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Behavioral Studies of Chemoreception by the Pacific White Shrimp Litopenaeus Vannamei: Testing Attractability and Palatability of Proprietary Chemical Mixtures that Augment Feed Pellets Used in Shrimp Aquaculture

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BEHAVIORAL STUDIES OF CHEMORECEPTION BY THE PACIFIC WHITE

SHRIMP *LITOPENAEUS VANNAMEI:* TESTING ATTRACTABILITY AND

PALATABILITY OF PROPRIETARY CHEMICAL MIXTURES THAT AUGMENT FEED

PELLETS USED IN SHRIMP AQUACULTURE

by

FARIDA ELSAYED

Under the Direction of Charles Derby, PhD

ABSTRACT

Litopenaeus vannamei or Pacific white shrimp is the most widely farmed crustacean in the world. Shrimp are commonly fed feed containing 30-40% soybean meal or other plant-based feeds that are more economically and environmentally sustainable than animal-based feed. However, plant-based pellets are less palatable and less chemically attractive compared to animal material. Based on that, current research and practice includes the addition of specific marine animal meals in order to enhance palatability and attractability of plant-based shrimp feed. Yet, it is not sustainable or economically achievable to continue relying on marine animal meal. In the herein study, the effect of proprietary chemical mixtures designed by our research group as feed additives was examined based on their attractability and palatability in comparison to krill meal, a highly attractive and palatable supplement for shrimp feed. In palatability assays, total amount of pellets was measured before and after one-hour and three-hour periods of feeding in group-housed animals. In attractability assays, responses of shrimp were measured based on the number of probes and grabs on the source (airstone) of the stimulus being released. Each diet-set used contained different concentrations of krill meal and synthetic chemical mixtures. Results demonstrated these chemical mixtures enhance attractability and palatability of soybean based feed in *L. vannamei* when compared to krill meal. Furthermore, the addition of a proprietary mixture (= "premix") improved responses in the attractability assays when compared to stimuli that did not contain the premix. Overall, results support the hypothesis that synthetic chemical mixtures can improve palatability and attractability of soybean meal based shrimp feed. This work could provide a reference for the development of synthetic chemoattractants and chemopallatants for the aquaculture of shrimp.

INDEX WORDS: Pacific white shrimp, attractability, mixtures, palatability, chemoreception,

aquaculture.

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DEDICATION

I dedicate my thesis work to my family and friends. My parents, Amira and Hesham, have been the ultimate source of support and encouragement. It would have been impossible without them. My brother, Omar, constantly reminding me that it was still possible to smile and laugh even through the hurdles of graduate school.

I also dedicate this thesis work to my friends from both worlds. A special thank you goes to Mariusz, Lucia, Rania, Angelica, Ahmed, Philipos, and Alexandre for the endless support, advice, laughter and complete understanding of the hardships of Graduate School. I could not have pushed through without them, especially during the hard times.

Finally, I dedicate this work to my undergraduate family who constantly reminded me why I am doing this.

ACKNOWLEDGEMENTS

I would like to give a special thank you to my laboratory members: MiNa, Sarah, Kash and Vivian for constantly helping me with various aspects of my research. I would also like to thank my advisor and PI, Dr. Charles Derby, for guiding me throughout the whole process. Finally, I would like to thank Dr. W. Walthall and Dr. M. Schmidt, for serving on my M.S. thesis committee.

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1 INTRODUCTION

Human global consumption and demand of seafood has been increasing considerably over the past few decades. In 2010, it was estimated that global seafood consumption would rise to 17.0 kg per capita by 2020 (Merino et al. 2010). The current issue of overfishing is of serious concern due to potential impact on marine ecosystems as well as on future sources of food for humans (Amaya et al. 2007). Overfishing of small pelagic fish species has especially raised concerns since these fish species are essential in marine and terrestrial food webs (Gamboa-Delgado et al. 2016).

As a result of this growing demand, aquaculture has attempted to advance quickly to alleviate some of the strain on the oceans. In addition, it provides alternative methods for maintaining seafood supply to meet growing global demand (Sanchez et al. 2012, Jung et al. 2013). Humans directly consume almost two thirds of global fishery production. One third is used to produce fishmeal and fish oil, which are intensely utilized in livestock and aquaculture industries for protein and lipid sources. Aquaculture in particular has constantly relied on fishmeal and fish oil due to their nutritional profiles that have been shown to increase attractability and palatability in several marine and freshwater animal species. Although aquaculture is an alternative method for seafood supply, its reliance on and consistent use of animal-based meals and extracts has been recognized to be ecologically unsustainable (Deutsch et al. 2006, Naylor et al. 2000, Merino et al. 2012).

Due to this growing awareness, considerable attention has been given to plant-derived alternatives for the partial and possibly the total replacement of fishmeal and fish oil in terms of protein content, cost-effectiveness, sustainability and availability. Consequently, soybean products and derivatives have been given much attention based on these factors (Davis et al.

2004, Qihui et al. 2015, García-Ortega et al. 2016). Suarez et al. (2009) observed that soybean meal could replace fishmeal by 40% with respect to protein content in feeding trials utilizing Pacific white shrimp. In most cases, plant-based meals used as the only constituents in shrimp feed have been found to be relatively ineffective without additional supplementation. This is due to unbalanced amino-acid profiles (especially methionine and lysine), anti-nutritional or toxic compounds, indigestible carbohydrates and low palatability profiles (Bulbul et al. 2016). Hence, it becomes a necessity for aquaculture to utilize animal-based extracts, which have been shown to contain strong chemoattractants, to supplement plant-meal based feed. Previous studies have characterized crustacean feeding stimulants to include dissolved amino acids and low molecular weight compounds such as nucleotides, nucleosides, organic acids and quaternary ammonium compounds (Carr et al. 1996, Holland and Borski, 1993).

Behavioral studies have been underestimated due to the employment of other methods that typically include parameters such as growth rate as the only relevant factors in aquaculture. Yet, these parameters do not explain underlying mechanisms of crustacean feeding behavior, which could be informative in assessing the effects of chemostimulants on enhancement (or the lack thereof) of consumption. Utilizing knowledge of feeding stimulants as well as feeding behavior of crustaceans can be applied in the improved design of feed additives that are not necessarily animal-based (e.g. krill meal and squid meal). It has been shown that chemostimulants may enhance feed consumption through attractability by virtue of olfactory processes and palatability by enhancing phases of consumption (Holland and Borski, 1993, Samocha et al. 2004,Sanchez et al. 2005, Smith et al. 2005, Suresh et al. 2011,Derby et al. 2016). Thus, it is necessary to acknowledge the importance of how crustaceans respond to chemicals in food. Crustaceans evolved to have a wide variety of chemoreceptors, sensory neurons that

respond to chemical stimuli, to differentiate between different chemical signals and cues. In the case of allocating and selecting food, crustaceans rely on certain cues that include a vast and complex array of molecules, some of which are currently known while most are not well understood. Since *L.vannamei* is an aquatic decapod crustacean, the most important feeding cues are normally small hydrophilic compounds, with some lipophilic compounds being relevant (Derby, 2000). Based on many years of studies, certain chemicals that initiate feeding behavior have been characterized for crustaceans in general. Common metabolites serving as feeding stimulants include dissolved amino acids, nucleosides, nucleotides, quaternary ammonium compounds and organic acids. In addition, interspecific differences in response to feeding stimulants have been observed e.g. spiny lobsters respond to different feeding stimulants compared to shrimp (Carr, 1988). Since olfactory processes in crustaceans have been shown to mediate the response and detection of chemical stimuli, the most well studied chemosensors in decapod crustaceans are the aesthetasc sensilla, which represent olfaction. Aesthetasc sensilla are modified hair-like structures that are innervated by olfactory neurons and are located on the antennules. Other relevant chemosensory sensilla, distributed chemosensilla, contain chemo- and mechano-sensory neurons, which are mostly located on the antennules, second antennae, mouthparts and legs of decapod crustaceans. Hence, the involvement of these parts in locating relevant food molecules and, manipulating food is directly related to the activity these chemoreceptors (Derby and Weissburg, 2014). Once a chemostimulant is detected by antennular chemosensors, chemosensors on the antennules and legs allow the animal to search and move towards the source of food molecules. Once the leg touches the source, the food particle is grabbed and moved to the mouthparts. Such behavior has been observed when animals were

introduced to krill meal, animal-based extracts, and other known chemostimulants (Carr et al. 1996;Derby, 2000; Derby and Weissburg, 2014; Derby et al. 2016).

Studies conducted on crustacean feeding behavior using krill hydrolysate as a supplement added to soybean meal based diets determined that krill meal is a source of free amino acids and small peptides, thus a strong chemoattractant. Palatability and attractability experiments carried out in many studies utilizing different crustacean species have shown similar results (Córdova-Murueta and García-Carreño 2002, Smith et al. 2005, Sanchez et al. 2005,). Other stimulants such as squid meal also have similar chemostimulatory effects (Tantikitti 2014). Hence, krill meal has been commonly used as a high-end feeding enhancer in shrimp feed. Yet, from a sustainability standpoint, it is still a major issue since krill is under stringent conservation protection, costly to acquire and unsustainable (Nunes and Sabry-Neto, 2011).

Since krill meal and other animal-based extracts are currently not considered sustainable sources of feed-enhancers, other strategies that employ the development of mixtures or combinations of several chemical ingredients in diets, which ensure that animals consume maximal amounts to sustain growth are in order (Sanchez et al. 2005). The approach of utilizing artificial chemical mixtures to supplement shrimp has been applied before. Hartati and Briggs (1993) showed that *P.monodon* responded well to taurine and yeast extracts over all diets tested. They suggested that *P.monodon* might be most responsive to taurine due to the presence of taurine-specific antennular chemoreceptors.

In the past, palatability and attractability assays have been employed to study the effects of chemostimulatory compounds on fish and crustacean feeding behavior where positive results were observed, such as searching for food, food allocation and ingestion (Carr et al. 1984, Holland and Borski 1993, Walker et al. 2005). Based on this idea, different chemicals of may

yield different responses and thus can possibly be manipulated in a laboratory setting to test for

the best balance of major chemostimulants (Carr et al. 1996, Forster et al. 2010, Salze et al.

2010). Our research group has carried out the development of artificial chemical mixtures, based

on knowledge of feeding stimulants previously discussed herein. First, proprietary mixture M1

was developed and tested for attractability in comparison to krill extract. Results showed that M1

could induce responses from concentrations as low as 10 μM. At 10 mM, M1 elicited responses

similar to 10 μg/ml of krill extract (Figure 1). Based on these results, three other proprietary

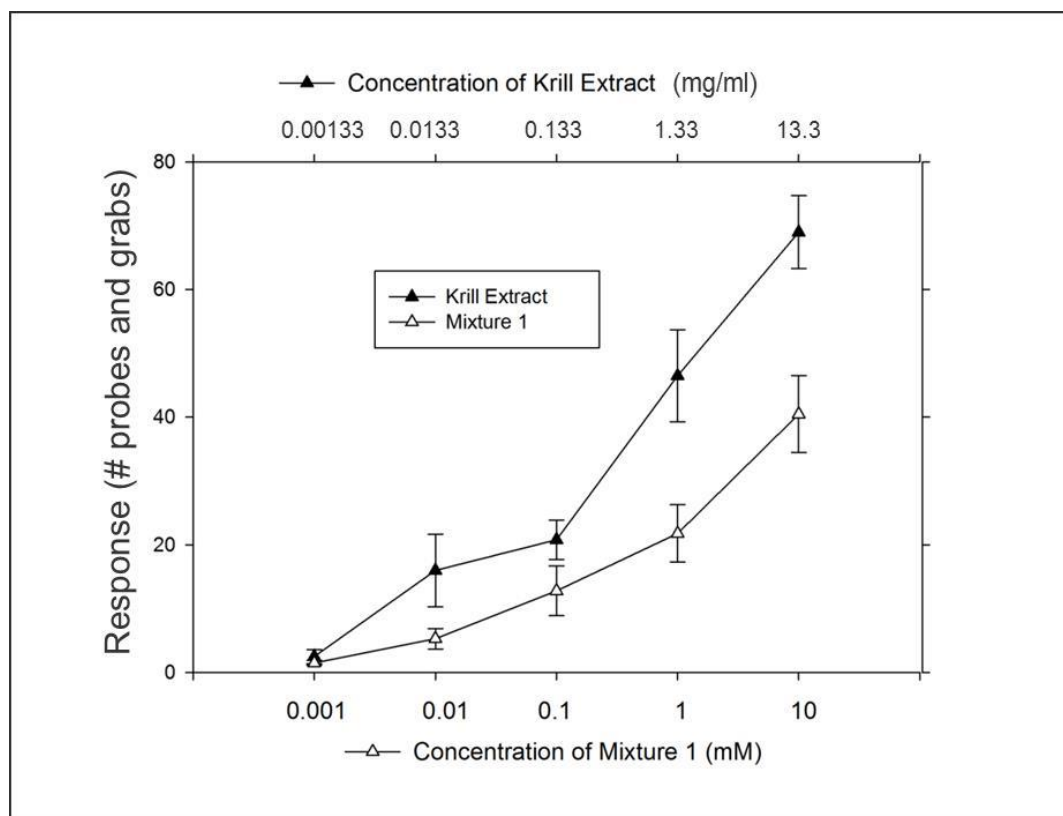mixtures were developed (Table 1) (Derby unpublished).

Figure 1. Concentration-Response plot for Krill extract and M1.
Concentration-Response function of M1 and krill extract. Values expressed in mean± SEM # of probes
a/o grabs in 4 min in 6 aquaria, each containing 20-25 animals taken from a population with a mean mass
of 7.2 g (range, 5–10 g). (Derby unpublished data).

Table 1 Attractability assay results for proprietary mixtures M1,M2,M3 and M8
Attractability (n=6 tanks, mean)

Relative Response (% of 10^{-3}M M1)			
Mixture Type	10^{-3}M	10^{-4}M	10^{-5}M
M1	100	78.6	25.9
M2	82.1	33.8	33.8
M3	87.1	22.4	33.8
M8	60.7	38.8	25.9

In this study, behavioral studies, palatability and attractability assays, were utilized to assess the effect of proprietary chemical mixtures, M3 and M8, on feeding behavior of the Pacific white shrimp, *Litopenaeus vannamei.* M3 and M8 were chosen based on the fact that they are simpler and less costly mixtures. In addition, they were shown to be as effective as M1 and M2 at low concentrations. Furthermore, feeding responses to different diets with or without our proprietary mixtures were compared to krill meal in those same diets. We also compared the presence of a proprietary premix in combination with our chemical mixtures due to its known enhancing properties. It should be noted that Belzenger et al. (2015) showed that protection (via heat lipid coating) of proprietary amino acid premix in shrimp feed greatly enhanced feeding and growth in different shrimp species by reduction of leaching rate and nutrient loss by approximately 40%.

We hypothesized that less complicated and less expensive artificial chemical mixtures can ultimately substitute for animal-based additives as effective feeding stimulants when used to supplement moderately unpalatable basic feed pellets for *L. vannamei* aquaculture.

2 OBJECTIVE

The purpose of this study is to test the palatability and attractability of proprietary synthetic chemical mixtures, M3 and M8, designed by our laboratory as possible future alternatives to krill meal, which has been previously shown to be an effective feeding enhancer .Yet, krill meal itself is a costly and unsustainable additive in crustacean feed. Hence, the development of synthetic, less costly and economically sustainable chemical mixtures can help towards a more ecologically sustainable and environmentally friendly future in aquaculture. My hypotheses are as follows: 1) Plant-based feed pellets supplemented with proprietary chemical mixtures, M3 and M8, enhance consumption, and 2) M3 and M8 can emulate the effects of krill meal as a chemoattractants, especially when a proprietary premix is present. In summary, it is possible that artificial chemical mixtures can be effective feeding stimulants and attractants when used as additives to moderately unpalatable plant-based basic feed pellets for the Pacific white shrimp, *L. vannamei.*

3   MATERIALS AND METHODS

3.1   Animal housing and conditions

Pacific white shrimp, *L. vannamei,* were grown to small juveniles (approx. 3.5g each) at Integrated Aquaculture International's Shrimp Nutrition Center feed laboratory in Kekaha, HI, USA. Animals were shipped to Georgia State University, Neuroscience Institute. Animals were placed in holding aquaria (74 cm (l) x 30 cm (w) x 28 cm (h); 0.22 m^2 bottom area). The tank was filled with 60L of artificial seawater at 32-35 ppt that was filtered and aerated. Tanks were kept at 25-27°C. Light: Dark cycle was 12 hour: 12 hour. Initially, there were approximately 36 animals per tank (570 g/m^2). Animal feed consisted of commercial shrimp feed (Uni-President Enterprises Corp., Tainan, Taiwan). Animals were fed once daily and weekly given squid mantle.

3.2   Feeding enhancers: Krill Meal, M3, and M8

Three feeding enhancers were tested. One was krill meal, which is standardly used as a high-end feeding enhancer in the shrimp aquaculture industry. The krill meal used in our experiments was Qrill™ Antarctic Krill Meal (Qrill™ AQUA) from Aker BioMarine (Oslo, Norway). According to the manufacturer, Qrill™ AQUA is made from *Euphausia superba* that is caught by a continuous trawling system; the catch is preheated and cooked on board, then the water is separated out along with the oil, and the meal is then dried by a conventional drier on board to about 6% moisture. The other two feeding enhancers were two proprietary mixtures, M3 and M8, fabricated by Dr. Charles Derby. These are composed of off-the-shelf chemicals of reagent grade.

3.3 Production of pellets

All experimental diets were manufactured at Integrated Aquaculture International's Shrimp Nutrition Center feed laboratory in Kekaha, HI, USA. The formulation of the pellets is shown in Tables 1 and 2. Diets were manufactured by grinding and combining ingredients in a mixing bowl followed by addition of oil and water. The diets were then pelleted by forcing the diet mixture through a meat grinder with a 3-mm die attachment (Hobart Corporation, Troy, OH, USA). The resulting strands were cooked in steam for 20 min and then dried in a forced air oven at approximately 80°C until completely dry (ca. 12 h). The dry diets were chopped down to the required size (3 mm x 5 mm) and stored in a freezer prior to being used. The pellets had a bulk density of 610.2 g/L. Feed samples were analyzed for percent gelatinization by subjecting one portion of the sample to gelatinization by autoclaving at 121°C and 15 psi for 1 hr. The gelatinized starch (a filler) was then digested by glucoamylase enzyme, and the resulting glucose was measured on a glucose analyzer. This step is standard in commercial pellet production since it makes pellets harder. A duplicate portion of the sample was treated with the glucoamylase enzyme without autoclaving, and the glucose formed from pre-existing gelatinized starch was measured on the glucose analyzer to calculate percent gelatinized starch in the sample. Percent gelatinization is the amount of gelatinized starch in the sample expressed as a proportion of total starch in the sample. The results of this analysis showed that our procedure of post-pellet conditioning produced >85% starch gelatinization.

3.4 Behavioral assays

All tests were carried out during daylight of the Light: Dark cycle. All tests were run blind, i.e. the experimenter did not know the identity of stimulus or pellet being tested. All diet sets,

D1-D4, D6 and D8 (Table 1) and SD1-SD6 (Table 2), have been used to conduct both attractability and palatability assays in group-housed and individually housed animals. This work will be discussed in the Discussion section. The work presented herein is only a portion of the whole project.

3.4.1 Ingestion assays for group-housed animals

Ingestion assays were performed of two durations: a one-hour test and a three-hour test. Each test had its own set of controls. Both used the same housing conditions and feeding schedules. The same observer carried out both tests. The observer carried out both assays using the same general protocols.

3.4.1.1 Ingestion assay for group-housed animals: One-hour assay

Adult animals were tested in groups of nine, with each group housed in its own tank out of six aquaria. Pellets labeled D1-D4, D6, and D8 were tested (Table 1). D4 is the control, which contains 0% krill meal. Prior to testing, 100 pellets were counted and weighed per diet type per tank. Each pellet weighed in the range of 50-75 mg. An average total weight per 100 pellets was 6.40 g. All pellets were stored and capped in centrifuge tubes in a refrigerator at 4 °C.

Each experiment included adding the prepared 100 pellets of a given diet type to an aquarium. This was repeated in all six aquaria allowing 3 min between additions to aquaria to allow ample collection time. Animals were left to feed for 60 min. Pellets were then collected using a hand-held fine mesh net. Pellets were separated from fecal matter and other materials. The pellets were then left to dry overnight at 35 °C in an oven. Finally, the dried pellets were weighed again. This protocol was repeated for each of the six diets.

To correct for pellet weight changes (leaching and water circulation) that are unrelated to the feeding activity of the shrimp, the same protocol was used excluding the animals. Pre-weighed and pre-counted pellets were left in aerated tanks for 60 min. Pellets were then left to oven-dry at 35°C overnight and then weighed. The change in dry weight was calculated including a correction for the control. Data were expressed as mean $\pm$ SEM (n=6 aquaria) for each pellet type.

3.4.1.2 Ingestion assay using group-housed animals: Three-hour assay

The same protocol was applied for the three-hour assay. The differences include: 1) 200 pellets were counted instead of 100 with an average total weight of 12.94 g, 2) each aquarium had eight animals and 3) animals were left to feed for 180 min. Control protocols were identical in both the one-hour and the three-hour assays. The differences were the ones mentioned previously. Controls were tested in tanks with no shrimp.

Table 2. Diet compositions of D1-D4, D6 and D8 used in palatability assays expressed in relative %.

	Diet					
Ingredient (%)	**D1 (6% krill meal)**	**D2 (3% krill meal)**	**D3 (1% krill meal)**	**D4 (0% krill meal)**	**D6 (5% M8)**	**D8 (5% M3)**
Wheat flour	32.1	33.2	33.9	34.2	32.5	32.5
Soybean meal	28.2	29.1	29.7	30	28.5	28.5
Poultry protein concentrate*	25.7	26.5	27	27.3	25.6	25.6
Krill meal/Mixture	6	3	1	0	5	5
Monocalcium phosphate	2.87	2.94	3	3.03	2.9	2.9

Menhaden oil	2.77	2.86	2.92	2.95	2.79	2.79
Lecithin	1.02	1.05	1.07	1.08	1.03	1.03
Sodium alginate	0.94	0.97	0.99	1	0.95	0.95
Vitamin & mineral premix	0.28	0.29	0.3	0.3	0.28	0.28
Cholesterol	0.16	0.16	0.17	0.17	0.16	0.16

3.4.2 Attractability assays for group-housed animals

3.4.2.1 Stimulus Delivery

Animals ranging in weight from 5–10 g were tested as a group in their holding aquaria (see section *3.1*). A peristaltic pump (Minipuls® 3, Gilson, Middleton, WI, USA) was used and calibrated to introduce stimuli at 2 ml/min. Stimuli were introduced through a tube connected with a terminal airstone situated near bottom front of the aquaria. Two outlets of the pump were used: one for the background stimulus (SW) and the other for an experimental stimulus. For each test, SW was accessible for 5 min, followed by the experimental stimulus for 5 min, during which behavioral observations were recorded.

3.4.2.2 Stimulus preparation

Stimuli were prepared by grinding 1 g of each of 6 diets (SD1-SD6) into a fine powder for 5 min using a mortar and pestle. The powder was then added to 100 ml of seawater (10mg/ml) and stirred on a stir plate for 1 h. The mixture was then filtered and stored at -20°C in 1 ml aliquots in 1.5ml Eppendorf tubes. This procedure was repeated for each diet. Diet compositions are displayed in Table 2.

3.4.2.3 Behavioral quantification

Behavior was quantified as the number of probes and grabs on the airstone. 'Probe' is defined as an animal actively touching the airstone with legs, and 'Grab' is defined as an animal using its paired legs or mouthparts to hold the airstone. Second antennae touching was not a behavior included in this study due to breakage/shortening commonly observed in shrimp held in small tanks. Each behavior was counted as a single incident as long as the animal sustained that behavior. A second event was counted when an animal stopped probing or grabbing the airstone for more than 2 s and then probed or grabbed again. These events were quantified for each minute of observation. "Response" is quantified as the number of probes and number of grabs in the last 4 min of stimulation, i.e. the experimental stimulus minus the number of probes and grabs in the last 4 min of stimulation with SW. The response was considered a group measure since individual animals were not tracked. The 4-min time period was used rather than 5 because of a ca. 1 min delay between valve switching of the peristaltic pump and the new stimulus reaching the airstone. Each aquarium contained 20-25 juvenile animals with each aquarium being used as a replicate. Therefore, the sample size was six (n=6). No more than two stimuli were tested per aquarium per day, with at least 60 min between tests. Each of the experiments (see below) was run with the same number of animals per aquarium and with each stimulus. A total of 6 different stimuli were tested. Results are graphed as normalized mean ± SEM of the number of probes and grabs as % of the control (SD1: 2% dilution) for each stimulus.

3.5   Statistics

Data were analyzed using Statistica. Data were expressed as normalized mean± SEM (n=6 aquaria) as % of the control (D4 and SD1: 2% Dilution) for each pellet type and each stimulus. Statistical analyses were done using non-parametric Friedman one-way ANOVA

(Diet/ Stimulus) and if significance was found, post-hoc Wilcoxon matched pairs signed ranks test post hoc tests ($\alpha = 0.05$) followed. A p-value <0.05 was considered significant.

Table 3. SD Diet compositions expressed in %.

Ingredient	SD1	SD2	SD3	SD4	SD5	SD6
Soybean Meal	30	30	30	30	30	30
Poultry Protein Concentrate	27.3	27.3	27.3	27.3	27.3	27.3
Monocalcium Phosphate	3	3	3	3	3	3
Menhaden Oil	3	3	3	3	3	3
Premix	1.1	1.1	1.1	1.1	1.1	1.1
Sodium Alginate	1	1	1	1	1	1
Vitamin/Mineral/Cholesterol	0.5	0.5	0.5	0.5	0.5	0.5
Wheat Flour	34.2	29.2	29.2	19.2	33.2	20.2
Krill Meal	0	**5**	0	0	0	0
M3	0	0	5	5	1	1
Premix	0	0	0	5	0	1
Silica/CornStarch/Bentonite/Wheat Flour	0	0	0	5	0	12
Total	100	100	100	100	100	100

4   RESULTS

4.1   Ingestion assays: Artificial mixtures, M3 and M8, enhance pellet palatability and thus consumption rate in both 1 h and 3 h tests.

Shrimp were tested in groups to determine if krill meal, M3 or M8 affected feeding consumption over one-hour and three-hour assays.

For the 1 h assay, shrimp consumed 30-40% of the fixed number/weight of pellets for each diet type (Figure 2). Addition of 6% krill (29.6 % increase compared to 0% krill meal control) significantly increased palatability of pellets and therefore consumption in a 1 h period.

On the other hand, addition of 3% and 1% krill meal did not show any significant differences in consumption compared to the control (D4), which could be explained by the short duration for consumption. In comparison to pellets containing krill meal, D8 (5% M3) showed higher consumption rates than all pellets except for D1 (6% krill meal), which showed a 8.4% higher consumption rate compared to 5% M3 (D8). In addition, D6 (5% M8) proved to be more palatable than D2 (3% krill meal), D3 (1% krill meal) and D4 (0% krill meal), but slightly less palatable compared to D8 (5% M3) and D1 (6% krill meal). This shows that D1>D8>D6 in the 1 h feeding assay. Non-parametric Friedman one-way ANOVA yielded a significant effect (χ^2 (N=6, df=5)= 19.06699), $P < 0.00187$), and Wilcoxon matched pairs signed ranks ($\alpha = 0.05$) showed that 6% KM=5%M3 >5% M8=Control=1% KM= 3% KM).

The 3 h assay showed feeding trends more clearly due to the longer duration of consumption. As shown in Figure 3., 6% krill meal consumption increased to 82.5% compared to the control (D4) and D8 (5% M3) increased about 92.5% compared to the control. Hence, in contrast to the 1 h assay, addition of 5% M3 increased consumption rate the most compared to all other diets groups. These results were also supported in attractability assays performed in a previous study (Derby et al. 2016). Addition of 5% M8 showed consumption rates higher than pellets with 3%, 1% krill meal and the control, yet the difference was not statistically significant. Non-parametric Friedman one-way ANOVA showed a significant effect (χ^2 (N=6, df=5)= 28.09524), $P < 0.00004$), and Wilcoxon matched pairs signed ranks ($\alpha = 0.05$) showed that 6% KM=5%M3 >5% M8=Control=1% KM= 3% KM). D2 (3% krill meal) showed inconclusive results in both the 1 h and 3 h feeding assays. These results, from 1 h and 3 h feeding assays, show that M3 and M8 have increased palatability and in the case of M3, significantly. Also, M3

showed its capacity to mimic the chemo stimulatory effects of krill meal depending on the concentrations, with M3 being a superior palatant compared to M8.
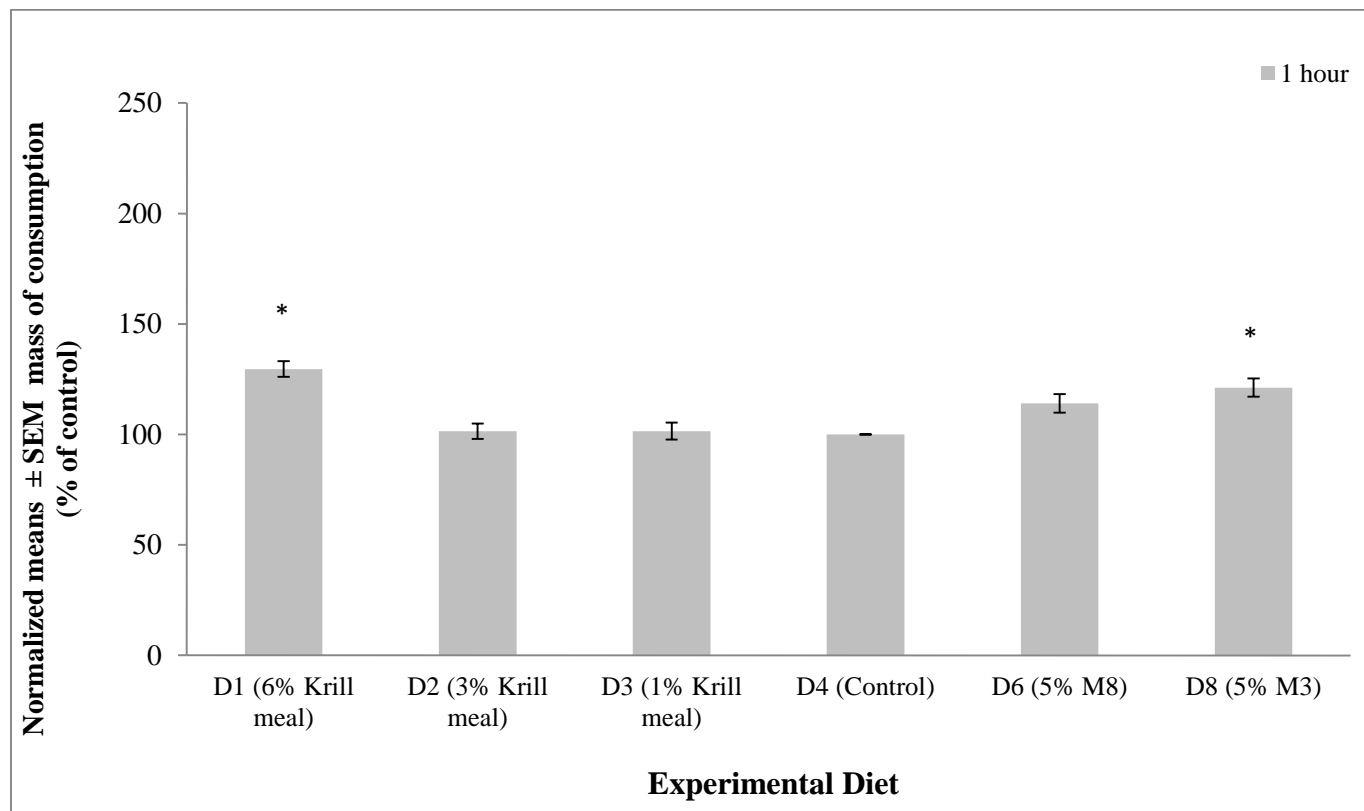
4.1.1 One-hour feeding assay

Figure 2. One-hour palatability assays for group-housed animals.
Bar graph of normalized means ± SEM of post-consumption pellet weights (g) presented as % of control (D4). N=6 aquaria. 100 pellets with a mean total mass of 6.40 g were added to each tank and shrimp were left to feed for 1 h. Non-parametric Friedman one-way ANOVA shows a significant effect (χ^2 (N=6, df=5)= 19.06699), $P < 0.00187$), and post-hoc Wilcoxon matched pairs signed ranks ($\alpha = 0.05$) showed that 6% KM=5%M3 >5% M8=Control=1% KM= 3% KM).
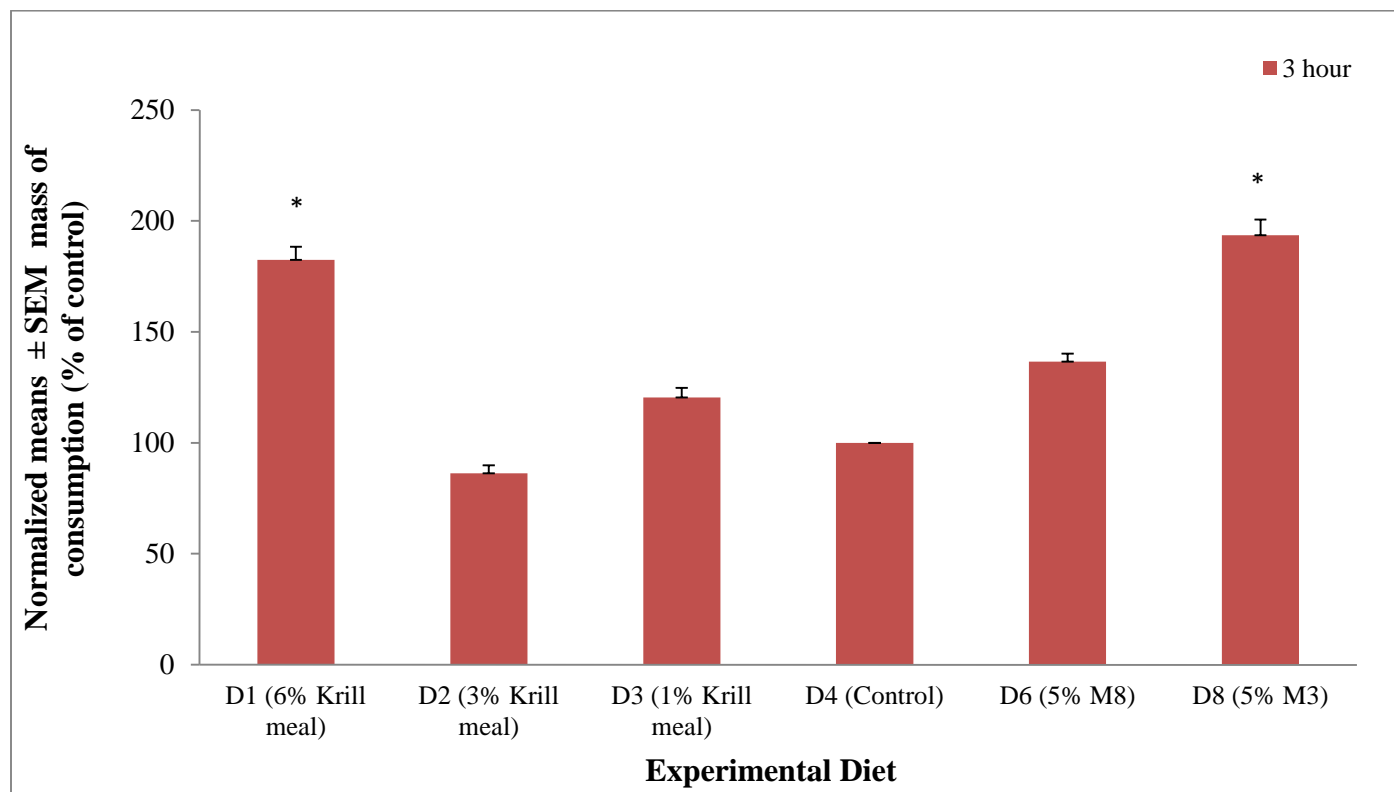
4.1.2   Three-hour feeding assay

Figure 3. Three-hour palatability assays for group-housed animals.
Bar graph of normalized means ± SEM of post-consumption pellet weights (g) presented as % of control (D4). N=6 aquaria. 200 pellets with a mean total mass of 12.94g were added to each tank and shrimp were left to feed for 3 h. Non-parametric Friedman one-way ANOVA shows a significant effect (χ^2 (N=6, df=5)= 28.09524), $P < 0.00004$), and Wilcoxon matched pairs signed ranks post-hoc ($\alpha = 0.05$) showed that 6% KM=5%M3 >5% M8=Control=1% KM= 3% KM).

4.2   Attractability Assays for group-housed animals demonstrate the efficacy and attractability of artificial mixtures in comparison to krill meal with or without the presence of Premix

Attractability assays were performed to assess the effectiveness of M3 at two concentrations, 1% and 5%, in comparison to 5% krill meal. In addition, the proprietary premix effectiveness was evaluated in combination with and without 1% M3 and 5% M3 (Figure 4). Figure 4 shows normalized means (as % of the control) of probes and grabs calculated for each stimulus at

different dilutions. These results demonstrate that M3 can be as effective as and, in at least one

case (1% M3), more effective than krill meal extract at 5%. Furthermore, the presence of premix

improved the efficacy of the relevant stimuli. Furthermore it should be noted that M3 did not

exhibit a typical concentration-response relationship, at least for the two concentrations that were

tested. In fact, it was surprising that the lower concentration of M3 with and without premix

induced more probes and grabs compared to 5% M3 with or without premix and compared to the

control (SD1). Since 3% krill meal additive showed inconclusive results in the feeding assays

and previous attractability assays, it was not tested again (Derby et al. 2016). Generally, these

results indicate that even at lower percentages, artificial mixture M3 can be as effective as krill

meal. The addition of premix further improves attractability in all cases regardless of mixture

concentration. Friedman one-way ANOVA did not show statistically significant differences with

respect to the 2% dilution stimuli set. A statistically significant difference was found for the 1%

dilution stimuli set (χ^2 (N=12, df=5)= 18.23809, $P = 0.00267$), and Wilcoxon matched pairs

signed ranks (5%M3 + Premix>control=5% KM=5%M3=5% M3 + Premix=1% M3 + Premix).
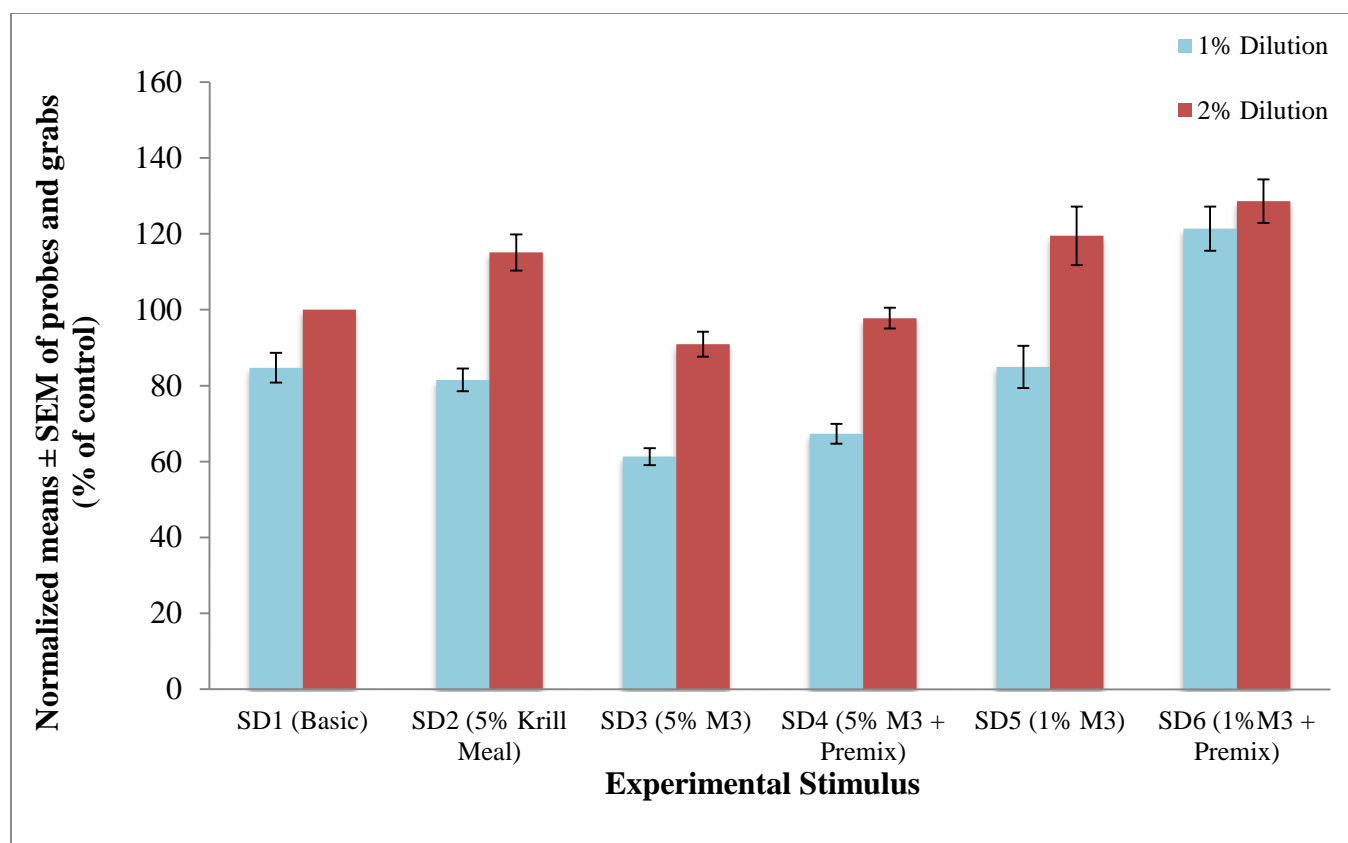
Figure 4. Response (probes a/o grabs) to experimental stimuli (SD1-SD6) at 1% and 2% dilutions.
Responses of aqueous extracts of experimental stimuli (SD1-SD6) at 1% and 2% dilutions. Values expressed mean ± SEM of the # of probes and grabs in 4 min for 6 aquaria, each containing 20-25 animals. Friedman one-way ANOVA did not show statistically significant differences with respect to the 2% dilution stimuli set. A statistically significant difference was found for the 1% dilution stimuli set (χ^2 (N=12, df=5)= 18.23809, P = 0.00267), and Wilcoxon matched pairs signed ranks (5%M3 + Premix>control=5% KM=5%M3=5% M3 + Premix=1% M3 + Premix).

5   DISCUSSION

Previous studies reported the effects of marine animal extracts on feeding performance of shrimp when added to commercial plant-based shrimp feed pellets. Results indicated that these extracts improved feeding performance by enhancing attractability via appetitive behavior stimulation and/or augmenting palatability i.e. increasing consumption (Sanchez et al. 2005, Smith et al. 2005, Nunes et al. 2006, Suresh et al. 2011).

In this study, these effects were also demonstrated for krill meal. Additionally, we examined the efficacy of different concentrations of krill meal in terms of palatability and the effectiveness of two artificial chemostimulant mixtures, M3 and M8.

Here, we used Pacific white shrimp, *L. vannamei*, due to the fact that it is the most widely cultured shrimp species in commercial aquaculture in the world. We also used krill meal based on current knowledge of its effects on attractability and palatability in shrimp feed (Smith et al. 2005, Suresh et al. 2011, Qihui et al. 2013). Attractability and palatability assays in group-housed animals were performed. Based on these studies, two hypotheses were evaluated: 1) plant-based feed pellets supplemented with our proprietary mixtures enhance consumption and 2) one proprietary mixture, M3, can emulate the effects of krill meal as a chemoattractant, especially when premix is present.

The first hypothesis was tested using palatability assays, which demonstrated that krill meal is a strong palatant when added to commercial soybean meal based feed pellets. Animals consumed more pellets in 1 h and 3 h time frames (Figures 2 and 3, respectively) depending on the relative concentration of krill meal and M3 and M8. With respect to the M3 and M8, only one concentration (5% of each) was tested herein. D8 (5% M3) showed promising results in comparison to D2 (6% krill meal) and the other diets. As for D6 (5% M8), it proved to also stimulate feeding behavior that was similar to D8 (5% M3), yet the consumption rate was slightly lower. Both M3 and M8 proved to be relatively palatable compared to 6% krill meal, which was the most palatable of all the additives. When compared to 0%, 1% and 3% krill meal, both M3 and M8 were more palatable. Furthermore, it should be noted that the differences between diets were not as clear in the 1 h assay when compared to the 3 h assay results. This may have been due to the short

duration the animals were allowed to feed. Krill meal or extract has been previously shown to enhance feeding in shrimp. The reason may lie in the idea that important feeding stimulants (such as free amino acids, nucleosides, nucleotides, organic acids and quaternary ammonium compounds) are present in krill meal and/or extract (Carr and Derby, 1986, Heinen, 1980, Holland and Borski, 1993, Suresh et al. 2011, Nunes et al. 2006).

Three h palatability assays were more conclusive in this respect since they allowed animals sufficient time to search and feed. Pellets with 3% krill meal did not increase consumption in a dose-dependent manner in both the 1 h and 3 h assays. These results are supported by attractability assay results demonstrated in a previous study conducted by our group (Derby et al. 2016). Furthermore, these results were in line with our previous study with respect to group-housed assays. Similarly, 3% krill-meal did not exhibit the dose-response demonstrated by the attractability and individual housed assays. Additionally, group-housed 3 h palatability and individual-housed assays showed that krill meal is a dose-dependent palatant from concentrations as low as 1% up to 6%. It is not well understood as to why this occurred even though animals did consume 3% krill meal pellets in individual assays. Suresh et al. (2011) demonstrated the same effects utilizing blue shrimp *Litopenaeus stylirostris*. They demonstrated that consumption rates did not exhibit a dose-dependent response when 3% krill-meal was added to poultry-based feed pellets in 1 h group-housed assays yet showed typical dose-dependence when added to plant-meal based feeds.

Finally, these unusual responses may have been caused by multiple factors such as pellet hardness, size, leachate, water quality and/or human error during pellet processing. Williams et al. (2005) discussed that leaching from pellets is partially related to the amount of binders included in feed pellets. Even though all pellet types used in the

palatability assays were composed of the same ingredients with slight amount differences, it is possible that these slight variations may have contributed to the responses observed in this study.

Furthermore, our mixtures showed different results in the 3 h assays compared to the 1 h feeding assay. Although D8 (5% M3) was more palatable compared to 6% krill meal, D6 (5% M8) was more similar to 1% krill meal in terms of consumption yet more palatable than 3% krill meal. The differences between M3, M8 and 6% krill meal in the 3 h assay were greater compared to the 1 h assay. Again, this may have been due to time limitations rather than qualitative aspects. These same effects were demonstrated by MiNa Choe and Charles Derby (unpublished data) using attractability assays, where D1 (6% krill meal) at 1% and 2% dilutions exhibited the highest attractability. D8 (5% M3) showed to be less attractive but the difference was not significant. In general, based on the results shown here and in a previous article, krill meal is an effective chemoattractant that can be used to enhance consumption rates and amounts of plant-based shrimp feed (Derby et al. 2016). Similarly, artificial proprietary mixtures, M3 and M8, showed promising results in terms of palatability when compared to a strong chemoattractant such as krill meal. The basic feed pellets used in this study, which served as the controls, contained 27% marine-protein-free feed concentrate, 30% soybean meal, and 34% wheat flour. From the results shown herein and the attractability results obtained by (MiNa Choe and Charles Derby, unpublished data), it appears that all known aspects of feeding behavior have been observed with respect to krill meal, M3 and M8 i.e. olfactory processes mediated by antennular aesthetasc sensilla and distributed chemoreception mediated by distributed chemosensilla in mouthparts, legs, and antennules (Derby, 2000). Lee and Meyers (1997) and many other scientists demonstrated

that mixtures could be more stimulating than single compounds by additive or synergistic interactions. These results were obtained using behavioral (and electrophysiological experiments), where all behaviors known to be mediated by olfaction and distributed chemoreception were observed.

The second hypothesis was tested by conducting attractability assays using aqueous solutions of 6 diet formulations (Table 3) to compare the effects of krill meal as a chemo attractant with M3 (1% and 5%). M8 was not tested due to practical reasons; M3 was chosen based on its effectiveness (palatability assays). One h leachates of intact pellets for all 6 diets were used since in a previous study (Derby et al. 2016), we found that 5-min leachate extracts did not yield conclusive results. Based on the results in the current study, artificial mixture M3 (1%) with and without premix at 2% and 1% dilutions proved to be more effective than 5% krill meal at the same dilutions. To our surprise, 5% M3 (with and without) premix showed lower attraction values when compared to 1% M3 and 5% krill-meal. Palatability assays (MiNa Choe and Charles Derby, unpublished data) displayed the same results with respect to 5% M3. A possible explanation as to why this occurred could be that the animals were overwhelmed by 5% M3 without premix. It is possible that the feeding stimulants in M3 can induce stronger feeding behavior at lower concentrations, and that higher concentrations (without premix) could have slightly deterred the animals. It is hypothesized that if higher concentrations of M3 (without premix) were to be tested, response would fall below control values and vice versa.

Hence, the assumption is that M3 induces synergistic effects by activating different receptors by different compounds and that at higher concentrations, M3 is less effective (Carr and Derby 1996, Coman et al. 1996).

In addition, premix has been shown to enhance the effects of both krill meal and M3 (1% and 5%). This is possibly due to premix's amphoteric properties. Leaching may have been reduced by the presence of premix, prolonging the residence time of the compounds in the mixture (Blezinger et al.2015). Furthermore, it has been documented that many species of crustaceans, especially, shrimp, perceive phospholipids, which have amphoteric properties, as attractive chemostimulants (Shanker et al. 2010). This could explain the improved responses seen with diet mixtures that included premix in their composition; yet it does not explain why 5% M3 did not show the expected dose-dependent effect. It is hypothesized that higher concentrations of premix with higher concentrations of M3 could further reduce responses below other stimuli including controls, since the premix prolongs the residence time of the compounds in the mixture. More work is needed to verify these effects.

In summary, shrimp showed positive responses to both M3 in comparison to krill meal. M3 in particular exhibited higher attractability and palatability compared to M8 and 6% krill meal. This study has shown what was previously defined in the literature: krill meal is a strong chemoattractant for *L.vannamai*. Here we showed that artificial chemical mixtures might be able to emulate these effects. Thus, it is feasible to design artificial chemical mixtures that are more palatable and attractive to shrimp. Scientists are actively looking into techniques that can enhance plant-based meal feeds for shrimp. For example, solid-state fermentation of soybean meal by yeast (*S. cerevisiae*) has been shown to enhance nutrient digestibility and nutritional value (increasing crude protein and lipid contents while decreasing fiber content) of shrimp feed as well as other feeds (Sharawy et al. 2016). The use of yeast extract has been shown to be a strong feeding attractant for *P. monodon* (Hartati and Briggs, 1993). Zhao et al. (2015) also described that 45% of fishmeal can be replaced by

yeast extract for *L. vannamei* diets without affecting digestibility and growth. Most of the literature discusses partial replacement rather than total replacement; hence, the novelty of the work presented in this article is that no fishmeal was included in any of the diets or stimuli. Hence, our proprietary mixtures provide the necessary components to increase attractability and thus ingestion of soybean-meal based pellets without the use of fishmeal. This work has shown that it is possible to design effective chemoattractants for shrimp that are more sustainable and cost effective compared to animal extracts and meals.

Future work may include improvements in the study design with inclusion of more animals and other economically relevant shrimp and fish species. Additionally, other artificial chemical mixtures may be developed based on M3 and M8 to have a better understanding of which compounds are more relevant in terms of attractability and palatability. Finally, in order to fully conclude that M3 and M8 are strong chemostimulants for *L. vannamei*, feed supplemented with these mixtures will be tested in aquaculture facilities (iAqua) that hold higher numbers of animals, which will shed light on the practicality (economically and biologically) of these mixtures. Currently, growth rates of animals given feed supplemented with M3 and M8 are being compared to the growth rates of animals given basic feed. Results are promising so far.

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