trophic Aquaculture (IMTA) system. In such systems, "fed" species (those that are fed directly) can be coupled with one or more "extractive" species (those that can utilize downstream nutrients) to improve nutrient utilization in a system while yielding an additional product(s) and corresponding sources of income (Neori et al. 2007).

CHAPTER 2

INTRODUCTION

The whiteleg shrimp *Litopenaeus vannamei* is one of the most widely farmed marine animals in the world. Nutrient requirements of this species have been well-studied and a variety of pelleted diets for varying life stages and culture conditions are available commercially (Tacon et al. 2002; Kuhn et al. 2010). However, *L. vannamei* is a natural omnivore and scavenger (Moss and Pruder 1995; Varadharajan and Pushparajan 2013) and will readily consume natural biota when present in a culture system. Pond biota (Moss and Pruder 1995; Moss et al. 2001), including natural live organisms (Porchas-Cornejo et al. 2011), bioflocs (Burford et al. 2004; De Schryver et al. 2008; Kuhn et al. 2009; Crab et al. 2012), and functional probiotics (Liu et al. 2009), can provide many required compounds that have been shown to improve growth and survival of shrimp when compared to providing formulated feeds exclusively.

These observations suggest that *Litopenaeus vannamei* has the potential to utilize nutrients that become available within an Integrated Multi-Trophic Aquaculture (IMTA) system. IMTA has the potential to improve nutrient utilization in a system while diversifying the species produced (Troell et al. 2009). With careful consideration of trophic interactions and system design, IMTA couples "fed" species (those that are proffered feed directly) with one or more "extractive" species (those that can utilize downstream waste materials as nutrient sources). Water quality enhancement and reuse can also be realized through IMTA systems. Kang et al. (2003) found that by adding sea

cucumbers to abalone systems, levels of inorganic nitrogen were reduced. As a result, growth and survival of abalone were improved and heating costs were reduced in winter via reduced water exchange. Martinez-Cordova *et al.* (2011) demonstrated that the integration of black clams and algae with shrimp production systems sufficiently removed particulate and dissolved wastes to allow reuse of the culture water. This benefit is very attractive as regulatory agencies and eco-conscious consumers increasingly seek minimal waste output to the environment and reduction in use of finite water resources.

Research and commercial scale IMTA projects worldwide have combined a variety of culture methods and species, often with promising results. Fish (Chopin et al. 1999; Neori et al. 2000; Irisarri et al. 2013; Al-Hafedh et al. 2015) and shrimp (Martinez-Cordova et al. 2011, Muangkeow et al. 2011) have previously served as primary fed species due to their popularity and use of high protein feed. Extractive species often include suspension feeders and deposit feeders that utilize dissolved organic and solid waste products. This group includes bivalves like mussels (Irisarri et al. 2013), clams (Martinez-Cordova et al. 2011), oysters (Jones et al. 2002), sea cucumbers (Kang et al. 2003; Kim et al. 2015), and filter feeding fish like tilapia (Muangkeow et al. 2011), among others. Other beneficial extractive species include seaweeds (Chopin et al. 1999; Neori et al. 2000; Yokoyama and Ishihi 2010; Irisarri et al. 2013), microalgaes (Martinez-Cordova et al. 2011), and even terrestrial plants (Lange *et al.* 2013) that can assimilate dissolved nutrients present in the water column.

A previous study (Chapter 1) suggested that egesta (fecal pellets) from co-cultured sea urchins (*Lytechinus variegatus*) could serve as an alternative feed for the shrimp *Litopenaeus vannamei*. When proffered a formulated diet, adult *Lytechinus*

variegatus produce negatively buoyant, mucous-covered egesta pellets of approximately 1 mm diameter. These egesta are composed of undigested feed ingredients interspersed with the urchin's digestive microbes (Dennis 2014, Hakim et al. 2015), which serve to aid the urchin in the digestion of many complex carbohydrates, proteins and lipids (Hakim et al. 2016). One or more of the members of these gut microbiota could possibly have probiotic properties in addition to any nutritional benefit derived from the egesta. Shrimp feeds containing probiotics have proven to be very effective in contributing to or enhancing existing digestive enzyme activity (Moss et al. 2001; Liu et al. 2009) as well as aiding in disease control by outcompeting and displacing known pathogenic organisms in the gut (Li et al. 2009; Thompson et al. 2010).

Potential candidates for use in an IMTA system must be carefully considered based on knowledge of their feeding behaviors, nutritional needs, and waste production so that highly efficient assimilation of any input of egesta can be achieved. In this study, we will determine if urchin egesta consumed in combination with commercial feed could improve growth, feed conversion, body composition, and survival of *L. vannamei*. We will also evaluate the level of urchin egesta production required to sustain shrimp growth at commercially-relevant densities.

METHODS

Experiment 1

Culture Conditions

The experiment was conducted using a 4000 L recirculating system equipped with a Polygeyser DF-3 biological filter (Aquaculture Systems Technologies, LLC, New

Orleans, LA, USA), a SMART high-output 80 W UV sterilizer (Emperor Aquatics Inc., Pottstown, PA, USA), and a TF500 double-venturi protein skimmer (Top Fathom, Hudsonville, MI, USA) used for foam fractionation. The system consisted of 36 interconnected 76 L glass tanks with a bottom surface area of 0.18 m^2 and a working volume of approximately 69 L each. Each tank contained a plastic floating mesh basket (ca. 0.12 m^2 interior surface area) with a false floor intended to house sea urchins separately from shrimp while allowing any egesta from the urchins to sink to the bottom for consumption by the shrimp (Figure 1).

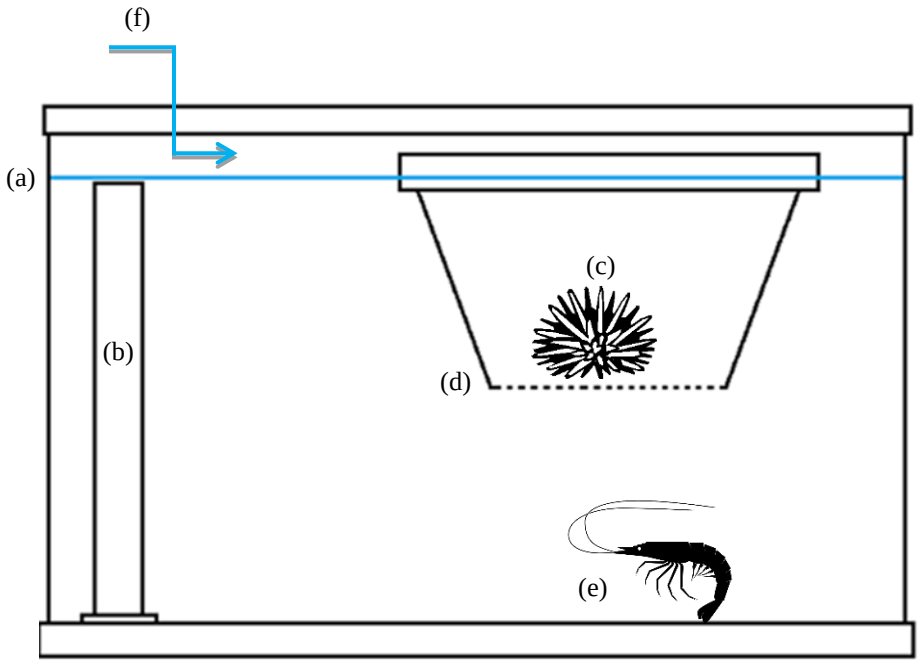

Figure 1: Schematic diagram of an experimental tank used in experiment 2. (a) water level; (b) standpipe; (c) mesh basket for urchin housing; (d) false floor; (e) area containing shrimp; (f) water input from system.

Total ammonia nitrogen, nitrite, nitrate, pH and alkalinity levels were checked weekly using saltwater test kits from Aqua Pharmaceuticals, LLC (Malvern, PA, USA) for ammonia and nitrogen and La Motte Company (Chestertown, MD, USA) for alkalinity. Photo-period, water temperature, and salinity were held constant (12:12 light: dark, 25±1 °C, 32.0±1.0 ppt salinity).

Litopenaeus vannamei stock were initially acquired from Shrimp Improvement Systems (FL, USA) and transported to the E.W. Shell Fisheries Center in Auburn, AL. They were then transported in aerated coolers to the University of Alabama at Birmingham in Birmingham, AL. Animals were held at a density of approximately 50 individuals per tank in the described experimental system and proffered a maintenance ration of commercial feed (Ziegler Bros., Inc., described below) once daily for two weeks prior to stocking.

Groups of four juvenile shrimp (average group weight: 2.76±0.69 g) were assigned to one of seven treatments and stocked into separate tanks with four replicates per treatment. Each group was proffered a set ration of a 2.0 mm short cut commercial shrimp feed containing 40% protein, 9% lipid, and 3% fiber (Zeigler Bros., Inc.), either without or with co-cultured sea urchins in the baskets above producing egesta (Table 1). In treatments where urchins were the fed species, four urchins (average group weight: 100.5±0.70 g) were proffered a formulated, cold-extruded feed (Hammer et al. 2012) containing 29% protein, 32% carbohydrate, 7% lipid, 6% fiber, and 26% ash at 3% of the initial basket biomass daily. One treatment group of shrimp were proffered a designated 100% level of shrimp feed that represented a total of 35.57±0.96 g over the course of the experiment, or approximately 0.17 g feed per individual per day divided into two

feedings at 9:00 AM and 4:00 PM. The 100% ration was based on an estimated 0.8 g of weight gain per shrimp per week at a FCR of 2. Other treatment groups were proffered reduced rations of shrimp feed (Table 1).

Table 1: Treatment groups for Experiment 1. Each treatment is classified by the presence (Egesta +) or absence (Egesta -) of fed sea urchins producing egesta and a full or reduced ration of commercial shrimp feed (Zeigler Bros., Inc.). Shrimp feed ration is presented as fed per individual shrimp. Estimated egesta ration was calculated based on a daily 3 g urchin feed ration and an estimated urchin absorption efficiency of 72%, divided between four shrimp in each replicate. n=4 per replicate.

TREATMENT GROUP	SHRIMP FEED RATION (%)	SHRIMP FEED RATION (g/day)	ESTIMATED EGESTA RATION (g/day)
EGESTA +	100	0.17	0.21
EGESTA +	60	0.10	0.21
EGESTA +	20	0.03	0.21
EGESTA +	0	0	0.21
EGESTA -	100	0.17	0
EGESTA -	60	0.10	0
EGESTA -	20	0.03	0

Termination of experiment

The experiment was terminated after 8 weeks. Urchins were placed on paper towels outside of the water for at least 30 seconds to remove excess water before being weighed. Shrimp were blotted with paper towels to remove excess water before being weighed. Shrimp and urchins were individually weighed to the nearest 0.001 g. Final mean individual weight for each urchin or shrimp replicate was calculated as *[sum of individual weights in each replicate (g)]/number of surviving individuals per replicate]*. Shrimp weight gain was calculated as *[final mean individual weight (g) - initial mean individual weight (g)]*. Total shrimp biomass harvested per m^2 was calculated as *[final group weight (g)/ tank bottom surface area (m^2)]*. Urchin weight gain was calculated as *[final group weight (g) - initial group weight (g)]*.

Urchin Egesta Collection

The amount of egesta falling through the bottom of the urchin basket was measured to estimate the daily amount of egesta (wet and dry) available to the shrimp. After the termination of Experiment 1, shrimp were removed and urchins were left in baskets and proffered urchin feed as usual. Three baskets were selected and nested into a secondary basket lined with 100 µm Nytex mesh to catch any egesta falling from the urchins while maintaining adequate water exchange. Baskets remained in place for 24 hours and there were 12 daily collections extending over 4 days. Material collected on the Nytex mesh was rinsed from the mesh with RO water and transferred into a pre-weighed sieve. Approximate wet weight of the egesta was determined. Samples were then dried overnight at 30 ˚C in a mechanical convection oven (Economy Model 18EM, Precision Scientific) to a constant dry weight. Absorption efficiency of urchins proffered the urchin feed was calculated as *{[dry urchin feed proffered (g) - dry egesta produced (g)/dry urchin feed proffered (g)] *100}.* Hammer et al. (2004) found that dry matter absorption efficiencies of urchins proffered formulated diets did not differ significantly between samples obtained at 5 and 10 weeks. Accordingly, it was assumed that the dry matter absorption efficiency of the urchins did not change throughout the experiment, and thus the nutrient quality of the egesta remained stable over the course of the experiment. Total dry matter available to shrimp via the egesta was calculated as *{[mean daily urchin feed (g)* fed days] * [1- absorption efficiency]}*. Food conversion ratio (FCR) for shrimp was calculated as *[total dry matter shrimp feed proffered (g) + calculated dry matter egesta (g)] / shrimp wet weight gain (g).*

Shrimp Biochemical analysis

All individual shrimp were cut into three pieces and dried at 50˚C in a gravity convection oven (DVS 600, Yamato Scientific America, Inc.) to a consistent dry weight. Dry matter content was calculated as *[dry weight (g)/wet weight (g)]}* 100*. Moisture content was calculated as *[100-dry matter content (%)]*. Individual shrimp were ground into a powder using a Wiley mill (Thomas Scientific) fitted with a 40-mesh screen. Protein and carbohydrate levels were determined in all available individual shrimp (n=12-16 per treatment) via colorimetric analysis (Lowry et al., 1951, and Dubois et al., 1956, respecively). Ash was determined in all available individual shrimp (n=12-16) using a muffle furnace at 550˚C for 4 h and calculated as *{[final weight (g)/initial weight (g)] * 100}*. Six individual shrimp were chosen randomly from each treatment and their lipid levels were determined via gravimetric analysis (Folch 1957).

Considering a 40% protein feed (as is composition provided by Zeigler) and sea urchin egesta with a protein composition of 16.7% (Chapter 1), mean protein available per shrimp was calculated as {*[feed total (g)* 0.4]+[egesta total (g)* 0.167]}/individual shrimp per replicate*. Net apparent protein retention of shrimp in each treatment was calculated as *{mean weight gain (g)* [mean whole body protein composition (%)* 0.01]}/ mean protein proffered per individual (g).*

Statistics

Mean values of the response variables determined for the different dietary treatments were compared using one-way ANOVA followed by a Tukey HSD test for *post-hoc* analysis. A p-value ≤ 0.05 was considered significant.

Experiment 2

Culture conditions

Juvenile shrimp (mean individual weight 0.36±0.02 g) were stocked into tanks that were part of the recirculating aquaculture system as described in Experiment 1. Each tank contained one urchin basket and water conditions were maintained as described previously (Experiment 1). Shrimp were housed at densities of 4, 8, 12, and 16 per tank, equivalent to approximately 23, 45, 68, and 90 individual shrimp per m^2. There were 5 replicates (tanks) per density treatment.

Eight small urchins (ca. 140.4±0.68 g total biomass) were stocked into each basket and proffered a formulated, cold-extruded feed containing 29% protein, 32% carbohydrate, 7% lipid, 6% fiber, and 26% ash at 3% of total urchin biomass daily, representing ca. 4.17±0.02 g of feed proffered per day. The total feed provided to the urchins in each basket during the 8-week experiment was ca. 229.41±0.87 g, or 28.68±0.11 g per individual. Shrimp were not proffered feed directly and only sea urchin egesta that fell passively into the bottom of the tank were available for consumption. Total dry matter available to shrimp via the egesta was calculated as *{[mean daily urchin feed (g) * fed days] * [1- absorption efficiency]}*. Dry matter FCR for shrimp was calculated as *[egesta dry matter available (g)/ shrimp weight gain (g)]*.

Termination of Experiment

The experiment was terminated after 8 weeks. Urchins were placed on paper towels outside of the water for at least 30 seconds to remove excess water before being individually weighed. Shrimp were blotted with paper towels to remove excess water

before being individually weighed. Individual shrimp and urchins were weighed to the nearest 0.001 g and total group weight was calculated as the sum of all individual weights in a replicate tank. Weight gain was calculated *[final weight (g) – initial weight (g)]*. Total shrimp biomass harvested per bottom surface area (m^2) was calculated as *[final group weight (g)/ tank bottom surface area (m^2)]*.

Statistics

Mean values of the response variables determined for the different dietary treatments were compared using SPSS Statistics 23 (IBM Corp.) using one-way ANOVA followed by a Tukey HSD test for *post-hoc* analysis. A p-value ≤ 0.05 was considered significant.

RESULTS

Experiment 1

Shrimp and Urchin Weight Gain

At the termination of the experiment, individual weight gain of shrimp provided urchin egesta did not differ significantly from those proffered a full ration of shrimp feed (Table 2). Consumption of urchin egesta combined with commercial shrimp feed resulted in significantly larger shrimp when compared to shrimp proffered commercial shrimp feed alone at the same level of inclusion. Urchin final group weight gain did not differ among treatments and had a mean of 10.96±1.80 g.

Table 2: Weight gain and harvested biomass of shrimp proffered increasing rations of shrimp feed (SF), with or without urchin egesta (UE) over an 8-week growth trial. Values represent the mean ± SEM. Values with different letter superscripts within a column indicate significant differences determined with one-way ANOVA and a Tukey HSD test for *post-hoc* analysis ($p \leq 0.05$; n=4 replicates per treatment).

TREATMENT GROUP	**MEAN INDIVIDUAL INITIAL WEIGHT (g)**	**MEAN INDIVIDUAL WEIGHT GAIN (g)**	**HARVESTED BIOMASS (g/m^2)**	**FCR**
20% SF	0.71±0.01	$2.65±0.06^{a}$	$55.50±5.61^{a}$	$0.96±0.16^{ab}$
60% SF	0.68±0.02	$7.18±0.37^{b}$	$150.80±3.71^{b}$	$0.77±0.01^{a}$
100% SF	0.70±0.02	$9.46±0.39^{bc}$	$194.27±20.14^{bc}$	$1.04±0.13^{ab}$
UE ONLY	0.68±0.02	$10.37±0.35^{cd}$	$245.61±7.76^{cd}$	$1.03±0.04^{ab}$
20% SF + UE	0.69±0.02	$12.40±0.72^{de}$	$290.83±16.42^{de}$	$1.01±0.06^{ab}$
60% SF + UE	0.68±0.02	$14.09±0.75^{e}$	$304.73±11.96^{de}$	$1.20±0.04^{ab}$
100% SF + UE	0.69±0.01	$14.72±0.68^{e}$	$318.53±12.53^{e}$	$1.39±0.06^{b}$

Survival

Mean shrimp survival did not differ among treatments and ranged from 75-100%. Mortality was exclusively due to shrimp jumping through gaps in tank lids onto the floor, rather than actual response to dietary treatment. Survival in all urchin treatments was 100%.

Urchin Egesta Collection

The mean dry matter absorption efficiency of urchin groups proffered the formulated urchin feed was 71.6±0.01% SEM (n=12). Approximately 45.0 g of dry matter from the egesta was estimated to be available to each replicate tank of shrimp throughout the experiment.

Shrimp Biochemical Analysis

There were no significant differences in carbohydrate or protein composition among shrimp representing the different treatments. Lipid composition in shrimp did not

differ significantly from the control (full ration shrimp feed) except when shrimp were proffered a 60% ration of shrimp feed exclusively. When shrimp feed and urchin egesta were available together, increasing the ration of shrimp feed did appear to support a trend of increasing lipid deposition. Lipid composition was significantly higher in shrimp proffered urchin egesta in addition to a 60% or 100% shrimp feed ration than in shrimp proffered urchin egesta alone. Mean ash composition and mean moisture content appeared to decrease with increasing individual weight (Table 3).

Table 3: Mean dry matter proximate composition of individual shrimp grown with a control (100%) or a reduced (60% or 20% of the control) shrimp feed (SF) ration without or with available urchin egesta (UE). Values represent the mean percent ± SEM. Values with different letter superscripts within a column indicate significant differences determined with one-way ANOVA and a Tukey HSD test for *post-hoc* analysis ($p \leq 0.05$; n=6-16 replicates per treatment).

TREATMENT GROUP	PROTEIN	CARBOHYDRATE	LIPID	ASH	MOISTURE
20% SF	61.91±3.93	1.35±0.09	5.30±0.18^{ab}	15.85±0.35^{a}	79.30±0.42^{a}
60% SF	60.85±1.13	1.26±0.09	4.95±0.21^{a}	15.25±0.18^{ab}	76.97±0.18^{b}
100% SF	59.13±1.23	1.20±0.10	6.10±0.14^{bcd}	14.37±0.32^{bc}	76.43±0.37^{bc}
UE only	63.07±1.20	1.18±0.09	5.46±0.26^{ab}	13.32±0.32^{cd}	75.48±0.60^{bc}
20% SF + UE	61.60±0.75	0.90±0.08	5.72±0.18^{abc}	13.14±0.22^{cd}	75.46±0.33^{bc}
60% SF + UE	62.03±1.08	1.10±0.08	6.68±0.22^{cd}	13.00±0.25^{d}	74.81±0.34^{c}
100% SF + UE	55.51±2.37	1.05±0.05	6.94±0.35^{d}	12.30±0.34^{d}	75.33±0.36^{bc}

Apparent Net Protein Retention

Shrimp consuming sea urchin egesta in the absence of commercial shrimp feed had the highest protein retention among treatments, significantly higher than the values of all other treatments. Values for urchin egesta alone and urchin egesta with a low ration (20%) of shrimp feed were significantly higher than those proffered any level of shrimp feed exclusively. Protein retention decreased as additional shrimp feed was proffered to shrimp in addition to urchin egesta (Figure 2).

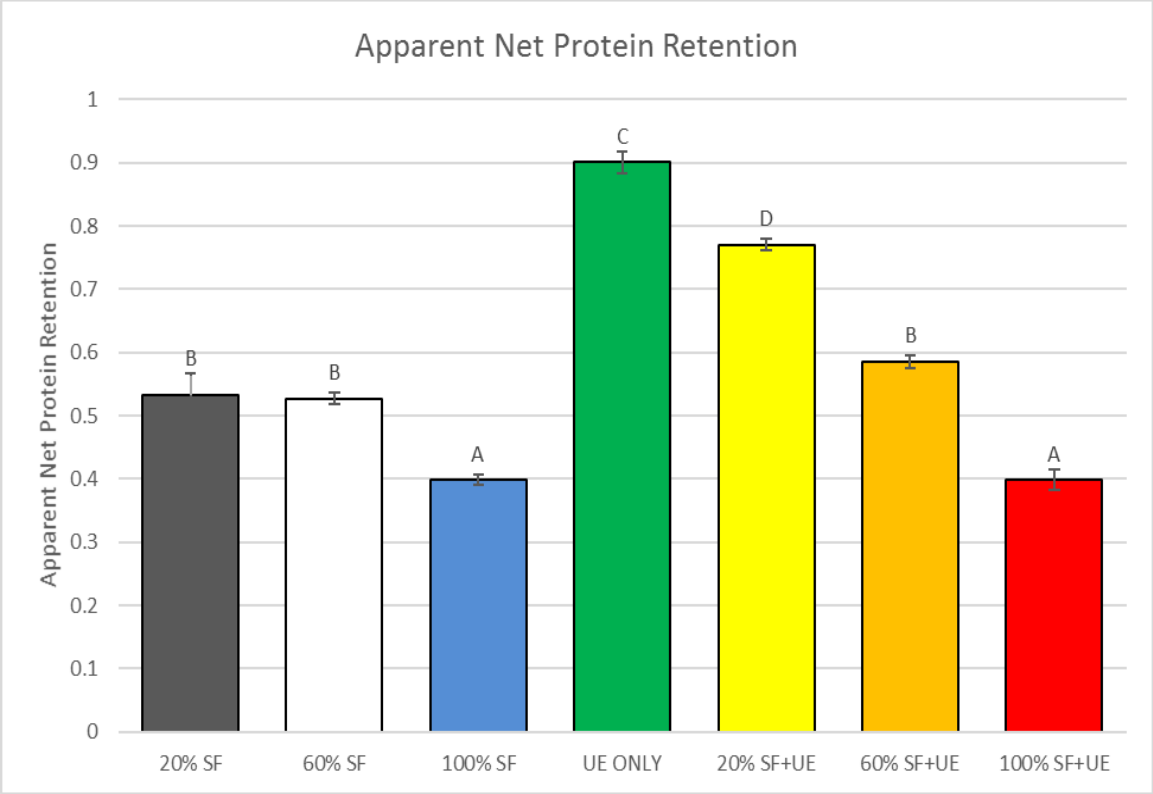

Figure 2. Apparent net protein retention of shrimp proffered full or reduced rations of shrimp feed (SF), with or without urchin egesta (UE). Error bars represent SEM. Differing letters above columns represent significant differences determined with one-way ANOVA and a Tukey HSD test for *post-hoc* analysis ($p \leq 0.05$; n=4 replicates per treatment).

Experiment 2

Shrimp Biomass

Increasing shrimp density with no increase in the number of urchins produced successive significant increases in harvested biomass until the 90 individuals/m^2 treatment was reached. Mean individual weight gain of shrimp decreased with increasing density. (Table 4).

Table 4: Growth responses and harvested biomass of shrimp at different densities in polyculture with sea urchins. Values represent the mean ± SEM. Values with different letter superscripts within a column indicate significant differences determined with one-way ANOVA and a Tukey HSD test for *post-hoc* analysis ($p \leq 0.05$; n=5).

DENSITY	***MEAN INDIVIDUAL INITIAL WEIGHT (g)***	***MEAN INDIVIDUAL WEIGHT GAIN (g)***	***HARVESTED BIOMASS (g/m^2)***	***FCR***
23 SHRIMP/M^2	0.37±0.01	$6.55±0.38^{a}$	$145.16±5.51^{a}$	$2.66±0.10^{a}$
45 SHRIMP/M^2	0.35±0.01	$5.73±0.18^{ab}$	$250.60±9.82^{b}$	$1.55±0.06^{b}$
68 SHRIMP/M^2	0.37±0.01	$4.94±0.14^{bc}$	$341.91±10.29^{c}$	$1.14±0.04^{c}$
90 SHRIMP/M^2	0.36±0.01	$3.95±0.11^{c}$	$365.90±10.06^{c}$	$1.09±0.04^{c}$

Urchin Egesta

The mean dry matter absorption efficiency of urchin groups proffered the formulated urchin feed was 71.59±0.01% SEM (n=12, Experiment 1). Approximately 65.6 g of dry matter from the egesta was estimated to be available to each replicate tank of shrimp throughout the experiment.

Survival

Mean shrimp survival did not differ significantly among treatments and ranged from 93-97%. Mortality was exclusively due to shrimp jumping through gaps in tank lids onto the floor, rather than actual response to dietary treatment. Water flow loss to one tank resulted in the loss of one replicate group of urchins in the 45 individuals/m^2 density treatment on day 29. These urchins were replaced with a group of urchins from the same cohort to allow for continued egesta production. Thus, all shrimp treatments received approximately the same amount of egesta. Survival of urchins in all other replicates of all other treatments was 100%.

DISCUSSION

Weight gain increased as proffered feed ration increased. Shrimp provided with fresh natural urchin egesta exclusively in integrated culture exhibited equally high weight gain and had significantly higher net apparent protein retention when compared to shrimp proffered the full ration of commercial shrimp diet. This suggests that the composition of the urchin egesta was adequate to promote high rates of growth and had suitable nutrients to support new tissue development, despite the results of a previous experiment (Chapter 1) which found that dried urchin egesta contained limited quantities of protein (16.67±0.37%) and lipid (3.99±0.21%) and comparatively high concentrations of ash (47.61±0.57%) and insoluble protein and fiber (30% calculated by difference) when compared to the shrimp feed.

The nutritional benefits related to consumption of the sea urchin egesta are not known. Shrimp showed increased weight gain when urchin egesta supplemented commercial shrimp diets for all rations. This growth enhancement suggests shrimp have genetic growth potential that can be realized when provided with appropriate factors for growth. The amount of dry matter estimated to be available to individual shrimp proffered egesta in Experiment 1 (ca. 10.7 g) was greater than that provided by a full ration of shrimp feed exclusively (ca. 7.9 g), but proffered protein was lower in the urchin egesta treatment (ca. 1.8 g) and the urchin egesta + 20% shrimp feed treatment (ca. 2.5 g) than the shrimp feed treatment (ca. 3.5 g). Given the proximate protein and energy levels of urchin egesta accompanied by high weight gain in groups proffered urchin egesta suggest these egesta must provide some growth enhancement factor(s) that confers highly efficient nutrient utilization or protein retention.

We hypothesize the presence of a distinct, live microbial community in the urchin egesta consumed by the shrimp could have contributed to the additional weight gain and protein retention of the shrimp. Urchins rely heavily on microbial support in the gut digesta to support processing of many complex carbohydrates, proteins, and lipids (Hakim et al. 2016) which remain active after egestion and continue their metabolic processes (Dennis 2014; Hakim et al. 2015). Shrimp have been shown to utilize enzymes acquired from the diet, resulting in enhancements in total enzyme activity and weight gain (Moss et al. 2001; Liu et al. 2009), so it is possible that the digestive benefits of the microbes within the egesta were conferred to the shrimp in these experiments. It is also possible that the presence of the egesta contributed to other available sources of recycled nutrients, such as shrimp feces or natural productivity in the tanks, which could have contributed to this high growth and apparent net protein retention of shrimp fed urchin egesta.

Protein and carbohydrate composition of the shrimp carcass did not differ among treatments. These results contrast in part with those of Wasielesky et al. (2006) who found that body composition was influenced by protein content of feed and availability of natural productivity. Thus, fresh natural urchin egesta may provide enough nutrition to be considered a complete diet for shrimp without causing noticeable deficiencies or have some nutritional benefits that may spare deficiencies; these explanations are supported by the observations in pond water by Moss et al. (2006). Lipid composition in shrimp proffered urchin egesta was similar in those proffered the commercial shrimp feed, but increasing feed ration did result in increasing levels of body lipid when egesta was also available.

Shrimp in Experiment 2 were provided only with urchin egesta as a food source. In general, mean weight and biomass were inversely related. Although there were not enough treatments to determine a true plateau, the biomass gain increased as the number of shrimp increased and seemed to plateau upon reaching the two higher density treatments. It appears that shrimp weight gain is dependent on the amount of egesta available, suggesting that additional urchin egesta contains nutrients important to shrimp growth, and that minimal daily nutritional requirements or the presence of growth factors are necessary to promote maximal weight gain. Providing additional egesta through increases in urchin biomass would presumably allow for increased total weight gain to some maximal point. A density limit for maximum growth under the selected density conditions of our investigation appears to have been reached somewhere between 45 and 68 individuals per m^2. Considering the initial urchin biomass, approximately 8.8-11.7 g of initial urchin biomass per shrimp under these conditions was required to support highest growth in this size class. Feed rate, temperature, density and size of urchins or shrimp, and many other factors would likely influence the ideal biomass ratio of these organisms. Additionally, this estimate could be reduced or higher growth could be achieved if producers included some level of supplementation of commercial diets to complement the urchin egesta.

Integration of these two species in an IMTA system has promise when considering the significant growth of shrimp proffered urchin egesta. Such a system could have practical benefits for both producers and consumers concerned with economic and environmental sustainability. Through the utilization of extractive species, water quality can be improved after being used in the production of a primary fed species,

allowing it to be reused in the culture system or discharged with low environmental impact (Kang et al. 2003; Martinez-Cordova et al. 2011). In doing this, secondary products are also produced from the same culture system, which can increase producer stability in a fluctuating market. These secondary extractive species can also show enhanced growth when compared to extractive species cultured alone (Chopin et al. 1999). Continued improvements to overall growth outcomes in this integrated system may involve proffering urchin and shrimp feeds specifically formulated to be used system-wide to minimize waste residues that cannot be remediated *in situ*. Additionally, extractive species at different trophic levels could be added to remove remaining compounds in the system, such as algae and plants that can utilize dissolved nitrogenous compounds (Chopin et al. 1999; Neori et al. 2000; Yokoyama and Ishihi 2010; Martinez-Cordova et al. 2011; Irisarri et al. 2013; Lange et al. 2013).

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APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 04-Apr-2016

TO: Watts, Stephen A

FROM: Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: NOTICE OF APPROVAL

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 04-Apr-2016.

Protocol PI: Watts, Stephen A

Title: Evaluation of a Stacked Polyculture System Involving the Sea Urchin Lytechinus Variegatus and the Pacific Whiteleg Shrimp Litopenaeus Vannamei

Sponsor: UAB DEPARTMENT

Animal Project Number (APN): IACUC-10043

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Institutional Animal Care and Use Committee (IACUC) | Mailing Address:
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