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Behavioral response to selected feed attractants and stimulants in Pacific white shrimp, *Litopenaeus vannamei*

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Abstract

Nine commercial feeding attractants and stimulants for *Litopenaeus vannamei* were evaluated by observation of behavioral responses in animals allotted in one Y-maze aquarium apparatus. In the validation phase, fishmeal–Brazilian origin (FMBO); fishmeal–Peruvian origin (FMPO); blood meal (BM), meat and bone meal (MBM), squid meal (SM), fish oil (FO) and fish solubles (FS) were evaluated. There was also a control without stimulatory raw material. The tested materials were included at 3% in neutral gelatin pellets (wet basis). In each behavioral observation, two different ingredients were offered at the same time in equal quantities, being allotted in the end of each chamber's arm. In Phase II after system validation, the following commercial attractants were tested: 80% crude protein (CP) vegetable dried biomass (VDB_{80}), 68% CP vegetable dried biomass + glutamate + betaine (VDB_{68}), complex of amino acids (alanine, valine, glycine, proline, serine, histidine, glutamic acid, tyrosine and betaine) with enzymatically digested bivalve mollusk (CAA), condensed fish soluble protein (CFSP), squid liver meal (SLM), betaine (Bet), dried fish solubles–low biogenic amines (DFS_{LB}), dried fish solubles–high biogenic amines (DFS_{HB}) and whole squid protein hydrolysate (WSPH). Attractants were used at a 3% level wet basis with neutral gelatin, without any additional ingredient source available. The best four commercial attractants from this phase (CAA, CFSP, SLM and WSPH) were compared under 0.5% and 1.0% levels. In Phase I of the study, a higher percentage of choices were observed for FMPO and FMBO. BM and FO were the least chosen ingredients. In Phase II, the worst results were observed for Bet, DFS_{HB} and, mainly, for VDB_{80} and VDB_{68}. When two-by-two comparisons were performed, results suggested that CFSP and CAA were the best commercial attractants tested. In the last phase, both CFSP and CAA at 1.0% level were significantly more chosen by shrimp than CFSP (0.5%), SLM (0.5 or 1.0%) or WSPH (0.5 or 1.0%). At both 0.5% and 1.0% levels, shrimp spent a similar amount of time feeding on CFSP and CAA. However, only CAA was statistically better than SLM and WSPH together. Further work is required to better elucidate the chemical drivers of chemostimulation for *L. vannamei* for each one of the attractants tested.

Keywords: Attractants; Stimulants; Feed additives; Food preference; *Litopenaeus*

1. Introduction

As inclusion of fishmeal is confronted in commercial shrimp feeds, the role of chemoattractants becomes

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crucial to preserve feed attractiveness. High inclusion of low-cost vegetable protein sources, such as soybean meal, in shrimp diets is often linked with poor performance. This can be the result of decreased feed consumption led by low food attractability and palatability. Identifying the chemosensory stimuli which shrimp normally find palatable may assist in improving ingestion of formulated diets (Holland and Borski, 1993). Arousal of shrimp feed intake can also minimize leaching of feed nutrients caused by the animal's slow feeding behavior (Peñaflorida and Virtanen, 1996).

Behavioral, physiological, and electrophysiological responses have been employed in testing chemosensitivities of decapods (Heinen, 1980). Most aquatic chemical signals identified so far among aquatic animals are soluble molecules relatively small, where amino acids predominate. Evidence that mixtures can be more stimulatory than single compounds, by either additive or synergistic interactions, has accumulated in both behavioral and electrophysiological studies (reviewed by Lee and Meyers, 1997).

Specialized literature on dietary attractants and stimulants for marine shrimp is scarce. Smith et al. (2005) examined squid, crustacean and krill meals, fish and krill hydrolysates and a betaine product as stimulants in black tiger shrimp *Penaeus monodon* diets. These authors concluded that *P. monodon* showed a significantly greater preference for feeds containing crustacean or krill meal. Huang et al. (2003) conducted an experiment to investigate the dietary selectivity in Chinese shrimp, *Fenneropenaeus chinensis*. The experimental diets contained one of the following ingredients: fish flesh, shrimp flesh, clam foot or polychaete worm. A commercial formulated diet and a mixed diet were also utilized. The results showed that specific growth rates were highest in the mixed diet fed group, and food conversion efficiencies were highest at polychaete worm fed group. The authors stated that Chinese shrimp possess the ability to discriminate different diets.

Artificial diets for crustaceans must be chemically attractive to induce their location and feeding, and addition of small amounts of chemostimulants might increase ingestion rates and improve growth, survival, and food conversion (Carr, 1988). The present work was carried out to evaluate nine commercial attractants and/or stimulants in *Litopenaeus vannamei* diets. Behavioral responses of individuals allotted in a Y-maze aquarium apparatus were monitored. The objectives were (1) to know whether the Y-maze apparatus is a valid methodology to evaluate attractiveness in *L. vannamei*; and (2) to measure and compare feeding stimuli of commercial attractants fed to this species.

2. Materials and methods

2.1. Shrimp and conditioning period

Attractability behavior assays were carried out with juvenile shrimp *L. vannamei* ranging in size between 6 and 12 g in body weight. Animals were collected from a commercial shrimp farm and transported alive to the laboratory. Animals were then stocked in a 1.000-l tank equipped with a biological filter and continuous aeration. Prior to any behavioral evaluations of attractability, shrimp were first subjected to an acclimation period of 1 week to adapt to laboratory rearing conditions. During the conditioning period, animals were fed *ad libitum* with a 35% crude protein diet (Camaronina 35 hp, Agribrands Purina do Brasil Ltda., São Lourenço da Mata, Pernambuco, Brazil).

2.2. Shrimp behavioral evaluations

To start attractability sessions, shrimp were transferred to a 50-l glass aquarium equipped with a rubber carpet laid over a biological sand filter fixed on the tank bottom. To stimulate more rapid responses in feeding arousal, animals were starved for 24-h prior to any behavior observations. Attractability bioassays began by stocking one starved animal at a time in a double choice aquarium containing filtered and disinfected seawater at 33‰ salinity.

The aquarium resembled a Y-maze and measured 1.3 m × 0.3 m × 0.4 m (length × width × height). The Y-maze apparatus was equipped with an acclimation chamber, isolated by a removable glass guillotine and a rubber bottom to facilitate shrimp locomotory activity (Fig. 1). During each session, no water exchange was used to avoid the reotaxis influence over the animal's orientation towards the food source. Aeration was only used right before the delivery of the ingredient in water. For each observation, two ingredients were compared. They were offered separately in similar amounts (2 g), placed individually in the perimeter of one of the Y-maze apparatus arms. Prior to behavioral evaluations, shrimp was stocked in the acclimation chamber and allowed to acclimate to the Y-maze system for 10 min.

For each ingredient tested, one different specimen of *L. vannamei* was used. In case food was not detected within a 7-min time limit, observation was interrupted and the animal replaced by another acclimated specimen. After each session, water was discarded and completely replaced by new filtered seawater at 78 l of total aquarium volume.

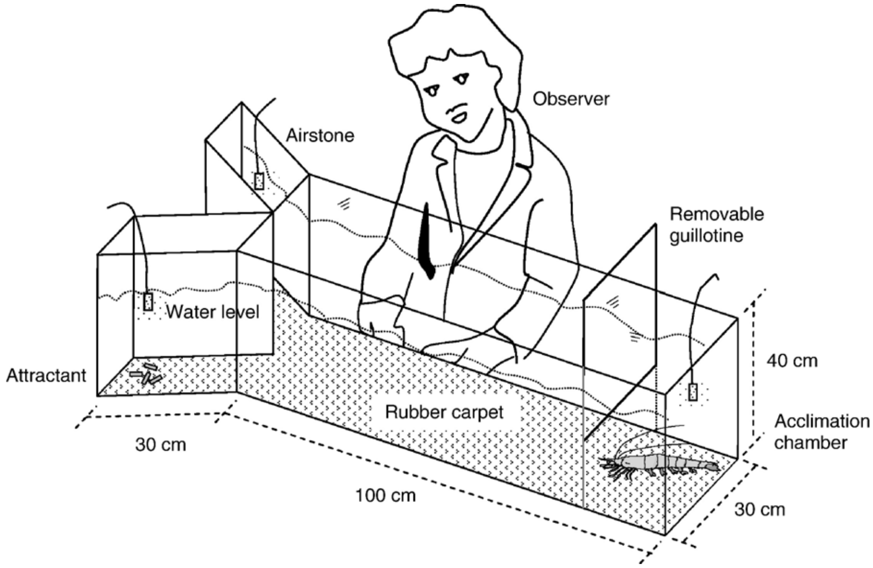

Fig. 1. Schematic drawing of the Y-maze device showing the position of the observer, guillotine, aeration points and attractants.

2.3. Measurement of behavioral responses to feeding stimuli

Shrimp feeding stimulus was measured visually by one observer positioned on the right side of the Y-maze aquarium (Fig. 1). Observations were made during the day, in the presence of artificial light and lasted less than 30 min per specimen. The whole observation area was covered by a dark plastic liner to minimize disturbance to shrimp behavior. Feeding behavior was categorized according to the following responses proposed by Lee and Meyers (1997) with modifications: (1) detection (*detec*): after the guillotine withdrawn, time elapsed between the immersion of attractants in water and the start of shrimp's perception of chemical signals, such as moving of the antennules, mouth parts and pereiopods; (2) orientation and locomotion (*orie* + *loco*): time shrimp spent to find the chosen ingredient after *detec*; shrimp starts the movement forward or backward the feed, walking or moving quickly from one place to another, searching calmly or intensively the feed; and (3) feeding activity (*feed*): if food consumption occurred, time spent during the feed intake process.

When feed consumption was observed for the selected ingredient, it was scored as a positive choice. Refusal to feed on the gelatin pellet after *orie*+*loco* occurred was accounted as rejection. The percentages of positive choices and rejections for each tested ingredient or attractant in relation to all other tested products were calculated according to the following expressions: positive choice (%) = (total number of choices/total number of comparisons) × 100; and rejection (%) = (total number of rejections/total number of choices) × 100.

2.4. Experimental design

The study was divided into three phases, including an initial stage (Phase I) to validate the methodology and apparatus used (Table 1). For Phase I, seven different stimulatory raw materials (SRM) were compared against each other: (1) fishmeal–Brazilian origin (FMBO); (2) fishmeal–Peruvian origin (FMPO); (3) blood meal

Table 1
Detail of different study phases used in feeding response evaluations with *L. vannamei*

Details	Study phase I	II	III
Purpose of feeding response evaluation	Validate methodology with raw stimulatory animal by-products (terrestrial and marine origin)	Compare commercially available feeding attractants	Compare selected attractants from Phase II under reduced dosages
Inclusion level	3%	3%	0.5% and 1.0%
Negative control	Neutral gelatin	Neutral gelatin and soybean meal	Neutral gelatin and soybean meal
Number of pairwise comparisons per ingredient	5	5	7
Total number of comparisons/ sessions (duration)	159 (23.75 h)	225 (37.39 h)	168 (9.14 h)

(BM); (4) meat and bone meal (MBM); (5) squid meal (SM); (6) fish oil (FO); and (7) fish solubles (FS). There was also a control (CON) without SRM. SRM were included at 3% wet basis and tested for at least five different specimens of *L. vannamei*. Animals were discarded after each session.

Phase II of the study consisted of comparing nine commercially available attractants for shrimp at a 3% level wet basis. Each commercial attractant was tested with five different specimens of *L. vannamei* of 9.2± 2.47 g body weight. Attractants used in this phase were as follows: (1) 80% crude protein (CP) vegetable dried biomass (VDB_{80}); (2) 68% CP vegetable dried biomass +glutamate+betaine (VDB_{68}); (3) complex of amino acids (alanine, valine, glycine, proline, serine, histidine, glutamic acid, tyrosine and betaine) with enzymatically digested bivalve mollusk (CAA); (4) condensed fish soluble protein (CFSP); (5) squid liver meal (SLM); (6) betaine (Bet); (7) dried fish solubles–low biogenic amines (DFS_{LB}); (8) dried fish solubles–high biogenic amines (DFS_{HB}); and (9) whole squid protein hydrolysate (WSPH).

The four best attractants from Phase II were selected and compared in Phase III at 0.5 and 1% inclusion levels wet-basis. Each inclusion level and individual attractant was tested with seven different specimens of *L. vannamei* of 9.4± 3.47 g body weight. In Phases II and III, ingredients were compared against each other, as well as against a negative control. In this case, soybean meal at 3% inclusion wet basis was used as the negative control (CON), without any additional ingredient source available.

Table 2
Number of comparisons and percentages of positive choices and rejections of individual stimulatory raw materials (SRM) offered to *L. vannamei* juveniles (6–8 g)

Attractant*	Number of Comparisons	Positive choices**, *** (%)	Rejection****
CON	35	2.9^{g}	100.0
MBM	44	54.5^{af}	8.3
SM	44	59.1^{ad}	0.0
FMPO	41	75.6^{a}	0.0
FMBO	41	65.9^{ac}	0.0
BM	35	45.7^{bcdef}	25.0
FO	35	25.7^{b}	44.4
FS	41	58.5^{ae}	8.3
	Chi-square *P*	<0.001	–[#]

Each comparison represents the response of one animal submitted simultaneously to two SRM in a Y-maze apparatus.

* Control (no addition of SRM; neutral gelatin plus water), MBM (meat and bone meal), SM (squid meal), FMPO (fishmeal–Peruvian origin), FMBO (fishmeal–Brazilian origin), BM (blood meal), FO (fish oil) and FS (fish solubles).

** Positive choice (%)=(number of choices/number of comparisons)×100.

*** Values in column which do not share the same superscript letter are statistically different by the *z*-test ($P<0.05$).

**** Rejection (%)=(number of rejections/number of choices)×100.

[#] Not applicable.

Table 3
Frequency of positive choices (%) of individual stimulatory raw materials (SRM) in *L. vannamei* juveniles (6–8 g) stocked in a Y-maze apparatus

Attractant[a]	Control	MBM	SM	FMPO	FMBO	BM	FO	FS
CON	–	100 (5)	100 (5)	100 (5)	100 (5)	100 (5)	80 (5)	100 (5)
MBM	0 (5)	–	75 (8)	80 (5)	75 (8)	20 (5)	20 (5)	38 (8)
SM	0 (5)	25 (8)	–	50 (8)	63 (8)	20 (5)	20 (5)	40 (5)
FMPO	0 (5)	20 (5)	50 (8)	–	20 (5)	20 (5)	0 (5)	38 (8)
FMBO	0 (5)	25 (8)	38 (8)	80 (5)	–	80 (5)	0 (5)	20 (5)
BM	0 (5)	80 (5)	80 (5)	80 (5)	20 (5)	–	40 (5)	80 (5)
FO	20 (5)	80 (5)	80 (5)	100 (5)	100 (5)	60 (5)	–	80 (5)
FS	0 (5)	38 (8)	60 (5)	63 (8)	80 (5)	20 (5)	20 (5)	–

Frequency of choice of the SRM is presented as the value in row compared against the SRM value in column. Data in parentheses indicate number of comparisons conducted for each comparison.

[a] Control (no addition of SRM; neutral gelatin plus water), MBM (meat and bone meal), SM (squid meal), FMPO (fishmeal–Peruvian origin), FMBO (fishmeal–Brazilian origin), BM (blood meal), FO (fish oil) and FS (fish solubles).

2.5. Preparation of gelatin pellets

Each SRW and commercial attractant was weighed to 1.73 g and individually mixed with 50 ml of water and 7.5 g of neutral gelatin. This solution was homogenized in a magnetic stirrer with heating during 5 min. After mixture, the solution was then transferred to a small container with plastic straws intended to give a cylindrical shape which was then kept under refrigeration until gelatinization. Gelatin pellets were then expelled from straws and sliced to a size of 5.5 mm in length. All pellets were kept frozen at −20 °C until use.

2.6. Statistical analyses

For each Phase, positive and rejection data derived from comparisons carried out among all ingredients or attractants were submitted to the chi-square analysis. Whenever possible, paired comparisons were conducted with the *z*-test if differences were statistically significant ($P<0.05$). The times for detection (*detec*), orientation

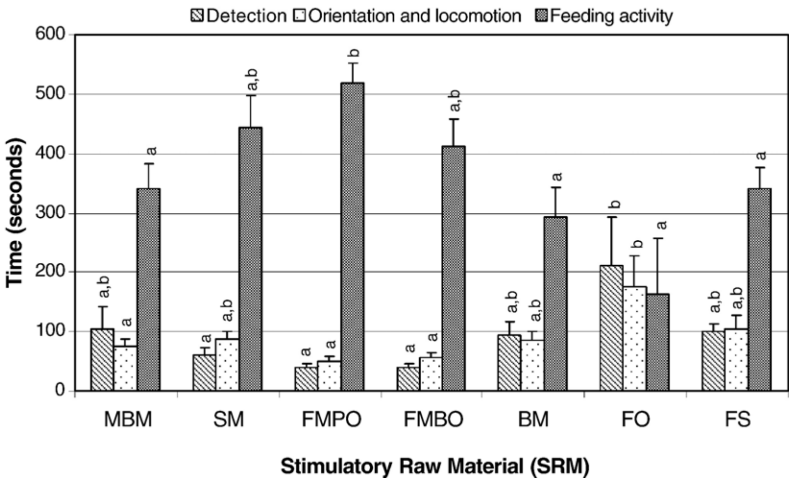

Fig. 2. Time *L. vannamei* juveniles spent in detection, orientation and locomotion and feeding activity of SRM (stimulatory raw material). Data presented as mean±S.D. of SRM against the control diet (neutral gelatin plus water). Bar values which do not share the same superscript are statistically different by Turkey's Multiple Range Test ($P<0.05$). MBM, meat and bone meal; SM, squid meal; FMPO, fishmeal–Peruvian origin; FMBO, fishmeal–Brazilian origin; BM, blood meal; FO, fish oil, and; FS, fish solubles.

and locomotion (*orie+loco*) and feeding activity (*feed*) were subjected to one-way or two-way ANOVA, followed by Turkey's Multiple Range Test if the null hypothesis was rejected ($P<0.05$).

3. Results

3.1. Validation of the Y-maze methodology

Water quality during behavioral sessions with *L. vannamei* were kept at 4.6±0.47 mg/L dissolved oxygen, 27.9±0.15 °C temperature and 31.3±1.03‰ salinity. The negative gelatin pellet (CON) was significantly less chosen by shrimp than any of the SRM tested ($P<0.05$; Table 2). A higher percentage of positive choices was observed for FMPO and FMBO which were significantly different from the values reported for BM and FO (Table 2). Blood meal (BM) and FO exhibited the poorest results for positive choices. Additionally, the percentage rejection was low for all SRM tested except for FO. There were no significant differences between the positive choices for FMPO and FMBO and those reported for FS, SM and MBM. Fishmeal–Peruvian origin (FMPO), FMBO and SM were not rejected by *L. vannamei* in any occasion.

Paired comparisons carried out between the SRM confirm these results (Table 3). The negative control gelatin pellet (CON) was chosen only once when compared with FO. Fishmeal–Peruvian origin (FMPO), FMBO and SM scored the highest choices among all SRM tested. FMPO always prevailed in shrimp choices

Table 4
Number of comparisons and percentages of positive choices and rejections of nine commercial attractants fed to *L. vannamei* juveniles (9.2±2.47 g)

Attractant*	Number of comparisons	Positive choices (%)**, ***	Rejection (%)****
CON[#]	45	20.0[f]	22.2
VDB_{80}	45	35.6[ef]	37.5
VDB_{68}	45	40.0[def]	27.8
CAA	45	66.7[ab]	0.0
CFSP	45	73.3[a]	3.0
SLM	45	62.2[abcd]	0.0
Bet	45	42.2[cde]	15.8
DFS_{LB}	45	53.3[abcde]	8.3
DFS_{HB}	45	46.7[bcde]	19.0
WSPH	45	60.0[abcd]	0.0
	Chi-square *P*	<0.001	–[†]

Attractants were tested at a 3% level wet basis. Each result represents the response of one animal exposed simultaneously to two attractants in a Y-maze apparatus.

* VDB_{80}: 80% CP vegetable dried biomass; VDB_{68}: 68% CP vegetable dried biomass+glutamate+betaine; CAA: complex of amino acids (alanine, valine, glycine, proline, serine, histidine, glutamic acid, tyrosine and betaine) with enzymatically digested bivalve mollusk; CFSP: condensed fish soluble protein; SLM: squid liver meal; Bet: betaine; DFS_{LB}: dried fish solubles–low biogenic amines; DFS_{HB}: dried fish solubles–high biogenic amines; WSPH: whole squid protein hydrolysate.

** Positive choice (%)=(number of choices/number of comparisons)×100.

*** Values in the column which do not share the same superscript are statistically different by the *z*-test ($P<0.05$).

**** Rejection (%)=(number of rejections/number of positive choices)×100.

[#] Control, 3% soybean meal+neutral gelatin.

[†] Not applicable.

Table 5
Frequency of positive choices (%) of nine commercial attractants fed to *L. vannamei* (9.2±2.47 g) stocked in a Y-maze apparatus

Attrac[a]	CON[b]	VDB_{80}	VDB_{68}	CAA	CFSP	SLM	Bet	DFS_{LB}	DFS_{HB}	WSPH
CON[b]	–	40	20	0	0	20	40	20	20	20
VDB_{80}	60	–	40	20	0	20	60	40	40	40
VDB_{68}	80	60	–	20	20	20	40	40	40	40
CAA	100	80	80	–	60	80	40	60	40	60
CFSP	100	100	80	40	–	60	60	80	80	60
SLM	80	80	80	20	40	–	60	60	80	60
Bet	60	40	60	60	40	40	–	20	40	20
DFS_{LB}	80	60	60	40	20	40	80	–	60	40
DFS_{HB}	80	60	60	60	20	20	60	40	–	20
WSPH	80	60	60	40	40	20	80	60	80	–

Five comparisons were carried out for each pair of attractants. Frequency of choice of the attractant is presented as the value in each row compared against the attractant value in each column.
[a] Commercial attractant: VDB_{80}: 80% CP vegetable dried biomass; VDB_{68}: 68% CP vegetable dried biomass+glutamate+betaine; CAA: complex of amino acids (alanine, valine, glycine, proline, serine, histidine, glutamic acid, tyrosine and betaine) with enzymatically digested bivalve mollusk; CFSP: condensed fish soluble protein; SLM: squid liver meal; Bet: betaine; DFS_{LB}: dried fish solubles–low biogenic amines; DFS_{HB}: dried fish solubles–high biogenic amines; WSPH: whole squid protein hydrolysate.
[b] 3% soybean meal+neutral gelatin.

except when compared with SM. When FMPO and FMBO were compared, shrimp chose the former more often (Table 3). In general, SRM of terrestrial sources were less preferred by *L. vannamei* than from marine origin.

Analysis of variance indicated significant differences in time spent in detection, orientation+locomotion and feeding among SRM tested (Fig. 2; $P<0.05$). Shrimp spent longer to detect FO than all other SRM tested except MBM. Conversely, a shorter period was required to detect FMPO and FMBO when compared to MBM. Shrimp spent longer time in *orie+loco* when fed FO than all other SRM, except when exposed to BM and FS. Shrimp spent significantly more time feeding on FMPO than when fed MBM, FO and FS ($P<0.05$).

3.2. Evaluation of commercial attractants and stimulants

In shrimp feeding responses to commercial attractants, a statistically higher number of positive choices was achieved with CFSP when compared to VDB_{80}, VDB_{68}, Bet, DFS_{HB} and CON ($P<0.05$; Table 4). On the other hand, there were no significant differences between the

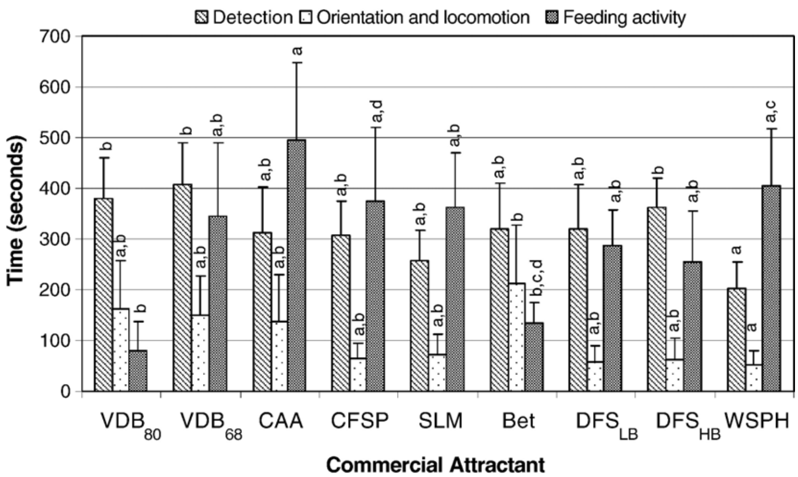

Fig. 3. Time *L. vannamei* juveniles spent in detection, orientation and locomotion and, feeding activity of nine different commercial attractants (mean± S.D.) against a control ingredient (neutral gelatin+3% soybean meal). Bar values which do not share the same superscript are statistically different by Turkey's Multiple Range Test ($P<0.05$). VDB_{80}: 80% CP vegetable dried biomass; VDB_{68}: 68% CP vegetable dried biomass+glutamate+betaine; CAA: complex of amino acids (alanine, valine, glycine, proline, serine, histidine, glutamic acid, tyrosine and betaine) with enzymatically digested bivalve mollusk; CFSP: condensed fish soluble protein; SLM: squid liver meal; Bet: betaine; DFS_{LB}: dried fish solubles–low biogenic amines; DFS_{HB}: dried fish solubles–high biogenic amines; WSPH: whole squid protein hydrolysate.

Table 6
Number of comparisons and percentages of positive choices and rejections of four selected commercial attractants fed to *L. vannamei* juveniles (9.4±3.47 g)

Attractant *	Inclusion level (%)	Positive choices **, *** (%)	Rejection ****
CON #	3.0	8.9^{e}	20.0
CAA	0.5	51.8^{ab}	0.0
	1.0	67.9^{a}	2.6
CFSP	0.5	44.6^{bc}	8.0
	1.0	71.4^{a}	5.0
SLM	0.5	21.4^{de}	8.3
	1.0	30.4^{cd}	0.0
WSPH	0.5	17.9^{de}	20.0
	1.0	21.4^{de}	8.3
	Chi-square *P*	<0.001	– †

Attractants were challenged at 0.5% or 1.0% level. Each result represents the response of one animal submitted simultaneously to two attractants in a Y-maze apparatus. A total of 56 observations were carried out for each paired comparison.

* CAA: complex of amino acids (alanine, valine, glycine, proline, serine, histidine, glutamic acid, tyrosine and betaine) with enzymatically digested bivalve mollusk; CFSP: condensed fish soluble protein; SLM: squid liver meal; WSPH: whole squid protein hydrolysate.

** Positive choice (%)=(number of choices/number of comparisons)×100.

*** Values in the column which do not share the same superscript are statistically different by the *z*-test ($P<0.05$).

**** Rejection (%)=(number of rejections/number of positive choices)×100.

Soybean meal+neutral gelatin.

† Not applicable.

CFSP positive choices and those verified for CAA, SLM, DFS_{LB} and WSPH. Level of shrimp rejection for CFSP reached 3%.

Gelatin pellets with the complex of amino acids (CAA) were significantly more chosen by shrimp than VDB_{80}, VDB_{68}, Bet and CON. However, the differences between CAA positive choices and those found for CFSP, SLM, DFS_{LB}, DFS_{HB} and WSPH were not statistically significant ($P<0.05$; Table 4). No rejection was observed for the complex of amino acids (CAA).

Squid liver meal (SLM) and WSPH were significantly more chosen by shrimp only when compared with VDB_{80} and CON. Nevertheless, SLM and WSPH results for positive choices were not significantly different than those verified for VDB_{68}, CAA, CFSP, Bet, DFS_{LB} and DFS_{HB} (Table 4). Furthermore, no rejection was observed for both SLM and WSPH.

Dried fish solubles–low biogenic amines (DFS_{LB}) showed a significantly higher positive choice only when compared with CON (soybean meal+neutral gelatin). Although positive choices for DSF_{LB} did not differ from other commercial attractants tested (Table 4), it exhibited an 8.3% shrimp rejection. The worst results were observed for Bet, DFS_{HB} and, mainly, for VDB_{80} and VDB_{68}. These commercial attractants presented a high rejection by shrimp, which ranged from 15.8% to 37.5%. Moreover, shrimp choices for VDB_{80} and VDB_{68} were not significantly different from those verified for the negative control ingredient (no attractant; $P>0.05$).

When two-by-two comparisons were performed (Table 5), results suggested that CFSP and CAA were the best commercial attractants evaluated. Considering a minimum frequency of choices of 60%, CFSP and CAA were chosen preferentially in 8 and 7 out of 9 comparisons, respectively. Gelatin pellets containing condensed

Table 7
Frequency of choice (%) of four selected commercial attractants challenged at 0.5 or 1.0% level with *L. vannamei* juveniles (9.4±3.47 g) stocked in a Y-maze apparatus

Att a	CON b	$CAA_{0.5}$	$CAA_{1.0}$	$CFSP_{0.5}$	$CFSP_{1.0}$	$SLM_{0.5}$	$SLM_{1.0}$	$WSPH_{0.5}$	$WSPH_{1.0}$
CON	–	14.3	0.0	28.6	28.6	28.6	14.3	28.6	28.6
$CAA_{0.5}$	85.7	–	28.6	42.9	28.6	71.4	42.9	71.4	42.9
$CAA_{1.0}$	100.0	71.4	–	57.1	42.9	71.4	71.4	71.4	71.4
$CFSP_{0.5}$	71.4	57.1	42.9	–	14.3	57.1	28.6	42.9	42.9
$CFSP_{1.0}$	71.4	71.4	57.1	85.7	–	71.4	71.4	71.4	71.4
$SLM_{0.5}$	71.4	28.6	28.6	42.9	28.6	–	42.9	71.4	42.9
$SLM_{1.0}$	85.7	57.1	28.6	71.4	28.6	57.1	–	71.4	71.4
$WSPH_{0.5}$	71.4	28.6	28.6	57.1	28.6	28.6	28.6	–	57.1
$WSPH_{1.0}$	71.4	57.1	28.6	57.1	28.6	57.1	28.6	42.9	–

Seven comparisons were carried out for each combination of attractant and inclusion level. Frequency of choice of selected attractant is presented as the value in each row compared against the attractant value in each column.

[3]Frequency of choice of the attractant in the column when compared with the attractant in the row.

a Commercial attractant: CAA: complex of amino acids (alanine, valine, glycine, proline, serine, histidine, glutamic acid, tyrosine and betaine) with enzymatically digested bivalve mollusk; CFSP: condensed fish soluble protein; SLM: squid liver meal; WSPH: whole squid protein hydrolysate.

b Control: soybean meal+neutral gelatin.

Table 8
Time *L. vannamei* juveniles spent in detection (*detec*), orientation and locomotion (*orie+loco*) and, feeding activity (*feed*) when fed four selected commercial attractants (mean±S.D.; $n=7$) at two inclusion levels (0.5% or 1.0%) compared with a control ingredient (neutral gelatin+3% soybean meal)

Time required for	Attractant	Time (seconds)[a, b]	
		0.5% level	1.0% level
detec	CAA	307±91	291±51
	CFSP	312±80	330±90
	SLM	364±80	269±48
	WSPH	309±57	274±75
orie+loco	CAA	91±46 aX	70±39 bX
	CFSP	85±50 aX	150±50 aY
	SLM	86±55 aX	64±34 bX
	WSPH	64±22 aX	48.3±29 bX
feed	CAA	191±33 acX	281±65 aY
	CFSP	224±56 aX	248±56 acX
	SLM	105±19 bX	204±39 bcY
	WSPH	153±36 bcX	170±45 bX
ANOVA *P*	detec	orie+loco	feed
Attractant	Ns	0.003	<0.001
Level	Ns	Ns	<0.001
Attractant×level	Ns	0.021	0.035

CAA: complex of amino acids (alanine, valine, glycine, proline, serine, histidine, glutamic acid, tyrosine and betaine) with enzymatically digested bivalve mollusk; CFSP: condensed fish soluble protein; SLM: squid liver meal; WSPH: whole squid protein hydrolysate.

[a] For the same variable, means in a column which do not share the same lowercase letter are statistically different by Turkey's Multiple Range Test ($P<0.05$).

[b] For the same variable, means in a row which do not share the same uppercase letter are statistically different between them by the Turkey's Multiple Range Test ($P<0.05$).

fish soluble protein (CFSP) was not preferentially chosen only when confronted with CAA.

In the opposite end, commercial attractants with the worst results were VDB_{80} and VDB_{68}. These attractants showed very low frequency of choice (20% or 0%) when compared individually with other attractants (Table 5). Gelatin pellets with 80% CP or 68% CP vegetable dried biomass+glutamate+betaine (VDB_{80} and VDB_{68}, respectively) were more chosen by shrimp only when compared with the control ingredient (soybean meal) or with Bet.

The time elapsed until attractant detection by shrimp was significantly lower for WSPH when compared with DFS_{HB}, VDB_{80} or VDB_{68} ($P<0.05$; Fig. 3). However, differences were not significant between WSPH and DFS_{LB}, Bet, SLM, CFSP and CAA ($P>0.05$). Time shrimp spent in *orie+loco* was significantly lower when WSPH was compared with Bet ($P<0.05$; Fig. 3). There were no statistically significant differences for all other comparisons among attractants evaluated ($P>0.05$).

Finally, shrimp spent longer feeding on CAA when compared with VDB_{80} or Bet ($P<0.05$; Fig. 3). Accordingly, the time of *feed* for CAA was not statistically different from VDB_{68}, CFSP, SLM, DFS_{LB}, DFS_{HB} and WSPH ($P>0.05$). The time shrimp spent feeding on the whole squid protein hydrolysate (WSPH) was only statistically higher than VDB_{80} ($P<0.05$).

3.3. Selected commercial attractants and stimulants under reduced dosages

Both CFSP and CAA at 1.0% level ($CAA_{1.0}$) were significantly more chosen by shrimp than $CFSP_{0.5}$, $SLM_{0.5}$, $SLM_{1.0}$, $WSPH_{0.5}$ and $WSPH_{1.0}$ (Table 6; $P<0.05$). The results of positive choices for $CFSP_{0.5}$ and $CAA_{0.5}$ were not as clear as those reported for the 1.0% inclusion level, particularly for $CFSP_{1.0}$, $SLM_{0.5}$, $WSPH_{0.5}$ or $WSPH_{1.0}$. These were chosen as only a few times as the CON and no statistical differences were reported between them.

When $CFSP_{1.0}$ was confronted with $CAA_{1.0}$ there was a slightly higher preference for $CFSP_{1.0}$ (Table 7). This trend was kept even when all comparisons were considered. However, no statistical analysis was possible in this case.

Statistical differences with higher responsiveness were observed for *feed* (Table 8). Hence, at lower inclusion levels, *feed* appeared to be a better indicator of attractiveness in *L. vannamei* than *detec* and *orie+loco*. When tested at 1.0% level, $CFSP_{1.0}$ showed an increased *orie+loco* compared to other attractants tested (CAA, SLM and WSPH). The time required for *detec* of $CFSP_{1.0}$ was significantly higher than for $CFSP_{0.5}$.

At 0.5% level, $CFSP_{0.5}$ and $CAA_{0.5}$ showed a similar time of *feed* (Table 8). In this case, it seemed that $CFSP_{0.5}$ was a better attractant than $CAA_{0.5}$ since only the first has produced a significantly higher time of *feed* when compared to $SLM_{0.5}$ and $WSPH_{0.5}$ ($P<0.05$). On the other hand, at 1.0% level, the opposite was observed. $CFSP_{1.0}$ and $CAA_{1.0}$ had again comparable results of feeding activity but only $CAA_{1.0}$ was statistically better than $SLM_{1.0}$ and $WSPH_{1.0}$ together.

4. Discussion

4.1. Validation phase

The very low percentage of positive choices and the 100% rejection observed for the CON (gelatin+water) have fully demonstrated the suitability of the negative control in attractiveness studies with *L. vannamei*.

The major components of feeding stimuli reported over the years have been relatively small, water-soluble chemicals, such as amino acids, mainly taurine, glycine, arginine, glutamic acid and alanine; small peptides, amines, nucleotides and nucleosides, and quaternary ammonium bases, such as betaine (Lee and Meyers, 1997). Protein-rich ingredients of marine origin, such as fishmeal and squid meal contain these substances which are feeding effectors for shrimp (Smith et al., 2005). Hence, the present results for FMPO and FMBO were expected and confirm the superior ability of marine fishmeal to stimulate *L. vannamei* chemoreceptors.

Fat- or oil-based materials are generally immiscible in water and therefore are not optimal chemicals to attract, stimulate or trigger shrimp feeding responses. The minimal content of water-soluble compounds in FO makes it poorly effective as a feeding activator for fish or crustaceans feeds (Zimmer-Faust, 1989). Therefore, the results reported herein for FO demonstrate it provided no or only a minimum feeding stimuli for *L. vannamei*.

Comparable positive feeding responses observed for FMPO and SM agree with previous studies which indicate their beneficial effect in stimulating feed intake and (or) growth (Fenucci et al., 1980). Results from the present study reiterate the ability of SM to stimulate shrimp feeding responses.

The quality and nutrient composition of fishmeal may vary highly according to the freshness and type of the raw material and the processing conditions adopted. Raw material freshness is best described by the content of biogenic amines in fishmeal. Shrimp feeds containing fishmeal with increased levels of biogenic amines caused a decrease in feed intake (Ricque-Marie et al., 1998; Mundheim et al., 2004). In our study, the higher preference of *L. vannamei* for FMPO in comparison with FMBO suggests that FMBO had a poorer freshness condition, able to inhibit shrimp feeding activity to some level. FMBO is obtained from local fish processing by-products. Therefore, a higher degree of spoilage and hydrolysis may be expected compared to whole fish processed FMPO.

Proteins of terrestrial origin, such MBM, contain less of these appetizing small water-soluble substances (Lim and Dominy, 1991). Lee and Meyers (1997) have reported that some feedstuffs and chemical compounds can function as feed intake inhibitors, especially those with high ammonia level. Ammonia is generally volatile when subjected to thermal action during the drying process. In some cases, the volatile nitrogenous content is used as an indicator of freshness of dietary feedstuffs and by-products included in animal feeds.

In view of the fact the validation phase results are in reasonable agreement with the results reported in previous works (Fenucci et al., 1980; Lim and Dominy, 1991; Holland and Borski, 1993; Lee and Meyers, 1997; Smith et al., 2005), the use of the Y-maze chamber may be considered as a valid methodology to evaluate chemical feeding effectors in *L. vannamei*.

4.2. Commercial attractant evaluation

When overall results are analyzed, it can be seen that CFSP, CAA and WSPH stimulated higher feeding responses in *L. vannamei* in relation to other commercial attractants tested. On the other hand, VDB_{80} and VDB_{68} produced the worst results for almost all experimental indicators observed. The other remaining attractants, i.e., SLM, Bet, DFS_{LB} and DFS_{HB}, can be categorized as medium performance feeding stimulants.

Fish solubles are produced as a by-product of fish canning and fish oil production industries. During the processing of fish to recover the oil fraction a mixture of water and oil is produced. This mixture is then centrifuged to remove the oil. The residual aqueous phase can be drawn off and much of the water evaporated from it to produce condensed fish solubles (as CFSP) or dried fish solubles (as DFS_{LB} and DFS_{HB}). Fish solubles are high in water-soluble vitamins and have been used as a source of unidentified growth factors in poultry and swine feeding (Soares et al., 1970). As with water-soluble vitamins, fish solubles are also high in water-soluble substances with boosted feeding stimuli response, such as amino acids (Smith et al., 2005). So the higher attractiveness for CFSP observed in our study is supported by the accepted premise of feeding attractants for aquatic animals. On the other hand, results for DFS_{LB} and DFS_{HB} were not as good as those for CFSP. This may indicate that the drying processing to which DFS_{LB} and DFS_{HB} were subjected has possibly damaged their chemicals with attractiveness abilities.

Fish solubles–high biogenic amines (DFS_{HB}) showed a higher level of rejection than DFS_{LB} and it was chosen relatively less than the latter. Cadaverine, histamine and putrescine are foul-smelling molecules produced by protein hydrolysis during putrefaction of animal tissue. Depending on the concentration it may be a toxic diamine, being the decarboxylation product of the amino acids lysine, histidine and arginine, respectively (Pike, 1993). Differences in fishmeal quality due to variability in raw material freshness may be evaluated by the content of biogenic amines left in fishmeal (Aksnes and Mundheim, 1997). Therefore, the present results suggest that raw material freshness affects the bioefficacy of the commercial attractant produced.

As previously mentioned analyses of natural fluids or of extracts of attractive natural materials to crustaceans have generally shown that major stimulants are substances of low molecular weight. They have chemical properties consistent with the hypothesis that they are amino acids or closely related substances (Heinen, 1980). In addition, Holland and Borski (1993) have suggested there are low-molecular-weight compounds other than amino acids (e.g., nucleotides, tertiary amines, peptides) that contribute to the effectiveness of the <1000 MW fraction. There are also palatability factors in the whole (shrimp-head offal) extract that add to the low-molecular-weight stimulatory compounds to create the total palatability of the natural extract.

The superior results of attractiveness for the commercial complex of AA used in our work confirm the chemosensory ability of amino acids to elicit positive feeding responses in *L. vannamei*. Further, when CAA's results were contrasted with those obtained for betaine, it evidenced that amino acids pools are better attractants for *L. vannamei* than isolated ones.

However, the present results for betaine do not agree with Smith et al. (2005) who found that betaine included at 2% in the diet produced a significantly higher total apparent daily feed intake in *P. monodon*. Although that disagreement can be attributed to the different methodologies carried out in the two studies, it is pointed out that two distinct sources of betaine were used. The betaine used by Smith et al. (2005) contained additional amino acids from hydrolysed protein, whereas the source used in the present study was composed of betaine alone. So the specific technological processes employed to manufacture betaine products may have strong impact on its stimulatory effects.

The increased feeding responses that WSPH and SLM have provided in the present study indicate that whole or liver squid are effective attractants for *L. vannamei*. Results also indicate that the protein hydrolysis process to which WSPH was submitted has produced even better responses. These observations agree with the positive responses reported in other studies with squid meal (Fenucci et al., 1980; Akiyama, 1986; Holland and Borski, 1993), but contrast with Smith et al. (2005). These latter authors observed that when more squid meal was included in *P. monodon* feed there was a decrease in total daily feed intake. In the present study, the better results for WSPH are supported by Refstie et al. (2004), with Atlantic salmon, Córdova-Murueta and García-Carreño (2002), with *L. vannamei*, and Anggawati et al. (1990), with *P. monodon*.

The poor stimulatory effects of SRM from plant sources (VDB_{80} and VDB_{68}) in our study suggest that (1) sources of vegetable origin have no or very low stimulatory power; (2) high crude protein levels are not necessarily indicators of attractiveness; and (3) the supplementation of vegetable sources with certain amino acids (glutamate and betaine) may prove useless to stimulate feeding responses in *L. vannamei*.

4.3. Feeding responses to reduced dosages with commercial attractants

Feeding attractants have been usually included in shrimp and prawn diets at levels ranging from 0.5% to 5.0%, depending on the attractant type and the composition of the diet (Hartati and Briggs, 1993). Smith et al. (2005), for instance, observed in *P. monodon* better responses for krill hydrolysate, fish hydrolysate, crustacean meal and krill meal when they were included in the diet at 1%, 2%, 5% and 5%, respectively. Felix and Sudharsan (2004) tested glycine betaine included at 0.5%, 1.0% or 1.5% in diets for *Macrobrachium rosenbergii*. As a general rule, lower dietary inclusion levels of additives are preferred, although this will depend on their cost, market availability and nutritional composition.

On the other hand, the "growth enhancer" approach allows higher dietary inclusion levels of ingredients with boosted gustatory properties. In this context, up to 15% of the growth promoting ingredient can be included in the shrimp diet (Córdova-Murueta and García-Carreño, 2002). Here, it is expected that the so-called enhancer has both feeding stimuli and nutritional impact on the diet.

The positive response observed for CAA in the present study is supported by Coman et al. (1996) who found that the mixture of amino acids tested was an effective stimulant to *P. monodon*. These authors highlight that such strong response is consistent with synergistic interactions of mixtures. However, it was seen in our work a level effect in regards to CAA's efficacy. The best results were only observed when CAA was used at 1.0% level. So these findings suggest that no matter how effective an attractant is if its inclusion is below the optimum level, shrimp stimulatory feeding responses will be weaker.

$CFSP_{1.0}$ has proven itself as the best commercial attractant evaluated in the present work for *L. vannamei*, followed near by $CAA_{1.0}$. According with Carr and Derby (1986), the chemical and physical characteristics owned by powerful stimulatory substances are the following: low molecular weight, high water solubility, a source of N, non-volatility, and exhibit acidic or basic properties. Assuming as a valid premise that the ability of an attractant to elicit positive feeding responses may be better estimated at lower inclusion levels, the longer time spent in feeding activity seen for $CFSP_{0.5}$ is useful.

However, the much longer time required for shrimp in orientation and locomotion when fed $CFSP_{0.5}$ warrants further examination.

When challenged by lower inclusion levels (0.5% or 1.0%), SLM and WSPH did not show responses as good as those verified in the previous phase (3.0% level). Hence, a reduction in inclusion level may be a useful way to compare two or more attractants. Chemical analyses are further required to attain a more detailed understanding of the factors that caused the inferior results for SLM and WSPH in the challenging phase. Perhaps the amino acid profile in CAA and CFSP contained more stimulatory effectors for *L. vannamei* than in SLM and WSPH. This is a question that remains to be evaluated that is presently under consideration in our laboratory.

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