Vol. 23: 115–126, 1995 | DISEASES OF AQUATIC ORGANISMS Dis aquat Org | Published October 26

Taura syndrome in *Penaeus vannamei*: demonstration of a viral etiology

K. W. Hasson[1,*], D. V. Lightner[1], B. T. Poulos[1], R. M. Redman[1], B. L. White[1], J. A. Brock[2], J. R. Bonami[3]

[1] Department of Veterinary Science, University of Arizona, Tucson, Arizona 85721, USA
[2] State of Hawaii, Aquaculture Development Program, Dept. of Land and Natural Resources, Honolulu, Hawaii 96813, USA
[3] Laboratoire de Pathologie Comparée, UM2, Place E. Bataillon, F-34095 Montpellier Cedex 5, France

ABSTRACT: Taura syndrome (TS) was hypothesized to be caused by a virus and proven experimentally by meeting the criteria of Rivers' postulates. This was accomplished through 3 serial infectivity studies utilizing specific-pathogen-free *Penaeus vannamei* as the host for the TS virus (TSV). Test animals were infected via intramuscular injection with either a crude or cell-free suspension of the virus. The source of the crude homogenate was TSV-infected Ecuadorian *P. vannamei*, which were collected during August 1993. Both types of viral inocula caused cumulative mortalities of 73 to 87% among treatment groups. Diagnosis of TS was based on histological analysis of moribund shrimp collected during each experiment. All moribund shrimp, collected between 1 to 3 d post-injection, demonstrated moderate to severe pathognomonic TS lesions. Both gross external and histological lesions, characteristic of chronic phase TS, were observed in 25 to 100% of all survivors. Virions with a buoyant density of approximately 1.337 g ml^{-1}, icosahedral morphology, and a diameter of 31 to 32 nm, characteristics which suggest that TSV is a member of either the Picornaviridae or Nodaviridae, were recovered from the dead shrimp collected during each of the 3 infectivity studies. Comparisons of TSV samples isolated from naturally infected *P. vannamei* from Hawaii (USA) and Ecuador indicate that the same virus was responsible for the TS epizootics in both of these shrimp growing regions.

KEY WORDS: Taura syndrome · Taura syndrome virus · TSV · Rivers' postulates · Penaeid shrimp

INTRODUCTION

Since its discovery in 1992, determination of the etiology of Taura syndrome (TS) has eluded investigators. The disease often causes mortalities of 80 to 85% among infected, pond-reared *Penaeus vannamei* populations (Lightner et al. 1994). 'Per os' transmission studies conducted with *Penaeus stylirostris* have shown this species to be TS resistant. However, it is presently unknown if a carrier state exists in this species (Brock et al. 1995). TS lesions are primarily observed in the cuticular epithelium and subcuticular connective tissues of the gills, mouth, esophagus, stomach, hindgut, appendages and general body of *P. vannamei* when examined using routine histological methods (Lightner et al. 1995). Typical TS lesions are characterized by necrosis and nuclear pyknosis of the cuticular epithelium and subcutis within the aforementioned target organs. Such lesions often appear 'peppered' or 'buckshot' laden due to the presence of karyorrhectic nuclei and multiple, variably staining and sized, intracytoplasmic inclusion bodies (Lightner et al. 1995). Survivors of both naturally occurring and induced TS epizootics typically have grossly visible, multifocal melanized lesions of the cuticle and histological lesions characterized by hemocytic infiltration and melanization (Brock et al. 1995, Lightner et al. 1995). At present, the only reliable and accepted method for making a definitive diagnosis of TS is through histological demonstration of pathognomonic lesions (Brock et al. 1995, Lightner et al. 1995).

The history, economic importance, clinical signs, histopathology, and lesion ultrastructure of TS have

*E-mail: aquapath@ccit.arizona.edu

been reviewed in recent publications (Brock et al. 1995, Lightner et al. 1995). The geographical range of TS has expanded to include most of the shrimp growing areas of Ecuador, as well as single or multiple sites in Peru, Colombia, Honduras, Brazil, Hawaii (USA), Guatemala, Mexico, and part of the southeastern United States. The disease was first recognized in samples from shrimp farms located at the mouth of the Taura River in the province of Guayas, Ecuador (Jimenez 1992, Lightner et al. 1994, Wigglesworth 1994, Brock et al. 1995). The cause of TS was initially attributed to fungicide toxicity resulting from the use of 2 systemic fungicides, Tilt® (Ciba-Geigy) and Calixin® (BASF), on banana plantations adjacent to affected shrimp farms (Lightner et al. 1994, Wigglesworth 1994). However, these fungicides do not induce histological lesions characteristic of TS when experimentally administered to *P. vannamei* by injection, 'per os' or through waterborne exposure (Lightner et al. 1994, Wigglesworth 1994, Brock et al. 1995). In addition, TS epizootics of pond-reared *P. vannamei* have been reported and confirmed in areas where bananas are not grown and neither of these chemicals is employed (Wigglesworth 1994, Brock et al. 1995). A recent static renewal study demonstrated that Benlate O.D.® (DuPont), another commonly used fungicide in TS affected regions, was also found to be incapable of inducing TS in *P. vannamei* after continuous waterborne exposure to 3 different concentrations (1, 0.1, and 0.01 ppm) for a period of 30 d (Lightner et al. in press).

During summer 1994, 'per os' infectivity studies, originally conducted in Hawaii and later repeated at the University of Arizona's Aquaculture Pathology Center (UAZAPC), demonstrated that TS could be induced in specific-pathogen-free (SPF) *Penaeus vannamei* after 5 to 6 d of feeding on minced, TS infected tissue (Brock et al. 1995). Analysis of TS infected shrimp tissues by transmission electron microscopy (TEM) showed the presence of putative cytoplasmic virus particles (Brock et al. 1995, Lightner & Redman unpubl. data). Further evidence that TS was virus-caused was demonstrated at UAZAPC during a subsequent infectivity study in which SPF *P. vannamei* were intramuscularly injected with diluted filtrates (10^0, 10^{-2}, 10^{-4}) prepared from previously frozen, TS positive shrimp carcasses. This study demonstrated that the TS agent was smaller than 0.45 µm, transmissible by injection, resistant to freezing and thawing, and highly virulent at both low and high concentrations (Brock et al. 1995). Icosahedral viral particles (approx. 31 nm in diameter) were isolated from carcasses of moribund and dead shrimp collected during the study utilizing a modified IHHNV (infectious hypodermal and hematopoietic necrosis virus) purification protocol (Bonami et al. 1990) and observed by TEM (Hasson unpubl. data). Based on these findings, Brock et al. (1995) hypothesized that the disease was virus induced, that the virus was possibly a member of the family Nodaviridae, and named the agent Taura syndrome virus (TSV). Further studies at UAZAPC showed that numerous virus particles demonstrating the same size and shape could be isolated and purified from naturally infected *P. vannamei* originating from both Ecuador and Hawaii (J. R. Bonami unpubl.). To confirm the viral etiology of TS, 3 sequential infectivity bioassays were conducted at UAZAPC with the objective of fulfilling the criteria of Rivers' postulates (Rivers 1937). This report describes those studies, which demonstrate that TS is caused by a newly recognized penaeid virus.

MATERIALS AND METHODS

Experimental shrimp. Approximately 5000 SPF *Penaeus vannamei* postlarvae (Mexican strain) were air-shipped to the UAZAPC, from the Oceanic Institute, Hawaii. The SPF stock was developed as previously described by Wyban et al. (1992) and Pruder et al. (1995). The postlarvae were reared using a modification of the methods reported by Williams et al. (1992) until they reached the early juvenile stage (1.75 to 2.1 g). In each of the 3 bioassays performed, 2 to 4 glass aquaria (90 l each) were stocked with 15 shrimp $tank^{-1}$, and shrimp were acclimated for 24 h prior to being injected. Each bioassay was conducted for a period of 4 to 5 d, during which the shrimp were fed a daily ration of Rangen No. 4 pellet at a rate of 4% of the overall biomass divided between 2 feedings.

All aquaria were disinfected (200 ppm chlorine from calcium hypochlorite) for 2 d prior to use, fresh-water rinsed, and filled with 85.5 l of prepared sea water (Forty Fathoms Bio-Crystals Marinemix, Marine Enterprises International, Inc., Baltimore, MD, USA). Water chemistries/conditions were monitored on Days 0, 2, and 4 during each of the 3 studies and had the following values or ranges: pH 8.5, 24 ppt salinity, 25 to 28°C, 0.25 to 0.5 mg l^{-1} total ammonia, and 0.10 to 0.25 mg l^{-1} nitrite. Each aquarium was individually outfitted with two 1 l biological filters containing crushed oyster shell and granular activated carbon, with water recirculation provided by an airlift to each filter Aeration was generated by an electric air pump and delivered to each tank by 3 teflon air lines (2 for the airlifts and 1 for water aeration). Control aquaria were physically isolated from the virus exposed treatment aquaria and the top of each was sealed with a plastic sheet to contain aerosols. Individual nets and pipettes (for shrimp and water sampling) were assigned to each aquarium to maintain sanitary conditions and prevent cross-contamination.

Inoculum preparation and injection. A crude homogenate of TS infected *Penaeus vannamei*, viral suspensions purified by sucrose density gradient centrifugation, and appropriate control solutions (described below) were utilized as inocula in 3 sequential bioassays (Fig. 1). Test shrimp were intramuscularly injected (0.01 or 0.02 ml) in the third abdominal segment using a sterile 1 ml tuberculin syringe. Each member of a given control or treatment group received only 1 injection of inoculum of the same lot of either control solution or viral preparation. Injected shrimp were temporarily transferred to a bucket containing 10 l of siphoned aquarium water with aeration. The treated shrimp were thus maintained until all 15 experimental shrimp had been injected and were then returned to their aquarium along with the siphoned water. Prior to initiating each of the 3 bioassays, 8 to 10 randomly selected shrimp were preserved in Davidson's AFA fixative and their SPF status verified by routine histology following standard methods (Bell & Lightner 1988).

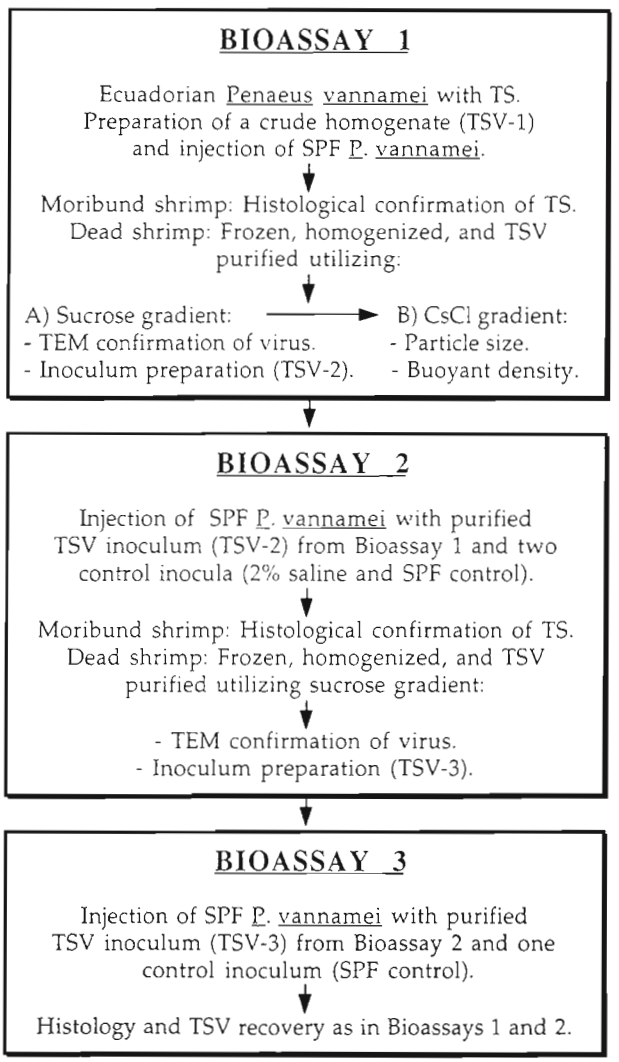


Fig. 1. Experimental design of 3 sequential infectivity studies performed with the objective of demonstrating a viral etiology for Taura syndrome utilizing specific-pathogen-free (SPF) *Penaeus vannamei* as the host for the virus

Bioassay 1: injection of a crude homogenate: A crude inoculum (TSV-1) was prepared from frozen (–80°C), TS positive *Penaeus vannamei*, which originated from a shrimp farm in Ecuador and were collected during August 1993. Approximately 60 g of shrimp heads was minced, diluted 1:3 with sterile TN buffer (0.02 M Tris-HCl, 0.4 M NaCl, pH 7.4) and homogenized (Fig. 1). The homogenate was clarified by 3 low-speed centrifugation steps [746 × *g* (10 min), 4302 × *g* (10 min), and 30 590 × *g* (30 min)] and the resulting supernatant diluted 1:10 with sterile 2% saline (NaCl) to produce a crude inoculum. Thirty SPF *P. vannamei* (1.75 g avg wt) were injected with 0.01 ml of inoculum (TSV-1) and then equally distributed between two 90 l aquaria. The shrimp were observed twice daily for disease signs over 4 d, at which time the experiment was terminated and survivors harvested. During the course of the study, moribund shrimp and all survivors at termination were preserved in Davidson's fixative (Bell & Lightner 1988). Records of daily mortalities and observations were maintained. Any shrimp found dead were frozen (–80°C) and later processed for virus recovery and preparation of the treatment inoculum used in Bioassay 2.

Bioassay 2: first preparation and injection of purified virus: Approximately 20 g of TS infected *Penaeus vannamei* carcasses, collected between 24 to 72 h post-injection (PI) during Bioassay 1, was minced and homogenized with 14 ml TN buffer (Fig. 1). Viral purification, utilizing a 15 to 40% (w/w) linear sucrose density gradient, was conducted according to the methods of Bonami et al. (1990) with slight modifications. Briefly, all ultracentrifugation steps were performed at 233 000 × *g* for the periods described in Bonami et al. (1990). Fractions containing virions from the sucrose density gradient were isolated using an Auto Densi-flow fraction collector (Buchler Instruments), diluted with TN buffer, and pooled. The suspension was centrifuged at 233 000 × *g* for 3.5 h to pellet the virus, which was then resuspended in 150 µl of TN buffer, and the presence of virus was confirmed by TEM. Approximately 120 µl of the final viral suspension was saved for CsCl gradient centrifugation to further purify the virus. To prepare the treatment inoculum (TSV-2), 10 µl of the viral suspension was diluted 1:100 with sterile 2% saline in distilled water.

Two different control inocula were prepared. A homogenate of uninfected shrimp was prepared using approximately 20 g of SPF *Penaeus vannamei* carcasses and then processed for virus recovery as de-

scribed above (Bonami et al. 1990). The resulting virus-free and cell-free suspension was diluted 1:100 with sterile 2% saline and is hereafter referred to as the SPF control inoculum. The second control inoculum consisted of heat sterilized 2% saline in distilled water.

A total of 60 SPF *Penaeus vannamei* (1.80 g avg wt) were each injected with 0.02 ml of either the purified virus inoculum (TSV-2) or 1 of the 2 control inocula. Thirty shrimp were injected with the purified virus inoculum and divided equally between two 90 l aquaria. In like fashion, 15 shrimp were injected with 2% saline and another 15 with the SPF control inoculum. Control groups were maintained in separate aquaria. Observations, preservation of dead shrimp by freezing, and fixation of moribund shrimp were performed as described for Bioassay 1. The experiment was terminated on Day 5 PI and survivors were preserved for histological analysis using Davidson's fixative.

Bioassay 3: second preparation and injection of purified virus: Approximately 17 g of shrimp carcasses, collected 24 to 72 h PI during Bioassay 2, was homogenized with 12 ml TN buffer and processed for virus isolation and purification as previously described. As in Bioassay 2, 10 µl of the final viral suspension was diluted 1:100 with 2% saline to prepare a cell-free viral inoculum (TSV-3). Samples of the undiluted suspension were analyzed by TEM to confirm virus presence.

Fifteen SPF *Penaeus vannamei* (2.1 g avg wt) were injected with 0.02 ml of the treatment inoculum as in the previous experiment (Fig. 1). To serve as controls, 15 shrimp were injected with the SPF control inoculum prepared and used in Bioassay 2. The experiment was conducted for 5 d with all samplings conducted as before. The resulting dead treatment shrimp (12.5 g) were processed for virus recovery and analyzed by TEM following the same protocol previously described.

Histopathology. All moribund shrimp sampled were injected with Davidson's fixative, processed, and stained with hematoxylin and eosin-phloxine (H&E) using routine histological methods of Bell & Lightner (1988). Sections were analyzed by light microscopy for the presence of pathognomonic TS lesions within the organs and tissues normally affected by the disease. Lesion severity, graded from 1 to 4, was determined according to a modified grading method of Bell & Lightner (1987). TS-negative shrimp received a severity grade of 0; mild, focal TS lesions were assigned a grade of 1; moderate, locally extensive to multifocal lesions received a grade of 2 to 3; and severe, multifocal to diffuse TS lesions were assigned a grade of 4. As lesion severity often varied between tissue types within a given sample, the overall severity grade per shrimp was based on its most severely affected organ or tissue.

Buoyant density of virus particles. Approximately 120 µl of viral suspension, which was purified by sucrose density gradient centrifugation prior to Bioassay 2 (Fig. 1), was overlaid onto a 25 to 50% CsCl density gradient (w/w) and centrifuged at 233 000 × *g* for 16 h. Fractions containing virions from the CsCL gradient were processed and the virus isolated following the protocol of Bonami et al. (1990). The resulting pelleted virus was resuspended in 100 µl of TN buffer and the presence of virus particles confirmed by TEM. Determination of the refractive index and buoyant density of the virus was done following the methods of Bonami et al. (1990).

Transmission electron microscopy. Sucrose- and CsCl-gradient-purified viral suspensions were examined by TEM to establish the presence of virions and determine whether other pathogens or cells were present in the preparations. Viral suspensions were negatively stained on carbon-collodion-coated, 300 mesh copper grids utilizing 2% phosphotungstic acid (pH 6.5) as described by Bonami et al. (1990). All observations were made using either a Hitachi H-500 or a Jeoul 100CX transmission electron microscope. Two suspensions of CsCl-gradient-purified virions, one prepared from the resultant dead treatment shrimp of Bioassay 1 (Ecuadorian isolate) and another from naturally infected Hawaiian *Penaeus vannamei* (Hawaiian isolate), were negatively stained as previously described and photographed at a magnification of approximately 26 000×. A stock solution of tobacco mosaic virus (TMV, 10 mg ml^{-1}) was diluted 1:20 with TN buffer and mixed 4:1 with the Hawaiian virus sample prior to preparing the grid. Addition of TMV to the Hawaiian virus sample served as an internal size reference as TMV has a known diameter of 18 nm (Francki et al. 1991). A 3 mm diffraction grating replica, containing 2160 parallel lines mm^{-1} (no. 606, Ted Pella, Inc., Redding, CA, USA), was also photographed at approximately 26 000× and used to calculate the actual magnification of each electron micrograph. Thirty viral particles per geographic isolate were measured to determine both the mean values and ranges of the particles' maximum (point to point) and minimum (side to side) diameters using the corrected magnification value. Dimensions of the Hawaiian viral isolate were also determined by comparison to TMV

RESULTS

Time course of mortality

In each bioassay, peak mortalities occurred among treatment shrimp, injected with either the crude TS shrimp homogenate (TSV-1) or purified virus inocula (TSV-2 and TSV-3), within 24 to 48 h PI and then

Table 1 *Penaeus vannamei*. Results of 3 infectivity studies summarizing daily and cumulative percentage mortalities, shrimp lesion severity, and prevalence of experimentally induced Taura syndrome (TS) in SPF juvenile shrimp. Each treatment individual received a single intramuscular injection of either a crude TS positive tissue homogenate (TSV-1) or 1 of 2 cell-free viral suspensions (TSV-2 and TSV-3). Control inocula consisted of either sterile 2% saline or a purified cell-free suspension made from SPF *P. vannamei* (SPF control). All shrimp removed from the tanks (dead or moribund) were counted as mortalities. Lesion severity was graded (G) from 1 (mild, focal) to 4 (severe, multifocal to diffuse)

Inoculum/Replicate	Daily mortality						Cumulative % mortality on Day 4 or 5	No. examined by H&E histology (no., day sampled)	No. with TS and lesion severity (G)
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5			
Bioassay 1									
TSV-1/a	0	4	4	3	0	–	11/15, 73%	5, Day 0	0 of 5
								1, Day 1	1 of 1, G: 2–3
								2, Day 2	2 of 2, G: 2–4
								1, Day 4	0 of 1
TSV-1/b	0	6	5	1	0	–	12/15, 80%	5, Day 0	0 of 5
								3, Day 1	3 of 3, G: 3–4
								1, Day 4	0 of 1
Bioassay 2									
2% saline	0	0	0	0	0	0	0/15, 0%	8, Day 0	0 of 8
								8, Day 5	0 of 8
SPF control	0	0	0	0	0	0	0/15, 0%	8, Day 5	0 of 8
TSV-2/a	0	2	7	0	2	0	11/15, 73%	1, Day 1	1 of 1, G: 3–4
								2, Day 2	2 of 2, G: 3–4
								4, Day 5	0 of 4
TSV-2/b	0	5	5	2	0	0	12/15, 80%	3, Day 1	3 of 3, G: 3–4
								2, Day 2	2 of 2, G: 3–4
								3, Day 5	0 of 5
Bioassay 3									
SPF control	0	0	0	0	0	0	0/15, 0%	10, Day 0	0 of 10
								10, Day 5	0 of 10
TSV-3	0	4	4	3	2	0	13/15, 87%	3, Day 1	3 of 3, G: 4
								3, Day 2	3 of 3, G: 2–4
								1, Day 3	1 of 1, G: 3
								2, Day 5	0 of 2

rapidly declined (Table 1, Figs. 2 to 4). Cumulative mortalities ranged from 73 to 87% among each treatment population. No mortalities occurred among the control populations injected with either 2% saline or the SPF control inoculum.

Clinical and gross signs

Clinical signs of induced TS were characterized by lethargy, atactic swimming, empty midguts due to anorexia, opaque musculature, and laterally recumbent posture just prior to death. Some of the infected shrimp were observed to swim to the surface, rotate dorsal to ventral, and sink back down to the bottom, where they remained momentarily motionless. This behavior was repeated several times until the shrimp would remain immobile on the bottom with death ensuing within 1 to 2 h. The majority of the dead and moribund shrimp were found to have a soft cuticle, suggesting that ecdysis had occurred; however, cast molts were not observed in the treatment aquaria. Upon termination of each bioassay, 25 to 100% of the treatment survivors had multifocal, melanized cuticu-

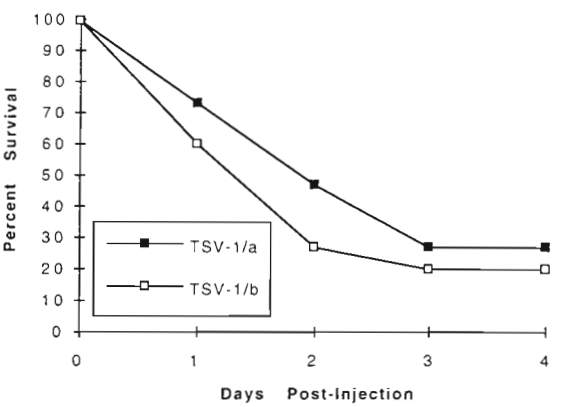


Fig. 2. *Penaeus vannamei*. Daily percentage survival of Bioassay 1 SPF juvenile shrimp injected with a crude homogenate (TSV-1) prepared from TS infected *P. vannamei* originating from Ecuador. TSV-1/a and TSV-1/b: treatment replicates

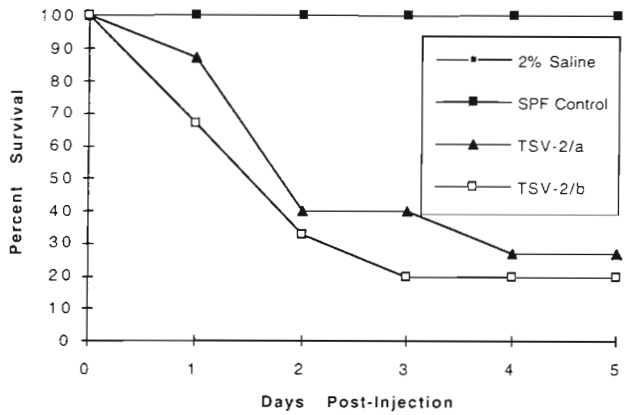


Fig. 3. *Penaeus vannamei*. Daily percentage survival of Bioassay 2 SPF juvenile shrimp injected with either a sucrose-gradient-purified TSV suspension (TSV-2) prepared from Bioassay 1 TS fatalities, a sucrose-gradient-purified virus-free suspension (SPF control) prepared from SPF *P. vannamei*, or sterile 2% saline. TSV-2a and TSV-2/b: treatment replicates. Percent survival curves of the SPF control and 2% saline injected groups are identical (100% survival) and are represented by a single horizontal line

lar lesions identical to those seen on pond-reared shrimp that had survived a TS epizootic (Fig. 5a, b). In many instances, such shrimp were observed to completely resolve these melanized lesions with the next molt (Fig. 5c). Both the saline and/or the SPF control inoculum injected shrimp (Bioassays 2 and 3) behaved and fed normally throughout each experiment.

Histopathology

Histological analysis of moribund treatment shrimp, sampled on Days 1 to 3, revealed moderate to severe (grade 2 to 4) TS lesions within 3 or more known target tissues, including the cuticular epithelium of the general body, appendages, gills, mouth, esophagus, stomach, and hindgut (Table 1). The lesions were characterized by necrosis and nuclear pyknosis of the cuticular epithelial cells and subcutis. Present in these lesions were multiple, lightly to darkly staining, basophilic spheres of varying diameters, which were interpreted as intracytoplasmic inclusion bodies and karyorrhectic nuclei (Figs. 6 to 8). Moribund shrimp sampled within the first 24 h PI typically displayed severe, multifocal TS lesions within the gills. Mild TS lesions were noted within a nodule of hematopoietic tissue located in the epigastric region of a few severely infected shrimp. Consistent with the declining mortality rates and observation of melanized external lesions, none of the virus exposed shrimp sampled at termination of each bioassay (Table 1) displayed any active TS lesions. However, chronic phase lesions, characterized by focal melanization and hemocytic infiltrates, were noted in some of the treatment survivors. Moderate to severe lymphoid organ lesions, characterized by poorly to highly vacuolated spheroids and similar to those described by Bonami et al. (1992), were commonly observed in treatment survivors, but absent from the control animals. Analysis of all control shrimp sampled on Day 0 and at termination (Day 5) demonstrated that they were not infected with TS at the inception of the studies and remained free of the disease for the duration of each bioassay.

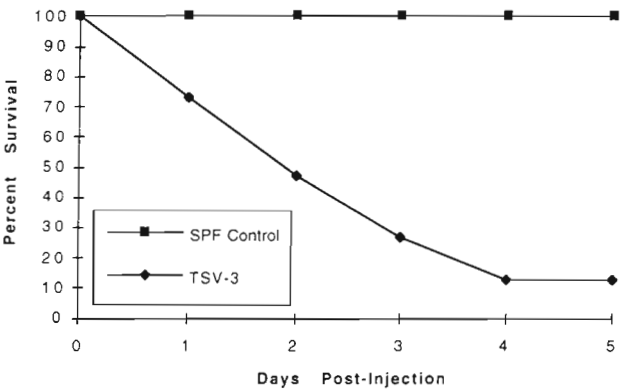


Fig. 4. *Penaeus vannamei*. Daily percentage survival of Bioassay 3 SPF juvenile shrimp injected with either a sucrose-gradient-purified TSV suspension (TSV-3) prepared from Bioassay 2 TS fatalities or a sucrose-gradient-purified virus-free suspension (SPF control) prepared from SPF *P. vannamei*

Virus particle size and buoyant density

TEM of the viral suspensions purified by sucrose density gradient centrifugation showed that each preparation was cell-free and contained numerous hexagonal viral particles indicative of cubic symmetry and icosahedral morphology. CsCl gradient centrifugation of the sucrose-gradient-purified virus, recovered from Bioassay 1 shrimp, produced a single opaque band in the gradient. Analysis of this fraction and that of the Hawaiian virus suspension by TEM revealed that both preparations contained large numbers of icosahedral viral particles of similar size. Mean values for the maximum (point to point) and minimum (side to side) diameters of the viral particles of both samples (n = 30) were calculated based on magnification of the electron micrographs determined using a diffraction grating replica. The Ecuadorian and Hawaiian viral particle diameters were equivalent in size, with a mean point to point length of 31 nm and a mean side to side length of 27 to 28 nm (Table 2, Fig. 9a and inset). Determination of the Hawaiian virus particle dimensions through comparison with TMV

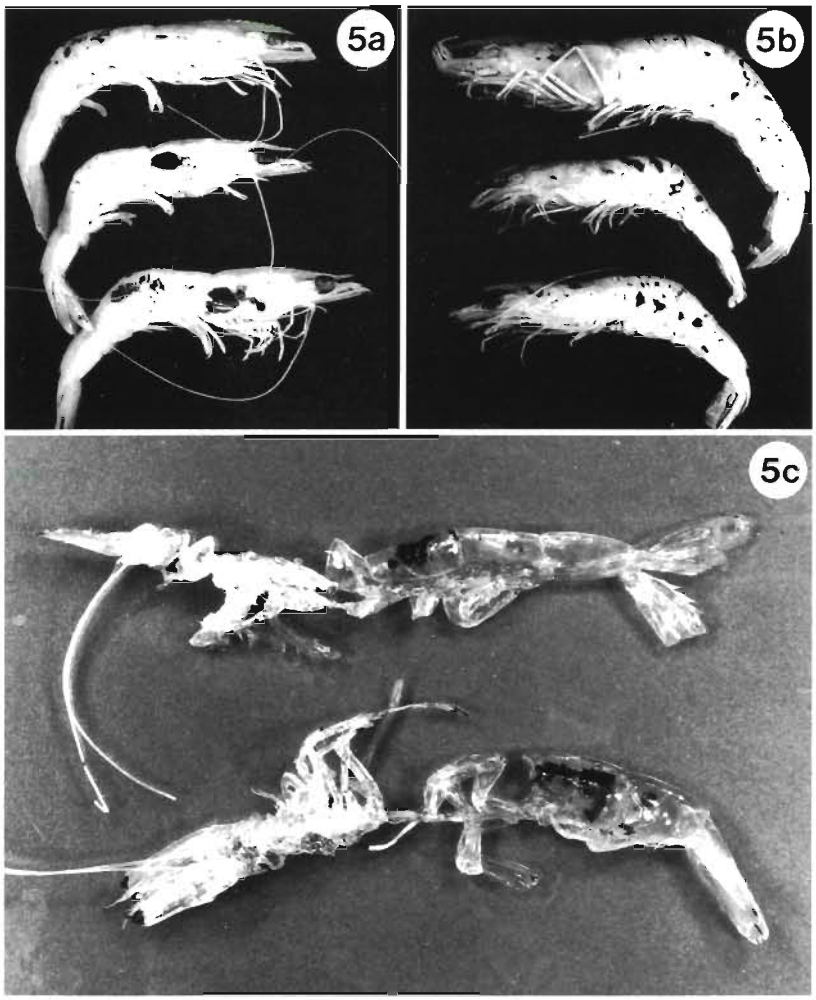
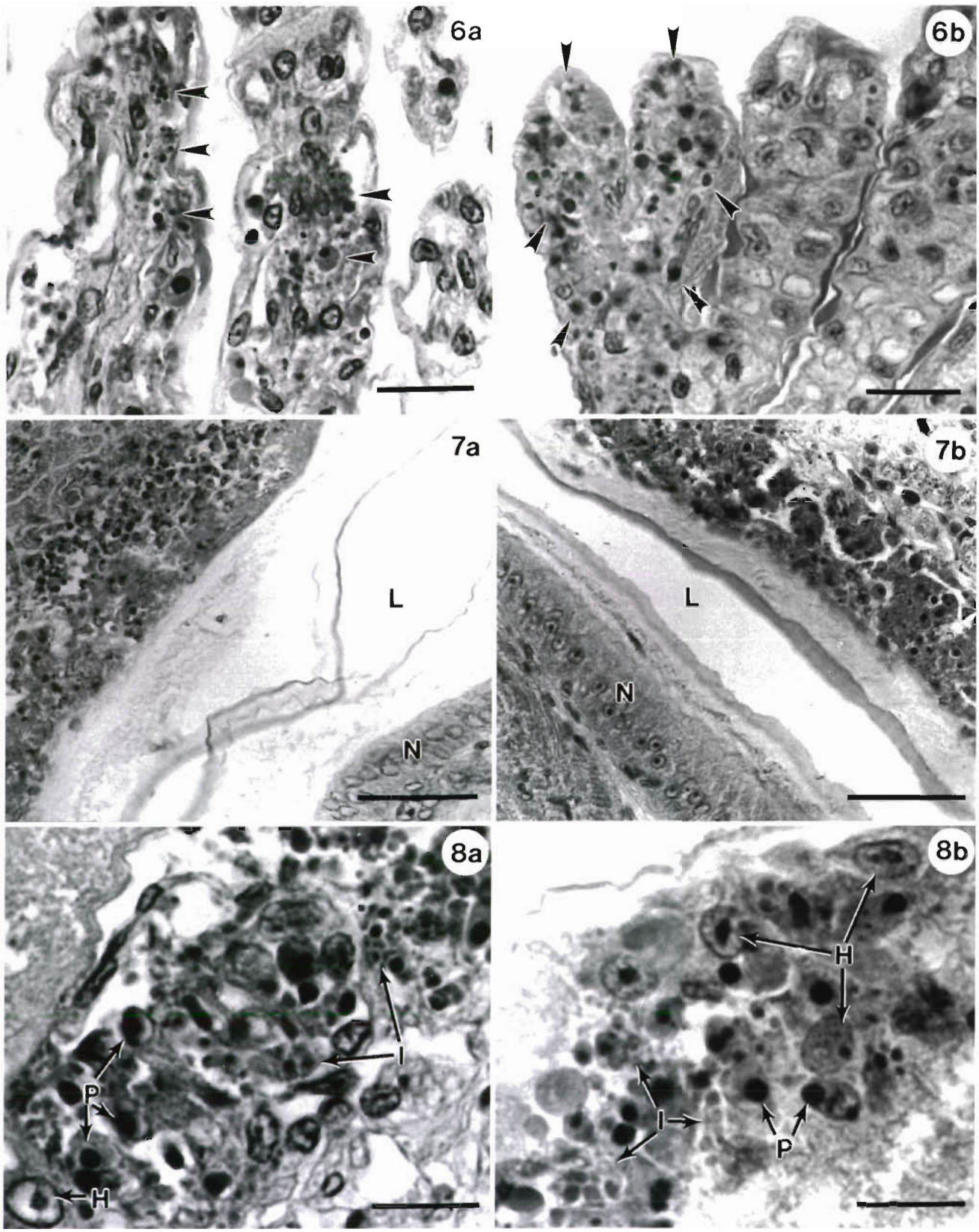


Fig. 5. *Penaeus vannamei*. Juvenile shrimp with gross external lesions that are characteristic of chronic phase TS. (a) Survivors of experimentally induced TS, 4 d after injection with a crude TS inoculum (TSV-1). Note multifocal, melanized cuticular lesions. (b) Ecuadorian, pond-reared shrimp collected during a TS epizootic. Multifocal, melanized cuticular lesions are evident and are similar in distribution and appearance to those seen on the shrimp in (a). (c) Cast-off molts with prominent melanized lesions. These molts were recovered from an aquarium containing virus-injected *P. vannamei* which had survived the infection and were recovering

(Fig. 9b) showed a negligible difference to the values determined using the diffraction grating replica. Utilizing this method showed diameters to be slightly larger, with a mean point to point length of 32 nm and a mean side to side length of 28 nm (Table 2). Refractive index analysis established the Ecuadorian virus as having an average buoyant density of 1.337 g ml^{-1}.

DISCUSSION

The results of previous studies conducted in Hawaii and at the UAZAPC during summer 1994 were suggestive of an infectious etiology for TS and attributed the disease to a putative viral agent named Taura syndrome virus (TSV) (Brock et al. 1995). The purpose of the present study was to determine if TS has a viral etiology by fulfillment of the criteria of Rivers' postulates, which stipulate that the viral agent must be isolated from a diseased host and that it be capable of inducing the same disease, with all of its characteristics, when injected in the form of a cell-free extract into a disease-free member of the same species (Rivers 1937). The series of experiments reported here demonstrated that viral particles can be isolated and purified from *Penaeus vannamei*, with either naturally acquired or experimentally induced TS infections. In each bioassay, the successful isolation and preparation of cell-free viral suspensions was confirmed by TEM analysis. Observations of virions with icosahedral shape, a diameter of 31 to 32 nm, and approximate buoyant density of 1.337 g ml^{-1} corroborate the previously reported findings of Brock et al. (1995). Furthermore, intramuscular injection of cell-free suspensions of the isolated virus induced both clinical signs and histopathological lesions that are characteristic of TS (Figs. 5 to 8). Peracute to acute phase TS lesions were induced within 3 or more of the known organ systems targeted by TS in all moribund shrimp sampled within 72 h PI during each bioassay, with lesion severities ranging from grade 2 to 4 (Table 1). Peak mortalities occurred within 48 h PI among all virus-exposed treatment groups and cumulative mortalities, ranging from 73 to 87%, were similar in magnitude to mortality figures reported from shrimp farms that had experienced TS epizootics (Wigglesworth 1994, Brock et al. 1995, Lightner et al. 1995). That the TS agent was unaffected by the inherent dilution factor, caused by serial passage through 3 groups of test shrimp, indicates that it replicated in the exposed hosts and, thus, rules out the possibility of a toxic etiology. Complete absence of mortalities or histological lesions among the control shrimp (Bioassays 2 and 3), injected with sterile saline or with the acellular, virus-free suspension prepared from SPF shrimp, provides further evidence that the viral agent causes the disease. Based


6a
6b
7a
L
N
7b
L
N
8a
I
P
H
8b
H
I
P

Figs. 6 to 8. *Penaeus vannamei*. Histological sections of juvenile shrimp with TS showing lesions which are pathognomonic for the disease and present a characteristic 'peppered' or 'buckshot' appearance. Fig. 6. Gill lamellae displaying (a) experimentally induced and (b) naturally acquired peracute to acute TS lesions of the cuticular epithelium. Affected foci contain necrotic cuticular epithelial cells. The predominant features of the lesions are the multiple, pale to darkly staining, often spherical inclusions of varying diameters (arrowheads), that are pyknotic and karyorrhectic nuclei and cytoplasmic inclusion bodies. Hematoxylin and eosin. Scale bars = 20 µm. Fig. 7. Sagittal sections of the esophagus displaying (a) experimentally induced and (b) naturally acquired peracute to acute TS lesions of the cuticular epithelium. Normal cuticular epithelium (N) is adjacent to locally extensive TS lesions [upper left half of (a), upper right half of (b)]. L: Esophagus lumen. Hematoxylin and eosin. Scale bars = 50 µm. Fig. 8. Higher magnification photomicrographs of TS lesions in the cuticular epithelium of pleopods from shrimp with (a) experimentally induced or (b) naturally acquired TS disease. Both sections illustrate the characteristic 'peppered' or 'buckshot' appearance of TS lesions. At this magnification, pyknotic nuclei (P) and hypertrophied nuclei (H), as well as multiple, variably staining, cytoplasmic inclusions (I) are readily apparent. Hematoxylin and eosin. Scale bars = 15 µm

on our present findings, we conclude that a viral etiology for TS has been firmly established and that the naming of the agent as Taura syndrome virus by Brock et al. (1995) is justified. Furthermore, demonstration of a viral etiology helps to explain the rapid spread of TS into shrimp growing regions (i.e. Hawaii, Colombia, southeastern U.S., Mexico and 2 hatcheries in Ecuador) where fungicides, previously suspected as causing the disease, were not in use. As the viral isolates from Hawaii and Ecuador were shown to be identical in size and morphology, we believe that the same virus is responsible for the TS epizootics that have occurred throughout the Americas. One of the possible reasons for the rapid dissemination of TSV is the highly stable nature of the virus. This is evidenced by the recovery of viable, infectious virions from dead shrimp showing advanced post-mortem change, the virus' ability to withstand the physical stresses incurred during viral purification, and its capacity to endure long-term freezing and multiple freeze/thaw cycles (the TSV positive carcasses from Ecuador had been frozen for approximately 1 yr prior to being used in Bioassay 1). Although experimentally induced and naturally occurring TSV epizootics result in excessive losses, cumulative mortalities among affected *P. vannamei* populations do not appear to greatly exceed 85 to 90%. This suggests that a small percentage of each population is resistant to infection, or that they are capable of recovering from the disease. The attributes or characteristics which either confer resistance or enable some shrimp to recover from the disease are unknown and require further investigation.

Treatment shrimp with experimentally induced TS were observed to be lethargic, anorexic, soft bodied, and immobile prior to death, clinical signs which are similar to those of naturally infected farm-raised *Penaeus vannamei* (Brock et al. 1995). However, reddening of the abdomen and tail fan, commonly seen in naturally occurring TS infections and attributed to infection mediated expansion of the chromatophores, was not observed in our laboratory studies. We believe that this condition may be linked to the diet of pond-reared shrimp and the incorporation of carotenoids resulting, directly or indirectly, from the consumption of phytoplankton. Absence of such natural foods in our experimental system and the feeding of a pelleted ration probably account for the lack of chromatophore pigmentation and reddening of the tail in our experimentally induced infections.

The observation of moribund, TS infected animals swimming slowly to the tank's surface, rotating dorsal to ventral, and sinking back to the bottom, has not been previously reported for this disease. This behavior is a clinical sign of IHHNV infected *Penaeus stylirostris* (Lightner et al. 1983); however, IHHNV lesions were not detected in any of the moribund treatment shrimp or those surviving to termination. As a result, it appears that this behavior may also be characteristic of terminal TS infections in *Penaeus vannamei*.

In all 3 bioassays, chronic phase TS lesions among 25 to 100% of the treatment survivors were essentially identical, both grossly and histologically, to those seen

Table 2. Comparison of the maximum (point to point) and minimum (side to side) particle diameters between Ecuadorian and Hawaiian virus isolates (n = 30) as determined by TEM of negatively stained (2% PTA), CsCl gradient-purified samples. TMV: tobacco mosaic virus

Lengths measured	Diffraction grating replica method (nm)		TMV method (nm)
	Hawaiian virus	Ecuadorian virus	Hawaiian virus
Point to point			
Min	30	30	31
Max	34	34	34
Mean	31	31	32
Side to side			
Min	26	24	26
Max	30	30	31
Mean	28	27	28

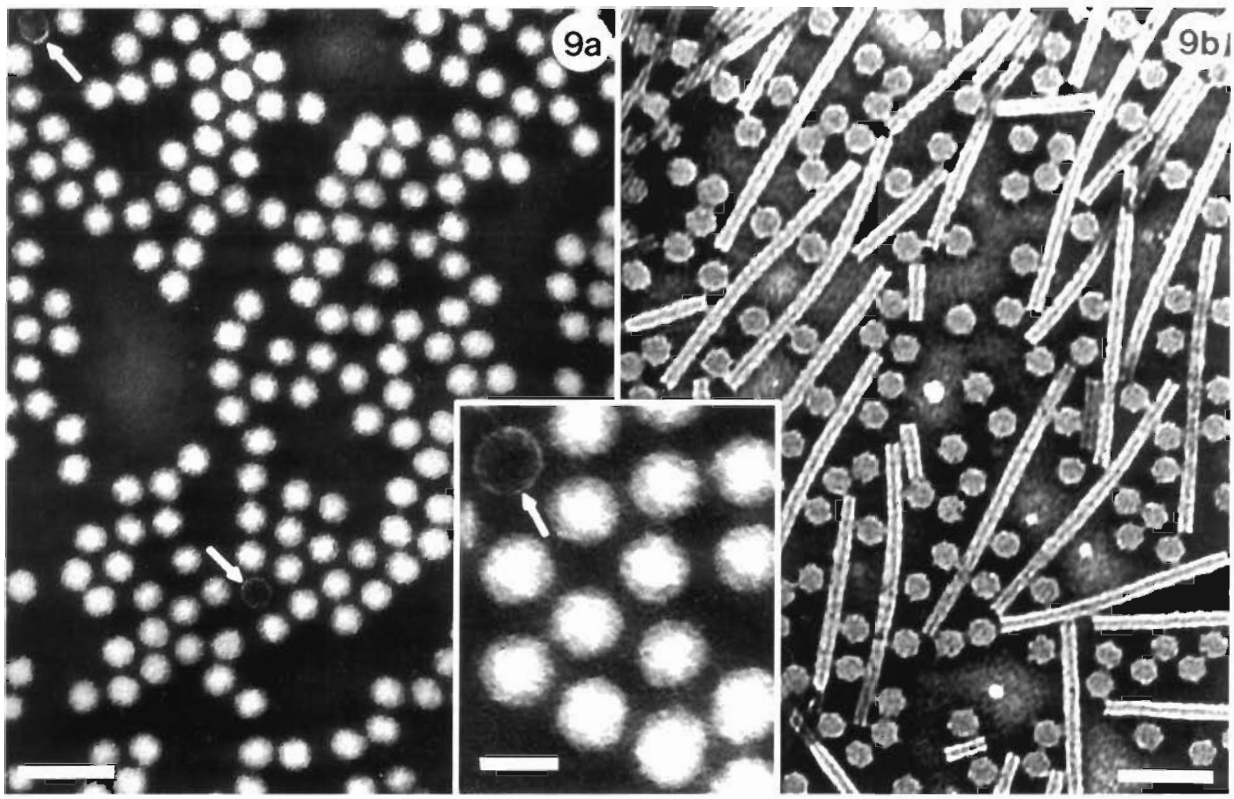


Fig. 9. Transmission electron micrographs illustrating CsCl-gradient-purified viral isolates from both Ecuador and Hawaii. (a, inset) Ecuadorian viral isolate showing numerous full and a few empty (arrows) icosahedral viral particles with a diameter of 31 to 32 nm. (b) Hawaiian viral isolate mixed with tobacco mosaic virus. Negative stain (2% PTA). Scale bars = (a) 93 nm, (inset) 32 nm, (b) 96 nm

among pond-reared shrimp which had survived a natural TS epizootic. Based on histological observations of *Penaeus vannamei* with experimentally induced acute and chronic phase TS disease, it appears that TS lesion pathogenesis and remission among survivors follow a set pattern. Peracute to acute phase necrotic foci of cuticular epithelial cells are removed, possibly by circulating hemolymph and infiltrating hemocytes entering the lesion, followed by melanin deposition within the damaged area. During this process, the affected epithelial cell layer regenerates and melanized foci are either resorbed or cast off with the cuticle during the next molt. Based on this proposed course of the disease process, we now believe that chronic phase TS lesions are indicative of a recovery phase and the onset of tissue regeneration in survivors of a TS epizootic.

The observation of mild TS lesions within the hematopoietic nodules (HEO) of a few severely infected treatment shrimp has not been previously reported for naturally occurring cases of TS infection. We believe that these lesions may be a consequence of injection-induced disease, occurring in shrimp that are highly susceptible to TS and probably of minor significance.

Moderate to severe lymphoid organ lesions were observed in the majority of treatment survivors of each bioassay, but were not detected in the control shrimp. These lesions were characterized by poorly to highly vacuolated spheroids of the type associated with 4 known penaeid viral diseases, specifically those caused by lymphoid organ vacuolization virus (LOVV; Bonami et al. 1992), yellow head virus (YHV; Boonyaratpalin et al. 1993), rhabdovirus of penaeid shrimp (RPS; Nadala et al. 1992), and lymphoidal parvovirus (LPV; Owens et al. 1991). Shrimp surviving a TS infection may be sufficiently weakened to permit activation of additional viral infections, such as LOVV, which was reported by Jimenez (1992) to be commonly found in shrimp with naturally occurring TS infections. However, another possible explanation is that the morphological changes seen in the lymphoid organ (LO) are a result of phagocytosis and clearing of TSV particles, spent hemocytes, and necrotic cell debris from the hemolymph by the LO cells. Thus, spheroid formation may be directly caused by TSV or it may be represen-

tative of a generalized physiological response mounted by the host during the final stages of a TS infection. Once a TSV-specific gene probe is developed, *in situ* hybridization analysis of these shrimp should help to clarify the nature of these lesions.

Waterborne exposure and 'per os' transmission, resulting from cannibalism of infected carcasses by previously healthy shrimp, appear to be among the natural modes of TSV transmission (Brock et al. 1995, Brock unpubl. data). The aquatic insect *Trichocorixa reticulata* (commonly known as the water boatman) may also be involved in the spread and transmission of TSV. Reports of excessive numbers of these insects in TSV infected shrimp ponds have been received from Ecuador, Honduras, and Colombia's Pacific coast (D. Garriques, El Rosario, Ecuador, pers. comm., Wigglesworth, Ecuadminsa, Ecuador, pers. comm., R. Bador, Acuanal, Colombia, pers. comm.) and observed by 2 of the present authors (D. V. Lightner & J. A. Brock). Preliminary studies conducted by some of the shrimp growers in Ecuador (D. Garriques & J. Wigglesworth pers. comm.) indicate that elimination of these insects prevents the onset of TS epizootics, suggesting that *T. reticulata* may serve as a source or vector of TSV. Confirmation of such an epizootiological pathway could hasten the development of management strategies for controlling this disease.

Based on the limited physiochemical information presently known about this intracytoplasmic virus, TSV shows characteristics that justify its tentative placement within either the Picornaviridae or the Nodaviridae (Francki et al. 1991). Further studies are under way to better define the structure, genomic make-up, pathogenesis, and host range of TSV. The possibility that asymptomatic survivors of a TS epizootic are carriers of or latently infected with TSV and capable of vertically transmitting the virus are topics which urgently need to be investigated. To this end, work on both a TSV polyclonal antibody and TSV-specific gene probes are in progress. Development of these potentially sensitive diagnostic tools may permit rapid detection of TSV and further our understanding of this new viral pathogen of penaeid shrimp.

Acknowledgements. This research was supported by grant no. 88-38808-3320 from the U.S. Dept of Agriculture, CSRS, to the Gulf Coast Laboratory Marine Shrimp Farming Consortium. The authors thank Hernan Aubert, Rodney Williams, Carlos Pantoja, Fred Nelson, and Jeff Garza (UAZAPC) for their input and/or assistance in conducting these experiments. Gratitude and thanks to David Garriques (El Rosario, Guayaquil, Ecuador), Dr John Wigglesworth (Ecuadminsa, Guayaquil, Ecuador), and Dr Regis Bador (Acuanal, Bogota, Colombia) for their cooperation and support. Special thanks to David Bentley and Beth Huey (UAZ) for TEM technical assistance, and to Dr Sam Stern (Dibsa, Guayaquil) for providing the TSV infected shrimp initially used in this study.

LITERATURE CITED

Bell TA, Lightner DV (1987) IHHN disease of *Penaeus stylirostris*: effects of shrimp size on disease expression. J Fish Dis 10:165–170

Bell TA, Lightner DV (1988) A handbook of normal penaeid shrimp histology. World Aquaculture Society, Baton Rouge, p 6

Bonami JR, Lightner DV, Redman RM, Poulos BT (1992) Partial characterization of a togavirus (LOVV) associated with histopathological changes of the lymphoid organ of penaeid shrimp. Dis aquat Org 14:145–152

Bonami JR, Trumper B, Mari J, Brehelin M, Lightner DV (1990) Purification and characterization of the infectious hypodermal and haematopoietic necrosis virus of penaeid shrimps. J gen Virol 71:2657–2664

Boonyaratpalin S, Supamattaya K, Kasornchandra J, Direkbusaracom S, Aekpanithanpong U, Chantanachoolin C (1993) Non-occluded baculo-like virus, the causative agent of yellow head disease in the black tiger shrimp (*Penaeus monodon*). Gyobyo Kenkyu 28: 103–109

Brock JA, Gose R, Lightner DV, Hasson KW (1995) An overview of Taura syndrome, an important disease of farmed *Penaeus vannamei*. In: Browdy CL, Hopkins JS (eds) 'Swimming through troubled water'. Proceedings of the special session on shrimp farming. World Aquaculture Society, Baton Rouge, p 84–94

Francki RIB, Fanquet CM, Knudson DL, Brown F (1991) Classification and nomenclature of viruses. Fifth report of the international committee on taxonomy of viruses. Springer-Verlag, Vienna

Jimenez R (1992) 'Síndrome de Taura' (resumen). Aquacultura del Ecuador, Guayaquil 1:1–16

Lightner DV, Hasson KW, White BL, Redman RM (in press) Chronic toxicological and histopathological studies with benomyl in *Penaeus vannamei* (Crustacea: Decapoda). Aquat Toxicol

Lightner DV, Jones LS, Ware GW (1994) Proceedings of the Taura Syndrome Workshop: executive summary; submitted reports, and transcribed notes. Jan 21–22, 1994. Univ of Arizona, Tucson

Lightner DV, Redman RM, Bell TA (1983) Infectious hypodermal and hematopoietic necrosis, a newly recognized virus disease of penaeid shrimp. J Invertebr Pathol 42: 62–70

Lightner DV, Redman RM, Hasson KW, Pantoja CR (1995) Taura syndrome in *Penaeus vannamei*: histopathology and ultrastructure. Dis aquat Org 21:53–59

Nadala ECB, Lu Y, Loh PC, Brock JA (1992) Infection of *Penaeus stylirostris* (Boone) with a rhabdovirus isolated from *Penaeus* sp. Gyobyo Kenkyu 27:143–147

Owens L, DeBeer S, Smith J (1991) Lymphoidal parvovirus-like particles in Australian penaeid prawns. Dis aquat Org 11:129–134

Pruder GD, Brown CL, Sweeney JN, Carr WH (1995) High health shrimp systems: seed supply — theory and practice. In: Browdy CL, Hopkins JS (eds) 'Swimming through troubled water' Proceedings of the special session on shrimp farming. World Aquaculture Society, Baton Rouge, p 40–52

Rivers TM (1937) Viruses and Koch's postulate. J Bacteriol 33: 1–12

Wigglesworth J (1994) 'Taura syndrome' hits Ecuador farms. Fish Farmer 17(3):30–31

Williams RR, Dehdashti B, Lightner DV (1992) Performance of an aquatic multispecies system in evaluating the effects

of a model microbial pest control agent on nontarget organisms. J Toxicol Environ Health 37:247–264

Wyban JA, Swingle JS, Sweeney JN, Pruder GD (1992) Development and commercial performance of high health shrimp using specific pathogen free (SPF) broodstock *Penaeus vannamei*. In: Wyban JA (ed) Proceedings of the special session on shrimp farming. World Aquaculture Society, Baton Rouge, p 254–259

Responsible Subject Editor: J. E. Stewart, Dartmouth, Nova Scotia, Canada

Manuscript first received: March 10, 1995
Revised version accepted: May 4, 1995