Hurricane Mitch: Development of Immunological Tools to Assess the Health Status of Shrimp

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Development of Immunological Tools to Assess the Health Status of Shrimp

By

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Background

Hurricane Mitch introduced several stressors to both aquacultured and feral shrimp populations. These stressors included influxes of anthropogenic pollutants, water turbidity, rapid salinity changes, and dissolved oxygen disturbances. Stressors are known to increase the susceptibility of animals, including invertebrates (Lacoste and others, 2001), to infection. Since the late 1990s, the white spot syndrome virus (WSSV) is a primary pathogenic agent in Central and South America (Lightner and Redman, 1998; Jory and Dixon, 1999).

White spot disease is pandemic and affects shrimp, crabs, crayfish, and other crustaceans. Although there are specific diagnostic assays for WSSV in infected tissues (Durand and others, 1996; Lee and others, 2000; Peng, and others, 1998; Tang and Lightner, 2000; Poulos and others, 2001), no diagnostic assays that reflect of invertebrate health or immune status have been developed. Assessments of immune status would be especially useful in the face of stressors that compromise immununological health. Within stressed shrimp, as with other animals, viruses that are better able to infect would preferentially replicate, thereby enhancing virulence and increasing the number of strains infective to invertebrate populations. For humans, viral pathogens that evolve to be more adaptive to host immune defense systems are responsible for recurrent epidemics.

Devastating disease outbreaks and mass mortalities result from infection by WSSV. Corresponding risk factors, as well as risk reduction factors, have been identified for culture situations (Corsin and others, 2001). Multivariate and univariate analyses of 158 risk factors for WSSV outbreaks in a rice-shrimp farming system showed higher incidence in ponds closer to the sea, in shrimp that were smaller than average size 1 month after stocking, and with the use of commercial feed. One important risk reduction factor was the presence of high numbers of shrimp with bacterial infections, which may recruit and stimulate hemocytes (blood cells) into immune defensive reactions. A second factor was the presence of dead post larvae at stocking, which may reflect those shrimp having succumbed to bacteria. The thrust of these findings is that immunological stimulation of shrimp populations exposed to bacteria enhanced overall survival. The question of interest then was what components mediated the enhanced survival.

To determine the overall “immune” or health condition of humans and agriculturally important species, available bioindicators include changes in blood cell numbers, body temperature, and the presence of acute phase proteins in serum. However, techniques to determine immunity are much less well developed for invertebrates. Further delineating the invertebrate immune defense system is a critical step toward pinpointing the measurable indicators by which shrimp health can be assessed. In this way, shrimp producers would be able to gauge invertebrate health, which is especially important when there are co-occurrences of stressors or pathogens.

Marker enzymes such as peroxidases, esterases, and phenoloxidase (PO), have been employed in the few such studies undertaken with invertebrates. Our investigations of the activation of crayfish hemocytes using flow cytometry (Cardenas and others, 2000) and the measurement of color change of PO in the presence of molecules from common pathogens (Cardenas and others, 2000) and insecticides (J. Jenkins, USGS, unpublished data) have demonstrated the measurable fluctuations by PO. Moreover, key molecules

involved in the enzyme cascade for pathogen recognition and activation have been isolated and characterized (Cardenas and Dankert, 1997, 2000).

Capitalizing on this progress, we proposed to construct biological tools to enable the direct assessment of the health status of invertebrates. Such tools make possible a color indicator system (IS) design. The IS would be designed to be a rapid detection system to quantitatively reflect shrimp health. The data obtained from the IS would be used in conjunction with visualized animal conditions and phenotypes, and would be the first step in providing shrimp culturists and gatherers of wild larvae with the ability to relate animal health to the presence of stressors or pathogens.

Introduction

Shrimp aquaculture is a prominent industry in the Gulf of Fonseca, which is shared by Honduras, Nicaragua, and El Salvador. The main cultured species is *Penaeus vannamei*, a tropical white shrimp species distributed in the Pacific Ocean, but wild larvae, mainly *P. stylirostris* and *P. vannamei*, are used as well (Carbonell and others, 1998). Along with the rapid growth in the shrimp industry, pathogenic viruses (see Lightner and Redman, 1998) have flourished. Numerous strategies have been employed to reduce production losses due to viruses, including the use of improved culture practices, stocking "specific pathogen free" (SPF) species or stocks, and the use of immunostimulants to enhance nonspecific defense mechanisms. Delineating measurable shrimp health parameters by way of an IS could be integrated into each of these strategies and would be of interest to the governmental agencies responsible for the economically important shrimp aquaculture.

Immunity is a crucial determinant of fitness, and both external and internal variables both influence immune status. Aquaculture practices can lead to conditions that promote disease states, and intensive aquaculture itself is a source of anthropogenic stress on cultured species (Carbonell and others, 1998). As with Hurricane Mitch, catastrophic flood events causing an influx of pollutants, high sediment loads, and oxygenation disturbances to both natural and aquaculture farms result in stress (Rodrigues and others, 2001). Gender (Rolff, 2001), molt (Sequeira and others, 1995), life stage, and genetic make-up (Krishna and others, 1997) each influences the effectiveness of disease resistance in invertebrates.

The immune defense systems of crustaceans consist of cellular and humoral reactions. Three subpopulations of hemocytes are responsible for a number of the reactions, containment of the PO system, phagocytosis, degranulation and release of reactive oxygen intermediates (Song and Hsieh, 1994), and coagulation (Soderhall and Smith, 1986). Circulating hemocyte subpopulations shift with life stage and pathogen exposure, and with consequent changes in PO level contained per subpopulation (Sequiera and others, 1995). The humoral components of crustacean immune defense include the activity of soluble enzymes, either activated in circulating hemolymph, or released by cells that serve to detoxify toxic molecules or inhibit the physiology of invading pathogens (Cardenas and Dankert, 1997, 2000). Antimicrobial peptides, proteases and protease inhibitors, as well as lectin-like molecules exist in the white shrimp species *Litopenaeus vennamei* and *L. setiferus* (Gross and others, 2001; Sritunyalucksana and others, 1999; Nappi and Ottaviani, 2000).

The primary mediator of the cellular response to injury and disease in invertebrates is the pro-enzyme prophenoloxidase (proPO) activating system (Soderhall and others, 1994), where the proPO is released from hemocytes by an active degranulation process that can be stimulated by inflammatory agents such as lipopolysaccharide (LPS) or peptidoglycan (molecules of bacterial cell walls) and ß-1,3-glucan (molecules of fungal and yeast cell walls). Once released, ProPO is proteolytically converted, through cleavage of the enzyme at a specific site, to its active form PO, which is the central component of an enzyme cascade that has been identified in crustaceans (Soderhall and Smith, 1985; Cardenas and others, 2000). The active form of the enzyme then functions to produce antimicrobial effects, wound repair, encapsulation, and phagocytosis. The nucleotide sequence of the proPO molecule has been deduced (Sritunyalucksana and others, 1999).

The overall goal of the current project was to develop the fundamental components, or diagnostic reagents, by which a hand-held IS could be designed for the detection of the levels of PO enzyme in penaeid shrimp. Once the fundamental components are developed, the IS would be geared to become field-ready. The constituent of focus for this project was the PO and proPO enzymes because the general molecular structures are ubiquitous in shrimp and invertebrate species and central to their innate immune response. The enzymes are defined, soluble molecules that can be used to generate mammalian antibody proteins as fundamental components of the IS. These antibodies can be coupled to color reagents (such as an enzyme-linked secondary antibody, or a coloring enzyme directly coupled to an anti-PO antibody) so that color development would produce results within minutes. Known ranges of standard proPO and PO enzymes would be used for scaling. In conjunction with observations on environmental and animal conditions, this IS would be reflective of the health status of individuals or groups of individuals.

Methodology

Peptide Synthesis

The amino acid sequence for PO of black tiger shrimp, *Penaeus monodon*, deduced by Sritunyalucksana and others (1999), was used as the template for generating synthetic peptides. Two 10-mer peptides were selected for synthesis based upon (1) the probable immunogenicity of the peptide and (2) the proposed sequence that spans the proPO-PO cleavage site. Hydropathy profiles generated of the amino acid sequence of PO (not shown) selected two relatively high in hydrophilic sequences, as these are likely on the outside of the protein surface, and are thus more immunogenic (Clark and others, 1996). The two synthetic peptide sequences generated by SynPep Corp., Dublin, CA were:

Peptide 1: N-N-D-L-R-E-P-K-E-V or
asparagine – asparagine – aspartate – leucine – arginine – glutamine – proline – lysine – glutamine - valine

Peptide 2: E-A-P-P-S- V-A-T-R or
glutamine – alanine – proline – proline – serine – valine – alanine – threonine - arginine

The peptides were synthesized under solid phase Fmoc chemistry (Wang 1973; Brad Keymer, personal communication, SynPep Corp.).

Antibody Production

Each peptide (~10 mg) was coupled by glutaraldehyde cross-linking to keyhole limpet hemocyanin (KLH) at SynPep Corp. For the initial inoculation, two replicate New Zealand White rabbits were inoculated with 1 mg of peptide 1 or peptide 1 diluted to 1.0 mL and injected with 1.0 Freund's complete adjuvant. The remaining four boosters were given with 500 µg antigen (1.0 mL) with 1.0 mL Freund's incomplete adjuvant. Serum from each pair of rabbits injected with each peptide was not pooled, and antiserum from one of each pair was employed in experiments. Rabbits were bled three times for antiserum collection.

The antiserum directed against the amino acid sequence within the PO molecule (peptide 1) will be referred to as "anti-PO". The antiserum directed against the peptide that spans the pro-PO cleavage site will be referred to as "anti-proPO" (peptide 2). Before inoculation, serum for use as a negative control (pre-immune sera or "sham") was collected from each rabbit.

In order to confirm antigen to antibody binding and to estimate antibody titer, Enzyme-Linked ImmunoSorbent Assays (ELISA) (Spectra Shell Plate Reader, SLT Labinstruments, Durham, N.C.) were performed using goat-anti rabbit IgG coupled to alkaline phosphatase (GAR-AP; Sigma Chemical Company, St. Louis, Mo.), and color was developed using p-nitro phenyl phosphate (pNPP) according to manufacturer instructions. The ELISA results confirmed the "anti-PO" antibodies bound to the corresponding synthetic peptide at an antiserum dilution (highest) of 1:24,000; the "anti-proPO" antibodies could produce a signal at an antiserum dilution (highest) of 1:48,000 (not shown).

PO Preparations and Antibody Reactivity by ELISA

The PO preparations were obtained from isolated hemocytes and from pooled, centrifuged hemolymph from crayfish (*Procambarus clarkii*) and from shrimp (*Penaeus setiferus*) using buffers and anticoagulants (Soderhall and Smith 1985). In order to determine if the reactivity of the antibodies to the PO preparations could be altered by the presence of pathogenic molecules, reaction mixtures included bacterial LPS (10 µg/ml), Zymosan A extracted from fungal cells (Sigma; 10 : l of a 1% solution) (Cardenas and others, 2000), or shrimp extract (10 : l). Zymosan A was prepared by dissolving 10 mg ZYA in 1 mL of 10 mM imidazole, 150 mM NaCl, pH 7, and incubating at 24 degrees for 1 hr prior to centrifugation. The resultant supernate was used (0.01 mL) in the activation procedure. Soluble shrimp extract was prepared from adult shrimp (n>1) having succumbed to WSSV prepared from adult animals (courtesy of Gulf Ecology Division, U.S. Environmental Protection Agency, National Health and Environmental Effects Research Laboratory, Gulf Breeze, Fla). Extract was prepared by grinding shrimp with a 5/16 inch tissue borer and extracted from the ventral midsections of the animals.

In order to assess the reactivity of the two antisera against PO preparations or PO preparations plus pathogenic molecules, ELISA analyses were performed as described

earlier (Cardenas and Dankert, 1997). The "anti-PO" or the "anti-proPO" antisera were used as the primary antibodies, and GAR-AP was the secondary antibody allowing color development.

In order to establish nonspecific binding to PO by the secondary antibody (GAR-AP), the PO preparations or hemolymph samples were placed into ELISA wells (10 µL into 100 µL of buffer) and incubated at room temperature for 1 hr. To each well, 100 µL of blocking buffer (non-fat dry milk, 5%) was added and incubated for 5 min. The wells were then washed three times using 0.1% Tween detergent (Sigma), followed by incubation with 100 µL of GAR-AP at a final dilution of 1:10,000 in blocking buffer for 1 hr. ELISA plates were rinsed in 10 mM diethanolamine, pH 9.5, and 1 mM $MgCl_2$. Color was developed using pNPP and measured in an ELISA plate reader (Tecan) at a wavelength of 405 nm. These background readings were subtracted from values reported to negate background. Pre-immune serum reactions are reported as "sham" values (table 1).

Statistics

Comparisons of absorbance values were made at $P<0.00125$ using four independent variables: antibody type (anti-PO or anti-proPO), PO source (hemocytes or hemolymph), species (crayfish or shrimp), and treatment (unstimulated, LPS, Zymosan A, or WSSV) with the General Linear Means procedure (SAS).

Results

Overall, significant interactions were found between (1) antibody type (anti-PO or anti-propPO), species, and treatment type (p=0.0001), (2) PO source (hemocytes or hemolymph), species, and treatment (p=0.0001), and (3) species and treatment. Highlights and trends will be reported. Hemocytes generally showed more reactivity than hemolymph.

The absorbance values in table 1 indicate that the antibodies against both synthetic peptides could react with natural sources of PO and proPO. In order to standardize results to account for differences in the substrate, potential loss of activity in the alkaline phosphatase, or other reagent variability, the conditions established here were employed for the remaining experiments. For each set of experiments, the data from a similar matrix were used to establish the 100% values of absorbance, whereby data are expressed as a percentage of this value (tables 1-4; fig. 1-4), and the raw data are presented in fig. 1-4.

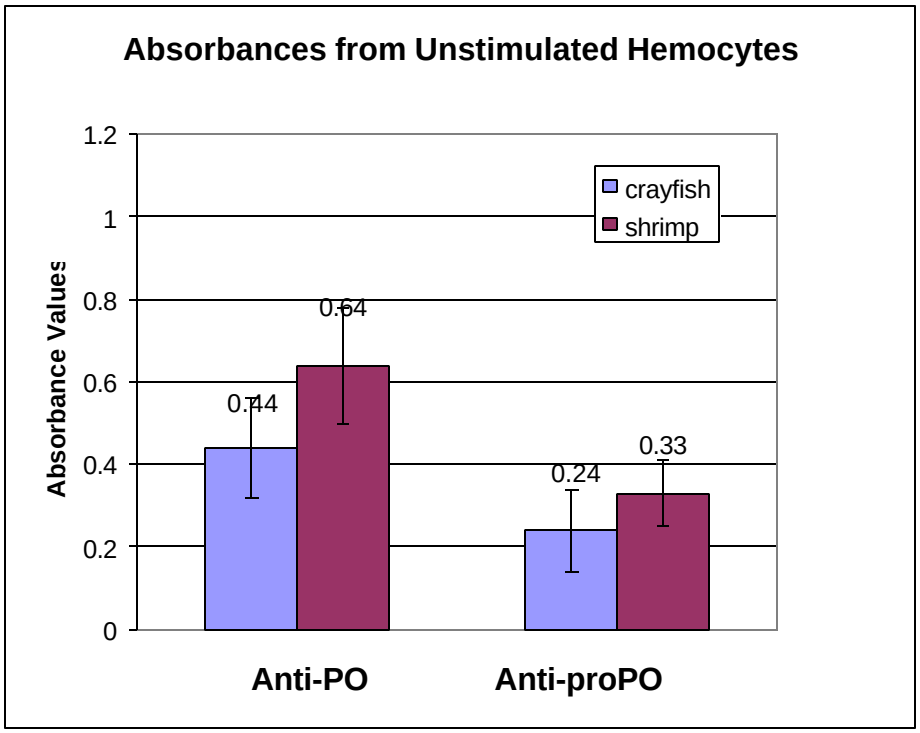
Table 1. Absorbance values[1] of ELISA performed using PO preparations from unstimulated hemocytes and hemolymph from crayfish (*Procambarus clarkii)* and shrimp *(Penaeus setiferus).*

	Crayfish		**Shrimp**	
	Hemocyte PO	**Hemolymph PO**	**Hemocyte PO**	**Hemolymph PO**
Anti-PO	0.44±0.12	0.24±0.09	0.64±0.14	0.47±0.10
Anti-proPO	0.24±0.10	0.32±0.10	0.33±0.08	0.46±0.11
Sham[2] PO	0.02±0.02	0.04±0.03	0.01±0.02	0.04±0.02
Sham proPO	0.01±0.02	0.01±0.01	0.01±0.01	0.04±0.03

[1]Data are expressed as means of absorbance values ± SEM, averaged from eight wells each.

[2]Sham denotes pre-immune rabbit serum. Results show that binding of secondary antibody is minimal.

With unstimulated hemocytes, anti-PO binding against PO from both species was significantly higher than binding by anti-proPO ($p<0.0001$), and shrimp signals were significantly higher than crayfish with anti-PO ($p<0.0001$) (fig. 1). Using unstimulated hemolymph, absorbances from binding by both antibody types were equivalent ($p=0.0346$) for both species.



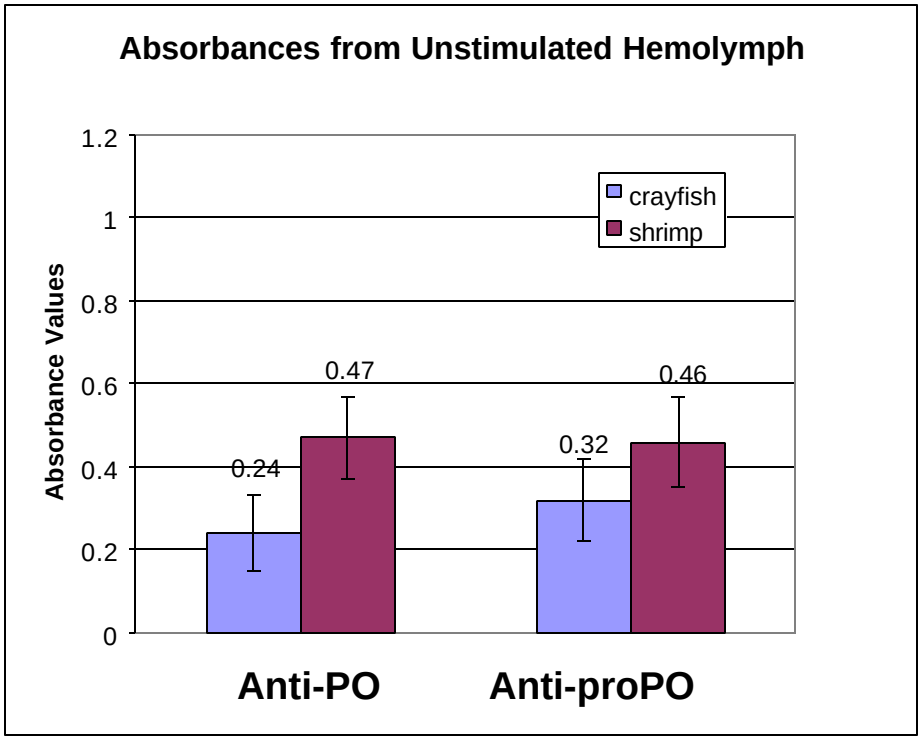


Figure 1. Absorbance values of PO and proPO from unstimulated hemocytes and hemolymph using Anti-PO and Anti-proPO antibodies in Enzyme-Linked Immunosorbent Assays.

The absorbance values in table 2 and graphics in fig. 2 indicate that exposure of crayfish and shrimp hemocytes to LPS (Gram negative bacterial cell wall preparations) increased the amount of binding by antibodies that are directed against the PO reactive sequence as compared with unstimulated control hemocytes, and that the binding by anti-proPO was decreased. Changes were more apparent for hemocytes and with anti-PO than with hemolymph preparations and anti-proPO that showed depressed binding, where no more than 47% binding occurred. Shrimp hemocytes stimulated with LPS showed significantly higher binding by anti-PO than crayfish cells ($p<0.0001$).

Table 2. Percentage of ELISA absorbance values [1] from PO preparations obtained after exposure to bacterial lipopolysaccharide (LPS) [2] compared to those without LPS and mean absorbance data.

	Crayfish		**Shrimp**	
	Hemocyte PO	**Hemolymph PO**	**Hemocyte PO**	**Hemolymph PO**
Anti-PO	120±14	90±10	132±21	105±11
Anti-proPO	45±8	34±12	22±13	47±9
Sham PO	NS[3]	NS	NS	NS
Sham proPO	NS	NS	NS	NS

Actual Mean Absorbance Values

	Hemocyte PO	**Hemolymph PO**	**Hemocyte PO**	**Hemolymph PO**
Anti-PO	0.528±0.017	0.216±0.009	0.845±0.029	0.494±0.011
Anti-proPO	0.108±0.008	0.109±0.012	0.073±0.010	0.216±0.010

[1] Data are expressed as a percentage of absorbance values ± SEM of the readings from an identical plate assayed without incubation with LPS (as in table 1).

[2] Incubations were made with LPS at 10 ng/mL (final concentration) for 60 min at room temperature.

[3] NS = absorbance values did not exceed 0.1 units.

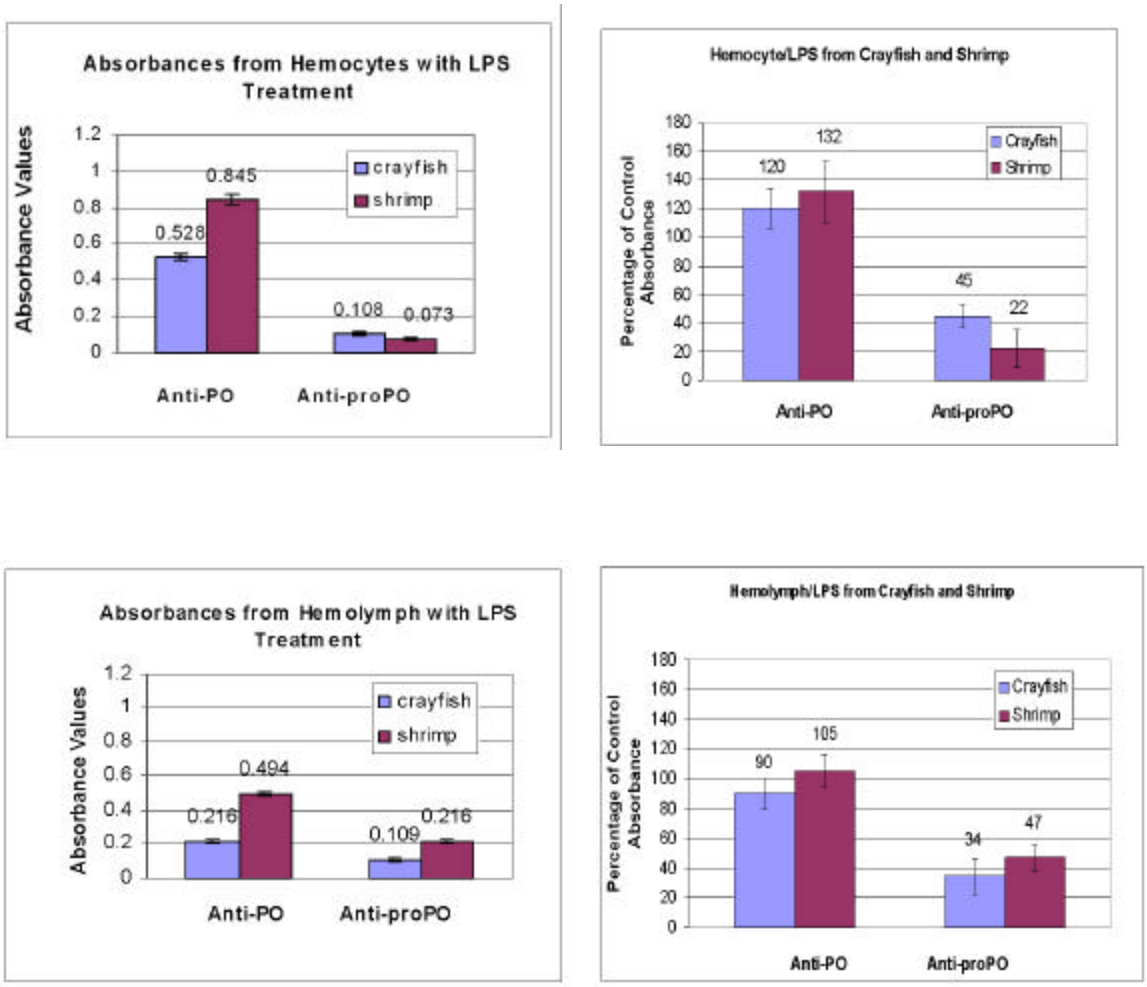


Figure 2. Actual absorbance values (left), or expressed as percentages (right) of unstimulated control treatment (table 1, fig. 1), of PO and proPO enzymes from hemocytes (top) and hemolymph (bottom) stimulated with LPS as measured with Anti-PO and Anti-proPO antibodies in Enzyme-Linked Immunosorbent Assays.

The absorbance values in table 3 and graphics in fig. 3 indicate that exposure to Zymosan A increased the amount of binding by antibodies that are directed against the PO reactive sequence as shown with hemocytes from shrimp, in particular. The highest level of binding in the study, and the greatest change among the treatments was shown with the Zymosan A-stimulated shrimp hemocytes with anti-PO, and with crayfish hemolymph with anti-proPO binding. Binding by anti-PO to hemocytes stimulated by Zymosan A was 1.5 times the level of control absorbance levels. Binding by anti-proPO to hemolymph was 1.35 time the level of control absorbance levels. The only anti-proPO binding in the study that showed levels higher than control were with this treatment (crayfish hemolymph with the Zymosan A).

Table 3. Percentage of ELISA absorbance values [1] using PO preparations obtained after exposure to fungal Zymosan A [2] and mean absorbance data.

	Crayfish		**Shrimp**	
	Hemocyte PO	**Hemolymph PO**	**Hemocyte PO**	**Hemolymph PO**
Anti-PO	80±13	67±8	154±17	135±11
Anti-proPO	23±7	42±10	16±6	29±8
Sham PO	NS[3]	NS	NS	NS
Sham proPO	NS	NS	NS	NS

Actual Mean Absorbance Values

	Hemocyte PO	**Hemolymph PO**	**Hemocyte PO**	**Hemolymph PO**
Anti-PO	0.352±0.016	0.161±0.008	0.986±0.023	0.635±0.011
Anti-proPO	0.055±0.007	0.134±0.010	0.053±0.005	0.133±0.009

[1] Data are expressed as a percentage of absorbance values ± SEM of the readings from an identical plate assayed without incubation with Zymosan A (as in table 1).

[2] Incubations were made with 10 : l Zymosan A as described in Methods at 24 degrees for 60 min.

[3] NS = abs values did not exceed 0.1 units.

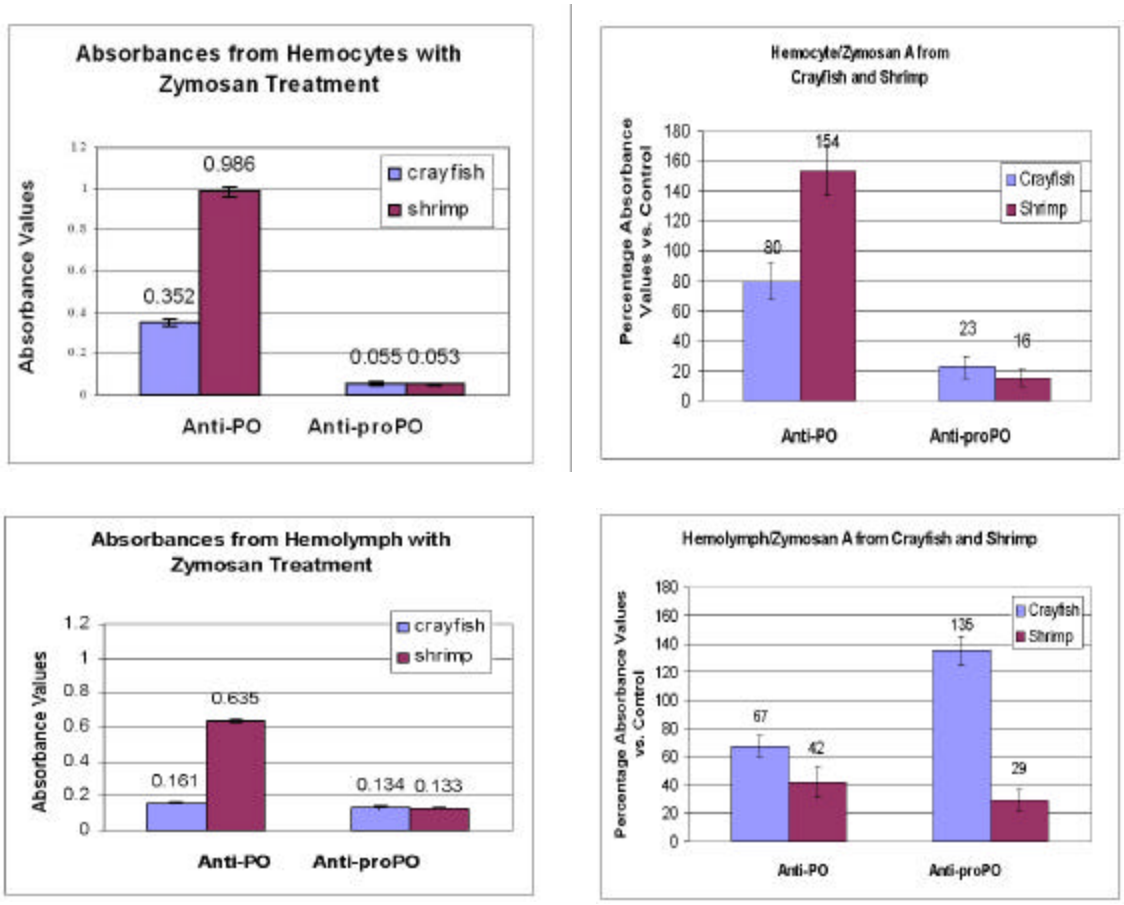


Figure 3. Actual absorbance values (left), or expressed as percentages (right) of unstimulated control treatment (table 1, fig. 1), of PO and proPO enzymes from hemocytes (top) and hemolymph (bottom) stimulated with Zymosan A as measured with Anti-PO and Anti-proPO antibodies in Enzyme-Linked Immunosorbent Assays.

The absorbance values in table 4 and graphics in fig. 4 indicate that exposure of crayfish or shrimp PO preparations to WSSV did not significantly increase or decrease the binding of antibodies against PO or proPO. Little change from control values was noted (at $P<0.00125$).

Table 4. Percentage of ELISA absorbance values [1] using PO preparations obtained after exposure to white spot syndrome viral extract (WSSV) [2] as compared with those without WSSV and mean absorbance data.

	Crayfish		**Shrimp**	
	Hemocyte PO	**Hemolymph PO**	**Hemocyte PO**	**Hemolymph PO**
Anti-PO	98±9	110±14	102±20	105±13
Anti-proPO	85±11	95±8	83±10	77±9
Sham PO	NS[3]	NS	NS	NS
Sham proPO	NS	NS	NS	NS

Actual Mean Absorbance Values

	Hemocyte PO	**Hemolymph PO**	**Hemocyte PO**	**Hemolymph PO**
Anti-PO	0.431±0.011	0.264±0.013	0.653±0.028	0.494±0.013
Anti-proPO	0.204±0.011	0.304±0.008	0.274±0.008	0.354±0.010

[1] Data are expressed as a percentage of absorbance values ± SEM of the readings from an identical plate assayed without incubation with WSV (as in Table 1).

[2] Incubations were made with 10 : l WSSV extract as per methodology section at 24 degrees for 1 hour.

[3] NS = abs values did not exceed 0.1 units.

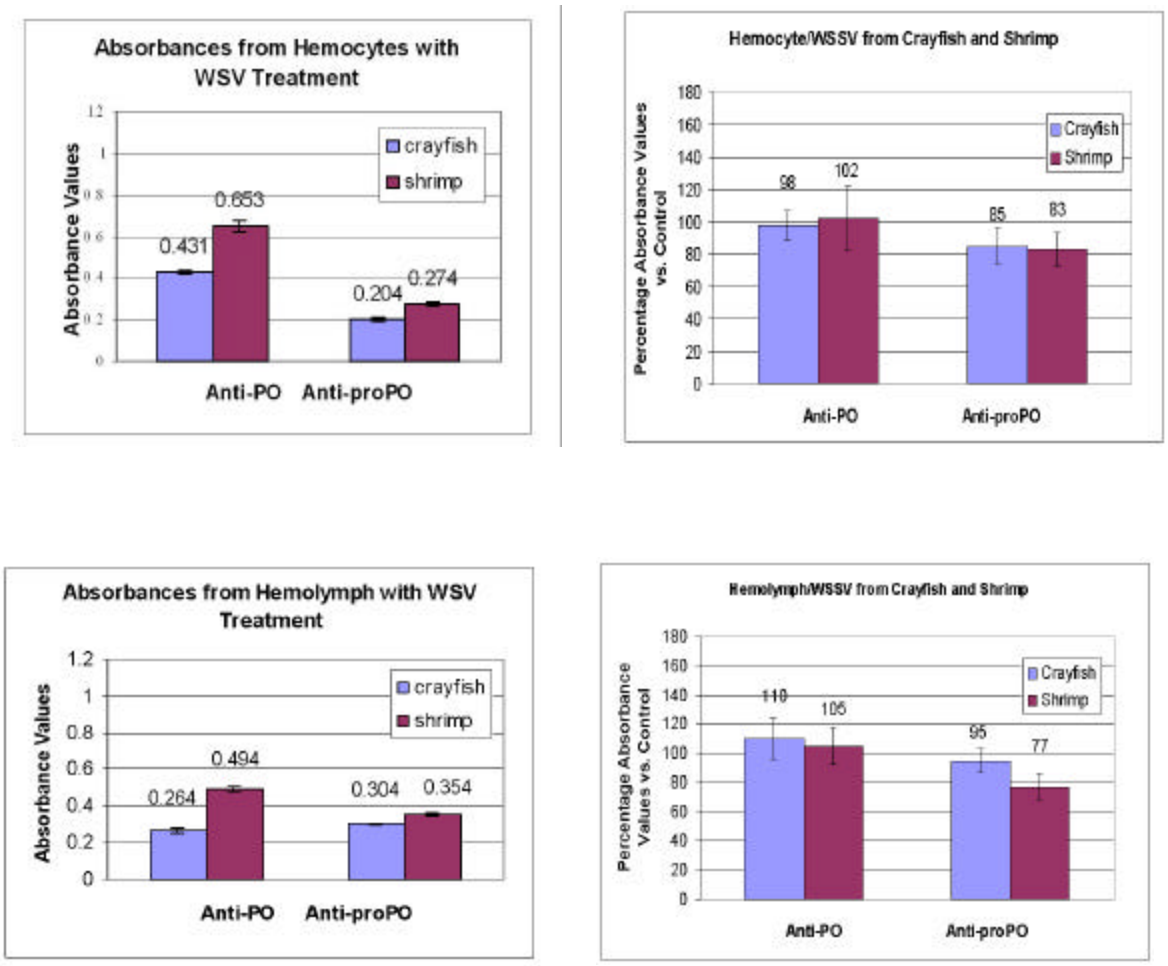


Figure 4. Actual absorbance values (left), or expressed as percentages (right) of unstimulated control treatment (table 1, fig. 1), of PO and proPO enzymes from hemocytes (top) and hemolymph (bottom) stimulated with white spot syndrome virus-infected shrimp extract as measured with Anti-PO and Anti-proPO antibodies in Enzyme-Linked Immunosorbent Assays.

Discussion

White spot disease is a devastating disease that can cross species barriers and is found worldwide. Mortality rates are usually very high and can reach 100% within 3 to 10 days from the onset of visible signs (Chou and others, 1995). Although there are several laboratory diagnostic assays for the WSSV, the uses of such assays are not suitable for predictive capabilities. The overall goal of this project was to develop immunologic reagents that can be used in a color indicator system (IS) for assessing the immune status of shrimp. The kit design would be easy-to-use, produce rapid results, and be adapted for field use. Delineating measurable shrimp health parameters by way of an IS could be integrated into each of the strategies already in use for reducing shrimp production loss due to pathogenic viruses, such as improving culture practices, stocking SPF shrimp species or stocks, and testing effectiveness of immunostimulants for increasing innate immunity.

This project began the process of diagnostic test development by synthesizing regions of the PO enzyme against which anti-PO and anti-proPO antibodies were developed. The "inactive" form of PO (proPO) has a higher molecular weight than active form, PO, due to an amino-terminal extension. Activation occurs by protease removal of this extension. The hypothesis was that the antibodies raised against synthesized PO and pro-PO would reflect enzyme presence of the PO and pro-PO, reactive in the presence of infectious agents. Exposure would be indicated by the rise or fall of the levels of either form of the enzyme.

The results of this study showed that the phenoloxidase (PO) system, the primary system for innate immunity in invertebrates, could be used to develop diagnostic reagents to assess invertebrate health status. The rabbit antibodies raised against important components of the shrimp phenoloxidase system can be used to detect PO in hemocytes and the hemolymph of *Procambarus clarkii* and *Penaeus setiferus* (table 1; fig. 1). Antibodies from rabbits not immunized with PO, the sham antibodies, showed only background levels of binding (table 1), thereby indicating that no significant cross-reactive antibodies to crustacean antigens naturally occur in rabbits. The anti-PO antibodies and anti-proPO antibodies reacted to accessible antigens in the positive controls. The secondary antibodies (goat-anti-rabbit) also did not cross-react with crustacean antigens. These results indicate the potential for further development of immunological reagents to other shrimp and invertebrate protein factors (such as LGBP, see below), and the likelihood of no complications by cross-reacting proteins.

In order to relate enzyme levels to the presence of pathogens, model pathogenic molecules from bacteria (e.g., LPS), fungus (e.g., Zymosan A), and WSSV (WSSV infected shrimp tissue) were incubated with hemocytes or hemolymph of *Procambarus clarkii* and *Penaeus setiferus* (tables 2,3,4; fig. 2-4). Changes were noted in the PO immune defense system as measured by antisera produced against proPO and PO.

In the unstimulated shrimp and crayfish control hemolymph and hemocytes, anti-proPO bound equivalently ($p=0.0346$), although when hemocytes were used as the PO source, binding was greater with anti-PO ($p<0.0001$). In this study, the greatest absorbances were noted with hemocytes, and binding by anti-PO was generally higher than by anti-proPO for both species. Generally, shrimp PO preparations generally elicited greater absorbance readings per specific reaction mixture (e.g. antibody,

treatment, and PO source). An exception was noted in the case of the high amount of anti-proPO binding in crayfish hemolymph having been activated with Zymosan A. There was a high amount of binding by anti-PO to shrimp hemocyte preparations with this same treatment (table 3, fig. 3).

The trend for higher anti-PO absorbance values and lower anti-proPO values was evident with the Zymosan A incubations, as with the LPS incubations. This apparent consistency of the lower signal by the pro-PO antibody could be due to the fact that if proPO is involved with an activation reaction, more PO than the inactive pro-PO is likely available for binding. The antibodies raised (anti-proPO) against the amino acid span across the cleavage site may not bind to activated enzyme because that sequence is not available in an activated molecule.

When comparing the absorbance readings using anti-PO with hemocytes from both species: unstimulated cells = WSSV < LPS = Zymosan ($p=0.00125$). Few differences were noted with hemolymph as the PO source. With hemolymph from both species using anti-proPO: Zymosan=LPS=unstimulated and WSSV=unstimulated=LPS – in other words, little change from controls was apparent when anti-pro PO was used with hemolymph. Additionally, few differences were noted with hemocytes in such a comparison with the anti-proPO antibody.

To demonstrate the level of stressor impact, notable changes from controls is desired. In this study, changes in absorbance values were less apparent with WSSV treatment than with LPS and Zymosan A. This may be due to the unknown dose of infective virus in those shrimp used for extract or possible degradation of proteins from that extract prior to ELISA. The assay might be better optimized using buffers specific to shrimp (Vargas-Albores and others, 1993) rather than crayfish, proteins.

However, the 1.5 fold level increase in absorbance using shrimp hemocytes and anti-PO shows the proteins reacted to fungal proteins (Zymosan A). Because more changes were noted with shrimp hemocytes throughout the study, the suggestion for diagnostic assay development is to employ shrimp hemocytes. However, for crayfish, the use of both hemocytes and hemolymph should be considered due to 1.2 fold increase in binding by anti-PO with LPS and the 1.35 fold increase in binding by anti-proPO with Zymosan A.

With regard to the choice of antibodies that were developed for diagnostics, for assays with both species, the anti-PO antibody showed the greatest changes in absorbance. However, the dynamics in level shifts can be highlighted, especially with crayfish (fig. 3) with binding by anti-proPO, as seen in figs. 2 and 3 with LPS and Zymosan A.

With future work, the development of a third antibody reagent is suggested. The invertebrate LPS and beta-1,3-glucan binding protein (LGBP), a hemolymph protein expressed during pathogen stimulation, is suggested for use as an antigen for a third antibody that could be used in the IS in conjunction with the anti-PO and anti-proPO. The third antibody would help to pinpoint the time of infection in the case of infected animals. During a WSSV infection, LGBP remains high throughout the infection, while PO expression initially increases, then proceeds downward, at which time virus is detectable by viral diagnostics (Roux and others, 2002). Because LGBP activates PO, this would add more information relevant with regard to pathogen infection. Detecting the temporal expression of each of these three proteins (PO, pro-PO, and LGBP) would

allow the investigator to pinpoint if an infective agent was present, and/or the stage (early or late) of the infection.

This study makes concrete steps in the development of an IS directed at measuring shrimp health status. This will assist producers in better integration of their farms into the coastal zone, with fewer impacts on the existing natural shrimp populations. During a WSSV infection, as with a bacterial infection (Sung and others, 2000), the PO level increases initially, then lowers, at which time virus is detectable by PCR (Roux and others, 2002) by in situ hybridization, or western blots and other methods (Durand and others, 1996; Lee and others, 2000; Peng and others, 1998; Tang and Lightner, 2000; Poulos and others, 2001). Levels of PO enzyme have been found to fluctuate with the presence of metals (Tujula and others, 2001; Cheng and Wang 2001), dredge spoils (Smith and others, 1995), ammonia-N (Cheng and Chen, 2002), and bacteria (Sung and others, 2000; Glupov and others, 1997; Soderhall and Smith 1985). Such environmental conditions may complicate results provided by the IS, so reliable marker development is necessary.

Results from the IS, when viewed logically in conjunction with other factors, will provide a good assessment on the health of shrimp. This would be considered a multiple bioindicator approach. Environmental data are important factors to consider, as are water quality, and clinical observations on pathological changes indicative of infection. With WSSV, clinical changes include white spots on the carapace, turbid hemolymph, necrotic hepatopancreas (Zhan and others, 1998). The results of the IS would be beneficial to culturists attempting to estimate a stage of WSSV outbreak, as there are three categorized forms of outbreaks, each with particular days till mass mortality is fully reached (Sudha and others, 1998). Melanization of carapace (indicating previous immunological stimulation) and size of the animals after stocking (Corsin and others, 2001) should be considered. Benefits of using a multiple bioindicator approach are purported with environmental health studies.

The quantitative IS would assay for the critical PO enzyme system that maintains invertebrate health and fighting pathogenic agents. Specific uses of a color indicator system for assessing invertebrate health status include:

1. Quick screening of feral collected postlarvae intended for use in intensive culture systems. Health assessments would occur prior to transporting individuals from the wild into a culture situation.
2. Screening of suspect animals prior to viral diagnostics. Such methods could include polymerase chain reaction (PCR), in situ hybridization, or western blots. These screenings, including testing for SPF shrimp, would enhance the control measures for culturing virus-free shrimp.
3. Assess different life stages, such as postlarvae, juveniles, and adults.
4. Support the breeding efforts toward pathogen-resistant strains of shrimp. Because invertebrates are poikilothermic, osmoregulatory animals, they are in close proximity to a multitude of stressors. The IS could assist in breeding individuals with higher levels of resistance.
5. Use IS in conjunction with other data observed. Such data include general observations such as notations on animal health and ecosystem condition. If a disease outbreak is ongoing, the IS could be used throughout to monitor enzyme levels, thus helping farm management.

6. Provide a pro-active approach for husbandry practices. The IS could be used periodically during normal operations in order to detect possible environmental affects. The change in PO levels would precede losses and allow the aquaculturists to better manage the population. Management practices could then be adjusted, thereby decreasing vigorous application of antimicrobials, or by increasing administration of immunostimulants (if available).
7. Estimate the health of the population. Multiple tests on a production facility or in a pond would allow for population measures.

Future steps to assure IS validity:

1. Repeat the experiments using 15 animals per treatment.
2. Optimize assay reagents composition per invertebrate species.
3. Optimize the antibody levels in the kits for ranges of expected antigen.
4. Isolate LMBP, produce a third antibody, and employ in the IS.
5. Expose selected stocks to specific pathogens or stressors and monitor PO system activation.
6. Test molt stages against the antibodies.
7. Perform the same studies with in vivo and in vitro sample preparations. In other words, use hemocytes exposed in vitro to pathogens, or use hemocytes from animals exposed to pathogens.
8. Quantify PO, pro-PO, and LMBP per life stage, per pathogen, and per sample preparation.
9. Test different life stages of shrimp and gauge known controls to those life stages.
10. Employ tests with different strains of shrimp, and from different geographic areas.

Fulfilling the above objectives will allow for the shrimp industry to act proactively with regard to shrimp health status that can be used as a biomarker for monitoring cultured populations or for screening wild shrimp populations. The IS is an early detection method that would employ quantifiable measures of shrimp health status.

This cooperative agreement addresses the goals of the USGS-BRD Hurricane Mitch Program by developing tools to assess the health of shrimp, with goals of sustainable shrimp farming, in the Gulf of Fonseca.

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Appendix 1: Raw Data

Table 1. Raw data for absorbances from ELISA using PO preparations from hemocytes and hemolymph from *Procambarus clarkii* and *Penaeus setiferus*.

	Anti-PO	Anti-pro PO	Sham PO	Sham pro-PO
	0.56	0.26	0.01	0
	0.37	0.21	0.06	0.01
	0.53	0.3	0.02	0
Hemocyte PO	0.56	0.09	0.03	0.01
P. clarkii	0.32	0.35	0.01	0.06
	0.23	0.24	0.01	0.01
	0.51	0.37	0.04	0.01
	0.43	0.13	0.01	0.01
	Anti-PO	**Anti-pro PO**	**Sham PO**	**Sham pro-PO**
	0.34	0.39	0.02	0.01
	0.29	0.33	0.04	0.02
	0.16	0.24	0.11	0.01
Hemolymph PO	0.1	0.26	0.03	0.01
P. clarkii	0.27	0.42	0.03	0.04
	0.13	0.51	0.02	0.01
	0.34	0.21	0.04	0
	0.28	0.27	0.05	0.01
	Anti-PO	**Anti-pro PO**	**Sham PO**	**Sham pro-PO**
	0.49	0.22	0.01	0
	0.77	0.41	0.01	0.02
	0.68	0.33	0	0.01
Hemocyte PO	0.67	0.29	0	0.01
P. setiferus	0.53	0.39	0.02	0.01
	0.44	0.32	0.05	0.01
	0.71	0.28	0	0
	0.82	0.45	0.01	0.01
	Anti-PO	**Anti-pro PO**	**Sham PO**	**Sham pro-PO**
	0.35	0.48	0.02	0.07
	0.62	0.52	0.02	0.01
	0.43	0.35	0.09	0.01
Hemolymph PO	0.32	0.44	0.03	0.06
P. setiferus	0.42	0.37	0.02	0.04
	0.44	0.53	0.04	0.02
	0.54	0.65	0.03	0.06
	0.45	0.38	0.03	0.08

Table 2. Percentage of ELISA absorbance values using PO preparations obtained after exposure to bacterial lipopolysaccharide (LPS) compared to those without LPS.

	Hemocyte PO *P. clarkii*	Hemolymph PO *P. clarkii*		Hemocyte PO *P. setiferus*	Hemolymph PO *P. setiferus*
	118	100		167	89
	100	96		120	99
Anti-PO	117	95	**Anti-PO**	145	115
	135	88		148	94
	129	95		132	112
	118	68		98	118
	103	86		128	97
	140	93		118	112
	36	29		7	57
Anti-pro PO	60	46	**Anti-pro PO**	33	38
	45	35		12	45
	50	28		18	39
	49	19		30	37
	39	56		9	52
	42	24		42	59
	40	35		23	49

Table 3. Percentage of ELISA absorbance values using PO preparations obtained after exposure to fungal Zymosan A as compared to those without Zymosan.

	Hemocyte PO *P. clarkii*	Hemolymph PO *P. clarkii*		Hemocyte PO *P. setiferus*	Hemolymph PO *P. setiferus*
	78	64		171	122
	59	64		146	138
	90	58		158	132
	79	72		177	136
Anti-PO	95	66	**Anti-PO**	123	146
	67	56		160	128
	94	74		145	126
	78	80		150	154
	18	56		18	15
	26	27		6	33
	20	46		11	38
Anti-pro PO	31	38	**Anti-pro PO**	14	26
	11	40		15	40
	23	35		23	27
	32	40		16	32
	21	54		24	23

Table 4. Percentage of ELISA absorbance values [1] using PO preparations obtained after exposure to white spot viral extract (WSV) 2 as compared with those without WSV.

	Hemocyte PO *P. clarkii*	Hemolymph PO *P. clarkii*		Hemocyte PO *P. setiferus*	Hemolymph PO *P. setiferus*
	92	125		92	88
	97	111		90	128
	116	130		132	98
Anti-PO	100	100	**Anti-PO**	84	103
	88	98		130	92
	98	108		107	111
	102	116		105	108
	88	88		78	112
	18	56		97	58
	26	27		77	84
	20	46		94	76
Anti-pro PO	31	38	**Anti-pro PO**	78	88
	11	40		86	80
	23	35		88	72
	32	40		65	76