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Effect of stressors on shrimp digestive enzymes from assays of feces: an alternate method of evaluation

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Abstract

The effect of change in feed and physical manipulation on the digestive system of white shrimp *Penaeus vannamei* (Boone, 1931) was examined for proteases secreted by the digestive gland and excreted in feces. Organisms were fed a commercial feed containing 45% protein and separated into two groups. One group served as the control and the other was physically manipulated weekly. Each group was subdivided into two groups, establishing four experimental subgroups, 50% of the organisms in each group used as controls and the others subjected to a change in feed by shifting from a 45% protein feed to a 35% protein feed from different brands to induce alimentary stress. Organisms were individually maintained and feces collected and analyzed on a daily basis for 2 months. Trypsin and chymotrypsin activities decreased in feces and mid-gut gland extracts of organisms fed the 35% protein feed and those physically manipulated during weighting. Two-way ANOVA demonstrated a greater effect of physical manipulation on trypsin and chymotrypsin activities than change in feed. One-way ANOVA demonstrated differences between the two periods analyzed (before and after the beginning of stressors). Composition of proteases in the mid-gut gland and feces were identical, as observed by S-SDS PAGE. Studies of enzyme activities in feces may be helpful, when studying organisms of short digestion periods where very active enzymes and low-efficiency digestion are expected, as in the case of decapods.

Keywords: Shrimp; Feed; Feces; Trypsin; Chymotrypsin; Stress

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1. Introduction

Response mechanisms to physical, chemical, and biological stresses may alter physiological functions, contributing to disease or even death. Experimental treatments impose different degrees of stress on organisms used for research purposes. Stress may be as varied as captivity, crowding, nutrition, or handling. Physical manipulation of organisms affects diverse systems, functions, and zootechnical variables. Growth is commonly affected by handling (Buurma and James, 1994). Other functions affected include reproduction (Schreck et al., 2001), digestion (Hajen et al., 1993), or excretion (Hunter and Uglow, 1993). Consequently, biased data or results may occur when evaluating a response to experimental factors.

We have previously shown that biochemical properties of food may affect the digestive system of shrimp (Ezquerra et al., 1997, 1999). Others have reported that physical manipulation of wild organisms can be a major stressor (Frisch and Anderson, 2000; Schreck et al., 2001; Cleary et al., 2002). Studies evaluating the effect of stressors and alternate non-invasive methods of evaluating physiological functions are needed.

Body fluids are useful as samples for evaluating metabolites that indicate physiological status of organisms. Saliva, feces, and urine have been used and validated (Von Borell, 2000; Brinez et al., 2003). They can be useful for different studies in laboratory and field research.

We evaluated two types of stress, alone and combined on the activity of the digestive enzymes, trypsin and chymotrypsin, recovered from feces. One stress was produced by changing the feed; the other by handling the specimens repeatedly.

2. Materials and methods

At the CIBNOR facilities, 12 specimens of white shrimp *Penaeus vannamei* (Boone, 1931), averaging 4.7 ($\pm$ 0.1) g, were raised individually in 70-l tanks, with aeration provided through air stones, temperature at 28 °C, and salinity at 37 ‰ . The shrimp were subjected to two types of stress: (1) alimentary stress, by shifting from a 45% crude protein feed to a 35% crude protein feed from two different brands, and (2) physical manipulation by handling the specimens repeatedly.

Prior to the experiment, organisms were fed twice daily at 0800 and 1700 h for 40 days with "Silver Cup" commercial feed (SC) containing 45% protein to increase size and become acclimated to experimental conditions. After the adjustment period, feces were collected 2 h after feeding by siphoning the feces from each tank and collecting it in a small-mesh sieve. Feces were collected from each tank on a daily basis (weekdays), for 2 months. Feces were gently rinsed with distilled water to remove excess of salt, and the sample from each specimen was placed in separate 1.5-ml test tubes. Tubes were ice-chilled during feces collection, and maintained at 4 °C until the start of enzyme extraction. On day 26 (from the start of feces collection), specimens were divided into four groups (Table 1). Group A (control group): not manipulated and no change in feed; Group B (one stressor): physical manipulation (weighed weekly) and no feed change; Group C (one stressor): feed change from 45% crude protein (Silver Cup

Table 1
Design of experimental groups

	No handling	Handling
No alimentary stress	A	B
Alimentary stress	C	D

Three specimens were used in each group and at least 20 samples obtained from each.

[SC feed]) to 35% crude protein (PIASA [PI feed]); and Group D (two stressors): weekly physical manipulation and feed change from SC to PI feed. During the entire experiment, at least 20 feces samples (10 before stressor period and 10 during stressors period) from each individual were collected and analyzed separately (more than 250 samples).

At the end of the experiment, the shrimp were weighted and killed by chilling, the digestive glands removed, weighed, and stored in individual 1.5-ml test tubes at −20 °C until used. Proximate analysis of the two commercial feeds was conducted with standard methods (A.O.A.C., 1990).

Enzymes from feces were extracted for assay, as described in Córdova-Murueta et al. (2003), by adding 300 μl distilled water or 200 μl when feces volume was less than half the tube, to each tube. The samples were homogenized and centrifuged for 30 min at 10,000 × *g* and 4 °C. Supernatant was decanted and stored at 4 °C.

Soluble protein in feces was determined by the Bradford method (1976), adapted to micro-assays, and using bovine serum albumin as the standard. For evaluation, 10 μl feces extract, 10 μl distilled water, and 200 μl Bradford reagent were mixed in a 96-well microplate in triplicate. Absorbance was recorded at 595 nm in a microplate reader (BIO RAD 550).

Specific activity of trypsin (EC 3.4.21.4) was assayed in a 96-well microplates. Triplicate samples were prepared with 10 μl feces extract and 200 μl substrate, consisting of 0.1 mM benzoyl-Arg-*p*-nitroanilide (BAPNA) in 50 mM Tris–HCl at pH 7.5, and 20 mM $CaCl_2$. Progress of the reaction at 405 nm was recorded every 30 s up to 3 min at 37 °C. Activity was evaluated using the ratio: (Abs 405 × ml reaction volume)/(8800 × mg protein), as described by García-Carreño et al. (1994). Chymotrypsin (EC. 3.4.21.2) activity was evaluated at 25 °C using 0.1 mM succinyl-$(Ala)_2$-Pro-Phe-*p*-nitroanilide (SAPNA) in 50 mM Tris–HCl at pH 7.5, and 20 mM $CaCl_2$ as substrate. The method and formula used for measuring trypsin activity was applied for chymotrypsin. One unit of activity for synthetic substrate is considered the amount of enzyme needed to hydrolyze 1 μM substrate in 1 min.

Each shrimp hepatopancreas (HP) was homogenized with sufficient distilled water to provide a 1.2 ml volume sample per test tube. Homogenates were centrifuged for 30 min at 4 °C and prepared in the same way as the assay of feces. HP extracts were assayed for soluble protein, and specific activities for trypsin and chymotrypsin. Assays of 1:40 (HP extract to distilled water) dilutions of HP extract were performed, following the same procedure as described for feces.

Substrate electrophoresis (S-SDS-PAGE) for composition of proteases in feces and HPs was conducted following the technique of García-Carreño et al. (1993). In brief, sodium dodecyl sulfate, 12% polyacrylamide gel electrophoresis (SDS-PAGE) was performed

according to the procedure of Laemmli (1970). Enzyme preparations, containing 2 mU chymotrypsin activity and diluted 1:1 with sample buffer, were loaded into individual gel wells at 4 °C in a vertical electrophoresis device. Molecular mass standards of 4 μl were loaded on each gel. Protease composition and molecular weight in a twin gel were studied after SDS-PAGE. Gels were immersed in 3% casein in 50 mM Tris–HCl at pH 7.5 for 30 min at 4 °C to diffuse the substrate into the gel. Temperature was then raised to 25 °C for 90 min. Gels were washed in water and immediately fixed and stained with Coomassie brilliant blue.

Statistical differences between groups for trypsin and chymotrypsin in feces, as in HP extract, were determined with two-way ANOVA after testing for parametric procedures. When differences were detected, HSD (Tukey's true significant differences) multiple comparisons test was performed. To compare trypsin and chymotrypsin activities, one-way ANOVA was used.

3. Results

Proximate composition of feeds is given in Table 2. According to the analytical results, protein, lipids, and ash content were higher for SC feed compared with PI feed (20%, 31%, and 35%, respectively).

Electrophoresis gels (not shown) confirmed that feces and HP proteinases extracts were identical in composition for every organism.

Table 3 shows values of variables assayed in feces and HP extracts, as affected by one of the stressors. Trypsin activity was significantly ($P < 0.05$) lower in feces and HP extract from specimens subjected to both stressors. Chymotrypsin activity was significantly lower in feces from organisms subjected to both stress, and in HP extract from handled specimens. No difference was found in HP extract from specimens subjected to changes in feed. No significant difference was found in the final weight of organisms, regardless of the treatment.

Figs. 1 and 2 show the interaction between both stressors in enzyme activities from feces and HP extract. For feces, chymotrypsin and trypsin activity had highest values for unstressed organisms (Fig. 1, see letter A in the four graphs). Activity was significantly lower when subjected to either stress (Fig. 1, see letters B and D in graphs 1 and 2 and letters C and D in graphs 3 and 4). For chymotrypsin and trypsin activities in specimens in Group B (handled and no alimentary stress), the activity was significantly lower than

Table 2
Approximate composition of dry experimental foods

Food	Moisture (%)	Protein (%)	Lipids (%)	Ash (%)	Crude Fiber (%)	Energy (kJ/g)
SC feed	6.5 ± 0.06	46.6 ± 0.1	7.19 ± 0.07	11.5 ± 0.02	1.9 ± 0.03	18.8 ± 0.04
PI feed	6.9 ± 0.06	36.8 ± 0.2	4.9 ± 0.04	7.4 ± 0.02	1.9 ± 0.09	19.4 ± 0.05
PI feed	6.9 ± 0.06	36.8 ± 0.2	4.9 ± 0.04	7.4 ± 0.02	1.9 ± 0.09	19.4 ± 0.05

Nitrogen free extracts (NFE) are considered the difference of 100%.
SC = Silver Cup® commercial feed; PI = Piasa® commercial feed.

Table 3
Average unit of chymotrypsin and trypsin activity in enzymatic extracts from shrimp feces (FE) and hepatopancreas (HP) under two stressor conditions and average final weights of shrimp

Measured variable	Effector*			
	Alimentary stress		Handling	
	No	Yes	No	Yes
Unit of chymotripsin (feces)	0.225 ± 0.03^{a}	0.137 ± 0.03^{b}	0.269 ± 0.05^{a}	0.094 ± 0.03^{b}
Unit of trypsin (feces)	0.147 ± 0.02^{a}	0.089 ± 0.02^{b}	0.169 ± 0.02^{a}	0.067 ± 0.02^{b}
Unit of chymotripsin (HP extract)	0.116 ± 0.03	0.127 ± 0.04	0.153 ± 0.03^{a}	0.090 ± 0.03^{b}
Unit of trypsin (HP extract)	0.189 ± 0.04^{a}	0.131 ± 0.04^{b}	0.234 ± 0.04^{a}	$0.086^{b} \pm 0.03$
Final average weight (g)	12.9 ± 2.5	11.9 ± 2.8	13.1 ± 2.8	11.7 ± 2.5

* Different letters in same row and factor means significant differences ($P < 0.05$).

Group A (control, no stress). Chymotrypsin and trypsin activity declined to the lowest value in Group C (no handling, but alimentary stress) and Group D (handling and feed change).

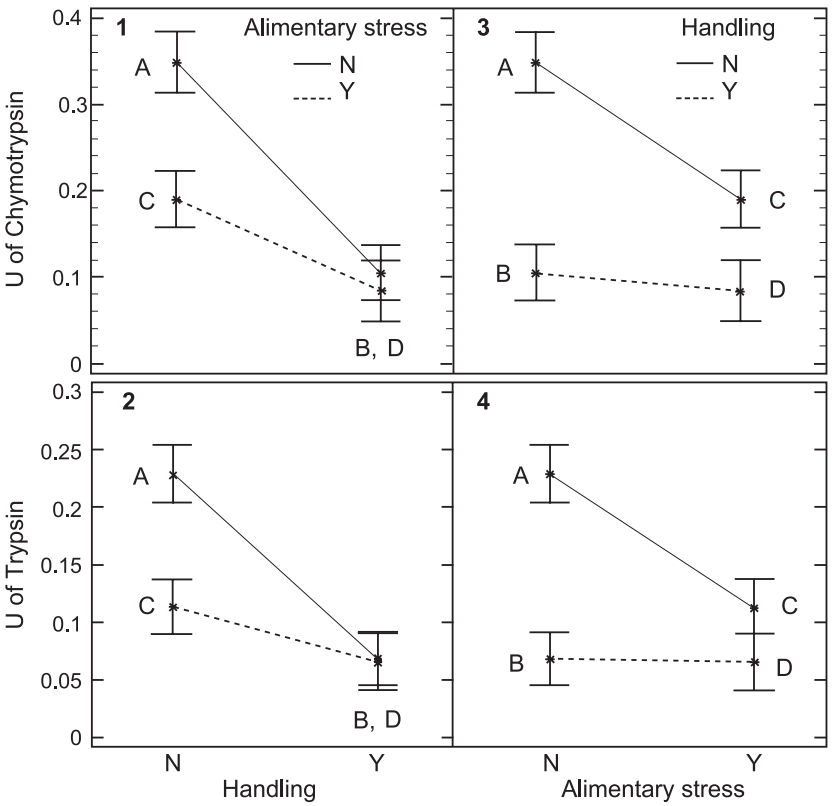

Fig. 1. Interaction plots ($P < 0.05$) for chymotrypsin and trypsin activity from feces extracts. N = no, Y = yes; A = no handled and no alimentary stress, B = handled and no alimentary stress; C = no handled and alimentary stress; D = handled and alimentary stress.

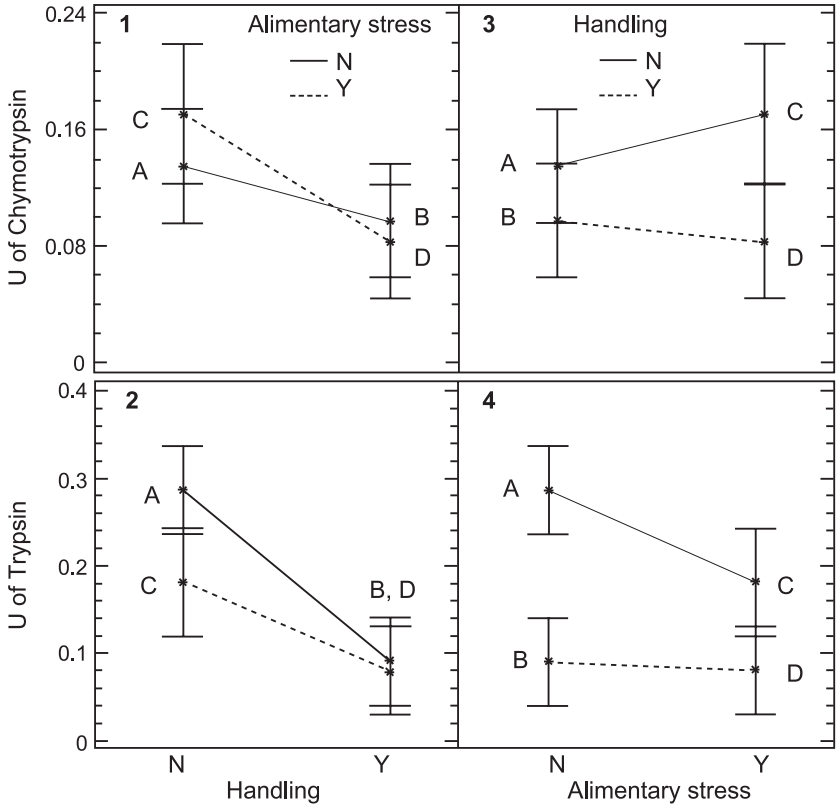

Fig. 2. Interaction plots ($P>0.05$) for chymotrypsin and trypsin activity from hepatopancreas extracts. N = no, Y = yes; A, B, C, and D are explained in Fig. 1 caption.

In HP extracts (Fig. 2), chymotrypsin and trypsin activity showed the same tendency as in feces, except for chymotrypsin activity in groups subjected to alimentary stress, where no significant differences were measured. Values for average enzymatic activity in each group are shown in Table 3.

Table 4
Average units of enzymatic activity of trypsin and chymotrypsin in experimental period measured in feces of individual shrimp before and after the beginning of stress period

Group	Trypsin		Chymotypsin	
	Before	After	Before	After
A	0.18 ± 0.02^{a}	0.28 ± 0.03^{b}	0.24 ± 0.02^{a}	0.43 ± 0.04^{b}
B	0.07 ± 0.01	0.07 ± 0.01	0.09 ± 0.01	0.12 ± 0.02
C	0.15 ± 0.02^{b}	0.04 ± 0.01^{a}	0.25 ± 0.03^{b}	0.03 ± 0.02^{a}
D	0.10 ± 0.01^{b}	0.03 ± 0.0^{a}	0.11 ± 0.01^{b}	0.05 ± 0.01^{a}

Different letters in same row and enzyme represent significant differences ($P<0.05$). Trypsin and chymotrypsin were evaluated with synthetic substrates.

Table 4 shows the average trypsin and chymotrypsin activity in feces in experimental groups before and after treatment. For Group A (control group), trypsin and chymotrypsin activity was higher during the second half of the study (testing the impact of stressors). For Group B (manipulated), no significant difference ($P>0.05$) between the two periods was found in trypsin and chymotrypsin activity. For Group C (alimentary stress), trypsin and chymotrypsin activity was significantly lower during treatment. For Group D (manipulated and alimentary stress), trypsin and chymotrypsin activity was significantly lower during treatment. Figs. 3 and 4 show data by individual specimen. Each bar is the average of 10 feces samples of each specimen (a, b, and c). In Fig. 3A, related to the activity of trypsin, specimen "a" did not follow the same tendency of the other two specimens (b and c). In Fig. 4 (B, related to the activity of chymotrypsin), specimen "c" did not follow the same tendency as the other two shrimp (a, b).

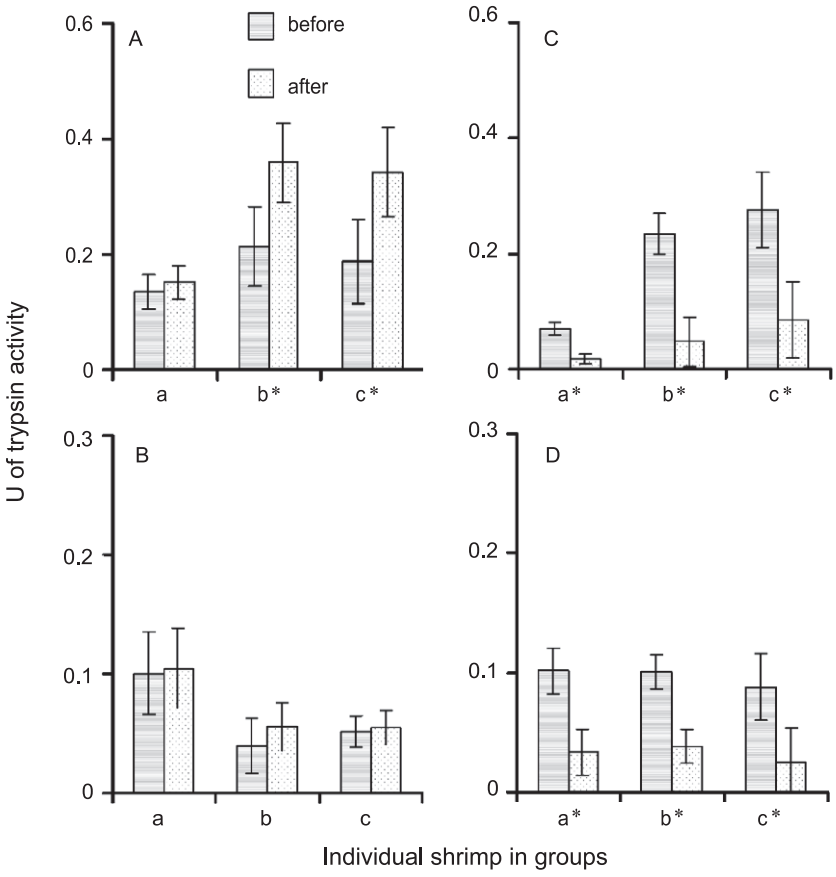

Fig. 3. Trypsin activity measured in feces extract before and after the start of the stress period in individual specimens. An asterisk means significant differences ($P<0.05$) between the before and after stress periods. Letters a, b, and c represent each individual within each group (A, B, C, and D), as explained in Fig. 1 caption.

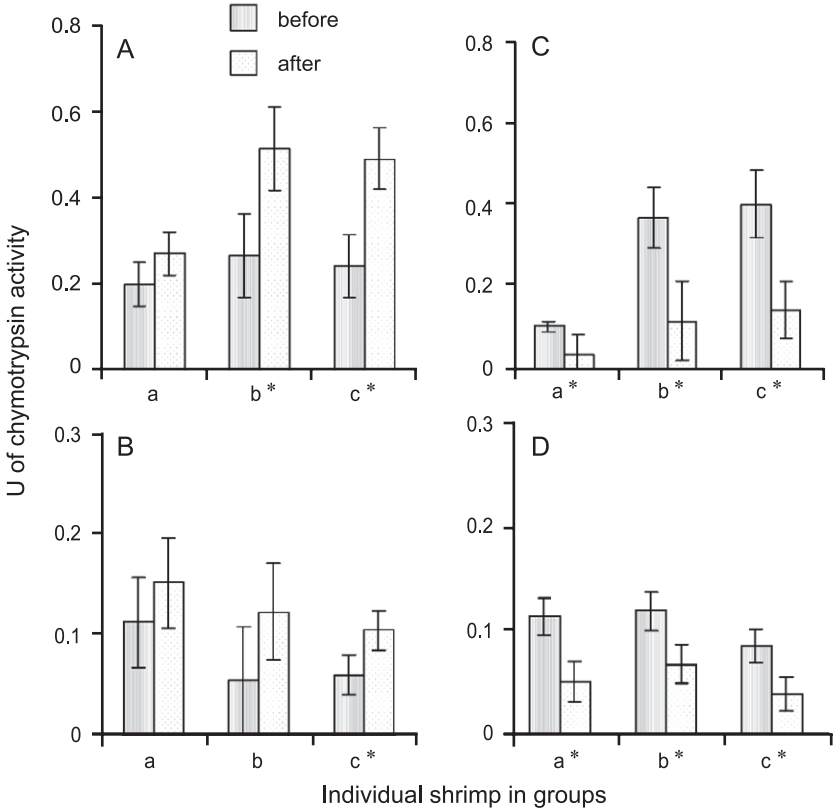

Fig. 4. Chymotrypsin activity measured in feces extract before and after the start of the stress period in individual specimens. An asterisk means significant differences ($P < 0.05$) between the before and after periods. Letters a, b, and c represent each individual with each group (A, B, C, and D), as explained in Fig. 1 caption.

Additionally, no correlation was found between molting and trypsin or chymotrypsin activity. During the experiment, every specimen, regardless of the experimental group, had several molts. It is noteworthy that some shrimp ate on the day of ecdysis. Also, feces were collected from some recently molted specimens. Regardless of the treatment, no differences were observed in average final weight of shrimp.

4. Discussion

In the past, killing a specimen is unavoidable to obtain tissue or organ samples when studying physiology. This approach limits potential of many research studies. Worst, it reduces the value of the data. Approaches to reduce stress and gain information about the extent of the stress are needed. We are interested in developing methods that avoid killing test specimens, to evaluate techniques to assess the extent of the effects of stressors on experimental organisms, and to be confident that the

alternative method of assay is reliable and comparable to the methods that kill the experimental organisms.

The commercial feeds used in this experiment were chosen to be different in formula and proximate composition, and to assure a response for different taste and nutritional value. As expected, test specimens responded to the stimulus of different levels of protein, lipids, and ash. The two brands of feed have been used for several years at the laboratory and superior performance of shrimp fed the SC brand was consistently observed. In this study, the number of organisms were kept low because each specimen provided multiple replicates (feces collected every day) that can be monitored, in a sufficiently long experiment, there is no limit to the number of samples taken from each specimen.

In previous work (Ezquerra et al., 1999; Córdova-Murueta and García-Carreño, 2002; Muhlia-Almazán and García-Carreño, 2002; Córdova-Murueta et al., 2003), food quality and composition was shown to affect enzymatic activity of digestive proteinase. Also shown for crustaceans, that alimentary stress is a stronger stressor of physiological processes than the molt cycle (Muhlia-Almazán and García-Carreño, 2002). This means that internal processes may be surpassed by external factors like alimentary stress (fasting or shifting to a different feed) or handling. Therefore, experimental designs should consider such external effects are possible and may be stronger than internal or other effects.

In this experiment, we observed that control Group A had higher proteolytic activity during the second period (after application of stressors in the experimental groups). This was considered the normal physiologic response (larger organisms, more proteolytic activity) for unstressed organisms. Taking this into account, we expected lower, or at most, equal proteolytic activity between the two phases of the experiment. This was what we observed in Groups B, C, and D.

According to data shown in Figs. 3 and 4, trypsin and chymotrypsin activity changed as a response to the two stressors. Most importantly, it allowed us to follow changes in each specimen and their variability, as observed in Table 3. Evaluation of protease activity is useful in many fields, such as ecological or biochemical ones. Applebaum and Holt (2003) found a relationship between chymotrypsin activity and alimentary stress (starvation) in red drum larvae, proposing that enzyme activity is an indicator of nutritional condition. We found that trypsin or chymotrypsin activity could be used as an indicator of the organism status under different conditions. Previously, we learned that alimentary stress affects enzyme activity in *P. vannamei* (Ezquerra et al., 1999; Muhlia-Almazán and García-Carreño, 2002; Muhlia-Almazán et al., 2003). However, such studies included handling of specimens. In another study, Córdova-Murueta (2002) found that differences in growth rate among shrimp fed different feeds were significant until several weeks after the experiment started. We suspect that this results from handling the subject specimens. We intend to determine what is the specific impact of handling on the digestive system of shrimp while, at the same time, evaluating other variables known to affect the digestive system. This study provided evidence that some stressors are stronger than others and that their interactions could be relatively simple to investigate. Handling had masked the effect of alimentary stress.

We confirmed by electrophoresis that there are no changes in proteinase composition recovered from feces over time in the same organism, compared with hepatopancreas enzymes at the end of the experimental period, as it presented by Córdova-Murueta et al.

(2003). These findings make feces a suitable body secretion to evaluate physiology of the shrimp digestive system and the effect of some stressors on it. Additionally, shrimp feces have been used for diagnoses of pathological conditions (Brinez et al., 2003), matching 100% with traditional histopathological methods.

The effect of stressors can be achieved by sampling feces. Feces sampling is a preferred approach for evaluating status of the digestive system when using digestive enzymes as indicators. Feces collection has other advantages. When sampling the hepatopancareas, uncertainty about what enzymes present in the extract cannot be resolved. The researcher cannot know with certainty if the enzymes analyzed were stored or secreted for digestion of food. Feces contain only secreted enzymes. By sampling the hepatopancreas, it is not possible to observe the effect of stressors in a single specimen because the experiment is usually an average of a group of shrimp. With this approach, the same specimen can provide an appropriate number of samples and the results can be compared for the same or similar tendencies in all specimens.

5. Conclusions

This study concludes that assays of feces are a suitable alternative source of information about digestive enzymes in shrimp to measure responses to some stressors, especially handling and alimentary stress produced by changing feed. Also, it provides a method for following a single specimen's response over an extended time period. Also the number of experimental organisms can be reduced.

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