NUTRITIONAL REQUIREMENTS OF BROWN SHRIMP, *Penaeus aztecus*

K. L. Shewbart, W. L. Mies and P. D. Ludwig
Ag-Organics Department
Dow Chemical U.S.A.
Lake Jackson, Texas

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ABSTRACT

Basic research was conducted in important areas of shrimp nutrition in order to formulate an artificial shrimp diet which would maximize growth. The essential amino acids of shrimp were determined and the amino acids of protein quantitized. The fat content of shrimp was analyzed as triglycerides, free fatty acids, and phospholipids. Carbohydrates were determined quantitatively by gas-liquid chromatography. The mineral content of shrimp was determined. Using information gained from the above analyses, growth trials were performed varying the percentages protein, energy, and minerals.

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SECTION I. INTRODUCTION

Recent proposals for the utilization of Texas marshlands for the purpose of shrimp mariculture have been met with enthusiasm. However, there are many problems yet to be worked out before shrimp mariculture may become an economically feasible industry.

Dow Chemical U.S.A., through the National Oceanic and Atmospheric Administration's Sea Grant Program, is cooperating in efforts to find solutions to these problems.

A major area of concern in the rearing of shrimp in pond cultures is the provision of an adequate artificial diet maximizing growth and survival. It was the objective of this group to study the problems of nutrition and to develop an artificial diet suitable for use in pond culture.

It was found that practically no basic research had been reported in the area of shrimp nutrition. A basic approach was felt to be the logical starting point for a thorough study of nutrition and, ultimately, formulation of a diet. Dietary needs of the shrimp, as determined from its physical makeup, were investigated in detail.

Since protein and the composition of that protein are some of the most important aspects of diet formulation, these problems were investigated first. Studies were begun to determine the essential amino acids of brown shrimp incorporating radioactive tracer techniques. From the results of this study, a least cost computer formulated protein source adequate in all essential amino acids could be formulated. Growth

studies were conducted to determine the percent protein in the diet which maximized growth.

A second important constituent of a viable diet was the lipid or fat content. It was felt that fat sources now being used did not reflect the lipid needs of shrimp. Analyses of the fatty acid content of shrimp bodies and stomach contents were performed to determine an adequate lipid source. By careful study of these analyses, it was hoped to determine an adequate lipid source which would maximize growth. Growth trials were to be run incorporating this lipid source.

Basic research towards providing an adequate dietary source of minerals was accomplished by determining, instrumentally, the chemical composition of P. aztecus. Growth trials were outlined, varying the percentages of those elements found to be present in greatest abundance.

Carbohydrate studies consisted of determining quantitatively, the sugars present in the blood and hepatopancreas of intermolt shrimp. Although it was hoped to run growth trials varying the amounts of carbohydrate in the diet, time did not permit a thorough examination of this area of research.

Finally, the response of P. aztecus to various attractants was determined. While the above studies were designed to develop a nutritional diet, this last experiment was performed to measure the acceptability of the diet. It was the purpose of this test to increase the attractiveness of the diet by the addition of small amounts of substances which are phagostimulants to shrimp.

It is felt that the work outlined above and the results which follow, form the basic groundwork for the formulation of an optimum diet for shrimp.

SECTION II. PROTEIN

One of the most important elements of a viable diet is protein, and it is generally believed that the biological value of protein depends upon its amino acid composition. Therefore, in order to provide a useful diet for shrimp at various life stages, the proper amino acids in the proper amounts must be present.

A. A Quantitative Analysis of the Amino Acids Present in the Protein of Brown Shrimp

In this investigation the amino acids of the protein of brown shrimp, Penaeus aztecus, were identified and analyzed quantitatively using recently developed gas-liquid chromatographic techniques (Gehrke and Leimer, 1971).

1. Materials and Methods:

Shrimp for these experiments were obtained from Pelican Bait Camp, Freeport, Texas. The heads, chitin, and gastrointestinal tracts of 60-80 mm juvenile brown shrimp were removed and discarded. The tail muscle remaining was ground into a fine powder with powdered CO_2. The powder was then homogenized with distilled water and treated with cold 5% trichloroacetic acid (TCAA). The sediment

was washed with TCAA and ethyl alcohol. Nucleic acids were removed by boiling with hot 5% TCAA for five minutes and washing with TCAA and water. Removal of carbohydrates, nucleic acids, and soluble amino acids was thus accomplished. Fats were extracted by refluxing overnight with ethyl ether and washing with ether. The samples were then dried in a stream of nitrogen for three hours and reground.

A sample of the purified protein was analyzed by the Kjeldahl method to determine the nitrogen content of the shrimp tail muscle.

A 25-mg. protein sample was hydrolyzed by the method outlined by Gehrke et al. (1968). The HCl was removed by vacuum evaporation to near dryness using a rotary evaporator at room temperature. The hydrolysate was then diluted with 0.1N HCl and passed through a 1½ cm. diameter x 5 cm. cation exchange column, containing 100/120 mesh Amberlite IR-120H resin to remove biological background. The eluant was evaporated and brought up to 25 ml. using 0.1N HCl.

A 2-ml. aliquot (2 mg.) of the protein hydrolysate was placed in a 3-ml. micro-reaction flask and the solvent evaporated by heating in an oil bath at 70°C. A stream of nitrogen was directed over the reaction flask to

aid evaporation. To the reaction flask were added 0.5 ml. bis (trimethylsilyl) trifluoroacetamide (BSTFA) and 0.5 ml. acetonitrile containing an internal standard, phenanthrene. The flask was heated at 145°C in an oil bath for 2.5 hours. A standard amino acid mixture was made up to contain approximately the same amount of amino acids as were present in the shrimp protein. The standard was reacted in the same manner as the sample, with internal standard also being added. The samples were run on a temperature programmed Varian Model 1200 gas-liquid chromatograph equipped with a hydrogen flame ionization detector. Separation was achieved using a 10 w/w% OV-11 on 100/120 mesh Supelcoport (6 ft. x 1/8 in. I.D.) gas chromatographic column. The temperature program started with an initial column temperature of 110°C, followed by 22 minutes at 2°C rise per minute, followed by a 4°C rise per minute up to 260°C.

The amino acids were identified by retention times. The quantities of the amino acids present in the tail muscle protein of juvenile brown shrimp were calculated from the peak areas of the gas-liquid chromatographic separation of their trimethylsilyl derivatives. The purified protein analyzed by the Kjeldahl method was 11.6% of the dry weight of the shrimp tails. The results of the quantitative analysis of the amino acids are presented in Table I-1.

TABLE I-1

Tail Muscle Content

Amino Acid	Gm AA/ 16 gm N	Gm AA/gm Live Body Weight*
Alanine	5.6	0.0122
Arginine	5.1	0.0111
Aspartic Acid	10.9	0.0350
Cystine	3.3	0.0072
Glutamic Acid	13.4	0.0292
Glycine	5.7	0.0124
Histidine	3.1	0.0068
Hydroxyproline	0.3	0.0007
Isoleucine	4.4	0.0054
Leucine	9.6	0.0157
Lysine	6.0	0.0159
Methionine	3.4	0.0074
Phenylalanine	5.6	0.0122
Proline	3.1	0.0068
Serine	5.0	0.0108
Threonine	5.3	0.0115
Tryptophan**	1.0	0.0022
Tyrosine	2.7	0.0059
Valine	5.0	0.0109

*Tail muscle 70% water, non-water portion 11.6% nitrogen.

**Tryptophan figure obtained from gas-liquid chromatography of base hydrolysate.

2. Discussion

Quantitative results obtained from this study of amino acid values compare favorably with other reported values for brown shrimp (Love and Thompson, 1966), and with other species of shrimp (Burkholder et al., 1966). It would therefore appear that the protein composition of P. aztecus from various locales does not vary appreciably. This is important, since it indicates that a satisfactory protein source for one species of shrimp would probably have widespread application towards other species. Furthermore, an artificial diet formulated in accordance with now known values of amino acids in shrimp would prove more valuable than the trial and error formulations now being tried. Quantitative amino acid values would also be an important first step in formulating a chemically defined diet.

3. Summary and Conclusions

The amino acid array of Penaeus aztecus was determined. It compared favorably with other published data.

B. Determination of the Essential Amino Acids of Brown Shrimp

Since protein is the largest and most expensive single constituent of a diet, it also seemed advisable to determine which of the amino acids were essential to the shrimp, i.e. could not be synthesized by the shrimp. An indirect isotopic method was employed to determine the essential amino acids of P. aztecus in this study. This method has been previously employed in insects (Kastings and McGinnis, 1958); marine flatfish (Cowey et al., 1970); and the prawn, Palaemon serratus (Pennant, Cowey and Forster, 1971).

1. Materials and Methods

Bait shrimp used in this experiment were of similar length as those used in the quantitative analysis. Uniformly tagged ^{14}C-glucose was obtained from New England Nuclear Corporation, having a specific activity of 192 m Curies per millimole glucose, radiochemical purity greater than 95%. The shrimp were injected in the muscle tissue directly behind the carapace with 20 microliters each of an isotonic solution containing the ^{14}C-glucose (0.1 m C).

Preliminary experiments were performed to determine the incubation period for maximum incorporation of the ^{14}C into the protein. Shrimp were killed at 2, 4, 8, 16, 24, 36, and 48-hour intervals after injection and the same weight of protein from each

time interval dissolved in Aquasol*. The radioactivity was determined in a Nuclear-Chicago Model 724 liquid scintillation spectrophotometer. These preliminary experiments indicated that a near maximum incorporation of the radioactivity occurred at 24 hours. Having determined the optimum incubation period, the shrimp in this experiment were returned to aerated aquaria for 24 hours after injection. The aquaria were equipped with Plexiglass covers with air inlet and outlet lines. The outlet air was passed through a dilute NaOH solution to trap any radioactive CO_2 as a safety precaution against release to the air. The shrimp were killed after the incubation period and the tail muscle was then treated in the same manner as that for the quantitative analysis, with the fats and nucleic acids being removed before hydrolysis. Following hydrolysis of a 25-mg. sample and recovery of the amino acids from the ion exchange column, the solvent was evaporated and the hydrolysate diluted up to 1 ml. with 0.1 N HCl.

Thin layer chromatography performed on Eastman cellulose chromatogram sheets was used to separate the amino acids in the protein hydrolysate. Four microliters of the protein hydrolysate were spotted two centimeters from

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*Available from New England Nuclear Corporation, Boston, Massachusetts.

the bottom and left-hand corner of the chromatogram sheet, and developed two-dimensionally by the solvent ascending technique. The chromatogram sheet was developed for 4 hours using a solvent containing n-butanol/acetic acid/water in a 4:1:5 volumetric mixture. Then the chromatogram sheet was rotated 90° counter-clockwise and developed 3.5 hours using an n-propanol/water solvent having a 7:3 volumetric mixture.

The sheets were removed and dried in an oven at 100°C for 5 minutes after development with each solvent. Identification of the individual amino acids was made both by position and color. A standard of the individual amino acids was developed in each solvent to give a hypothetical two-dimensional chromatogram. The separated amino acids were also sprayed with a polychromatic spray reagent (Moffat and Lyttle, 1959) to make positive identification.

Radioactivity of the separated amino acids was also determined by two different methods. The position of the radioactive amino acids was first determined by autoradiography. This consisted of pressing the chromatogram against a sheet of Kodak Royal Blue Medical x-ray film* sandwiched between two glass plates. The "sandwich" was wrapped with black cloth, kept in

*Available from Eastman Kodak Company, Rochester, New York

the dark for 8 days, and then developed. The presence of black spots on the developed film pinpointed the areas of radioactivity. A second method was employed in order not to overlook any lower amounts of radioactivity which may not have shown up after an 8-day exposure. Each of the amino acids was scraped off the chromatogram according to its R_f value and suspended in 20 ml. of a 4 w/w % CAB-O-SIL* toluene scintillation solution containing 5 gm. 2,2-diphenyloxazole (PPO)* and 0.3 gm. p-bis (4-methyl-5-phenylozazlyl) benzene (POPOP)* per liter of toluene (Snyder and Stephens, 1962). The radioactivity in each vial was determined in a Nuclear-Chicago Model 724 liquid scintillation spectrophotometer assayed at 10°C.

2. Discussion

The importance of the quality of the protein source cannot be overestimated, and it was for this reason that the essential amino acids were determined. The classical approach to the problem of identification of the essential amino acids, the deletion procedure, did not seem feasible in this case. This method is extremely time-consuming and involves the use of many different diets. Also, the deletion procedure would have been difficult to use, since at this time no purified (chemically defined) diet has been developed

*Reagents available from New England Nuclear Corporation, Boston, Massachusetts.

for P. aztecus. Thus, the indirect isotopic method previously described was used to determine which amino acids of the protein hydrolysate were able to incorporate ^{14}C from the injected radioactive glucose.

The thin-layer chromatograms on which the hydrolysate separation was performed did not separate all the amino acids completely. For example, there was practically no separation of threonine and tyrosine. Similarly, histidine-lysine and valine-methionine were not completely separated. However, there was no radioactivity in any of these spots, and they were all considered essential. Proline, alanine, and glutamic acid were quite well separated and all were highly radioactive, indicating that they could be readily synthesized from the fragments of glucose metabolism. Glycine, serine, and aspartic acid had lower specific activities than the above-mentioned nonessential amino acids, but it was not possible to give an accurate estimate of the specific activity of these amino acids. While they were well enough separated to give three distinct spots on the developed autoradiogram, separation was not total, and it was possible that in scraping off the entire spot and measuring the radioactivity, a small amount of radioactivity might have been contributed by another amino acid. However, since all three of these amino acids had about the same radioactivity, one could not have grossly influenced the

specific activity of another. Since a large specific activity of one amino acid relative to other amino acids may be interpreted either as the amino acid being easily synthesized from glucose metabolic fragments or as the amino acid turnover rate in the protein being large, it would not be possible to attach a great deal of significance to specific activity from this investigation. Table II-1, therefore, indicates only which amino acids show radioactivity and which do not.

TABLE II-1

The Essential Amino Acids of P. aztecus

Essential (no radioactivity)	Nonessential (radioactivity)
Arginine	Alanine
Histidine	Aspartic acid
Isoleucine	Cystine
Leucine	Glutamic acid
Lysine	Glycine
Methionine	Hydroxyproline
Phenylalanine	Proline
Threonine	Serine
Tryptophan*	
Tyrosine	
Valine	

*Tryptophan figure obtained from thin-layer chromatogram of base hydrolysate.

Tyrosine, by classical methods, has been shown to be nonessential in mice and insects, but did not incorporate ^{14}C. This result tends to confirm the assumption that tyrosine is synthesized from phenylalanine, an essential amino acid, and not from metabolic fragments.

3. Summary and Conclusions

The utilization of metabolic fragments of glucose metabolism in amino acid synthesis makes it possible to establish the essential amino acids for P. aztecus by radiochemical analysis. Nineteen amino acids were detected in this investigation; of these, eleven were essential in brown shrimp.

C. Effects of Various Percentages Protein on Growth and Survival of Brown Shrimp

In conjunction with the above studies, growth trials were performed varying the level of protein in an experimental diet. Since the quality of protein necessary to maximize growth had already been determined, it now seemed advisable to determine the quantity of protein necessary.

1. Materials and Methods

Shrimp for these studies were again obtained from Pelican Bait Camp. Six shrimp were individually weighed and measured, and introduced into 10-gallon aquaria. The aquaria were equipped with one-inch oyster shell

bottoms. Salinity was maintained at approximately 30.6 °/oo and temperature ranged from 25-28°C in each tank throughout the experimental period. The shrimp were fed at a rate of 8% of their biomass daily. Worm-like diets were prepared to contain 17.5, 22.5, 30.5, and 35.0% protein, respectively. A commercial shrimp chow containing 35% protein was also tested.

2. Discussion

Results of the growth trials are shown in Table II-2. It is not entirely possible to compare treatments with different population densities, as growth seems to be a function of density. Neither is it possible to analyze the results statistically, since only two replicates were run. However, a trend is apparent. Comparing diets 1-4, growth reaches a maximum between 22.5 and 30.5% protein. The 35% protein diet actually seems to decrease growth. It was difficult to compare Diet 5, the commercial shrimp chow, with the other diets due to poor survival. Duplication of the two treatments of Diet 5 was also poor. The superior growth of Diet 5 with 50% survival seems to bear out the theory that growth is a function of population density as well as diet.

TABLE II-2

Growth Trials for *P. aztecus* Varying Percent Protein

Diet	% Protein	% Survival	Initial Wt.[a] (g)	Final Wt.[a] (g)	Δg/Shrimp/Day[b]
1	17.5	100	20.40	22.26	0.014
	17.5	67	14.64	15.76	0.013
2	22.5	100	20.76	24.60	0.030
	22.5	100	22.56	25.62	0.024
3	30.5	100	22.20	25.38	0.025
	30.5	100	28.80	31.92	0.025
4	35	67	14.64	16.20	0.019
	35	100	26.28	27.42	0.009
5	35[c]	50	7.83	11.94	0.065
	35[c]	67	24.16	25.04	0.015

[a] Total weight

[b] 21-day growth trial

[c] Commercial shrimp chow

3. Summary and Conclusions

According to the results shown in Table II, growth as a function of percent protein reaches a maximum between 22.5 and 30.5. It appears that shrimp of this size cannot efficiently utilize diets containing higher percent protein. This seems reasonable in view of the fact that shrimp in their natural habitat feed on detritus which is often low in protein.

It is difficult to assess treatments where percent survival varies, as growth seems to be a function of population density.

SECTION III. FATS

The lipids of marine fish contain higher concentrations of the w3* series fatty acids than are contained in animal fats (Ackman, 1964). Since lipids of marine organisms do differ from vegetable and animal fats, some oils and tallows may be more nutritious than others in formulating diets for marine animals.

Increased amounts of linolenic acid, 18:3w3, and salmon oil, which contain a high proportion of w3 and w6 fatty acids, were shown to cause increased growth in trout when introduced at the 1% level in artificial diets (Castell et al., 1972).

Many of the fats now being added to artificial shrimp diets do not contain the higher polyunsaturates present in natural

*w3 defines the position of the double bond farthest from the carboxyl group as being three carbon atoms removed from the terminal methyl group.

diets, and it was felt that this area of diet development needed more attention. It has been stated in several cases that the ratio of polyunsaturated fatty acids in lipids of animals accurately reflects ratios of polyunsaturated fatty acids in their diets (Owen et al., 1972); (Ackman and Eaton, 1966); etc. If this statement is true for penaeid shrimp and superior growth could be obtained only by ingestion of marine oils or higher polyunsaturates, then changes must be made in artificial diets now being formulated for marine farming.

Several experiments were performed by Sick et al. (1972), varying the percent fat, protein, and carbohydrate in a number of artificial diets fed penaeid shrimp. However, since Sick et al. used a mixture of equal parts corn oil, vegetable and animal fat, and menhaden oil, it was difficult to determine which of these fats most influenced their growth studies. Kanazawa et al. (1970) tested several diets against a natural food, short-neck clam, in studies with Penaeus japonicus. Kanazawa had several variables, and again it was not possible to determine the effectiveness of the fat in these diets.

Thus, it was the purpose of this study first to analyze quantitatively the fatty acid composition of brown shrimp in order to evaluate potential fat sources for formulation of a shrimp diet; and second, to run growth trials with diets containing a suitable lipid source as indicated by previous analyses.

In addition to the above information, it was hoped that it might be possible to determine generally, from the fatty acid array, the natural food sources of juvenile P. aztecus and the changes in feeding habits throughout the life cycle.

A. A Quantitative Determination of the Fatty Acids Present in *P. aztecus* Lipid

1. Materials and Methods

All solvents were AR grade and had been dried and distilled previous to use. Juvenile brown shrimp were obtained from the Pelican Bait Camp, Freeport, Texas, within a few hours of capture. Female brown shrimp were obtained from Dow Chemical U.S.A. and were sacrificed immediately after spawning. Gastrointestinal tracts were removed and samples frozen until analyses were performed. Stomach contents were analyzed separately for total lipids. The tissues and exoskeleton were homogenized in a Waring Blendor and extracted repeatedly with acetone. The extract was taken up in hexane, dried over sodium sulfate, and weighed. Total phospholipid was obtained by extracting tissues with chloroform-methanol (2:1) by the method of Folch et al. (1956), and the extracts were analyzed for phosphorus (Allen, 1940). Free fatty acids and triglycerides were separated by column chromatography on columns prepared according to the method of McCarthy and Duthrie (1962). Triglycerides eluted from the column with ether, while the free fatty acids remained at the top of the column. Free fatty acids were eluted with 2% formic acid in ether. Column separation was confirmed by thin-layer chromatography.

Saponification was carried out in the usual manner, refluxing for one hour with alcoholic KOH. The solution was diluted to 50 ml. with water and the non-saponifiables extracted with hot petroleum ether and weighed after drying and removal of the solvent. The saponified mixture was acidified and the free fatty acids extracted with ether and dried. The ether was then evaporated and the fatty acids esterified using BF_3-CH_3OH (Morrison and Smith, 1964).

Fatty acid methyl esters were analyzed by gas-liquid chromatography on a Varian 1200 equipped with a hydrogen flame detector. The methyl esters were separated on a 10' x 1/8" 10% DEGS (polydiethylene glycol succinate) on Chromosorb W - AW (80/100) gas chromatographic column run isothermally at 180°C. Separation of overlapping peaks was achieved using a 5' x 1/8" column packed with 5% neopentyl glycol succinate (NPGS) (175°C isothermal) and a 10' x 1/8" 3% SE-52 on Chromosorb W gas chromatographic column. Identification of the peaks was carried out using commercially available standards and also standards prepared using mixed methyl esters from cod-liver oil (Ackman and Burgher, 1965). Peak areas were determined for quantization by measuring peak height x base at one-half height. Results were calculated as weight percentages.

2. Results and Discussion

The composition of lipid extracts from Penaeus aztecus is shown in Table III-1. Percent phosphorus was calculated from phospholipid extracted with chloroform-methanol solution. The phospholipid fraction is less than 30% as large as the triglyceride fraction, and indicates a large amount of fat stored as triglyceride. Only a total lipid analysis was performed on adult females. This investigation centered around the development of a lipid source for a diet intended to promote growth, not to maintain adults. Total lipid was also listed for the stomach contents of juvenile shrimp. However, due to the small sample sizes, a breakdown of phospholipid, triglyceride, and free fatty acids was not possible.

The fatty acid composition of the various lipid fractions is given in Table III-2. These analyses reveal a typical marine fatty acid pattern, with a large percentage of higher polyunsaturated fatty acids. The total lipid fatty acids of the stomach contents is also given in Table III-2. It is interesting to compare the amounts of fatty acids present in the stomach contents with the fatty acids from total lipids of brown shrimp, and note the similarities. It has been suggested that lower forms of animal life deposit dietary fat relatively unchanged. This seems to be

TABLE III-1

Lipid Content and Composition of *P. aztecus**

	Females	Juveniles	Stomach Contents (Juveniles)
Collection Date	5/9/72	6/12/72	6/12/72
Animals	8	112	42
Wet Weight (g.)	256	353.9	1.7
Total Lipid[a] (% Wet Weight)	2.0	2.1	0.6
Phospholipid		14.2[b]	
Free Fatty Acid[c]		6.2[b]	
Triglyceride[c]		52.8[b]	
Cholesteryl Ester[c]		0.7[b]	

*% wet weight (g.)

[a]Chloroform-methanol extract

[b]Percent of total lipid

[c]Acetone extract

TABLE III-2

Fatty Acid Composition of P. aztecus Lipids

Fatty Acid	Total Lipid: Spawned Female	Total Lipid: Juvenile	Total Lipid: Stomach Contents	Triglyceride[a]	Free Fatty Acid[a]	Cholesteryl Ester[a]	Phospholipid[a]
10:0	0.6	0.3	0.5	-	1.3	-	-
12:0	0.2	0.3	0.3	-	-	-	-
14:0	3.8	4.2	4.1	7.9	7.0	8.1	0.9
16:0	17.4	15.4	19.5	15.3	19.0	15.3	23.6
16:1 w7	10.3	7.7	7.6	13.1	10.6	13.0	6.0
18:0	10.2	10.8	10.4	6.1	13.8	12.0	11.6
18:1 w9	14.2	13.4	14.6	12.4	10.4	10.6	15.6
18:2 w6	2.1	2.9	3.4	3.4	5.1	6.6	3.7
18:3 w3	1.4	2.9	1.7	5.0	9.6	3.2	3.0
20:1 w9	3.8	3.5	4.5	4.4	1.6	6.1	2.0
20:2 w6	0.4	0.3	0.5	0.5	0.8	0.2	0.6
20:4 w6	0.4	0.3	0.3	0.3	-	-	1.2
20:5 w3	20.6	20.4	19.2	16.6	9.5	22.0	28.0
22:1 w9	1.8	1.5	1.5	1.8	0.8	1.1	0.6
22:3 w6	2.1	2.1	2.0	2.4	1.3	-	1.1
22:6 w3	12.0	10.0	10.4	6.0	8.2	2.8	10.8

[a]Juvenile shrimp bodies

the case in brown shrimp.

Differences were expected between the fatty acid content of the adult female shrimp and the juveniles in that their foods are probably different, the juveniles being taken from the estuaries and the adults from deeper waters. It may be significant that the juveniles have twice the amount of linolenic acid as do the adult females. Since the juveniles are still in a state of rapid growth, the higher unsaturates may be retained selectively to meet the demand for the biosynthesis of phospholipids required for metabolic processes and formation of cellular membranes.

It is important to note also from Table III-1 the dates of the catches as lipid fatty acid composition changes with the seasons. Total fatty acid composition in winter months will more closely resemble the phospholipid fraction due to depletion of the triglyceride fraction. Shrimp for these studies were taken in late spring-early summer, and fatty acid content at this time is a mixture of phospholipid and triglyceride. The fact that fatty acid content is continuously changing may cause confusion as to what would be

the best ratio of fatty acids to make available in a diet. However, it is well to remember that brown shrimp taken at this time are beginning their peak growth, and the fatty acid array at this time reflects this optimum growth.

3. Summary and Conclusions

Lipid of the brown shrimp, P. aztecus, was analyzed and the fatty acid content of the various fractions determined. The total lipid of juvenile brown shrimp was compared to that of the adult spawned female, and it was noted that twice the amount of linolenic acid was present in the lipid from juveniles as was present in adult females. It was concluded that a diet would probably benefit from the addition of linolenic acid.

B. The Effect of Linolenic Acid on the Growth of P. aztecus

1. Materials and Methods

Shrimp for these trials were obtained from Pelican Bait Camp, Freeport, Texas, a few hours after capture. Eight shrimp ranging in size from 3-4.5 g. were introduced into 10-gallon tanks and acclimated for two days before trials were begun. During the acclimation period any shrimp which died were replaced.

Tests were run in duplicate and shrimp were fed 8% of body weight daily throughout the twenty-one day test period.

The basal diet used for this test was a commercially available shrimp chow which was ground and re-extruded in worm form containing the varying percentages of reagent grade linolenic acid. An algenate binder was used to hold the ground diet together. A control diet, ground and extruded in worm form, was made up to contain only binder and feed, with no additional linolenic acid.

2. Results and Discussion

Table III-3 gives a comparison of the growth trials performed with these diets. Fat was not extracted from the original diet, as it was the purpose of this study to improve upon an already existing diet. Survival was good throughout the experiment and overall survival was 87.4%, with all tanks showing a net gain in weight. It is not possible to make direct comparisons between treatments with different percent survivals, due to effects from changes in population densities. The overall response to increasing levels of linolenic acid, however, was quadratic in nature. It can be shown from Table III-3 that the largest weight gains were obtained by the addition of 1% linolenic acid to the basal diet. When the shrimp fed the basal diet plus 0.5% linolenic acid (100% survival) were compared to the shrimp fed the basal diet (100% survival), an 8.3% increase in growth was observed. The shrimp fed the basal diet plus 1.0% linolenic acid (100% survival) grew 14% faster than did the shrimp fed the basal diet (100% survival). Comparison of the 1.0% linolenic acid (75% survival) to the shrimp fed the basal diet (75% survival) also shows a 12% advantage to the shrimp fed the basal diet plus 1.0% linolenic acid.

TABLE III-3

Comparison of Five Artificial Diets Varying Percent Linolenic Acid

Diet	% Survival	Initial Weight[a]	Final Weight[a]	△g/Shrimp/Day[b]	% Response Relative to Basal Diet[d]
Basal	75	33.54	39.00	0.043	108
Basal	100	41.37	47.47	0.036	90
0.5% Linolenic[c]	100	43.61	50.29	0.039	98
1% Linolenic	75	28.16	34.22	0.048	112
1% Linolenic	100	38.39	45.21	0.041	102
2% Linolenic	87.5	32.72	38.36	0.038	95
2% Linolenic	87.5	32.50	38.07	0.038	95
5% Linolenic	75	31.51	34.34	0.023	57
5% Linolenic	87.5	35.27	38.49	0.022	55

[a] Total weight (g.)
[b] 21-day growth trial
[c] Duplicate not run under same conditions
[d] Based on 0.040 average

When comparing the treatments where survival was 100%, it appears that the addition of 0.5% linolenic acid to the basal diet increases growth, with a 1% addition further increasing growth. However, the addition of 2% linolenic acid to the basal diet causes a slight decline in growth. This decline is accentuated with the addition of 5% linolenic acid to the basal diet.

It appears, therefore, that there is an optimum additive point somewhere between 1% and 2% linolenic acid.

Because individuals within treatment groups could not be individually identified, group average weights were used as the criterion of measurement. Thus with two replicates per treatment, no statistical difference could be demonstrated. The data, however, indicate a definite trend toward a more rapid growth when 1-2% linolenic acid is added to the diet.

The increase in growth with the addition of varying amounts of linolenic acid has been noted in trout, and 1% seemed to be the optimum in that case (Castell, 1972). It is somewhat surprising to find the optimums of trout and shrimp to be so close in this respect.

As was noted earlier, linolenic acid appears to be a good source of basic material for the synthesis of

higher fatty acids. However, since in many cases fatty acids seem to be absorbed with very little composition change in shrimp, further studies should be conducted to determine if natural products high in polyunsaturates, such as menhaden oil, could be utilized effectively for increased growth.

3. Summary and Conclusions

Linolenic acid was introduced into an artificial shrimp diet at levels varying from 0.5% to 5%. It was concluded that the optimum level of linolenic acid was between 1% and 2% of the total diet, with a decrease in growth suggested with the addition of amounts greater than 2%.

It is emphasized that while a trend is apparent, further work is necessary to optimize both the additive amounts of linolenic acid and the lipid source itself.

SECTION IV. MINERALS

In order to better understand the mineral needs of Penaeus aztecus, the elemental chemical composition of the shrimp was determined. This was compared to the mineral composition of sea water since P. aztecus is able to derive some minerals from the water, especially during molt. It was hoped that this comparison would yield clues to aid in the formulation of a proper mineral mix for a diet.

From the values obtained from the first portion of this study, a two-level, three-factorial experiment was designed where the ratio of two of the elements present in shrimp, calcium and phosphorus, were varied along with percent protein and energy content.

A. Chemical Composition

1. Materials and Methods

Ten shrimp, 80-90 mm., were obtained from Pelican Bait Camp, Freeport, Texas, and sacrificed. The gastro-intestinal tract was removed and the remainder chopped finely and ground to a paste in a Waring Blendor. This paste was analyzed by atomic absorption and chemical wet methods for elemental chemical composition.* The carapaces and chitinous materials from ten more shrimp were also removed and ground with 100 ml. of water in a Waring Blendor until homogeneous, and this liquid was then analyzed for elemental chemical composition.

*Analyses were performed by Central Laboratory, Dow Chemical U.S.A., Freeport, Texas.

2. Discussion

The results of the elemental analysis are listed in Table IV-1. Ca^{++}, K^{+}, Na^{+}, $CO_3^{=}$, and Cl^{-} appear to be the ions present in highest concentrations in whole shrimp samples. The principal compound present is $CaCO_3$. In the carapace, while $CaCO_3$ still appears to be the largest single compound present, $SO_4^{=}$ is present in large amounts, probably in conjunction with Mg^{++} and Ca^{++}. All of the above-mentioned ions, including $SO_4^{=}$, are present in large quantities in sea water, and it is possible that some of the mineral needs of P. aztecus can be satisfied by the minerals in sea water. In fact, sea water probably contains a great excess of these important ions, if compared only to the blood ionic values rather than to the whole shrimp. This is not the case for phosphorus which, while relatively high in shrimp bodies, is present only in trace quantities in sea water. Phosphates are important intermediaries in metabolic processes such as conversion of glycogen to lactic acid and the Krebs cycle. Thus, it cannot be assumed that shrimp can derive the necessary phosphate from sea water.

It seemed, therefore, that growth trials should be performed adding different amounts of phosphate to determine if growth improvement could be achieved.

TABLE IV-1

Comparison of Elementary Chemical Composition of *P. aztecus* and Sea Water

	Shrimp Samples		
	Whole*	Carapace only	Sea Water
	%		
Ash	3.91	7.38	3.26
Ca	22.7	30.9	1.2
CO_3	46.2	44.0	0.9
S[1]	2.4	11.7	2.7
Na	6.2	3.3	32.4
Cl	7.8	4.3	58.0
K	8.9	2.1	1.2
Mg	3.1	1.7	3.9
P[2]	2.4	1.4	Trace
SiO_2	0.3	0.6	Trace
Trace Metals			
	(ppm)		
Fe	460	108	.02
Mn	6	4	.01
Cu	6	10	.01
Zn	72	66	.005
Al	14	30	-

*Excluding stomach and gut

[1] As $SO_4^=$

[2] As $PO_4^=$

3. Summary and Conclusions

The mineral compound present in highest concentration is calcium carbonate. Other ions present in large quantities are K^+, Na^+, and Cl^-. These ions are also present in large amounts in sea water. Phosphorus, while present in fairly large amounts in the shrimp, is present in only trace amounts in sea water.

The mineral needs of shrimp are probably not taken care of entirely by osmotic regulation, and a diet would benefit from supplementation of some of these minerals.

B. Feeding Trials

1. Materials and Methods

The feeding equipment* was a replicated two-level, three factor, full-factorial experiment, designed to test the effects and first-order interactions of protein content, $Ca^{++}/PO_4^{=}$ ratio, and available metabolic energy. Eight diets were compounded by Ralston-Purina. The characteristics of the diets are shown in Table IV-2.

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*Growth trials performed by Contract Research Department, Dow Chemical U.S.A., Freeport, Texas.

TABLE IV-2

Calculated Composition of Experimental Diets

Diet No.	Protein (%)	Ca/PO_4	(Kcal/gm.)
1	40	2:2	4.07
2	40	2:2	4.20
3	45	2:2	3.94
4	45	1:2	4.15
5	40	1:2	4.23
6	40	1:2	3.72
7	45	1:2	3.87
8	45	2:2	3.93

Bait shrimp, 80-110 mm. long, were individually measured, weighed, and stocked 15 to the 30-gallon tank. The experiment was run for thirty days. Salinity was maintained at 30 °/oo and temperature ranged from 25-28°C.

2. Discussion

Results of this experiment appear in Table IV-3. The individual weights were analyzed by a computer program which calculated the arithmetic mean, mode, median, and standard deviations, and then plotted the individual values against their cumulative frequency. An analysis of the stocking and harvesting individual-measurement data failed to reveal any significantly greater growth of one segment of the

population than another in a given tank. Similarly, no particular segment of the population experienced significantly greater mortality than the average.

TABLE IV-3

Growth Trials Varying Percent Protein, Ca/PO_4, and Energy

Diet No.	Protein	Ca/PO_4	Energy	Growth Rate (Day)	% Survival
2	LP	1:1	HE	0.0249	67
1	LP	1:1	LE	0.0201	80
8	HP	1:1	LE	0.0188	73
5	LP	1:2	HE	0.0174	67
3	HP	1:1	HE	0.0156	87
7	HP	1:2	LE	0.0126	80
4	HP	1:2	HE	0.0114	77
6	LP	1:2	LE	0.0074	83

This experiment was not an unqualified success due to the lack of water stability of some of the foods. In an attempt to produce a low calcium-to-phosphate ratio, highly soluble sodium phosphate had been added to four of the diets. When these foods were immersed in sea water, they broke down within minutes and were thus less available to the shrimp. The growth rates of the low calcium-to-phosphate diets were considerably more

variable and generally lower than those observed with the high calcium-to-phosphate diets. However, the disintegration of the foods did not adversely influence survival. It appears that the low protein diets produced the largest growth. It seems possible that shrimp at this stage cannot effectively utilize high levels of protein. It has since been noted that the optimum protein level may be between 22 and 30%. Thus, both the 40 and 45% protein diets are probably excessive in percent protein.

3. Summary and Conclusions

It was difficult to draw conclusions from the trials due to the lack of water stability of the high $PO_4^{=}/Ca^{++}$ diets. It appears that both 40% and 45% protein levels may be too high to be effectively utilized by juvenile shrimp.

SECTION V. CARBOHYDRATES

The function of carbohydrates in crustacean metabolism has not been elucidated and several theories have been propounded as to its role. Scheer (1951) and his co-workers have come to the conclusion that the primary energy source of crustaceans is protein rather than carbohydrate and fat. This would indicate metabolic pathways not in general use by other animals. A more popular theory is that crustaceans utilize

carbohydrates in much the same manner as other animals, and that crustacean intermediary metabolism centers mainly around glycogen and the fatty acids.

It was the purpose of this study to find out more about the carbohydrates present in shrimp and incorporate this knowledge into an energy source for a shrimp diet.

1. Materials and Methods

The shrimp for these studies, varying in length between 80-100 mm., were obtained from a local bait camp and molt stage determined by the method of Drach (1939). Only intermolt shrimp were used and these were killed by quick freezing. The gastrointestinal tract and hepatopancreas were removed. The shrimp bodies were extracted with aqueous ethanol. The ethanol was removed by rotary evaporation and the mg. glucose determined by the anthrone method as reported for Emerita asiatica by Parvathy (1970).

The aqueous samples were filtered through an ion exchange column, Amberlite IR-120H, to remove biological background. The water was removed from the samples by rotary evaporation and the sugars were silanized by the method of Sweeley et al. (1963). TMS-derivatives were injected into a Varian 1200 gas chromatograph. Column conditions were:

Gas chromatographic column: 10' x 1/8"
4% SE-30 on 60/80 mesh silanized Diaport S
Column temperature: Programmed 160°-270°C
at 4°C/min.
Injector temperature: 225°C
Detector: 300°C
Nitrogen flow: 50 ml./min.

Carbohydrate standards were run and peaks were identified by retention time.

Glycogen was isolated from the hepatopancreas by treatment with 30% sodium hydroxide solution and precipitation of the glycogen with alcohol. Glycogen was determined quantitatively by the anthrone method mentioned above.

2. Discussion

Results of the blood sugar analyses are given in Table V-1. Total mg. of sugar present was 33.1 mg./100 g. shrimp bodies. Glucose was by far the largest constituent, with traces of ribose and maltose.

TABLE V-1

Blood Sugar Values for Intermolt P. aztecus

Sugars	mg/100 g. Shrimp
Glucose	30.9
Ribose	2.1
Maltose	0.1

It is surprising to note that more carbohydrate intermediates present in normal metabolic pathways were not measured. However, since these compounds are normally present as phosphates, it may be that they were too polar to be eluted from the GLC column at temperatures used in this experiment. Also, several peaks were present in the gas chromatogram which could not be identified and for which no standards were available. It can only be theorized as to what these compounds might be.

Results of the hepatopancreas analyses revealed 22 mg. glycogen/gm. sample. Glucose, isolated by thin-layer chromatography, was also present in the hepatopancreas, but no attempt was made at quantitative analysis. It appears from the comparatively large glycogen value that there is storing of sugar as glycogen taking place during the intermolt stage.

It remains to be seen, however, what effects varying levels of carbohydrates would have upon growth and survival of penaeid shrimp. Due to a lack of time available for this work, no growth trials were run testing various levels of carbohydrates. It does appear that since glucosamine and n-acetyl glucosamine are needed for chitin synthesis, a diet might benefit from additions of these compounds or their precursors.

3. Summary and Conclusions

Three sugars were found to be present in the blood of P. aztecus; glucose, ribose, and maltose. Glucose was present in greatest concentration, but in much smaller quantities than glycogen in the hepatopancreas. Thus, it appears that glycogen is stored during molt. A diet might benefit from the addition of carbohydrate.

SECTION VI. ATTRACTANTS

Chemoreception appears to play an important role in feeding among shrimp. Not only must a diet be made nutritious, it must also be made attractive to the shrimp; first, so that the shrimp may find it easily; and second, so that enough will be consumed for maximum growth. Thus, an attempt was made to assess the attractiveness of various additives to a shrimp diet.

A. Response of P. aztecus to Various Attractants

1. Materials and Methods

Bait-size shrimp, 80-90 mm., were obtained from bait camps previously mentioned. Five shrimp each were placed in 10-gallon tanks and allowed to acclimate for 2 days before trials were begun.

The trials were conducted for five days and observations were made of the shrimp's reaction at 30 seconds, and

continued activity at 1, 5, 15, and 30 minutes after feeding. One point was awarded to a diet each time a shrimp was observed feeding on it at the prescribed intervals.

During the first trials, a commercial shrimp diet was soaked in the solubles to be tested: mullet, squid, and clam. However, no differences in feeding activity were observed between the soaked feeds and the control.

The solubles were then incorporated into the diets and extruded as a worm-like feed. These diets are listed in Table VI-1.

TABLE VI-1

Diets for Attractant Studies

Diet	Description
FS-2	2% fish solubles
FS-5	5% fish solubles
S-2	2% shrimp solubles
S-5	5% shrimp solubles
C-2	2% clam solubles
C-5	5% clam solubles
O-2	2% oyster solubles
O-5	5% oyster solubles
Control	Bland diet - same composition as diets above, but lacking attractants.
L-5	5% lobster

Total points were calculated by the following formula:

Total possible points = No. of shrimp x No. of days x No. of time intervals

125 (5) (5) (5)

2. Results and Discussion

Results of three weeks of trials are given in Table IV-2.

TABLE VI-2

Attractiveness of Various Shrimp Diets

Tests	Diets				
Test 1	FS-2	FS-5	S-2	S-5	Control
	108	122	72	42	56
Test 2	C-2	O-2	O-5	L-5	Control
	61	48	55	16	56
Test 3	C-2	C-5	FS-5	Control	Commercial Shrimp Diet
	59	73	73	56	89

In Test 1, 5% fish solubles was the best attractant, outperforming its nearest competitor by about 13%. In Test 2, 2% clam solubles was the best attractant. In Test 3, the best attractants from Tests 1 and 2 were compared against a commercially available shrimp chow

and a 5% clam solubles diet which had not been previously tested. The commercial shrimp chow gave 22% higher results than the best diet prepared with solubles. Duplication of results from test to test varied somewhat, although replication of the control was good. Also, replication of C-2 in Test 2 with C-2 in Test 3 was good. Results of FS-5 from Test 1, however, were not in agreement with FS-5 from Test 3. Reasons for this discrepancy are unknown.

3. Summary and Conclusions

It appears that the higher the percent solubles in a diet, the better the acceptance. Since solubles are a cheap additive, this seems like an acceptable method for diet improvement.

B. Attractant Studies on the Brown Shrimp, *P. aztecus*, Using a Maze Situation

Difficulties were inherent in the attractant studies summarized above. There was no choice of one diet over another by the shrimp. The method was slow and required maintenance of a large number of tanks.

In order to reduce some of these problems and introduce as an internal standard the shrimp itself, a new attractant system was devised similar to one described by Shelton and Mackie (1971) for experimentation on the shore crab, *Carcinus maenas*. This method gave the test animal a chance

to demonstrate food preferences utilizing maze conditions. This study also had difficulties inherent in it. The design of the tank is shown in Figure 1. Shrimp seemed to enter the test chambers from the main chamber randomly, whether there was feed in the bait chamber or not. They seemed to show a preference to the right side, which was better lighted. An attempt was made to correct this situation by taping black paper to the box. This corrected the situation and the shrimp then showed no preference; neither did they show a preference when feed was dropped in one side or the other. This, it was felt, was due to the fact that the shrimp were handled excessively and were in an excited state. At the end of each trial, the shrimp were netted and returned to the main chamber. This caused so much excitement that feed dropped on them would not be noticed. The first test of the day, when the shrimp were calm, was the only test where a significant response to feed was shown.

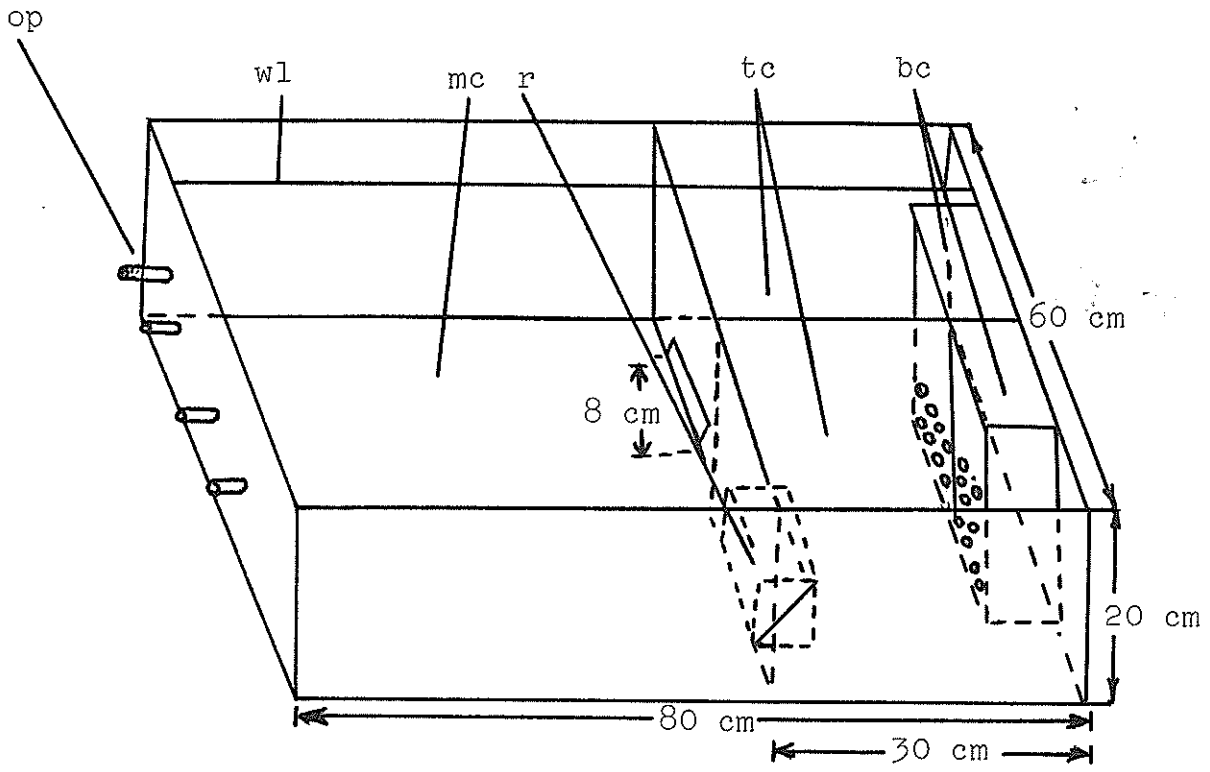

FIGURE 1. ATTRACTANT TANK: bc, bait chambers;
tc, test chambers;
mc, main chamber;
r, ramped entrance to test chamber;
wl, water level;
op, outlet pipe.

2. Summary and Conclusions

A better method for studying attractants is needed. A maze situation might possibly work, but much study would be needed to determine the parameters. Possibly these studies could be carried out in a darkened room with an acclimation period of several hours. At present, there is no good method for determining the attractiveness of various feed additives.

SECTION VII. SUMMARY AND CONCLUSIONS

1. Nineteen amino acids were identified as being present in the protein of P. aztecus. Of these, eleven are essential to the diet and cannot be synthesized by the shrimp.

2. Growth trials run, varying the percent protein in the diet, indicate an optimum protein level in juvenile P. aztecus between 22.5 and 30.5%.

3. Lipid of P. aztecus was analyzed and the fatty acid content determined.

4. Total lipid of juvenile brown shrimp was compared to that of the adult spawned female, and it was noted that twice the amount of linolenic acid was present in the lipid from juveniles as was present in adult females.

5. Growth trials run, varying the percent linolenic acid in the diet, indicate an optimum linolenic acid level between 1 and 2%.

6. Elemental chemical composition of P. aztecus was determined by both chemical and instrumental methods.

7. From data obtained from elemental analysis, the element present in largest quantities was calcium, followed by potassium in the body and sulfur in the carapace.

8. A series of growth trials varying percent protein, Ca/PO_4, and energy could not be interpreted as satisfactory due to a breakdown of some of the diets in water.

9. Glucose and its derivatives are the major carbohydrates present in shrimp.

10. Either clam or fish solubles would make the best attractant of attractants tested.

11. The higher the percentage of solubles, the better the attractive qualities of a diet.

12. A better method of evaluating attractants needs to be devised.

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